Identification of a novel opioid peptide (Tyr-Val-Pro-Phe-Pro) derived from human α_{s1} casein (α_{s1} -casomorphin, and α_{s1} -casomorphin amide)

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A new casomorphin pentapeptide (α_{s1} -casomorphin) has been isolated from the sequence of human α_{s1} -casein [α_{s1} -casein-(158–162)], with the sequence Tyr-Val-Pro-Phe-Pro. This peptide was found to bind with high affinity to all three subtypes of the κ -opioid receptor (κ_1 – κ_3). When amidated at the C-terminus, α_{s1} -casomorphin amide binds to the δ - and κ_3 -opioid sites. Both α_{s1} -casomorphin and its amide inhibit in a dose-dependent and reversible manner the proliferation of T47D

human breast cancer cells. This anti-proliferative activity was greater for α_{s1} -casomorphin, which was the most potent opioid in inhibiting T47D cell proliferation. In T47D breast cancer cells, other casomorphins have been found to bind to somatostatin receptors in addition to opioid sites. In contrast, α_{s1} -casomorphin and its amide do not interact with somatostatin receptors in our system.

INTRODUCTION

Casein peptide fragments (casomorphins) are a potential source of opioids in the breast. Indeed, both human and bovine caseins have been shown to contain, in their primary structure, peptides with opioid activity [1–3]. These peptides can be liberated by enzymic treatment of caseins both *in vitro* and *in vivo* [1,4–7]. Recently we have shown that α - and β -casein-derived peptides bind to opioid receptors and decrease the cell proliferation of T47D human breast cancer cells [8]. Furthermore, we showed that these peptides can equally interact with somatostatin receptors on the same cell line.

Caseins, which are synthesized under multi-hormonal control in the lactating mammary glands of mammals, comprise the major protein fraction in the milk in most species (80 % and 40 % of the proteins in ruminant and human milk respectively). Bovine milk contains four casein proteins, whose genes have been localized to bovine chromosome 4 [9]. They were found to be linked in the order α_{s1} -, β -, α_{s2} - and κ -casein [10–12]. These phosphoproteins form stable calcium-dependent colloidal particles, known as casein micelles. One of them, namely β -casein, which is the longest (209 amino acids in the bovine species, including five phosphoserine residues), is present in the milk of all species studied so far, and is the major component of human casein micelles. It accounts for 30 % of the total protein, and 70 % of the casein content, of human milk [13,14]. Its amino acid sequence has been determined [15], and the complete structural organization of the relevant gene was reported [16]. Evidence for exon skipping during the course of pre-mRNA splicing has been provided [17,18]. The existence of a human κ -casein, the phosphoglycoprotein known to ensure the stability of the casein micelles against precipitation by Ca^{2+} ions [19], was definitively proven 20

years ago [20]. Its amino acid sequence has been determined [21] and the relevant cDNA has been cloned and sequenced, starting from human mammary biopsy material [22].

The human casein system was considered, until recently, to be unusual in that it apparently lacked α_s -caseins [23,24], which comprise the major part (~ 50 %) of the bovine casein fraction [25]. However, the presence of α_{s1} -casein in human milk has been demonstrated recently ([26]; P. Martin, G. Brignon, V. Francois, J.-P. Furet and C. Leroux, unpublished work). From the primary structure of this protein, we have identified a pentapeptide [α_{s1} -casein-(158–162)] which might have opioid activity. In the present work we have synthesized the pentapeptide α_{s1} -casein-(158–162) (Tyr-Val-Pro-Phe-Pro). We show that it can displace radio-labelled opioids from their binding sites. Furthermore, we show that this new casomorphin can also decrease cell proliferation of the human T47D breast cancer cell line.

MATERIAL AND METHODS

Peptide synthesis

α_{s1} -Casomorphin (Tyr-Val-Pro-Phe-Pro) was synthesized by conventional peptide chemistry methods. t-Butoxycarbonyl (Boc) groups were used for protection of the α -amino groups, and a t-butyl group was used for protection of the phenolic hydroxy group. The dicyclocarbodi-imide/1-hydroxybenzotriazole method was used for coupling of the suitably protected amino acids. Removal of the t-butyl and Boc groups was achieved by using trifluoroacetic acid. The peptides were purified by semi-preparative HPLC on a reverse-phase C-18 Nucleosil column, with a 30 min linear gradient of 15–85 % methanol in 0.1 % aqueous trifluoroacetic acid.

