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European Journal of Pharmacology 296 (1996) 199–207

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The antiproliferative effect of opioid receptor agonists on the T47D human breast cancer cell line, is partially mediated through opioid receptors

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Received 13 April 1995; revised 17 October 1995; accepted 20 October 1995

Abstract

In the present study, we investigated the action of opioid receptor agonists on the proliferation of cells of the T47D human breast cancer cell line, grown in the absence of exogenously added steroids and growth factors. We found that the opioid receptor agonists ethylketocyclazocine, morphine, [D-Ala^2,D-Leu^5]enkephalin (DADLE), [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET) and etorphine inhibit dose dependently cell proliferation. The opioid receptor antagonist diprenorphine had no significant effect per se, but it was able to reverse the action of all opioid receptor agonists except morphine. In order to investigate the mechanism of action of opioids on T47D cells, we characterised the opioid receptors present on this cell line, by saturation binding, using radiolabelled [D-Ala^2,*N*-Me-Phe^4-Gly^5-ol]enkephalin (DAGO, μ-opioid receptor agonist), ethylketocyclazocine (κ_1-, κ_2-, μ- and δ-opioid receptor agonist), diprenorphine (κ_2-, κ_3-, δ- and μ-opioid receptor antagonist), DADLE (δ- and μ-opioid receptor agonist), and effectors. We identified opioid binding sites belonging mainly to the κ-type (κ_1, κ_2 and κ_3), a few δ-opioid receptor sites, but no μ-opioid receptors. Our results indicate that the inhibitory effect of opioids on T47D cell growth is mediated through κ- and δ-opioid receptors. The effect of μ-acting morphine might not be mediated through opioid receptors.

Keywords: Breast cancer cell (T47D); Cell proliferation; Opioid; Opioid receptor; Opioid receptor subtype

1. Introduction

The opioid system is one of the main inhibitory systems of an organism, decreasing the activity and/or growth of cells. Opioids act as antitumor agents in many in vitro and in vivo systems, inhibiting neoplasia by decreasing cell proliferation (Aylsworth et al., 1979; Ilyinsky et al., 1987; Kikuchi et al., 1987; Kita et al., 1992; Murgo, 1989; Scholar et al., 1987; Tsunashima, 1982; Von Hoff and Forseth, 1982; Zagon and McLaughlin, 1981a, b, 1983, 1984, 1989) in a dose-dependent and reversible manner. An interesting observation is that, in some cases, the opioid receptor antagonists naloxone and naltrexone also exert this antiproliferative or antitumor action (Ilyinsky et al., 1987; Kikuchi et al., 1987; Murgo, 1989; Tsunashima, 1982; Zagon and McLaughlin, 1981a, 1983, 1989). Furthermore, it was reported that μ-opioid receptors probably do not play a significant role in oncogenesis (Zagon et al., 1985).

Immunoreactive opioid peptides have been detected in a number of different tumors of neural and non-neural origin (Bostwick et al., 1987). Furthermore, more than 50% of metastatic breast tumours contain opioid immunoreactivity (Scopsi et al., 1989). High-affinity binding sites for different opioids have been identified in two human breast adenocarcinomas (Zagon et al., 1987), as well as in the MCF7 breast cancer cell line (Maneckjee et al., 1990). In this cell line, opioids (morphine, cyclazocine, [D-Ala^2, D-Leu^5]enkephalin – DADLE) inhibit cell proliferation through opioid receptors, in a dose-dependent manner. The antagonist

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SSDI 0014-2999(95)00703-2

naloxone totally reverses this effect. These results, together with the multiplicity of opioid receptors (see Fowler and Fraser, 1994, for a review), indicate a possible role of opioids in the growth of human breast cancer tumors.

In the present study, we analysed the effect of different opioids, with known selectivity towards opioid receptor sites, on the growth of T47D human breast cancer cells. We report a dose-dependent inhibition on cell proliferation by opioid receptor agonists, which, in most cases, was reversed by the antagonist diprenorphine. We furthermore identified high-affinity saturable opioid binding on these cells, mainly of the κ-type. Finally, we present evidence that, in the case of morphine, this antiproliferative effect might not be mediated through opioid receptors.

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

2.2. Cell growth conditions

Cells were plated in 96-well ELISA plates at an initial density of 10^4 cells, with 200 μl medium per well. All drugs were added to cultures 1 day after seeding (designated as day 0), in order to ensure uniform attachment of cells at the onset of the experiments. Cells were grown for a total of 6 days, with daily change of the medium containing opioid drugs. All added drugs were prepared shortly before use.

