Binding of Opioids to Human MCF-7 Breast Cancer Cells and Their Effects on Growth

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ABSTRACT

The well characterized human breast cancer cell line, MCF-7, has been shown to possess membrane receptors for various opioid ligands, and these compounds have been shown to modulate the growth of the cells in culture. Using specific radioligands for the receptor types, we were able to demonstrate that the MCF-7 cells possess multiple opioid receptor types. Relatively high-affinity-binding sites are present for the \( \mu \) - and \( \kappa \)-specific ligands, while lower affinity sites are present for the \( \delta \) - and \( \sigma \)-receptors. Opioid ligands specific for the different receptor types significantly inhibited the growth of the MCF-7 cells in a dose-dependent manner when grown in the presence of 10% fetal bovine serum. This inhibitory effect was reversed by the simultaneous administration of the opioid receptor antagonist, naloxone. However, the opioid effect appears to be restricted to the hormonally responsive fraction of the MCF-7 cell growth. Cells grown in the presence of charcoal-stripped fetal bovine serum are refractory to the effects of the opioids unless the media is supplemented with estradiol. The data presented here suggest an important regulatory role for opioids in the growth and development of human breast cancers.

INTRODUCTION

The induction and growth of human breast cancer appear to be directly related to the endocrine status of the host. The role of ovarian hormones in breast cancer development has been extensively reported (1). Recently, however, a number of other factors, such as glucocorticoids, thyroid hormone, vitamin D, insulin, prolactin, growth hormone, prostaglandins, epidermal growth factor, and endogenous brain peptides, have been shown to affect breast tissue (for extensive reviews see Medina et al. (2)), but the exact mechanisms by which they exert their effects are not completely understood (3).

Opioid peptides and their receptors have been implicated as neuromodulators in a variety of phenomena, most importantly for their analgesic and behavioral effects (4). Recent evidence points to their possible involvement in pathological states. The exact mechanisms involved in the actions of opioids in relation to oncogenic events are unknown. The exciting prospect of a link between pain-killing opioid drugs and the regression of hormone-associated cancers may have important chemotherapeutic implications in the treatment of these cancers.

Opioid compounds are known to alter cell function and growth (5, 6), and their growth-inhibiting properties in cultured neuroblastoma cells have been described (7). Long-term administration to mice with transplanted neuroblastoma tumors inhibits tumor growth and prolongs survival time. These antitumor effects were blocked by the concomitant administration of the opioid antagonist, naloxone (8-10). Met- and Leu-enkephalins have recently been shown to produce antimetastatic action in B16-BL6 melanoma as well as decrease the growth rate of the primary tumor (11). Growth of certain human glioma and neuroblastoma cells in culture and formation of a tumor colony by murine leukemia and melanoma cells in vitro were both decreased by human \( \beta \)-endorphin in a concentration-dependent manner (10). These effects were partially reversed by naloxone (10). There has also been some neuroendocrine evidence to suggest a role for opioids in the growth and development of mammary tumors. Pretreatment with naloxone has been shown to inhibit growth of dimethylbenzanthracene-induced mammary tumors in rats (12) and to cause complete regression in mice with spontaneous and s.c. transplanted mammary tumors (13).

Most of the known opioid drugs and peptides produce their wide spectrum of effects by interacting with at least one of four receptor types (\( \mu \), \( \delta \), \( \kappa \), and \( \sigma \)-receptors). In addition, recent biochemical and physiological studies have identified in mammalian brain a separate PCP\( ^{3} \)/N-methyl-D-aspartate binding site for PCP which is distinct from the \( \sigma \)-receptor (14). The human breast cancer cell line, MCF-7, has been extensively characterized and has been used to evaluate the effects of many other biological response modifiers on cell growth and proliferation (3). Characterization of the opioid receptor types in this human breast cancer cell line could prove useful in determining the role of opioids and their antagonists in the growth and development of human mammary cancers.

