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Antiproliferative and receptor binding properties of α- and β-casomorphins in the T47D human breast cancer cell line

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Abstract

In previous studies, we have shown that opioid agonists ([D-Ala^2,D-Leu^5]enkephalin (DADLE), [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET), ethylketocyclazocine and etorphine) bind to opioid binding sites and decrease cell proliferation of human T47D breast cancer cells. Furthermore, we provided evidence about a cross-reaction, also in the T47D human breast cancer cell line, of μ-acting opioids with type-II somatostatin receptors. Since a potential source of opioid activity in the breast might be casomorphin peptides (produced by the enzymatic degradation of α-casein and β-casein), we investigated the antiproliferative action of five different casomorphin peptides: α-casein-(90–95), α-casein-(90–96), β-casomorphin, β-casomorphin-(1–5) and morphiceptin. We show that all five peptides decreased, in a dose-dependent manner, cell proliferation. The general antagonist diprenorphine produced only a partial reversal of their action. Furthermore, we provide evidence that all peptides (except for morphiceptin) bind to δ- and κ-opioid binding sites of T47D cells with different selectivity. Finally, we show that these peptides are also partial competitors at the somatostatin receptors present in the same cell line.

Keywords: α-Casomorphin; β-Casomorphin; Opioid receptor; Receptor subtype; Somatostastin receptor; Breast cancer cell, T47D

1. Introduction

In previous studies (Hatzoglou et al., 1995b, 1996), we showed that opioid agonists ([D-Ala^2,D-Leu^5]enkephalin (DADLE), [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET), ethylketocyclazocine and etorphine) bind to opioid receptors and decrease cell proliferation of human T47D breast cancer cells. Furthermore, we provided evidence about a cross-reaction of μ-acting opioids with the type-II somatostatin receptor. Casein-peptide fragments (casomorphins) are a potential source of opioids in the breast. Indeed, both human and bovine caseins contain, in their primary structure, peptides with opioid activity (Meisel et al., 1989; Schlimme and Meisel, 1995; Teschemacher and Koch, 1991). These peptides can be liberated by enzymatic treatment of caseins, both in vitro and in vivo (Brantl et al., 1979; Lottspeich et al., 1980; Loukas et al., 1983; Meisel, 1986; Meisel et al., 1989). Since the breast epithelial cell is the major source of caseins, we assayed the possible antiproliferative activity of casomorphins in the T47D human breast cancer cell line.

In the present study, we provide evidence that α- and β-casomorphin-derived peptides bind to opioid receptors and decrease cell proliferation of T47D human breast cancer cells. Furthermore, we show that these peptides can equally interact with somatostatin receptors on the same cell line.

2. Materials and methods

2.1. 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

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RPMI medium, supplemented with 10% heat-inactivated foetal calf serum. They were cultured at 37°C, in a humidified atmosphere of 5% CO_2 in air.

2.1.1. Cell growth conditions

Cells were plated in 24-well ELISA plates at an initial density of 25×10^3 cells/well supplemented with 1 ml medium/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 4 days, with daily change of the medium containing drugs. Without addition of any drug, the doubling time of cells is 2.02 days. Therefore, the number of cells in control experiments was about 100 000 at day 4. All added drugs were dissolved, in phosphate-buffered saline, shortly before use.

2.1.2. Cell proliferation

Cell growth was measured by the tetrazolium salt assay (Mosmann, 1973). 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 to that of a standard curve of known numbers of T47D cells. All experiments were performed a minimum of three times, in triplicate.