2.3. [^{3}H]Thymidine incorporation

Cells were labelled for 24 h at 37°C with [^{3}H]thymidine (0.5 μCi/ml), washed twice with 100 μl phosphate-buffered saline and extracted with 100 μl 2 N NaOH. Thymidine incorporated into DNA was detected by washing cells with 10% trichloroacetic acid, 5% trichloroacetic acid, ethanol/ether (1:1, v/v), and the DNA-associated radioactivity was measured in a liquid scintillation system. Preliminary experiments showed that, under the conditions described in the present study, 24 h exposure to radiolabelled thymidine gave the highest specific DNA labelling.

2.4. Opioid binding conditions

Ligand binding assays in whole T47D cells were performed as described in Hatzoglou et al. (1994, 1995). For saturation and displacement binding experiments in whole cells, about 10^6 cells/well were used. Before binding, cells were washed twice with 2 ml of phosphate-buffered saline. Binding was performed in the same buffer, in a total volume of 0.4 ml, containing radioactive opioid, without (total binding) or with (non-specific binding) a 1000-fold molar excess of the same unlabelled opioid and effectors (see below). At least eight to ten different concentrations of radiolabelled opioid (varying from 1 to 50 nM) were used in duplicate during saturation binding. For displacement experiments, the effectors were used in concentrations 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, MI). The bound radioactivity was counted in a scintillation counter (Tricarb, Series 4000, Packard), with a 60% efficiency for Tritium. Binding was repeated at least 3 times (in duplicate). Non-specific binding under the described conditions ranged from 20 to 36%. For membrane binding of opioid ligands, we used our previously described techniques (Castanas et al., 1984, 1985a, b). The results were analysed by the Origin (MicroCal Co.) V 3.5 package, using equations described by Munson and Rodbard (1980).

2.5. Specific conditions for differential detection of various types of opioid binding sites

The conditions used for the differential detection of various opioid sites have been described in previous studies from our group (Castanas et al., 1984, 1985a, b). Briefly, δ-opioid sites were detected by the use of tritiated [D-Ala^2,D-Leu^5]enkephalin (DADLE), in the presence of the μ-selective effector morphiceptin. μ-Opioid sites were detected by the selective ligand [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAGO). The interaction of tritiated ethylketocyclazocine with κ_1-opioid binding site was assayed by performing the binding in the presence of 5 μM DADLE, which masks δ-, μ- and κ_2-opioid sites to which ethylketocyclazocine could bind with high affinity (Attali et al., 1982; Castanas et al., 1985b). Finally, diprenorphine binding (reacting mainly with δ-, μ-, κ_2- and κ_3-opioid sites) was measured under the same conditions as for ethylketocyclazocine. The interaction of the ligand with the κ_3-opioid site was estimated, in the presence of 5

μM DADLE, as described previously by our group (Castanas et al., 1985b). The specificity of opioid ligand interaction with each opioid site was confirmed by the use of specific effectors (morphiceptin for the μ-opioid site, [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET) for the δ-opioid site, and U 69593 for the κ_1-opioid site. The exact conditions of opioid ligand binding are summarised in Table 3.

2.6. Radiochemicals and chemicals

[^{3}H]Ethylketocyclazocine (S.A. 18 Ci/mmol) and [^{3}H]DADLE (S.A. 37 Ci/mmol) were bought from New England Nuclear Co. [^{3}H]diprenorphine (S.A. 29 Ci/mmol), [^{3}H]DAGO (S.A. 60 Ci/mmol) and [^{3}H]thymidine (S.A. 55 Ci/mmol) were from Amersham (UK). Unlabelled DAGO, DSLET and DADLE were from Sigma Chemical Co. (St Louis, MO). Ethylketocyclazocine was a gift from Sterling-Winthrop. Diprenorphine and etorphine were from Reckit and Coleman Co. Morphine and naloxone were from Francopia. All other chemicals were either from Merck (Darmstad, Germany) or from Sigma (St Louis MO).

3. Results

3.1. Effect of opioids on cell proliferation

As indicated in the introduction, opioid receptor agonists decrease cell proliferation in various systems, including breast cancer cells. In order to examine the effect of opioids on the proliferation of T47D cells, we chose the following agonists, which have different affinities for each class of opioid receptors (Castanas et al., 1985a, b): [D-Ala^2,D-Leu^5]enkephalin (DADLE) (δ- and μ-opioid receptor agonist), [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET) (δ-opioid receptor agonist), ethylketocyclazocine (has a strong interaction with δ-, μ-, κ_1- and κ_2-opioid sites), etorphine (has a high affinity for δ-, μ-, κ_2- and κ_3-opioid sites), and morphine as the prototype μ-opioid ligand. As shown in Figs. 1 and 2, and in Table 1, all opioid receptor agonists used produced a dose-dependent inhibition of cell proliferation, with IC_{50} values in the nanomolar range. The most potent inhibitor was morphine, followed by etorphine, DSLET, DADLE and ethylketocyclazocine (Table 1).