MATERIALS AND METHODS

Materials. [\( ^{3} \text{H} \)]Etorphine (30 Ci/mmol), [\( ^{3} \text{H} \)]EKC (45 Ci/mmol), [\( ^{3} \text{H} \)]PCP (5 Ci/mmol), and nonradiolabeled cyclazocine, PCP, and U-50,488H (Upjohn Company, Kalamazoo, MI) were provided by the National Institute on Drug Abuse (Bethesda, MD). [\( ^{3} \text{H} \)]DADLE (36 Ci/mmol) and [\( ^{3} \text{H} \)]DAGO (60 Ci/mmol) were purchased from American (Arlington Heights, IL). Unlabeled DAGO and DADLE were purchased from Peninsula Laboratories (San Carlos, CA). Morphine sulfate and naloxone were purchased from Mallinckrodt, Inc. (St. Louis, MO) and DuPont Company (Wilmington, DE), respectively. Levorphanol was a gift from Hoffman La Roche (Nutley, NJ).

Culture media, antibiotics, trypsin-EDTA solution, trypsin blue, and FBS were purchased from GIBCO (Grand Island, NY). 17\( \beta \)- Estradiol was purchased from Sigma Chemical Co. (St. Louis, MO). Dextran T-70 was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). CSS was prepared by incubation at 55°C for 30 min as described before (15).

Cell Culture. The human breast carcinoma cell line, MCF-7, which was originally isolated from a pleural effusion of a primary breast cancer patient (16), was obtained at passage 115. As described previously (15), cells were routinely subpassaged in Dulbecco's minimal culture media, antibiotics, trypsin-EDTA solution, trypsin blue, and 10% heat-inactivated fetal bovine serum; CSS, charcoal-stripped serum.
in order to ensure uniform attachment of cells at the onset of the experiments. The cells were grown for an additional 3 or 5 days for a total of 4 or 6 days in culture.

Cell growth was measured either by counting viable cells in a hemacytometer using trypan blue exclusion after brief trypsinization or by the tetrazolium salt assay for cell viability as described by Mosmann (17). Similar results were obtained using either assay. In the tetrazolium salt assay, cells were incubated for 4 h at 37°C with the tetrazolium salt, and the metabolically active cells reduced the dye to purple formazan. The absorbance was measured at 570 nm and compared against a standard curve of known numbers of MCF-7 cells (17, 18). All experiments were performed a minimum of three times.

Statistical Analysis. In some experiments, the significant differences were determined by analysis of variance.

Opioid Binding Assays. After being washed once with phosphate-buffered saline, cells were collected during the log phase of growth by gentle scraping and centrifugation. Cell homogenates were prepared by homogenization on ice with a Polytron (Brinkman Corp., Rexdale, Ontario, Canada) in 30 volumes (w/v) of 50 mM Tris-HCl, pH 7.4. The crude membrane preparation was obtained by centrifugation at 40,000 × g for 20 min, as previously described (19). The pellets were resuspended in Tris-HCl buffer, incubated at 0°C for 1 h, and recentrifuged. The pellet was resuspended again, recentrifuged, and finally resuspended in the appropriate reaction buffer at a final concentration of 50 × the original volume (w/v). Membrane protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Opioid binding to the intact membranes was measured as follows: In a final volume of 1 ml, aliquots of membrane preparations in 50 mM Tris-HCl buffer, pH 7.4, were incubated in triplicate for 60 min at 24°C with various concentrations of the [3H]-labeled opioid with or without excess of the specific nonradioactive ligand. The specific ligands used are indicated in the individual figure legends. At the end of the incubation period, bound radioactivity was measured by filtering the samples through Whatman GF/B glass fiber filters (Whatman International, Ltd., Maidstone, England) followed by three washes with 5 ml cold buffer. The filters were placed in 10 ml of Ultrafluor scintillation fluid (National Diagnostics, Manville, NJ) and counted in a Beckman scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Specific binding was calculated as the difference between total binding and nonspecific binding (in the presence of excess unlabeled opioid). Nonspecific binding was between 30 and 50% of total binding.