2.2. Binding conditions

Ligand binding assays with whole T47D cells were performed as described in Hatzoglou et al. (Hatzoglou et al., 1995a,b,1996). For saturation and displacement binding experiments with whole cells, about 10^6 cells/well were used. Before binding, cells were washed twice with 2 ml of phosphate-buffered saline (10 mM phosphate, 150 mM NaCl, pH 7.4). Binding was performed in the same buffer, in a total volume of 0.5 ml, containing radioactive opioids or [^{125}I][Tyr^{11}]somatostatin-14, without (total binding) or with (non-specific binding) a 1000-fold molar excess of the same unlabelled agent, or the various casomorphins, ranging from 10^{-11} to 10^{-6} M. The cells were incubated for 2 h at room temperature (18–22°C). At the end of the incubation period, the unbound radioactivity was eliminated by washing the cells twice with 2 ml cold buffer. Cells were removed from plates with 0.4 ml 2 N NaOH and mixed with 4 ml scintillation cocktail (SigmaFluor; Sigma, St. Louis, MO, USA). The bound radioactivity was counted in a scintillation counter (Tricarb, Series 4000, Packard) with a 60% efficiency for tritium. For somatostatin binding experiments, because an iodinated ligand was used, cells were removed from plates with 0.4 ml 2 N NaOH and the bound radioactivity was counted in a gamma counter (Tricarb Series, Packard) with a 95% efficiency for [^{125}I]iodine. Binding was repeated at least three times (in duplicate). The results were analysed by the Origin (MicroCal) V 3.78 package, using equations described by Inson and Rodbard (1980).

2.3. Radiochemicals and chemicals

[^{3}H]ethylketocyclazocine (S.A. 18 Ci/mmol) and [^{3}H][D-Ala^2,D-Leu^5]enkephalin (DADLE) (S.A. 37 Ci/mmol) were bought from New England Nuclear [^{125}I][Tyr^{11}]somatostatin (2000 Ci/mmol), [^{3}H]diprenorphine (S.A. 29 Ci/mmol) were from Amersham (UK). Ethylketocyclazocine was a gift from Sterling-Winthrop. Diprenorphine was from Reckit and Coleman. All casomorphin peptides were purchased from Sigma. All other chemicals were either from Merck (Darmstad, Germany) or from Sigma.

3. Results

3.1. Cell viability

After plating, we assayed the number and the viability of cells after at least 4 days of culture. We found that about 85–90% of the total number of cells were attached after 24 h. Furthermore, the doubling time of 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 time studied, there was no apparent change of cell viability, under basic conditions as well as after the addition of casomorphin peptides.

3.2. Effect of casomorphin peptides on cell proliferation

In the present study, we used five different casomorphin peptides. Their nomenclature and structure are presented in Table 1. Two of these peptides (α-casein-(90–95) and α-casein-(90–96)) are fragments of bovine α-casein (Loukas et al., 1983), while β-casomorphin, β-casomorphin-(1–5) and morphiceptin derive from the enzymatic degradation of β-casein (Brantl et al., 1979; Lottspeich et al., 1980; Chang et al., 1981). All tested peptides produced a concentration-dependent decrease of cell proliferation after 3 days of application (Table 2, Fig. 1). α-Casein-(90–95) and α-casein-(90–96) produce a 57% inhibition of cell proliferation. This effect was partially reversed by the addition of 10^{-6} M of the general opioid inhibitor diprenorphine. In contrast, β-casein-derived peptides (β-casomorphin-(1–5) and β-casomorphin) were less potent inhibitors of cell proliferation. Diprenorphine reversed partially the effect of β-casomorphin-(1–5) and completely the effect of β-casomorphin. Finally, morphiceptin inhibited cell proliferation, while its effect was not antagonised by diprenorphine.

The antiproliferative effects of casomorphins, as stated above, were partially reversed by the general opioid antag-

Table 1
α-Casein and β-casein fragments with opioid activity (casomorphins)

α-Casein-(90–95)	Arg-Tyr-Leu-Gly-Tyr-Leu
α-Casein-(90–96)	Arg-Tyr-Leu-Gly-Tyr-Leu-Glu
β-Casomorphin	Tyr-Pro-Phe-Val-Glu-Pro-Ile (human)
	Tyr-Pro-Phe-Pro-Gly-Pro-Ile (bovine)
β-Casomorphin-(1–5)	Tyr-Pro-Phe-Pro-Gly
Morphiceptin	Tyr-Pro-Phe-Pro-NH_2