To establish an opioid receptor-mediated mechanism of action of opiates on T47D cells, three criteria should be fulfilled: (a) it has to be dose-dependent (this was shown above); (b) opioid receptor antagonists must reverse the effect of agonists; and (c) opioid binding sites present on the cells must have an affinity compatible with the effect of opioids.

In the present study, we used the general antagonist diprenorphine, which at concentrations from 10^{-11} to

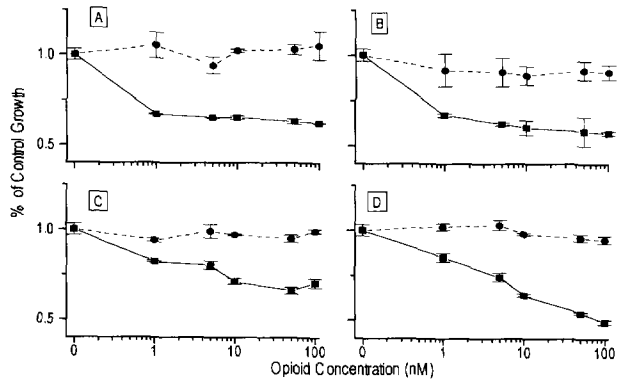

Fig. 1. Effect of opioid agonists on cell proliferation of T47D cells. Cells were incubated in the absence or the presence of the indicated concentrations of opioids (A = [D-Ala^2,D-Leu^5]enkephalin (DADLE), B = [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET), C = ethylketocyclazocine, D = etorphine) for 5 days, in the absence (solid lines) or in the presence (dashed lines) of 10^{-6} M diprenorphine. Cell growth was estimated by [^{3}H]thymidine incorporation as described in Materials and methods. Data presented are the means ± S.E. of three experiments in triplicate.

10^{-5} M had no effect on cell growth (not shown). As indicated in Fig. 1, the inhibitory effects of DSLET, DADLE, ethylketocyclazocine and etorphine on cell proliferation were completely reversed by the addition of 10^{-6} M diprenorphine. In contrast, in the case of morphine, diprenorphine had no effect. Indeed, the curves of morphine in the absence or in the presence of diprenorphine were superimposable (Fig. 2). These results indicate that, the action of DADLE, DSLET, ethylketocyclazocine and etorphine in T47D cells is mediated by opioid receptors. Nevertheless, we had to identify the presence of opioid binding sites.

3.2. Identification of opioid binding sites in T47D human breast adenocarcinoma cells

3.2.1. Validation of opioid binding on whole cells

In a recent paper from our group, we showed that opioid binding could be detected in whole cells derived

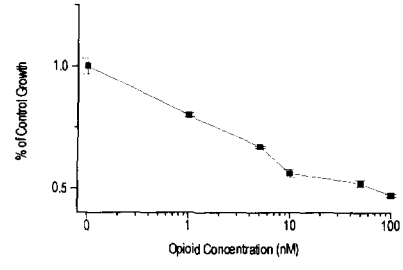

Fig. 2. Effect of morphine on cell proliferation of T47D cells. Cells were incubated in the presence of the indicated concentrations of morphine (solid line) in the absence or the presence of 10^{-6} M diprenorphine. The two curves were superimposable, as diprenorphine did not reverse the action of morphine (see Results, section 3.1). Data presented are the means ± S.E. of three experiments in triplicate.

Table 1
Effect of different opioid receptor agonists on cell proliferation

Opioid receptor agonist	% Maximum inhibition (±S.E.)	IC_{50} (nM)
DADLE	38±3.0	0.40
DSLET	43±2.7	1.06
Ethylketocyclazocine	34±2.5	0.78 (0.35, 10.2)
Etorphine	51±1.1	1.30
Morphine	53±0.7	1.02

Table presents the percent maximum inhibition of each opioid receptor agonist used, as compared to the growth of T47D cells without any added effector (control growth), and the IC_{50} of the applied opioid, derived from Fig. 1 and Fig. 2. In the case of ethylketocyclazocine, two numbers are given for the IC_{50}, depending on one- or two-site model.