Abbreviations used: DSLET, [D-Ser²,Leu⁵]enkephalin-Thr; DAGO, [N-Me-Phe⁴,Gly⁵-ol]enkephalin; Pen, penicillamine; DPDPE, [D-Pen²,D-Pen⁵]enkephalin; DADLE, [D-Ala²,D-Leu⁵]enkephalin.

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Cell cultures

The human breast cancer cell line T47D (originally isolated from a pleural effusion of breast adenocarcinoma) was obtained at passage 86. Cells were routinely grown in RPMI medium supplemented with 10 % (v/v) heat-inactivated foetal calf serum. They were cultured at 37 °C, in a humidified atmosphere of 5 % CO₂ in air.

Cell growth conditions

Cells were plated in 24-well ELISA plates containing 1 ml of medium/well at an initial density of 25×10^3 cells/well. All drugs were added to cultures 1 day after seeding (designated as day 0), to ensure uniform attachment of cells at the onset of the experiments. Cells were grown for a total of 6 days, with daily changes of the medium containing peptides. Without the addition of any drug, the proliferation doubling time of the cells was 2 days (see the Results section). Therefore the number of cells in control experiments was about 200 000 at day 6. All added drugs were dissolved in PBS shortly before use.

Cell proliferation

Cell growth was measured by the tetrazolium salt assay [28]. Cells were incubated for 4 h at 37 °C with the tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], and metabolically active cells reduced the dye to purple formazan. Dark blue crystals were dissolved with propanol. The absorbance was measured at 570 nm and compared against a standard curve produced with known numbers of T47D cells. All experiments were performed a minimum of three times, in triplicate.

Binding conditions

Ligand binding assays in whole T47D cells (about 10^6 cells/well) were performed as described by Hatzoglou et al. [29–31]. Before binding, cells were washed with 2×2 ml of PBS (10 mM phosphate, 150 mM NaCl, pH 7.4). Binding was performed in the same buffer (total volume of 0.5 ml) containing radioactive opioids, without (total binding) or with (non-specific binding) a 1000-fold molar excess of the same unlabelled agent, or the peptides used in the concentration range 1 pM–1 μ M. The cells were incubated for 2 h at room temperature (18–22 °C). At the end of the incubation period, unbound radioactivity was removed by washing the cells with 2×2 ml of cold buffer. Cells were removed from plates with 0.4 ml of 2 M NaOH, and mixed with 4 ml of scintillation cocktail (SigmaFluor; Sigma, St. Louis, MO, U.S.A.). The bound radioactivity was counted in a scintillation counter (Tricarb, Series 4000; Packard), with 60 % efficiency for ³H. Binding assays were repeated at least three times (in duplicate).

Preparation of rat brain membranes and measurement of opioid binding was performed as described previously by Loukas et al. [6]. Briefly, binding was performed in Tris/HCl buffer (10 mM, pH 7.4), in a final volume of 1.0 ml. The protein concentration was 300 μ g/assay. Binding was initiated by the addition of 2 nM [³H][D-Ser²,Leu⁵]enkephalin-Thr ([³H]DSLET) (a selective δ -opioid radioligand) or [³H][N-Me-Phe⁴,Gly⁵-ol]enkephalin ([³H]DAGO) (a selective μ -opioid ligand). Non-specific binding was estimated in the presence of 10 μ M naloxone. Peptide concentrations varied from 0.1 nM to 1 μ M. After the incubation, bound radioactivity was separated by filtration, under reduced pressure, through GF/D filters, previously soaked in Tris/HCl buffer, and rinsed twice with ice-cold buffer. The results were analysed using the Origin (MicroCal Co.) V 3.78

package, using equations described by Munson and Rodbard [32].