Two of these peptides (α-casein-(90–95) and α-casein-(90–96)) are fragments of bovine α-casein (Loukas et al., 1983), while β-casomorphin, β-casomorphin-(1–5) and morphiceptin derive from the enzymatic degradation of β-casein (Brantl et al., 1979; Lottspeich et al., 1980; Chang et al., 1981).

onist diprenorphine. This result indicates that more than one mechanism could be implicated in the action of these peptides. Recently, we (Hatzoglou et al., 1995a,1996) have shown that opiate alkaloids exert their antiproliferative effect on the T47D cell line by an interaction with opioid and somatostatin receptors. We therefore investigated a possible interaction of casomorphin peptides with these two membrane receptor systems.

3.3. Binding of casomorphin peptides to membrane receptor systems

3.3.1. Binding to opioid receptors

Fig. 2 presents the interaction of casomorphin peptides with different classes of opioid receptors. The results of the displacement studies are summarised in Table 3. We have found that:

(1) All casomorphin peptides, with the exception of morphiceptin, displaced [^{3}H][D-Ala^2,D-Leu^5]enkephalin (DADLE), which is, at nanomolar concentrations, a ligand of δ- and μ-opioid binding sites. In this particular cell line,

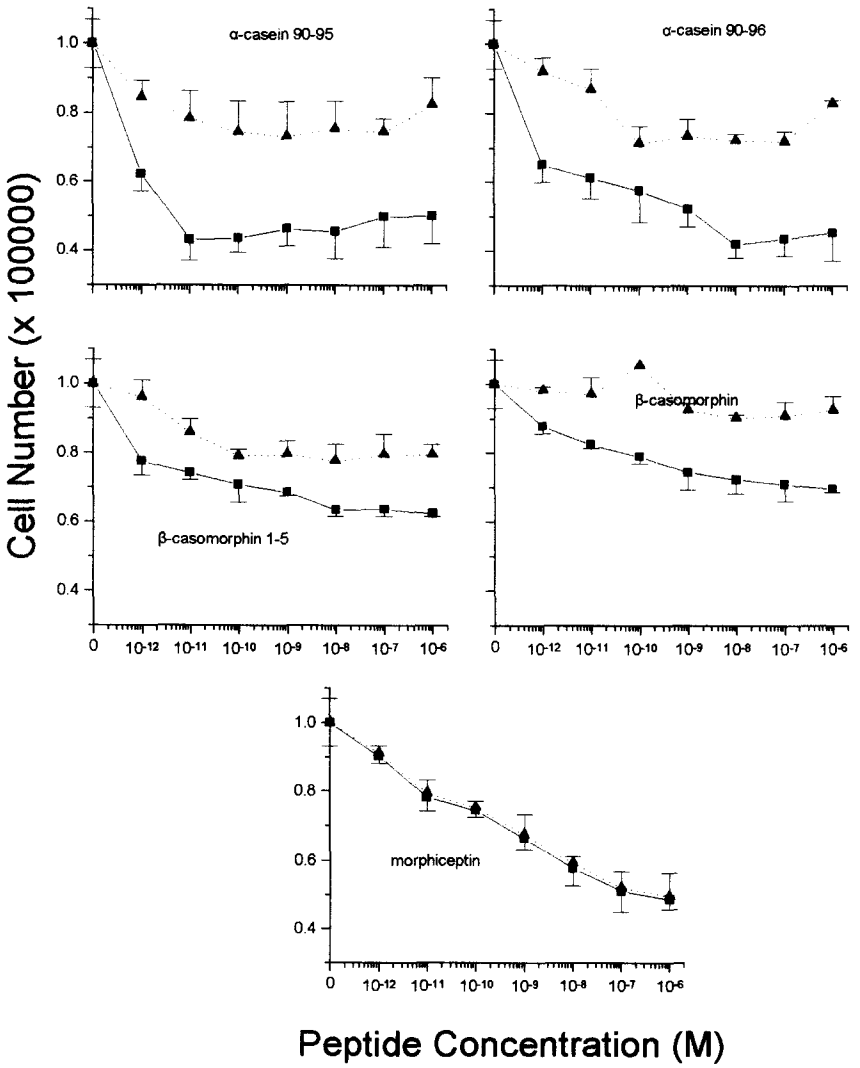

Fig. 1. Effect of α-casein- and β-casein-derived casomorphins with opioid activity on the proliferation of the human breast cancer cell line T47D. Figure presents the effect of different concentrations of each peptide in the absence (squares, plain line) or in the presence of 10^{-6} M of the general opioid antagonist diprenorphine (up triangles, dot line). Figure shows the mean ± S.E. of four experiments in triplicate.

Table 2
Effect of casomorphins on cell proliferation

Peptide	IC_{50} (M)	Maximum inhibition %	IC_{50} (M) + diprenorphine 10^{-6} M	Maximum inhibition %
α-Casein-(90–95)	$1.1\ 10^{-12}$	57	$1.2\ 10^{-12}$	27
α-Casein-(90–96)	$2.5\ 10^{-12}$	57	$1.1\ 10^{-11}$	29