from a well-differentiated human endometrial cancer (Hatzoglou et al., 1995). We tried to evaluate whether we could apply similar conditions to the T47D cell line, that is whether binding experiments could be performed in whole cells instead of membranes. Indeed, the preparation of cell membranes from cultured cells, although easy to perform, is time- and material-consuming. Table 2 presents the results of opioid binding to membrane preparations and on whole T47D cells. As indicated the affinity of opioid ligands for membranes or whole cells was comparable (3.4 versus 3.8 nM for DADLE, 1.8 and 38.7 versus 1.8 and 44.2 nM for ethylketocyclazocine, 0.5 and 10.2 versus 0.7 and 11.0 nM for diprenorphine in whole cells and membrane preparations, respectively). Furthermore, al-

Table 2
Comparison of opioid binding assays in membrane preparations and whole T47D human breast cancer cells

Ligand	Cells		Membranes	
	B_{max} (pM)	K_D (nM)	B_{max} (fmol/mg prot)	K_D (nM)
DADLE	8.9 ± 1.1	3.4 ± 0.5	3.1 ± 0.8	3.8 ± 0.2
Ethylketocyclazocine	80.3 ± 6.8	1.5 ± 0.4	22.5 ± 4.3	1.8 ± 0.3
	705 ±32.1	38.7 ± 4.9	351 ±40.5	44.2 ± 4.2
Diprenorphine	31.2 ± 3.8	0.5 ± 0.1	15.4 ± 2.8	0.7 ± 0.1
	205 ±38.4	10.2 ± 1.4	83.7 ± 9.2	11.0 ± 0.9

Table 3
Results of saturation binding, to whole T47D breast cancer cells, using different tritiated ligands and effectors

Ligand	Effector	K_D (nM)	B_{max} (pM)	Detected sites
[^{3}H]DADLE		3.5 ±0.21	8.3 ± 0.5	δ, μ
	Morphiceptin (1 μM)	3.6 ±0.23	7.9 ± 0.4	δ
	DSLET (0.1 μM)	ND	ND	μ
[^{3}H]DAGO		ND	ND	μ
[^{3}H]Ethylketocyclazocine		1.10 ± 0.07	71.5 ± 4.2	δ, μ, κ_1, κ_2
		33.0 ±1.32	726.0 ± 10.3	
	DSLET (0.1 μM)	1.20 ± 0.05	62.2 ± 3.5	μ, κ_1, κ_2
		25.3 ±2.15	730.0 ± 21.5	
	DSLET (0.1 μM) +	1.18 ± 0.02	63.5 ± 2.9	κ_1, κ_2
	Morphiceptin (1 μM)	26.2 ±1.77	727.0 ± 15.5	
	U69593 (1 μM)	1.22 ± 0.03	67.3 ± 3.8	δ, μ, κ_2
	DADLE (5 μM)	ND	ND	κ_1
		5.3 ±0.42	655.0 ± 14.7	
	DADLE (5 μM) +	ND	ND	-
	U69593 (1 μM)			
[^{3}H]Diprenorphine		0.7 ±0.02	22.0 ± 2.1	δ, μ, κ_2, κ_3
		8.3 ±0.93	202.0 ± 5.8	
	DSLET (0.1 μM)	0.5 ±0.01	14.5 ± 1.8	μ, κ_2, κ_3
		8.4 ±1.12	212 ±7.9	
	DSLET (0.1 μM) +	0.6 ±0.03	10.0 ± 2.9	
	Morphiceptin (1 μM)	9.2 ±1.01	199 ±7.2	κ_2, κ_3
	Ethylketocyclazocine (1 μM)	0.11 ± 0.02	21.2 ± 3.2	κ_3
	U69593 (1 μM)	0.8 ±0.02	23.7 ± 3.1	δ, μ, κ_2, κ_3
		7.9 ±0.78	197 ±5.4	
	DADLE (5 μM)	0.8 ±0.01	28.0 ± 1.9	κ_3

The sites detected under each condition are indicated. Table presents mean values ± S.E. of three experiments performed in duplicate. See Materials and methods (section 2.5) and Results (section 3.2.2) for details on opioid site characterisation.

though we could not calculate directly the corresponding number of opioid binding sites in membranes and whole cells, the ratio of binding capacities detected was similar. For example, the ratio of DADLE/ethylketocyclazocine sites was $8.9/80 = 0.113$ for whole cells and $3.1/22.5 = 0.138$ for membrane preparations. These data indicate that, under the conditions used in the present study, and in spite of the different nature of opioid ligands used (alkaloids, peptides), no significant degradation of the ligands occurred, during binding to whole cells. They further indicate that opioid binding site characterisation could be performed with whole cells, as the affinity of the ligand remained almost constant. Further experiments with varying temperature and incubation times showed that the best specific signal could be obtained by performing opioid binding for 2 h at room temperature (18–22°C). We used these conditions in all our studies thereafter.