Radiochemicals and chemicals

[³H]Ethylketocyclazocine (specific radioactivity 18 Ci/mmol), [³H]DSLET (specific radioactivity 41.5 Ci/mmol) and [³H][D-Pen²,D-Pen⁵]enkephalin (DPDPE; where Pen is penicillamine) (specific radioactivity 37 Ci/mmol) were bought from New England Nuclear. [³H]Diprenorphine (specific radioactivity 29 Ci/mmol) and [³H]DAGO (specific radioactivity 47.7 Ci/mmol) were from Amersham. Ethylketocyclazocine was a gift from Sterling-Winthrop. Diprenorphine was from Reckit and Coleman. All other chemicals were either from Merck (Darmstadt, Germany) or from Sigma.

RESULTS

Binding of α_{s1} -casomorphin and α_{s1} -casomorphin amide to opioid receptors

T47D cells

Figures 1 and 2 show the interactions of α_{s1} -casomorphin and its amide with different classes of opioid receptors present on the membranes of T47D cells. The results of the performed studies are summarized in Table 1.

[³H]Ethylketocyclazocine is an almost general ligand for opioid sites, interacting with δ -, μ -, κ_1 - and κ_2 -opioid sites. α_{s1} -Casomorphin displaced, with high affinity, [³H]ethylketocyclazocine from its binding sites on T47D human breast cancer cells. The displacement was complete, with a calculated IC₅₀ value of 0.1 ± 0.02 nM (Figure 1, left panel). The addition of 10 μ M [D-Ala²,D-Leu⁵]enkephalin (DADLE) during displacement experiments (which masks all sites to which ethylketocyclazocine could bind with the exception of κ_1), produced only minor effects on [³H]ethylketocyclazocine binding (IC₅₀ values of 0.1 and 0.7 nM in the absence and in the presence respectively of DADLE), indicating that α_{s1} -casomorphin interacts mainly with κ_1 -opioid binding sites (Figure 1, right, upper panel).

[³H]Diprenorphine is also an almost general opioid ligand, interacting mainly with δ -, μ -, κ_2 - and κ_3 -opioid sites. α_{s1} -Casomorphin partially displaced [³H]diprenorphine binding, with an IC₅₀ of 0.5 ± 0.02 nM. The addition of 10 μ M DADLE during [³H]diprenorphine binding, leaving only the κ_3 -opioid sites unmasked [33], produced a major shift of the displacement curve (Figure 1, right, lower panel), indicating that the peptide interacts with both the κ_2 - and κ_3 -opioid sites. The IC₅₀ for the κ_3 -opioid site could be calculated directly from the graph, and was found to be 5.6 pM. Furthermore, taking into consideration our previous results [31] on the affinity and concentration of κ_2 -opioid sites for diprenorphine (K_D , 8 nM; concentration, 75 pM), we could calculate the affinity of α_{s1} -casomorphin for the κ_2 -opioid site, giving a value of 0.9 nM.

Our previous results with T47D cells have shown that no μ -opioid receptors could be found [31]. In contrast, δ -receptors were identified. [³H]DPDPE is a selective ligand of δ -opioid sites. α_{s1} -Casomorphin shows a minor interaction with [³H]DPDPE binding (Figure 1, left panel), which does not permit a valid calculation of the IC₅₀.

Replacement of the C-terminus with an amide group produced major changes in α_{s1} -casomorphin binding affinity. As shown in Figure 2, α_{s1} -casomorphin amide cannot displace [³H]ethylketocyclazocine from its binding sites. On the contrary, we observed partial displacement of [³H]diprenorphine and [³H]DPDPE (IC₅₀ values of 0.12 and 1.7 nM respectively). The addition of 10 μ M DADLE during [³H]diprenorphine binding did not modify the

Figure 1 Displacement of radiolabelled opioids by α_{S1} -casomorphin in T47D human breast cancer cells