β-Casomorphin	$1.2\ 10^{-11}$	31	N.D.	8
β-Casomorphin-(1–5)	$2.3\ 10^{-11}$	38	$2.2\ 10^{-11}$	23
Morphiceptin	$2.3\ 10^{-10}$	52	$4.9\ 10^{-11}$	51

Table presents the calculated inhibitory concentration of casomorphin peptides. IC_{50} values were calculated after sigmoidal fitting of the curves presented in Fig. 1.

our previous results showed that this enkephalin analog interacts only with δ-opioid sites, as no μ-opioid receptors have been identified (Hatzoglou et al., 1996). The displacement of [^{3}H]DADLE was partial in all cases, ranging from 0% (morphiceptine) to 45% of the specific binding. The order of potency of peptides was: β-casomorphin-(1–5) > α-casein-(90–96) > α-casein-(90–95) > β-casomorphin ≫ morphiceptine. The above result was confirmed by the selective δ-opioid receptor agonist [^{3}H][D-Pen^2,D-Pen^5]enkephalin, which gave the same results as [^{3}H][D-Ala^2,D-Leu^5]enkephalin (DADLE) (not shown).

(2) [^{3}H]Ethylketocyclazocine, a ligand of δ-, μ-, κ_1- and κ_2-opioid receptors, was displaced only by α-casein-(90–95), β-casomorphin-(1–5) and β-casomorphin. This latter peptide was the only one to cause a complete inhibition of specific binding. The other two peptides produced partial inhibition of 78% and 37%, respectively, while α-casein-(90–96) and morphiceptine did not interact at all with ethylketocyclazocine.

(3) Only α-casein-(90–96) displaced completely [^{3}H]diprenorphine, a ligand of δ-, μ-, κ_2- and κ_3-opioid sites. β-casomorphin-(1–5) showed a 46% interaction, while the other casomorphins (α-casein-(90–95), β-casomorphin and morphiceptine) did not interact at all.

From the above interactions, taking into consideration the selectivity of the ligands for different classes of opioid receptors, we derived the selectivity of the different casomorphins for opioid binding sites in T47D human breast cancer cells. This selectivity is presented in Table 4. It is interesting that, besides their interaction with δ-opioid sites