3.2.2. Characterisation of opioid binding sites on T47D cells

Table 3 presents the results of the identification and characterisation of opioid sites by ligand binding experiments. Analysis of our findings showed the following.

(a) [^{3}H][D-Ala^2,D-Leu^5]enkephalin ([^{3}H]DADLE) bound to a single class of high-affinity sites (K_D 3.50 nM). The number of identified sites was small (B_{max} 6.5 pM). Taking into consideration the selectivity of DADLE, this binding could represent δ- and/or μ-opioid sites. Addition of [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET), a δ-opioid receptor agonist, during binding, abolished all specific binding, indicating a δ-selectivity. This result was confirmed by [^{3}H][D-Ala^2,*N*-Me-Phe^4-Gly^5-ol]enkephalin (DAGO) binding (a specific μ-opioid receptor ligand), in which case there was no specific binding. The addition of morphiceptin (a μ-opioid receptor agonist) during [^{3}H]DADLE binding did not modify either the affinity or the binding capacity of the detected site. We concluded therefore that opioid sites detected with [^{3}H]DADLE binding belong to the δ-type.

(b) On T47D cells, [^{3}H]ethylketocyclazocine binding showed biphasic binding curves, in Scatchard coordinates (Fig. 3 upper panel, Table 3). One component showed high affinity and low capacity (K_D 1.1 nM, B_{max} 71.5 pM), while the other shows low affinity but a higher capacity (K_D 33 nM, B_{max} 726 pM). Ethylketocyclazocine is a rather universal opioid receptor agonist that interacts, in other tissues, with δ-, μ- and two subtypes of κ-opioid sites (κ_1 and κ_2). We (Castanas et al., 1984, 1985b) and others (Attali et al., 1982) have shown that the addition of micromolar concentrations of DADLE during [^{3}H]ethylketocyclazocine binding, masks all opioid receptor sites except for κ_1-opioid sites. Therefore, when saturation binding was performed in the presence of DADLE (5 μM) the ligand

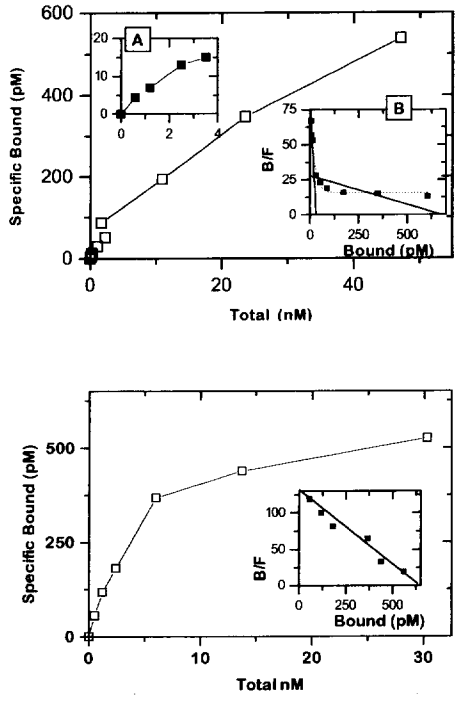

Fig. 3. Upper curve: saturation binding of [^{3}H]ethylketocyclazocine to whole T47D cells. Saturation binding was performed with whole cells, as indicated in Materials and methods. Figure presents the specifically bound [^{3}H]ethylketocyclazocine as a function of the total amount of radioactive opioid. Insert A presents a magnification of the initial part of the curve. Insert B shows the analysis of data in Scatchard coordinates. As indicated in the text and presented in Table 3, two binding sites were found. Lower curve: saturation binding of [^{3}H]ethylketocyclazocine to whole T47D cells in the presence of 5 μM DADLE. Saturation binding was performed as indicated in Materials and methods, in the presence of 5 μM [D-Ala^2,D-Leu^5]enkephalin (DADLE). Under these conditions, all opioid sites identified by ethylketocyclazocine are masked by DADLE, except for κ_1-opioid sites. The figure presents the binding isotherm of specific binding as a function of total radioactive opioid added. Insert presents the analysis of data in Scatchard coordinates. In this case, as indicated in the text and in Table 3, only one site was found, with κ_1-selectivity.

interacted mainly with κ_1-opioid sites. As shown in Fig. 3 (lower panel) and in Table 3, under these conditions, the Scatchard plot was linear and one class of κ_1-opioid sites was detected (K_D 5.3 nM, B_{max} 655 pM). Addition of 1 μM U69593 (a κ_1-opioid receptor agonist) during [^{3}H]ethylketocyclazocine binding produced a loss of the lower affinity binding, confirming its κ_1-selectivity. Addition of DSLET (a δ-opioid receptor agonist) provoked a minor inhibition of the high-affinity component. Finally, the simultaneous addition of U69593 and DADLE abolished all [^{3}H]ethylketocyclazocine specific binding. These results confirmed previous investigations (Attali et al., 1982; Castanas et al., 1985a, b) about the selectivity of ethylketocyclazocine binding.