Left panel: displacement of radiolabelled ethylketocyclazocine (■), diprenorphine (▲) and DPDPE (●) by α_{S1} -casomorphin. A total of 10^6 cells were used for displacement binding, which was performed at room temperature for 2 h in PBS. Radiolabelled opioid (~ 15 nM) was incubated alone (B_0) or in the presence of various concentrations of α_{S1} -casomorphin, ranging from 1 pM to 1 μ M. Non-specific binding was determined by the addition of 1 μ M of the same, non-labelled, opioid. Specific binding (B/B_0) was calculated using the formula $(B - NSB)/(B_0 - NSB)$, where B is total binding and NSB is non-specific binding. The broken lines represent the sigmoidal fitting used for the calculation of IC_{50} values. Right, upper panel: the same experiment as in the left panel was performed, using ethylketocyclazocine, in the presence of 10 μ M DADLE. In this case, DADLE blocks the interaction of the ligand with δ -, μ - and κ_2 -opioid sites, and therefore ethylketocyclazocine interacts only with κ_1 -opioid sites. Right, lower panel: the same experiment as above was carried out, but diprenorphine was used as the radiolabelled opioid. In this case, DADLE blocks the interaction of the ligand with δ -, μ - and κ_2 -opioid sites, permitting diprenorphine to interact only with κ_3 sites (see the Results section for further details).

Figure 2 Displacement of radiolabelled opioids by α_{S1} -casomorphin amide in T47D human breast cancer cells

The same conditions as in Figure 1 were used for binding, with the exception that α_{S1} -casomorphin amide was used as the displacing ligand. The interaction of this agent with the binding of radiolabelled ethylketocyclazocine (■), diprenorphine (▲) and DPDPE (●) is presented.

affinity of α_{S1} -casomorphin. These results indicate that α_{S1} -casomorphin amide interacts with δ - and κ_3 -opioid sites only, presenting a pharmacological selectivity completely different from that shown by the non-amidated peptide.

Rat brain membranes

As indicated in previous studies from our group, T47D human breast cancer cells do not present any μ -opioid receptors, while the concentration of δ -opioid binding sites is low [31]. In order to investigate a possible interaction of α_{S1} -casomorphin and α_{S1} -casomorphin amide with δ - and μ -opioid sites, we have performed displacement studies on rat brain membranes, which are rich in δ - and μ -opioid sites. Figure 3 presents the results of binding studies. With rat brain membranes, α_{S1} -casomorphin was a complete competitor of δ -opioid sites (IC_{50} 2.6 μ M), while α_{S1} -casomorphin amide displaced [3 H]DSLET only partially (IC_{50} > 10 μ M). On the contrary, with μ -opioid sites, α_{S1} -casomorphin amide completely displaced radiolabelled DAGO (IC_{50} 1 μ M), while α_{S1} -casomorphin showed little displacement. It is of interest to note, however, that the affinities of both peptides for opioid sites were much lower with rat brain membranes than with T47D human breast cancer cells (compare Figures 1, 2, and 3). These data are also summarized in Table 1.

Effects of α_{S1} -casomorphin and α_{S1} -casomorphin amide on cell proliferation

Cell viability

After plating, we assayed the number and the viability of cells for at least 6 days of culture. We found that 85–90 % of the total cells were attached after 24 h. Furthermore, the doubling time of the cells under basal conditions, without the addition of any drug, was 2.02 days. Cell viability was assayed routinely. Under our experimental conditions, and for the period of time studied, there

Figure 3 Displacement of radiolabelled opioids by α_{s1} -casomorphin and α_{s1} -casomorphin amide in rat brain membranes

Rat brain membranes (300 μ g) were incubated with 2 nM [3 H]DSLET (upper curves) or [3 H]DAGO (lower curves) in the presence of the indicated concentrations of α_{s1} -casomorphin (■) or α_{s1} -casomorphin-amide (●) for 1 h at room temperature. Non-specific binding was estimated in the presence of 10 μ M naloxone. The results (means of three different experiments performed in duplicate) are the ratio of specific binding in the presence to binding in the absence of the indicated concentration of each peptide (B/B_0).

was no apparent change in cell viability under basal conditions or after the addition of casomorphin peptides.

