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Modulation of the Estrogen-Regulated Proteins Cathepsin D and pS2 by Opioid Agonists in Hormone-Sensitive Breast Cancer Cell Lines (MCF7 and T47D): Evidence for an Interaction Between the Two Systems

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Abstract In many cancer cell lines, including breast, prostate, lung, brain, head and neck, retina, and the gastrointestinal tract, opioids decrease cell proliferation in a dose-dependent and reversible manner. Opioid and/or other neuropeptide receptors mediate this decrease. We report that only the steroid-hormone-sensitive cell lines MCF7 and T47D respond to opioid growth inhibition in a dose-dependent manner. Therefore, an interaction of the opioid and steroid receptor system might exist, as is the case with insulin. To investigate this interaction, we have assayed two estrogen-inducible proteins (pS2 and the lysosomal enzyme cathepsin D) in MCF7 and T47D cells. When cells were grown in the presence of FBS (in which case a minimal quantity of estrogens and/or opioids is provided by the serum), we observed either no effect of etorphine or ethylketocyclazocine (EKC) or an increase of secretion and/or production of pS2 and cathepsin D. However, when cells were cultured in charcoal-stripped serum and in the absence of phenol red, the effect of the two opioids is different: EKC decreased the production and/or secretion of pS2 and cathepsin D, whereas etorphine increased their synthesis and/or secretion. The differential effect of the two general opioids was attributed to their different receptor selectivity. Furthermore, the variations of the ratio of secreted/produced protein and the use of cycloheximide indicate that opioids selectively modify the regulatory pathway of each protein discretely. In conclusion, through the interaction with opioid and perhaps other membrane-receptor sites, opioid agonists modify in a dose-dependent manner the production and the secretion of two estrogen-regulated proteins. Opioids may therefore disturb hormonal signals mediated by the estrogen receptors. Hence, these chemicals may have potential endocrine disrupting activities. J. Cell. Biochem. 71:416–428, 1998.

Key words: opioids; cathepsin D; pS2; estrogen; cancer

Opioid agonists mediate a great number of different functions in the organism, including antinociception and the modulation of neuropeptide and hormone secretion. Recently, in a variety of cancer cell lines, including those of the breast [Hatzoglou et al., 1996a,b; Maneckjee et al., 1990], prostate [Kampa et al., 1997], lung [Maneckjee and Minna, 1990] , brain [Zagon et al., 1990; Zagon and McLaughlin, 1987, 1989], head and neck [Levin et al., 1997], retina [Isayama et al., 1991], and the gastrointestinal tract [Zagon et al., 1996a,b, 1997], opioids were found to decrease cell proliferation in a dose-dependent and reversible manner. This action is mediated by opioid [Hatzoglou et al., 1996b; Hytrek et al., 1996; Levin et al., 1997; Maneckjee et al., 1990; Maneckjee and Minna, 1990; Zagon et al., 1987a, 1989, 1990; Zagon and McLaughlin, 1989] and/or other neuropeptide receptors [Hatzoglou et al., 1995; Kampa et al., 1997; Maneckjee and Minna, 1990]. It is proposed that the opioid system might play a func-

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tional role in the control of cancer cells and potentially in the development of tumor growth [Gkonos et al., 1995; Noordzij et al., 1995].

In the breast, our group has shown that opioid alkaloids [Hatzoglou et al., 1996b] as well as opioid peptides derived from milk proteins [Hatzoglou et al., 1996a; Kampa et al., 1997] inhibit in a dose-dependent and reversible manner the growth of cancer cells through an interaction with opioid [Hatzoglou et al., 1996a,b] and somatostatin receptors [Hatzoglou et al., 1995, 1996a]. This effect was not found, on the other hand, in the BT20 cell line, which is hormone-independent [Panagiotou et al., 1997]. It was therefore concluded that a possible interaction of the opioid- and steroid-receptor system might exist, as was reported previously for the action of insulin [el-Tanani and Green, 1996a] or opioids [Maneckjee et al., 1990].