(c) [^{3}H]Diprenorphine (as well as [^{3}H]etorphine, which is no longer available), is also an almost univer-

sal ligand for opioid sites, having high selectivity towards δ-, μ-, κ_2- and κ_3-opioid sites (Attali et al., 1982; Castanas et al., 1984, 1985b). As shown in Fig. 4A and Table 3, Scatchard plots of [^{3}H]diprenorphine binding on T47D cells revealed two components: a high affinity (K_D 0.7 nM, B_{max} 22 pM), and a lower affinity (K_D 8.3 nM, B_{max} 202 pM) one. Addition of 5 μM DADLE (masking δ-, μ- and κ_2-opioid sites) permits the interaction of [^{3}H]diprenorphine only with κ_3-opioid sites. As shown in Fig. 4B, the Scatchard plot under these conditions was linear, suggesting one class of high-affinity binding sites of the κ3-type (K_D 0.8 nM, B_{max} 28 pM). [^{3}H]Diprenorphine binding in the presence of DSLET (δ-opioid receptor agonist), U69593 (κ_1-opioid receptor agonist), ethylketocyclazocine (δ-, μ-, κ_1- and κ_2-opioid receptor agonist) and DADLE (δ-, μ- and κ_2-opioid receptor blocker) (Table 3), confirmed our previous results in neural tissues (Castanas et al., 1985a, b) concerning the selectivity of the ligand.

From the above results, we confirmed the number and the pharmacological selectivity of the detected opioid sites, and determined the number and the affinity of κ_2-opioid sites, using computer simulation. Their B_{max} was estimated to be 75 pM, or 18660 sites per cell. These sites had a higher affinity for the agonist ethylketocyclazocine (K_D: ~ 1 nM) than for the antagonist diprenorphine (K_D: ~ 8 nM). The number and the selectivity of opioid receptors found on T47D cells are presented in Table 4.

The above results confirm that DSLET, DADLE, ethylketocyclazocine, and etorphine (given their selectivity for opioid binding sites) act through opioid receptors. Comparison of the 50% inhibitory concentration (IC_{50}) of the above opioids, presented in Table 1, with the affinities of the opioid sites, presented in Table 3, showed that both actions were exerted at nanomolar concentrations. This result, along with those presented in the previous paragraphs, argues for an opioid receptor-mediated action of the opioids used.

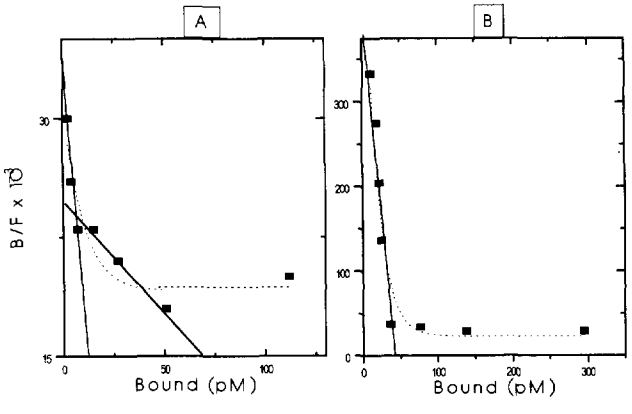

Fig. 4. Scatchard plots of [^{3}H]diprenorphine saturation binding to whole T47D cells. Saturation binding was performed as described in Materials and methods using [^{3}H]diprenorphine in the absence (total binding) or the presence (NSB) of one thousand fold excess of unlabelled diprenorphine. A = Scatchard plot of [^{3}H]diprenorphine binding. B = Scatchard analysis of [^{3}H]diprenorphine binding in the presence of 5 μM DADLE. Under these conditions, all opioid sites identified by [^{3}H]diprenorphine are masked by DADLE, except for κ_3 sites.

Table 4
Number of opioid sites on T47D human breast cancer adenocarcinoma cells

Receptor type	Subtype	Sites/cell
δ		2000 ± 100
μ		0
κ	κ_1	163000 ± 3500
	κ_2	19000 ± 600
	κ_3	10000 ± 500

The number of opioid receptors was calculated from the results of saturation binding presented in Table 3. The conversion factor for the calculation of the number of opioid sites per cell from B_{max} is 248.8. This factor is derived from Avogadro's number (6.22×10^{23} particles/mol), multiplied by 0.4 (conversion of pM to fmol/assay) and 10^{-21}, that is the fmol (10^{-15}) multiplied by the number of cells (10^6) per assay.