Cell growth

Figure 4 shows the effects of α_{s1} -casomorphin and its amide on cell proliferation of T47D human breast cancer cells. α_{s1} -Casomorphin at very low concentrations produced dramatic changes in cell proliferation (Figure 4, left panel), inhibiting cell growth by 80 %. Taking into consideration that the doubling time of T47D cells, under the conditions used in our study, is 2.02

days, it is concluded that α_{s1} -casomorphin inhibits cell growth completely, in a dose-dependent manner. The general opioid antagonist diprenorphine reversed the action of the peptide when the latter was present at concentrations lower than 5 nM. At higher peptide concentrations, the antagonistic effect of diprenorphine was blunted. This result indicates that α_{s1} -casomorphin might be at least 100 times more potent than diprenorphine. This was corroborated by findings presented above (see previous results and Figure 1). Indeed, our previous results [31] have shown that the affinity of diprenorphine for opioid sites on T47D cells is 8.3 nM, while the result presented in Figure 1 shows that α_{s1} -casomorphin has an IC_{50} of 0.5 nM. Similar results were found with α_{s1} -casomorphin amide. In this case, the inhibition of cell proliferation was less pronounced than for the carboxypeptide. Nevertheless, we found a 55 % inhibition of cell proliferation, which is comparable with the inhibitory actions of other α - and β -casomorphins in the same cell line [8]. In this case, the effect of diprenorphine was much less pronounced than for the carboxypeptide. Indeed, this opioid antagonist produced only a 10-fold shift of the α_{s1} -casomorphin amide curve (Figure 4, right panel).

Interaction of α_{s1} -casomorphin and its amide with somatostatin receptors on T47D cells

Previous results indicated that different casomorphin peptides interact with somatostatin receptors on the T47D human breast cancer cell line [8,30]. It thus was of interest to investigate whether such an interaction also occurs for α_{s1} -casomorphin. Our results (Figure 5) show that neither α_{s1} -casomorphin nor its amide displayed a significant interaction with somatostatin receptors on this cell line. Therefore their inhibitory action on cell proliferation is likely to be a result of their interaction with opioid receptors.

DISCUSSION

Human and animal caseins are rich sources of bioactive peptides (see [1,2] for reviews). From the enzymic breakdown of α - and β -caseins, multiple peptides with opioid activity have been isolated, characterized and synthesized [4–6,34]. α_{s1} -Casein, which was recently sequenced ([26]; P. Martin, G. Brignon, V. Francois, J.-P. Furet and C. Leroux, unpublished work), represents about 15 % of the total casein content of human milk. We have identified a pentapeptide [α_{s1} -casein-(158–162)] with the sequence Tyr-Val-Pro-Phe-Pro. This casein fragment is situated towards

Table 1 Affinity of α_{s1} -casomorphin and α_{s1} -casomorphin amide for opioid binding sites in T47D human breast cancer cells and rat brain membranes

The results are the calculated affinities from data presented in Figures 1–3, after sigmoidal fitting.

Ligand	Effector	Affinity (nM) α_{s1} -Casomorphin	α_{s1} -Casomorphin amide	Detected opioid sites
T47D cells				
[3 H]Ethylketocyclazocine	—	0.1	> 1000	δ , κ_1 , κ_2
[3 H]Ethylketocyclazocine	10 μ M DADLE	0.7	> 1000	κ_1
[3 H]Diprenorphine	—	0.5	0.12	δ , κ_2 , κ_3
[3 H]Diprenorphine	10 μ M DADLE	0.006	0.2	κ_3
[3 H]DPDPE	—	> 1000	1.7	δ
Rat brain membranes				
[3 H]DSLET	—	2600	> 10000	δ
[3 H]DAGO	—	> 10000	1000	μ

Figure 4 Effects of α_{s1} -casomorphin and its amide on the proliferation of T47D cells

Human breast cancer T47D cells (25×10^3 cells/well) were cultured for 6 days without or with the indicated concentrations of α_{s1} -casomorphin (left panel) or α_{s1} -casomorphin amide (right panel), in the absence (■) or in the presence (●) of $1 \mu\text{M}$ of the general opioid antagonist diprenorphine. Cell numbers are given relative to the number of cells in control experiments after 6 days of culture, which was 200 000 cells/well ($= 1.0$).