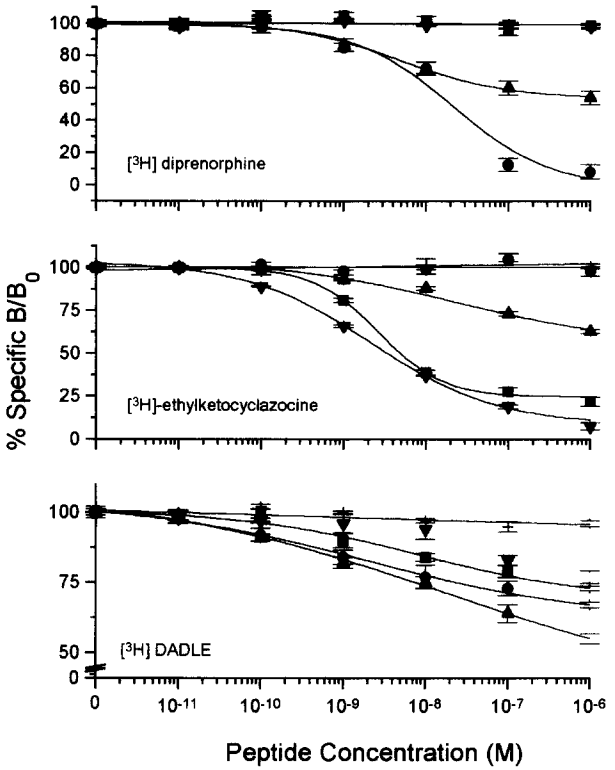

Fig. 2. Displacement of opioid ligands by casomorphins. T47D cells were incubated for 2 h with [^{3}H]diprenorphine (upper panel), [^{3}H]ethylketocyclazocine (medium panel) and [^{3}H]DADLE (lower panel) in the presence of the indicated concentrations of casomorphin peptides (α-casein-(90–95), squares; α-casein-(90–96), circles; β-casomorphin-(1–5), up triangles; β-casomorphin, down triangles; morphiceptin, crosses). The specific displacement of each ligand and the best-fitted line are presented. Means of two experiments in triplicate.

Table 3
Calculated inhibitory concentrations at 50% (IC_{50}) and maximum inhibition of specific binding of casomorphin peptides with different opioid ligands

Tritiated ligand	Ethylketocyclazocine		Diprenorphine		DADLE	
Peptide	IC_{50} (nM)	Maximum inhibition (%)	IC_{50} (nM)	Maximum inhibition (%)	IC_{50} (nM)	Maximum inhibition (%)
α-Casein-(90–95)	2.7	78	N.D.	0	3.4	27
α-Casein-(90–96)	N.D.	0	18.9	92	2.4	33
β-Casomorphin	2.42	93	N.D.	0	8.2	23
β-Casomorphin-(1–5)	62.2	37	4.9	46	7.8	45
Morphiceptin	N.D.	0	N.D.	0	N.D.	0

Table 4
Estimated interaction of casomorphins with different opioid binding sites in the T47D human breast cancer cell line

Peptide	δ	κ		
		κ_1	κ_2	κ_3
α-Casein-(90–95)	+	+ + +	–	–
α-Casein-(90–96)	+	–	–	+ + + +
β-Casomorphin	+	+ + + +	–	–
β-Casomorphin-(1–5)	+ +	–	+ +	–
Morphiceptin	–	–	–	–

According to our previous results (Hatzoglou et al., 1995b), T47D cells do not express μ-opioid binding sites. Therefore, the interaction of casomorphins with μ-opioid sites cannot be estimated.

(since μ-opioid sites are not present on this cell line, Hatzoglou et al., 1996), all casomorphin-peptides, except for morphiceptin, showed a significant interaction with κ-opioid binding sites.

3.3.2. Interaction with somatostatin receptors.

As shown in Fig. 1 and Table 2, inhibition of proliferation of T47D human breast cancer cells by casomorphins was partially reversed by the general opioid antagonist diprenorphine. Furthermore, these peptides showed a partial competition for opioid receptor binding of different opioid ligands (Fig. 2). To explain this partial interaction with opioid receptors, we investigated the possibility that casomorphins interact with somatostatin receptors. Indeed, recently, we have shown an interaction of some opioids with somatostatin receptors in this cell line (Hatzoglou et al., 1995b). As shown in Fig. 3, all five casein-derived peptides partially displaced somatostatin from its binding sites. This competition was at least 10-fold less pronounced than competition by somatostatin-14. The order of potency was: morphiceptine > α-casein-(90–95) > α-casein-(90–96) > β-casomorphin(1–5) > β-casomorphin. IC_{50} values for the above peptides were 1.3×10^{-8}, 8×10^{-8}, 8.9×10^{-8}, 1×10^{-7} and 4.7×10^{-7} M, respectively.