In order to investigate this interaction, we have assayed two estrogen-inducible proteins (pS2 and the lysosomal enzyme cathepsin D) in the two cell lines. We report here that opioid agonists modulate in a dose-dependent manner the intracellular and secreted forms of these two proteins, indicating a functional relationship of the two systems (steroids and opioids).

MATERIALS AND METHODS

Cell Cultures

The human breast cancer cell lines MCF7 and T47D were obtained between passages 104 and 120 and 18 and 33, respectively. Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (1/1) medium in six-well plates supplemented with 10% heat-inactivated fetal bovine serum (FBS) at a density of 12,500 and 10,000 cells/cm^2 for T47D and MCF7 cells, respectively. Alternatively, at least during 1 week prior to the experiments, they were supplemented with heat-inactivated charcoal-stripped FBS with 2 nM L-glutamine and 6 ng/ml insulin (DCC), and they were cultured in phenol red–free DMEM/F12 medium at a density of 25,000 cells/cm^2 (T47D) or 20,000 cells/cm^2 (MCF7), at 37°C in a humidified atmosphere of 5% CO_2 in air. Opioids were introduced daily, at the indicated concentrations, dissolved in culture medium, from a stock solution (10^{-3} M in acidified ethanol). All working solutions were prepared shortly before use. Medium containing drugs was replaced daily. In some experiments (see Results), cells were cultured in the presence of cycloheximide (10 μg/ml), which was introduced 3 days after the initiation of opioid treatment. All assays in the culture medium or in the cytosol were made at day 4 of opioid treatment, which corresponds to 1 day after the initiation of cycloheximide treatment.

Preparation of Cytosols

Cells were incubated for 3 days, as described above, in the absence or the presence of opioid agonists. Thereafter, medium was collected, and cells were washed twice with cold phosphate buffered saline (PBS), supplemented with 0.5 ml of receptor assay buffer (10 mM Tris-HCl, pH 7.4, containing 1.5 mM EDTA, 0.5 mM dithioglycerol, 10 mM sodium molybdate, and 10% glycerol), and centrifuged at 100,000*g* for 60 min. Each supernatant was then immediately assayed for pS2 and cathepsin D. Proteins were assayed with the method of Lowry et al. [1951].

Assays

Cathepsin D and pS2 were assayed in the cytosol and the culture medium with specific radioimmunoassay kits (ELSA-CATH-D and ELSA-pS2; CIS Biointernational, Gif-sur-Yvette, France). Intraassay variability ranged between 2.3 and 3.9%, while interassay variability was between 6.2 and 7.8%.

Analysis of the Results

Statistical analysis was performed with the Statgraphics Plus V 2.0 (Manugistics, Rockville, MD) and Origin V 4.1 (MicroCal Co., Northampton, MA) microcomputer programs.

Materials

17β-estradiol was from Sigma Co. (St. Louis, MO). Ethylketocyclazocine was a gift from Sterling-Winthrop. Diprenorphine and etorphine were from Reckit and Coleman Co. All other chemicals were either from Merck (Darmstad, Germany) or Sigma. Cell culture media were from Gibco (Grand Island, NY).

RESULTS

Effect of Opioid Agonists on the Proliferation of Estrogen-Treated Cancer Cells

When MCF7 (Fig. 1, left panels) and T47D cells (Fig. 1, right panels) were grown in the presence of varying concentrations of 17β-estradiol, a dose-dependent increase of cell proliferation was observed. When ethylketocyclazocine was added in the cell medium, we found a