The binding assays were performed a minimum of two times for each ligand used. All binding data were obtained under saturation conditions. Scatchard plots of the data were evaluated by LIGAND, a computer program developed by G. A. McPherson, which is a modification of the Munson and Rodbard program (20).

RESULTS

Opioid Receptor Measurements. Using [3H]etorphine, a potent narcotic agonist that is believed to interact with similar affinities at the μ-, δ-, κ-, and σ-opioid receptor types (21), the binding to intact membranes from MCF-7 cells was studied as a function of the radioligand concentration (Fig. 1). Saturation analysis revealed high-affinity, saturable binding; half-maximal binding was achieved at approximately 0.8 nM. Binding was linearly dependent on the protein concentrations of the membrane preparations (between 20 and 120 μg of protein). All subsequent binding studies were routinely performed within this linear range. Scatchard analysis of the binding data of cells grown to 50-70% confluence revealed binding to an apparent single class of sites (Bmax = 1000 fmol/mg protein; Kd = 3 × 10^-10 M; r = 0.9). At 95-100% confluence, Bmax = 300 fmol/mg protein; Kd = 4 × 10^-9 M.

Table 1 summarizes the effects of protein-modifying reagents on opioid binding in membrane preparations of MCF-7 cells. Many of the properties of opioid binding observed in this cell line are similar to those reported for membrane-bound receptors from rat brain (22). Opioid-binding activity was markedly decreased by heat (60°C for 15 min), trypsin (10 μg/ml for 15 min at 30°C), and proteinase K (10 μg/ml for 15 min at 30°C), suggesting that the sites are proteinaceous. Pretreatment with the sulfhydryl reagent, N-ethylmaleimide (0.5 mM) for 15 min at 30°C followed by quenching with dithiothreitol (2.5 mM) reduced specific binding by ~80%. Opioid-binding activity was also inhibited by the guanyl nucleotide GTP (50 μM GTP produced 90-95% inhibition relative to control binding), suggesting coupling to an inhibitory guanyl nucleotide-binding protein.

Using specific radioligands for receptor types (DAGO for the μ-receptor, DADLE for the δ-receptor, and U-50,488H for the κ-receptor), we were able to determine the subtypes present in the MCF-7 breast cancer cell line. These cells appear to possess multiple opioid receptor types. Scatchard analysis of the equilibrium binding of [3H]DAGO (μ-specific ligand) to intact membranes revealed two linear components with Kd values of 4.8 × 10^-9 M and 2.3 × 10^-6 M for the high- and low-affinity-binding components, respectively (Fig. 2A). Scatchard analysis of [3H]EKC binding to the membranes yielded a linear plot, suggesting binding to an apparent single class of high-affinity sites (Fig.
Fig. 2. Scatchard analysis of [\(^{3}\)H]DAGO binding (A) and [\(^{3}\)H]EKC binding (B) to MCF-7 cell membranes. Binding conditions were described in the legend to Fig. 1. Each experiment was repeated twice. [\(^{3}\)H]DAGO binding was carried out in the presence and absence of 1 \(\mu\)M unlabeled DAGO (specific \(\mu\)-agonist), and in the [\(^{3}\)H]EKC-binding reactions, 1 \(\mu\)M U-50,488H (specific \(\delta\)-agonist) was used. B/F, bound/free.

2B, \(B_{\text{max}} = 320 \text{ fmol/mg protein; } K_d = 1.7 \times 10^{-10} \text{ M; } r = 0.9\). In contrast, relatively low-affinity specific binding of [\(^{3}\)H]DADLE, a putative \(\delta\)-ligand which can also bind to the \(\mu\)-receptor with a lower affinity (21), was detectable in the MCF-7 cell membrane preparation under the binding conditions used in this assay (\(B_{\text{max}} = 7000 \text{ fmol/mg protein; } K_d = 2 \times 10^{-8} \text{ M; } r = 0.36\)). [\(^{3}\)H]PCP binding does not appear to occur in this breast cancer cell line.