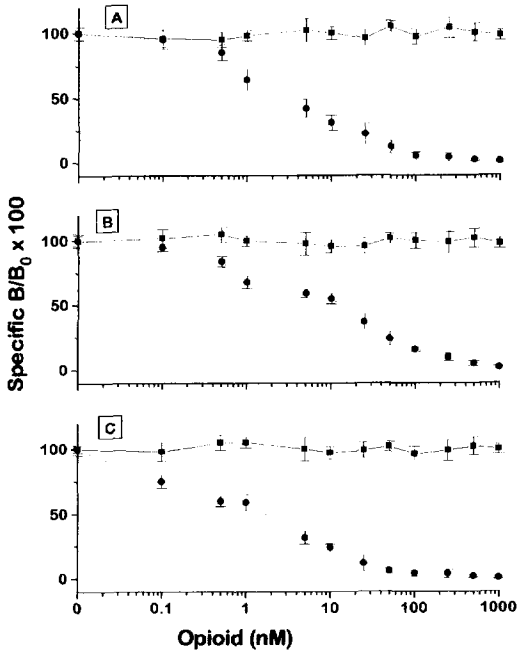

Fig. 5. Curves for the displacement by morphine of binding of the different radiolabelled opioids to T47D cells. One million of T47D human breast cancer cells were labelled with 100000 cpm of radiolabelled opioids (A = DADLE, B = ethylketocyclazocine, C = diprenorphine), in the absence, or the presence of the indicated concentrations of morphine (solid line, squares). For comparison, the homologous displacement of each tracer with the same non-labelled opioid is also presented (dashed line, circles). Non-specific binding was estimated by the addition in the incubation mixture of 10^{-6} M of the homologous opioid. Figure presents the specific B/B_0 fraction of binding. Data are the means ± S.E. of two experiments performed in triplicate.

3.3. Cross-reaction of morphine with opioid binding sites

Morphine had a strong, dose-dependent, antiproliferative effect on T47D cells (Fig. 2). This effect was not reversed by the opioid receptor antagonist diprenorphine. Furthermore, no μ-opioid sites were detected (Tables 3 and 4). Two different mechanisms could explain these results: (a) an interaction of morphine with other opioid sites, or (b) an interaction with membrane sites different from opioid receptors.

Fig. 5 shows the result of displacement studies with tritiated DADLE, ethylketocyclazocine and diprenorphine displacement by morphine, ranging from 10^{-10} to 10^{-6} M. No cross-reaction of morphine with opioid sites on T47D cells was detected. This result shows that morphine did not interact with other opioid sites on T47D cells and gives an explanation for the lack of diprenorphine inhibition of morphine action. These results raise the question whether morphine acts through a receptor-mediated mechanism, different from opioid receptors.

4. Discussion

Growing evidence indicates a possible role of opioid system-mediated agents in the control of breast cancer cell proliferation. Indeed, opioid receptors have been found and characterised in two cases of breast cancer (Zagon et al., 1987). Furthermore, 56% of a series of primary and metastatic breast tumors were found to contain opioid immunoreactivity (Scopsi et al., 1989). The opioid system is one of the inhibitory systems of an organism and therefore, from a physiological point of view, a good candidate for a possible inhibitory action on tumor growth and development. The establishment of long-term breast cancer cell lines has facilitated research into regulatory mechanisms of the cancer cell. In a recent paper, using the well-characterised cell line MCF7, Maneckjee et al. (1990) have shown that δ-, μ- and κ- (mainly κ_1-) opioid receptors could be identified on membrane preparations. In the same work, opiates and opioid peptide analogs decreased cell proliferation in a dose-dependent manner, and the addition of the opioid receptor antagonist naloxone, which had no effect per se, reversed this effect.

In the present study, we used the T47D cell line, also derived from human breast cancer, to assess a possible role of the opioidergic system. We report that opioid receptor agonists ([D-Ala^2,D-Leu^5]enkephalin (DADLE), [D-Ser^2,Leu^5]enkephalin-Thr^6 (DSLET), ethylketocyclazocine, etorphine), interacting through different receptor types, significantly inhibited the growth of T47D cells in culture, in a dose-dependent manner (Fig. 1). The inhibitory effect of DADLE, DSLET, ethylketocyclazocine and etorphine was completely reversed by the concomitant addition of the antagonist diprenorphine to the culture medium, in accord with previous studies (Maneckjee et al., 1990; Von Hoff and Forseth, 1982; Zagon and McLaughlin, 1981b). Diprenorphine did not show any effect on cell proliferation.