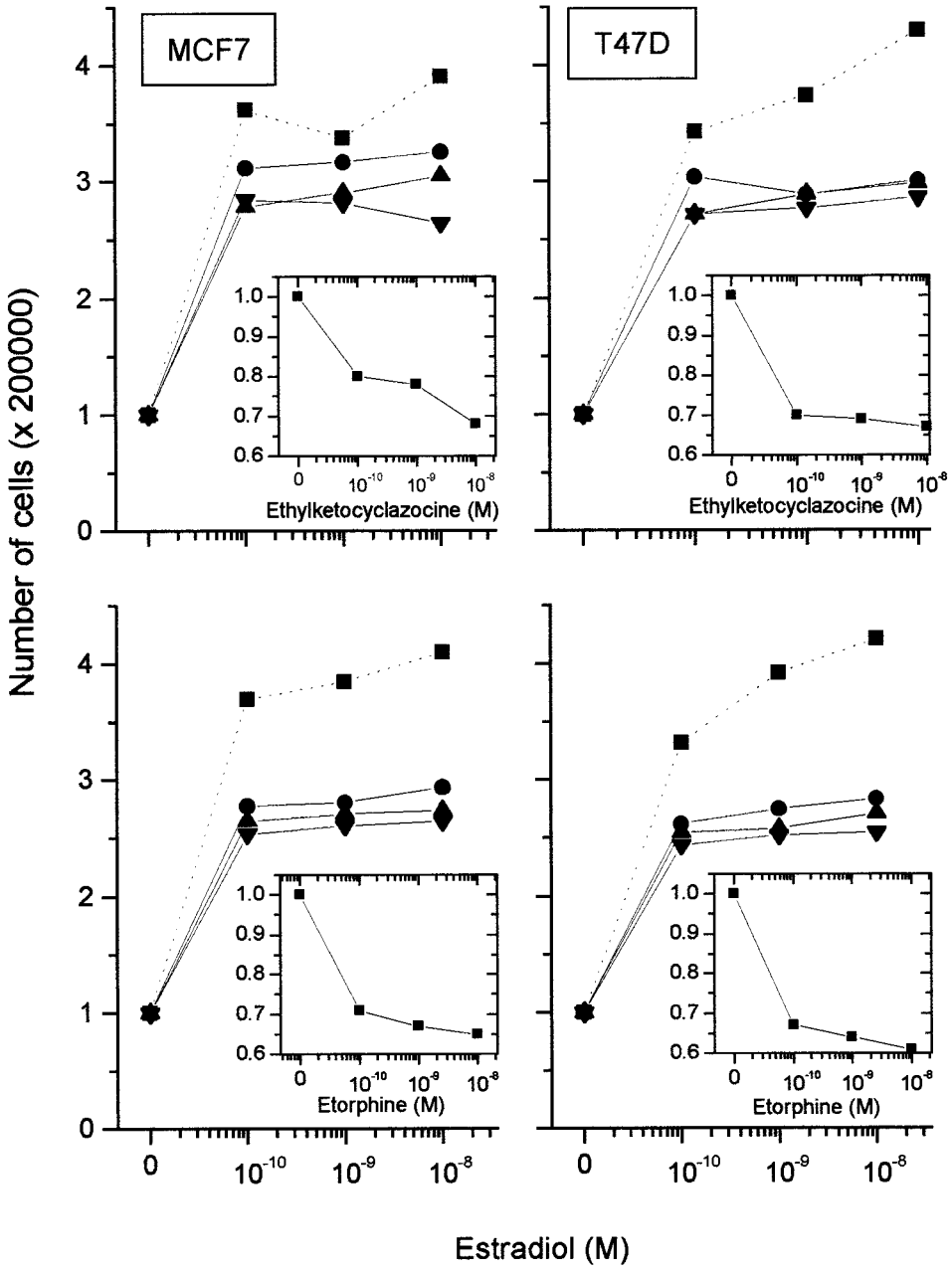

Fig. 1. Effect of opioid agonists on cell proliferation of estrogen-stimulated breast cancer cell lines. Cells (MCF7, left panels; T47D, right panels) were incubated for a total of 4 days in the presence of the indicated concentrations of 17β-estradiol alone (dotted line, squares) or in the presence (solid lines) of the opioid agonists ethylketocyclazocine (top panels) or etorphine (bottom panels). Different concentrations were used: circles, 10^{-10} M; up triangles, 10^{-9} M; down triangles, 10^{-8} M. Insets: The relative inhibition of cell proliferation in the presence of 10^{-8} M estradiol as a function of the concentration of the opioid agonist applied. Mean of three different experiments in triplicate.