4. Discussion

Previous studies have indicated that exogenous opioids exert their inhibitory action on cell proliferation of breast cancer cell lines through opioid and somatostatin receptors (Hatzoglou et al., 1995b, 1996; Maneckjee et al., 1990). From a physiological point of view, opioids could reach breast tumours through different pathways: (a) from the general circulation, as might be the case of β-endorphin, which has a sufficiently long half life (Scholar et al., 1987), (b) through their production by infiltrating lymphocytes (Kita et al., 1992), (c) through local production, either from tumour or stromal cells (Bostwick et al., 1987; Scopsi et al., 1989; Zagon et al., 1987), (d) by the fragmentation of α- or β-casein, yielding peptides with opioid activity (casomorphins) (for reviews, see Meisel et al., 1989; Schlimme and Meisel, 1995). In the present study, we investigated whether casomorphin peptides could inhibit cell proliferation of the well-differentiated T47D tumour cell line.

We assayed five casomorphins: α-casein fragments 90–95 and 90–96 (Loukas et al., 1983), β-casomorphin and β-casomorphin-(1–5) (Brantl et al., 1979; Lottspeich et al., 1980) as well as the amidated β-casomorphin-derived peptide morphiceptine (Chang et al., 1981). Our results show that all five peptides had an antiproliferative action on T47D cells. Preliminary and not yet published results show

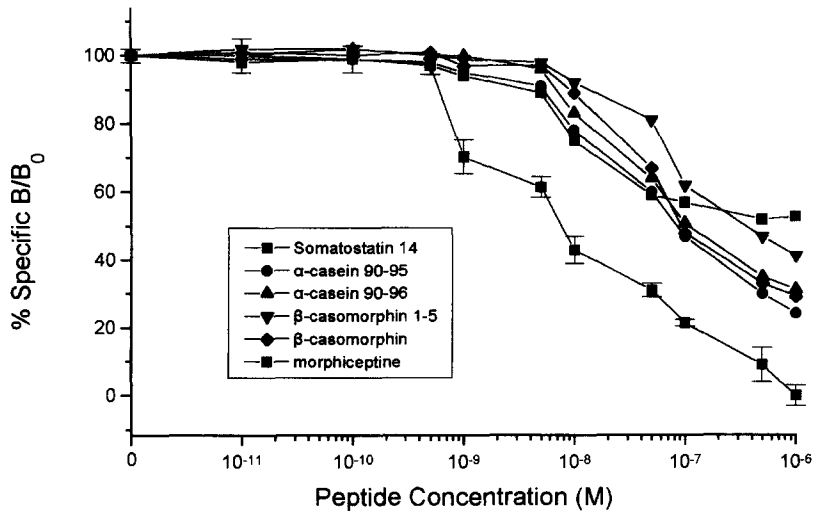

Fig. 3. Displacement of [^{125}I]Tyr^{11}-somatostatin-14 by somatostatin-14 and casomorphin peptides in the T47D breast cancer cell line. 12 fmol of [^{125}I]Tyr^{11}-somatostatin (about 50000 cpm) was incubated with the indicated concentrations of somatostatin-14 (squares), α-casein-(90–95) (circles), α-casein-(90–96) (up triangles), β-casomorphin-(1–5) (down triangles), β-casomorphin (diamonds) or morphiceptin (crosses). Figure represents the means of two experiments in triplicate. See Materials and methods for details of the binding experiments.