As shown in Figs. 1 and 2, DSLET, DADLE and ethylketocyclazocine inhibited T47D thymidine incorporation by about 40%. Etorphine and morphine inhibited growth by more than 50%. We cannot provide a plausible explanation for this partial inhibition. Nevertheless, in another study, Maneckjee et al. (1990), working with the MCF7 cell line, and assaying cell proliferation with another technique, found a comparable partial inhibition of cell growth by opioids. They indicated further that this effect was dependent on estrogens, and therefore in the MCF7 cell line inhibition of cell growth might be limited in the hormonally stimulated cell fraction. We have no evidence about such a dependence of the T47D cell line. Nevertheless, all our studies were performed in the presence of heat-inactivated, but not charcoal-stripped fetal calf serum. We are currently addressing this question by examining the action of opiates in the presence of different mitogens (steroids, growth factors or hormones), and by assaying the modification of respective receptors under their action.

The inhibitory action of opiates could be explained by their interaction with opioid receptors of the T47D cells. We report here the characterisation of opioid binding sites on whole T47D cells (Table 3). Previously published results from our group have shown that opioid binding sites could be equally well detected on membrane preparations and whole cells (Hatzoglou et al., 1994, 1995). In the present work we extend these findings to the T47D cell line. The results presented in Table 2 show that the affinity of opioid ligands is not changed when experiments are performed with membrane preparations or with whole cells. Furthermore, the ratio of binding sites identified by the three ligands was similar with membrane preparations and whole cells. This result indicates that no degradation of opioids occurred during binding, and that whole cells can be used for the identification of opioid sites.

Our results show that T47D cells possess different opioid binding sites (Table 3 and 4). Saturation and displacement binding studies indicated the presence of δ- and all three subtypes of the κ-receptor site (κ_1, κ_2, κ_3). Comparison of our findings with those reported by Zagon et al. (1987), in two primary human adenocarcinomas, shows similar affinities for ethylketocyclazocine and DADLE. In the same study, high-affinity μ-opioid sites were detected in only one case, supporting the observation by Zagon et al. (1985) that few tumors present μ-opioid sites which, probably, do not play a prominent role in neoplasia. Our results show that no

μ-opioid sites are present in T47D cell line, although they have been identified in another breast cancer cell line, the MCF7 (Maneckjee et al., 1990). This difference, as well as discrepancies regarding the number of binding sites and their affinities, could be attributed to the different biological characteristics of the two cell lines (T47D and MCF7), or the different conditions used in binding assays (see Hatzoglou et al., 1995, for a discussion). Nevertheless, the membrane binding of [^{3}H][D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAGO) in different buffers (phosphate-buffered saline, Tris) and salt concentrations confirmed the absence of specific binding and therefore the absence of μ-opioid binding sites (not shown).

These results confirm our hypothesis that the action of DADLE, DSLET, etorphin and ethylketocyclazocine is exerted through opioid receptors, as (a) these opioid receptor agonists interact preferentially with δ- and κ-opioid sites (Castanas et al., 1985a, b), which were the sites found on T47D cells; (b) the action of these opioid receptor agonists is exerted in the nanomolar range, comparable with the observed affinity for their binding sites; and (c) the addition of the antagonist diprenorphine reversed this opioid-related inhibition on cell proliferation.

Another singular result shown in the present study is that morphine, the prototype μ-opioid receptor agonist, exerted a dose-dependent inhibition of cell proliferation. Diprenorphine did not antagonise this effect (Fig. 2), suggesting that the action of morphine might not be mediated through opioid receptors. Indeed, no μ-opioid sites were found on T47D cells, sites to which morphine is preferentially bound (Table 4). Furthermore, morphine did not interact with other opioid sites (Fig. 5). A possible explanation for the antiproliferative action of morphine in T47D cells is that it acts through membrane protein or receptor systems other than opioid receptors. This hypothesis is consistent with the findings of Maneckjee and Minna (1990), which indicated that the inhibitory effect of morphine in lung cancer cell lines is reversed by nicotine, suggesting a possible interaction of opioid with the acetylcholine receptor system. Furthermore, recent studies (Kiefer et al., 1992; Xie et al., 1992; Yusuda et al., 1993) have shown that opioid receptors belong to the seven-loop membrane-GTP-coupled receptor superfamily, which includes other neurotransmitter receptors with homology to opioid sites. This possible interaction of morphine with other receptor systems is currently being investigated in our laboratory.

Acknowledgements

Work partially supported by University of Crete, Hellenic Anticancer Society, and Ministry of Health (KESY) grants.

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