dose-dependent inhibition of cell proliferation. This effect was more pronounced in the T47D than in the MCF7 cell line (Fig. 1, insets). Nevertheless, the effect of ethylketocyclazocine was completed for both cell lines at 10^{-9} M and reversed by the action of the general opioid antagonist diprenorphine (not shown). Similar results were found when ethylketocyclazocine was replaced by etorphine (Fig. 1, lower panels). These results indicate that opioids can modulate the growth of hormone-sensitive cell lines in the presence of estradiol. On the other hand, in the BT20 hormone-insensitive breast cancer cell line, no effect of opioid agonists on

cell proliferation was observed, indicating that the action of opioid agonists might be coupled to the effect of steroids [Panagiotou et al., 1997].

In order to further investigate the opioid-steroid interaction, we assayed the production and the secretion of two estradiol-regulated proteins in these two hormone-sensitive cell lines, namely pS2 and cathepsin D. We have used two experimental conditions:

1. Complete, phenol red–supplemented culture medium with 10% FBS. In this case, a minimal quantity of steroids is provided by the serum and the phenol red [Berthois et al., 1986].
2. Phenol red–free culture medium supplemented with 10% dextran-stripped FBS, insulin, and glutamine (DCC condition). In this case, no estrogen is present in the medium.

Throughout the present study, the assays of the two estrogen-regulated proteins, cathepsin D and pS2, were performed after a 3 day incubation with daily addition of opioids. The results are expressed as moles/milligram protein. We have preferred this expression, as previous works [Hatzoglou et al., 1996a,b; Maneckjee et al., 1990] have indicated an opioid-related decrease of cell number in culture. Considering therefore that the total amount of protein/cell might remain unchanged, we have expressed our results on this basis. Furthermore, a significant linear relationship between the cell number and the total measurable concentration of proteins was found in the two cell lines under the action of opioids.

Our results are as follows.

Effect of Opioids on the Production and Secretion of the Estrogen-Regulated Proteins pS2 and Cathepsin D

pS2

T47D cells (Fig. 2). Figure 2 shows the effect of opioid agonists on intracellular and secreted