Figure 5 Interactions of α_{s1} -casomorphin and α_{s1} -casomorphin amide with somatostatin binding sites

About 10^6 human T47D cells were incubated with 12 fmol of ^{125}I -[Tyr¹¹]somatostatin ($\sim 50\,000$ c.p.m.) in the absence (B_0) or in the presence of the indicated concentrations of α_{s1} -casomorphin (■) or α_{s1} -casomorphin amide (●). Results are means $\pm$ S.E.M. of two experiments performed in triplicate. See the Material and methods section for details of the binding experiments.

the C-terminal end of the molecule. Our interest was directed towards this peptide for the following reasons. (1) Its sequence has some similarity with those of endogenous opioid peptides, with a tyrosine at position 1 and a phenylalanine at position 4 (compare the new peptide, -Tyr-Val-Pro-Phe-Pro-, with [Met⁵]enkephalin, Tyr-Gly-Gly-Phe-Met-). (2) It presents an analogy with other casomorphin peptides, and especially with morphiceptin [compare the sequences of α_{s1} -casomorphin (Tyr-Val-Pro-Phe-Pro) and morphiceptin (Tyr-Pro-Phe-Pro)]. (3) In the amino acid sequence surrounding human α_{s1} -casomorphin in α_{s1} -casein, at the C- and N-terminal ends there is a proline and

a glutamine residue respectively [-Gln-(Tyr-Val-Pro-Phe-Pro)-Pro]. The Pro-Pro and Gln-Tyr bonds could be the site of attack by endopeptidases such as prolyl-endopeptidase, which specifically cleaves Pro-Xaa bonds, and others showing less specificity [35,36]. The action of such endopeptidases makes possible the release of the bioactive pentapeptide.

This pentapeptide, named α_{s1} -casomorphin, is shown here to have remarkable opioid actions. It displaces opioid ligands from their binding sites in the T47D human breast cancer cell line, interacting with high affinity with all three subtypes of the κ -opioid site (κ_1 - κ_3), and it also shows a significant but lower affinity for δ - and μ -opioid sites in the rat brain. The terminal amidation of α_{s1} -casomorphin modifies drastically its affinity for opioid sites. Indeed, α_{s1} -casomorphin amide shows no interaction with κ_1 - and κ_2 -opioid sites, while its interactions with κ_3 -, δ - and μ -opioid sites is enhanced. However, no interaction with somatostatin binding sites was observed for either α_{s1} -casomorphin or its amide, unlike all other casomorphins studied [8]. It is of interest that α_{s1} -casomorphin and α_{s1} -casomorphin amide show some discrepancies in affinity between rat brain membranes and T47D human breast cancer cells (see Table 1). This result could be explained by the following. (1) Binding was performed on membrane preparations in a hypotonic medium in the case of rat brain membranes, but in an isotonic medium and with viable whole cells in the case of T47D cells. It is known that ionic strength, and also the presence of selective ions, affects opioid binding in other systems [33,37,38]. (2) α_{s1} -Casomorphin is a peptide derived from human α_{s1} -casein, which is being tested in homologous (T47D cells) and heterologous (rat brain membranes) systems. It is possible, therefore, that the affinity of the human peptide for its homologous opioid receptors might be enhanced compared with binding in an heterologous system.

On comparing the calculated IC_{50} values of α_{s1} -casomorphin and its amide for displacement of radioactive ethylketocyclazocine, diprenorphine and DPDPE binding with those presented previously for bovine casein-derived opioid peptides in

the same system [8], it becomes obvious that these newly identified human casomorphins show a much higher affinity for opioid sites. Indeed, human α_{s1} -casomorphin is at least 20 times more potent in inhibiting ethylketocyclazocine binding than bovine β -casomorphin (IC_{50} s of 0.1 and 2.42 nM respectively) and 10 times more active than bovine β -casomorphin-(1–5) in inhibiting diprenorphine binding (IC_{50} s of 0.5 and 4.9 nM respectively). In addition, human α_{s1} -casomorphin amide is at least 25 times more potent than bovine β -casomorphin in inhibiting diprenorphine binding (IC_{50} s of 0.2 and 4.9 nM respectively), but almost equipotent with bovine α -casein-(90–96) with regard to its interaction with δ -opioid sites (IC_{50} s of 1.7 and 2.4 nM respectively). Furthermore, the affinities of α_{s1} -casomorphin and its amide are at least 100–1000 times greater than those reported for bovine α - and β -casomorphins [4–6,34]; see also [8] for a discussion). From this comparison it is concluded that human α_{s1} -casomorphin and its amide are, in the T47D human breast cancer cell line, the most potent casein-derived opioid peptides so far studied, comparable in their affinities with endogenous opioid peptides (see [33,38] for comparisons). On the other hand, our results on rat brain membranes (Table 1) show that α_{s1} -casomorphin amide is a rather selective μ -opioid agonist (as expected from the similarity of its primary structure with that of morphiceptin), with an IC_{50} value of 1 μ M (50 times less potent than morphiceptin [34]), while α_{s1} -casomorphin interacts preferentially with δ -opioid sites in this system, with an affinity at least 10 times higher than the reported affinities of bovine casomorphin peptides [4–6,34].