Apart from the direct binding studies using specific ligands, [\(^{3}\)H]etorphine binding was effectively competed by nonradioactive receptor type-specific ligands which demonstrate multiplicity of binding sites. Both normorphine (potent \(\mu\)-agonist) and U-50,488H (specific \(\kappa\)-agonist) (100 nM) inhibited [\(^{3}\)H]etorphine binding in membrane preparations of MCF-7 cells to give 113 and 90 fmol/mg protein specific binding, respectively. The opioid peptide, DADLE, exhibited very weak affinity for this binding site under the binding conditions used in this experiment. This finding is consistent with the observation of low-affinity-specific binding of [\(^{3}\)H]DADLE to MCF-7 membrane preparations.

Growth of MCF-7 Cells. When MCF-7 cells growing in the presence of FBS were treated with various opioids, growth over a 5-day period was severely retarded. As shown in Fig. 3, this effect was seen with drugs that use three classes of receptors (i.e., \(\mu\), morphine; \(\kappa\), cyclazocine; and \(\delta\), DADLE) and is dose dependent. All drugs were effective at concentrations as low as 10 nM, and maximum effects were reached at 50 nM.

These inhibitory effects were reversed by the simultaneous presence of naloxone (Fig. 4). Naloxone at concentrations as low as 5 nM was able to reverse the effect of morphine at 50 nm. When naloxone was combined with DADLE, cell growth was stimulated, resulting in a 20–30% increase in cell number compared to control cells grown in FBS in the absence of all drugs. Naloxone itself had no effect on cell growth under the conditions of these experiments. At concentrations above 50 nm, naloxone occasionally stimulated the cells to grow above the control level (data not shown).

The effect of the opioids appears to be limited primarily to the hormonally stimulated fraction of cell growth in this particular line of MCF-7 cells. As shown in Fig. 5, when hormones were removed from serum by stripping with dextran-coated charcoal, morphine no longer inhibited the baseline level of cell growth. However, when growth was enhanced with 17\(\beta\)-estradiol, the inhibitory effects of morphine were restored.

DISCUSSION

Treatment of patients with metastatic breast cancer is most frequently based on the estimation of estrogen receptors in the tumor; the role of estrogens in the modulation of growth of breast cancer is well established (1). However, recent research indicates the possible physiological role of additional factors in breast cancer development (2). The exact mechanisms underlying the actions of these factors in relation to oncogenic events are unknown. In recent years, human breast cancer cells maintained in long-term tissue culture have been used as models to study the mechanisms of action of growth factors in human breast cancer. In the present study, we have used the well-characterized human breast cancer cell line, MCF-7, to deter-

Fig. 3. Growth inhibition of MCF-7 cells by opioid peptides. MCF-7 cells were grown for a total of 6 days in the presence of 10% FBS. All opioid peptides were added 24 h after plating the cells in order to eliminate problems with drug effects on plating efficiency. Cell growth was assessed, after brief trypsinization, by counting viable cells by trypan exclusion using a hemacytometer. Data presented are the means ± SE (bars) of triplicate determinations. *Significantly different from control with no drugs (\(P < 0.05\)).

Fig. 4. Reversal of opioid inhibition by the antagonist naloxone. All drugs were added 24 h after plating cells in medium containing 10% FBS. Cell growth was assessed after a total of 5 days in culture by the tetrazolium salt assay described in "Materials and Methods." Data presented are the means ± SE (bars) of triplicate determinations. *Significantly different from control with no opioid (\(P < 0.05\)).

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mine its ability to bind various opioid ligands and subsequently to assess the ability of these compounds to modulate growth of these cells in culture. The physiological relevance of this approach has been strengthened by the recent report that 56% of invasive breast ductal carcinomas contain immunoreactive opioid peptides (23).

Since clinical regulation of the endorphinergic system has become increasingly useful to manage pain in patients with advanced cancer, it is important to determine whether these same drugs may help promote remission of hormone-dependent cancers and thus prolong life. The mechanisms by which these drugs inhibit growth of these tumors need to be investigated. These mechanisms may be direct, through the tumor cell of the receptor system, or indirect, such as through the release of hormones that can modulate the growth of the tumors (24–26).