that opioids with a major antiproliferative action block cells in G_0/G_1 phase (S. Panagiotou et al., in preparation). Nevertheless, the general opioid antagonist diprenorphine did not, in most cases, reverse completely this antiproliferative effect. Displacement studies with different opioid ligands of known selectivity towards distinct classes of opioid receptor showed that casomorphin peptides interacted, in our system, with δ-opioid receptors but mainly with κ-opioid receptors. In other systems, casomorphins interact also with different classes of opioid receptor: α-casein-(90–95) and α-casein-(90–96) interact mainly with δ- and μ-types of opioid receptor, with IC_{50} values of 3.6 and 5.2 μM for the δ-type, and 12 and 1.2 μM for the μ-type of opioid receptor, respectively (Loukas et al., 1983); β casomorphin and β-casomorphin-(1–5) interact equally with δ-opioid receptors (IC_{50} values 15 and 25 μM) and μ-opioid receptors (IC_{50} values 1.8 and 0.5 μM, respectively) (Lottspeich et al., 1980; Chang et al., 1981); finally, morphiceptin interacts only with μ-opioid receptors, with IC_{50} of 19 nM (Chang et al., 1981). The results of the present study indicate that casomorphins interact also with subtypes of the κ-opioid receptor in the T47D human breast cancer cell line. Indeed, as shown in Table 4, taking into consideration the selectivity of the ligands used, we propose that α-casein-(90–95) interacts mainly with the κ_1-opioid site, α-casein-(90–96) with the κ_3-opioid site, β-casomorphin with the κ_1-opioid receptor and β-casomorphin-(1–5) with δ- and κ_2-opioid binding sites with almost the same affinity. Morphiceptin (a μ-selective agonist) did not show in our system any interaction with opioid receptors. According to previous results, the T47D cell line does not express μ-opioid receptors (Hatzoglou et al., 1995b), explaining the lack of morphiceptin interaction for opioid sites. The action therefore of morphiceptin might be mediated through somatostatin receptors. Indeed, we have reported that morphiceptin binds to the type-II somatostatin receptor (Hatzoglou et al., 1995b). Interestingly, all casomorphins tested showed a significant interaction with somatostatin receptors in T47D cells (Fig. 3), indicating that these peptides might have a possible major physiological role in breast cancer.

Opioid and somatostatin receptors have been cloned recently and found to belong to the seven-transmembrane segments-G_i-coupled class of receptors (for reviews, see Reisine and Bell, 1993, 1995). An almost 65% homology between the different classes of opioid receptors has been found, while a 40% homology between opioid and somatostatin receptors has been detected (Reisine and Brownstein, 1995). The main transduction mechanism attributed to both classes is the inhibition of intracellular levels of cAMP. We propose that casomorphin peptides, acting through opioid or somatostatin receptors, might use this transduction pathway. This hypothesis is currently being tested in our laboratory.

An interesting observation in the present study is that casomorphins exerted their antiproliferative action at picomolar concentrations (Table 2), while their IC_{50} values for different classes of opioid and somatostatin receptors were in the nanomolar range (Table 3, Fig. 3). A possible explanation of this result might be that occupation of only a fraction of membrane receptors is sufficient for the initiation of the biological response. Alternatively, these peptides could interact with other membrane systems, besides opioids and somatostatin receptors. Both these possibilities are currently under investigation.

α-Casein- and β-casein-derived opioid peptides have been shown to be potent agonists in different systems implicated in nutrient uptake, post-prandial hormone secretion and immune response (for a review, see Schlimme and Meisel, 1995), and in gastrointestinal motility (Daniel et al., 1990). Furthermore, these peptides seem to cross different barriers in the body, including the brush-border and blood-brain barrier (Ermisch, 1992; Mahe et al., 1989; Nyberg et al., 1989; Pasi et al., 1993; Tome et al., 1987). In the breast, a major action of the epithelial cells is the production of caseins. Furthermore, during the tumour process, different proteases are released from the tumour cells (Janicke et al., 1993; Tandon et al., 1990). These enzymes could act on casein molecules and produce opioid-acting casomorphins. It remains to be investigated whether casein is secreted from human breast cancer cells and, additionally, whether such casomorphin production can be detected. This is currently under investigation.

The results of the present study, indicating an antiproliferative effect of casomorphins in breast cancer cells, suggest a possible local regulatory mechanism during breast neoplasia. Furthermore, casomorphin peptides could be, if they are well tolerated, a possible new and, perhaps, more physiological approach to cancer chemotherapy.

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