The above results on the pharmacological potency of α_{s1} -casomorphin and its amide were confirmed by their action on cell proliferation. Human α_{s1} -casomorphin, after 6 days, inhibited cell growth completely. Further studies from our group (S. Panagiotou, M. Kampa, F. Wallet, L'H. Ouafik, E. Bakogeorgou, P.-M. Martin and E. Castanas, unpublished work) have shown that this block occurs in the G_0 phase of the cell cycle. Replacement of the terminal carboxy group by an amide group resulted in a dose-dependent inhibition of cell growth with a maximal 55% inhibition, which is comparable with the inhibitory actions of other α - and β -casomorphins with the same cell line [8]. From the above results, and taking into consideration the affinities of α_{s1} -casomorphin and α_{s1} -casomorphin amide for the different opioid sites, we propose that κ_1 - and κ_3 -opioid sites (to which α_{s1} -casomorphin binds with the highest affinity) are likely to be the main receptors implicated in the opioid-dependent inhibition of cell proliferation, while the participation of other κ and δ sites might be accessory.

Recently we have shown that different bovine casomorphin peptides also inhibit the proliferation of T47D cells [8]. The results of the present study extend our previous investigations by adding a new, very potent, member of the casomorphin family, human α_{s1} -casomorphin. In the breast, a major action of the epithelial cells is the production of caseins. Furthermore, during the process of tumour formation, different proteases are released from the tumour cells [39,40]. These enzymes could act on casein molecules and produce opioid-acting casomorphins. It remains to be investigated whether α_{s1} -casein is secreted from human breast cancer cells, and additionally whether casomorphin production from this protein can be detected. This is currently under investigation. The results of the present study, indicating an anti-proliferative effect of α_{s1} -casomorphin and its amide in breast cancer cells, suggest a possible local regulatory mechanism during breast neoplasia. Furthermore, casomorphin peptides could, if

they are well tolerated, represent a possible new, and perhaps more physiological, approach to cancer chemotherapy.