The various neuropharmacological effects of opioid compounds, as well as those of the endogenous opioid peptides, are known to occur by interactions with a combination of four receptor types. We report here that MCF-7 cells possess membrane receptors for opioids. Using specific ligands, we were able to characterize these receptor sites and determine the effect of the ligand-receptor interaction on the in vitro growth of this cell line. The data show multiplicity of opioid receptor types in this cell line. Direct binding studies with the specific radiolabeled ligands, as well as competition studies with different ligands, indicate the presence of a relatively high number of binding sites for the μ- and κ-specific ligands. Each exhibited binding to a class of high-affinity sites. The δ-agonist, DADLE, showed lower affinity-specific binding to MCF-7 cell membrane preparations.

The results reported here also demonstrate that opioid ligands specific for the different receptor types significantly inhibit the growth of the MCF-7 cells in culture in the presence of 10% FBS in a dose-dependent manner. It is interesting that the putative δ-agonist, DADLE, which showed a relatively lower affinity binding to the membrane preparations, was able to retard the growth of the cells in culture to the same extent as the μ- and κ-agonists. This finding suggests that higher affinity δ-receptors may be present in a “masked” state under the binding conditions used in these assays or that the growth-inhibiting effect may be the result of cross-reactivity with the μ-receptors. The μ- and δ-receptor cross-reactivity has been reported in a number of systems (21).

The growth inhibitory effect of the opioid ligands is reversed by the simultaneous administration of the opioid receptor antagonist, naloxone. Such reversal has been demonstrated in several other systems both in vivo and in vitro (8–10) and may reflect both indirect and direct mechanisms of action of opioid peptides and their antagonists. The regression of growth of spontaneous and transplanted mammary tumors in C3H mice by intratumoral injections of naloxone suggests that the effect of this drug may be due to direct action against the tumor itself (12) involving the opioid receptor system. In addition, the opioid antagonist naloxone has been shown to have positive and negative effects on the growth of neuroblastomas in mice (9). These apparently conflicting effects result from dose-dependent action of the drug. Lower doses of naloxone reduced tumor incidence and delayed the appearance of the tumors, while higher doses reduced the time before tumor appearance and decreased survival time compared to the control, tumorbearing mice.

In our hands, naloxone reversed the growth inhibitory effects of morphine and DADLE on the cell and even caused a slight increase in cell number over controls in the presence of DA-DLE. At the concentrations used in our experiments (i.e., ≤50 nm), naloxone alone had no effect on cell growth. At higher concentrations (i.e., >50 nm) naloxone alone occasionally increased the cell number (data not shown). The lack of effect of naloxone alone in vitro may indicate an indirect mode of action of the opioid antagonists in controlling tumor growth in vivo. Naloxone is a potent inhibitor of prolactin secretion by the pituitary (24–26), and this hormone has been implicated in growth and maintenance of human breast cancer (15, 27).

In fact, the inhibitory effects of the opioids appear to be restricted to the hormonally responsive fraction of MCF-7 cell growth. When the drugs were administered to cells grown in the absence of the hormones present in the FBS, no inhibition was seen. Since the majority of human breast cancers are estrogen receptor positive and hence believed to be hormonally responsive, this observation may be of importance clinically. It will be necessary to determine the effects of these drugs on a number of estrogen receptor-positive as well as estrogen receptor-negative cell lines before more generalized conclusions can be drawn. It appears that addition of 10⁻⁴ M 17β-estradiol to the CSS increases the sensitivity of the cells to morphine-induced growth inhibition. We are currently examining the dose-response relationship of estrogens and morphine-induced growth inhibition. Whether the increased sensitivity to morphine in the presence of estrogens is an exclusive consequence of 17β-estradiol mitogenesis through the estrogen receptor or is a function of general growth stimulation by 17β-estradiol or any other mitogen for these cells, such as prolactin (15, 27), insulin-like growth factor I (28), or epidermal growth factor (29), remains to be determined. Whether these effects observed in culture translate to an “in vivo” system of solid tumors grown in nude mice needs to be determined.

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