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REFERENCES

- Meisel, H., Frister, H. and Schlimme E. (1989) *Z. Ernahrungswiss.* **28**, 267–278
- Schlimme, E. and Meisel, H. (1995) *Nahrung* **39**, 1–20
- Teschemacher, H. and Koch, G. (1991) *Endocr. Regul.* **25**, 147–150
- Brantl, V., Teschemacher, H., Henschen, A. and Lottspeich, F. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1211–1216
- Lottspeich, F., Henschen, A., Brantl, V. and Teschemacher, H. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1835–1839
- Loukas, S., Varoucha, D., Zioudrou, C., Strealy, R. A. and Klee W. A. (1983) *Biochemistry* **22**, 4567–4573
- Meisel, H. (1986) *FEBS Lett.* **196**, 223–227
- Hatzoglou, A., Bakogeorgou, E., Hatzoglou, C., Martin, P.-M. and Castanas, E. (1996) *Eur. J. Pharmacol.* **310**, 217–223
- Hayes, H., Petit, E., Bouniol, C. and Popescu, P. (1993) *Cytogenet. Cell. Genet.* **64**, 281–285
- Grosclaude, F., Joudrier, P. and Mahé, M.-F. (1979) *J. Dairy Res.* **46**, 211–213
- Ferretti, L., Leone, P. and Sgarrella, V. (1990) *Nucleic Acids Res.* **18**, 6829–6833
- Threadgill, D. W. and Womack, J. E. (1990) *Nucleic Acids Res.* **18**, 6935–6942
- Anderson, N. G., Powers, M. T. and Tollasken, S. L. (1982) *Clin. Chem.* **28**, 1044–1055
- Lönnerdal, B. (1985) *Am. J. Clin. Nutr.* **42**, 1299–1317
- Greenberg, R., Groves, M. L. and Dower, H. J. (1984) *J. Biol. Chem.* **259**, 5132–5138
- Hansson, L., Edlund, A., Johansson, T., Hernell, O., Strömqvist, M., Lindquist, S., Lönnerdal, B. and Bergström, S. (1994) *Gene* **139**, 193–199
- Martin, P. and Leroux, C. (1992) *Biochem. Biophys. Res. Commun.* **183**, 750–757
- Menon, R. S., Chang, Y.-F., Jeffers, K. F. and Ham, R. G. (1992) *Genomics* **12**, 13–17
- Waugh, D. F. (1971) in *Milk Proteins: Chemistry and Molecular Biology* (McKenzie, H. A., ed.), pp. 3–85, Academic Press, New York
- Chobert, J.-M., Mercier, J.-C., Bahy, C. and Hazé, G. (1976) *FEBS Lett.* **72**, 173–178
- Brignon, G., Chtourou, A. and Ribadeau-Dumas, B. (1985) *FEBS Lett.* **188**, 48–54
- Bergström, S., Hansson, L., Hernell, O., Lönnerdal, B., Nilsson, A. K. and Strömqvist, M. (1992) *J. DNA Sequencing Mapping* **3**, 245–246
- Chtourou, A., Brignon, G. and Ribadeau-Dumas, B. (1985) *J. Dairy Res.* **52**, 239–247
- Kunz, C. and Lönnerdal, B. (1990) *J. Pediatr. Gastroenterol. Nutr.* **10**, 454–461
- Ribadeau-Dumas, B. and Brignon, G. (1993) in *Progrès en Pédiatrie 10: Allergies Alimentaires* (Navarro, J. and Schmitz, J., eds.), pp. 27–39, Doin, Paris
- Johnsen, L. B., Rasmussen, L. K., Petersen, T. E. and Berglund, L. (1995) *Biochem. J.* **309**, 237–242
- Reference deleted
- Mosmann, T. (1973) *J. Immunol. Methods* **65**, 55–63
- Hatzoglou, A., Gravanis, A., Zoumakis, E., Margioris, A. and Castanas, E. (1995) *J. Clin. Endocrinol. Metab.* **80**, 418–423
- Hatzoglou, A., Ouafik, L'H., Bakogeorgou, E., Thermos, K. and Castanas, E. (1995) *Cancer Res.* **55**, 5632–5636
- Hatzoglou, A., Bakogeorgou, E. and Castanas, E. (1996) *Eur. J. Pharmacol.* **296**, 199–207
- Munson, P. J. and Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- Castanas, E., Bourhim, N., Giraud, P., Boudouresque, F., Cantau, P. and Oliver, C. (1985) *J. Neurochem.* **45**, 688–699
- Chang, K.-J., Killian, A., Hazoum, E. and Cuatrecasas, P. (1981) *Science* **212**, 75–77
- Loh, Y. P. (1984) *Annu. Rev. Neurosci.* **7**, 189–222
- Bond, J. S. and Butler, P. E. (1987) *Annu. Rev. Biochem.* **56**, 333–364
- Bourhim, N., Castanas, E., Giraud, P. and Oliver, C. (1993) *Mol. Neuropharmacol.* **3**, 139–146
- Castanas, E., Bourhim, N., Giraud, P., Boudouresque, F., Cantau, P. and Oliver, C. (1985) *J. Neurochem.* **45**, 677–687
- Janicke, F., Schmitt, M., Pache, L., Ulm, K., Harbeck, N., Höfler, H. and Graeff, H. (1993) *Breast Cancer Res. Treat.* **24**, 195–208
- Tandon, A. T., Clark, G. M., Chamnes, G. C., Chirgwin, J. M. and McGuire, W. L. (1990) *N. Engl. J. Med.* **322**, 297–302