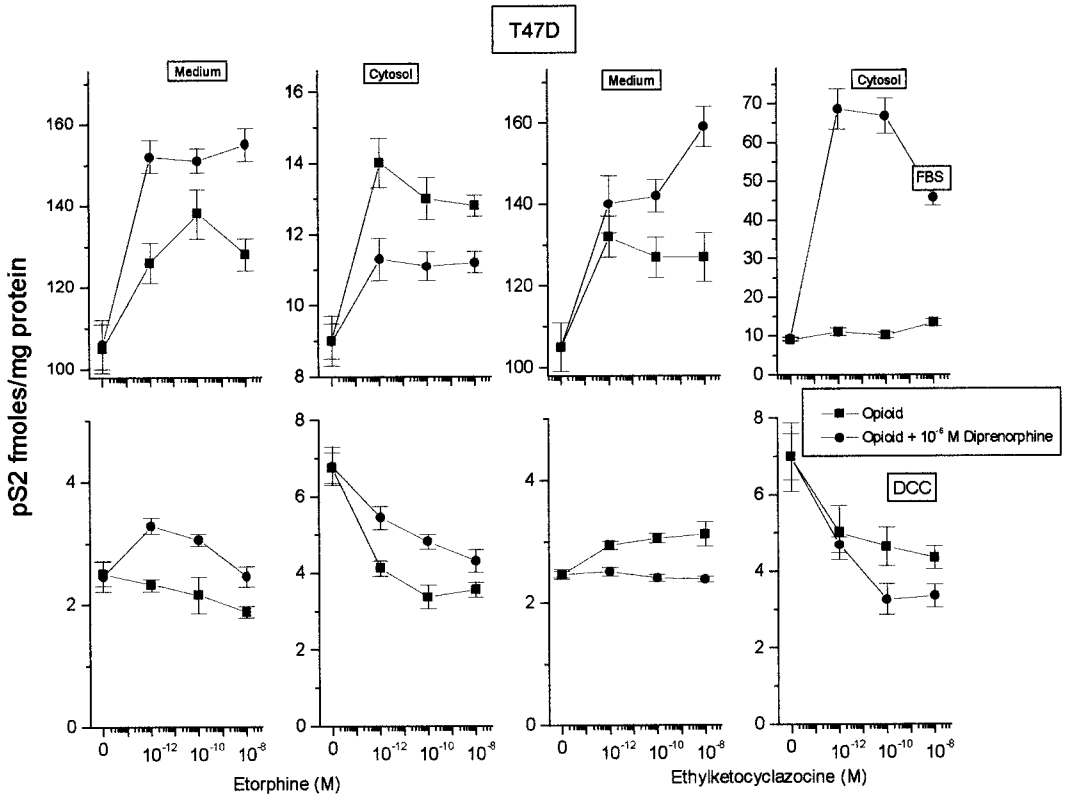

Fig. 2. Effect of opioid agonists ethylketocyclazocine (EKC) and etorphine on the concentrations of pS2 in the culture medium and the cytosol of T47D cells, cultured in 10% fetal bovine serum (FBS) or charcoal-stripped serum (DCC). Cells were incubated for 3 days in the absence (0) or the presence of the indicated concentrations of the opioid agonist etorphine or EKC alone (squares) or with the concomitant addition of the general opioid antagonist diprenorphine, 10^{-6} M (circles). Thereafter, media were collected, and cells were washed with cold PBS and supplemented with receptor buffer, as described in Materials and Methods. The cytosol was prepared by centrifugation at 100,000*g*. Mean ± SE of three experiments in duplicate.

pS2 in the human cancer cell line T47D. When cells are cultured for 3 days in the presence of 10% FBS, etorphine and ethylketocyclazocine increased the secretion of pS2 in the culture medium. On the contrary, minor effects of etorphine were observed in the cytosolic concentration of the protein, while no effect of ethylketocyclazocine was found. The observed effects might not be mediated through opioid receptors, as the addition of the opioid antagonist diprenorphine did not modify the effect of etorphine. On the contrary, in most cases diprenorphine enhanced the effect of the agonists. In DCC conditions, the cytosolic content of the protein was significantly decreased in the presence of etorphine and ethylketocyclazocine. This decrease is partially reversed by the addition of diprenorphine when etorphine is used and potentiated in the case of EKC, indicating a differential action of the two opioid agonists. It is interesting to note that the observed effects of opioids on the T47D pS2 production and secretion are exerted with calculated IC_{50}s about 10^{-10} M. This concentration is comparable with the affinity of the opioid agonists for their binding sites [Hatzoglou et al., 1996b].

MCF7 cells (Fig. 3). In the MCF7 cell line cultured in the presence of FBS, a 3 day application of etorphine produced a significant increase of the production and release of pS2, partially reversed by diprenorphine. On the contrary, only the production was enhanced by the opioid, while the release was decreased, when the cells were cultured in the presence of DCC. Similar results were observed when etorphine was replaced by EKC, but in that case the agonist effect was less well antagonized by diprenorphine. On the contrary, when MCF7 cells were cultured in the presence of DCC, only the production of pS2 was enhanced, while the secretion of the protein was diminished. This effect was partially antagonized by diprenorphine and was comparable in MCF7 and T47D cells (compare Figs. 2, 3).

Cathepsin D.

T47D cells (Fig. 4). In the T47D cell line, etorphine produced a biphasic effect on the secretion of cathepsin D. However, the intracel-

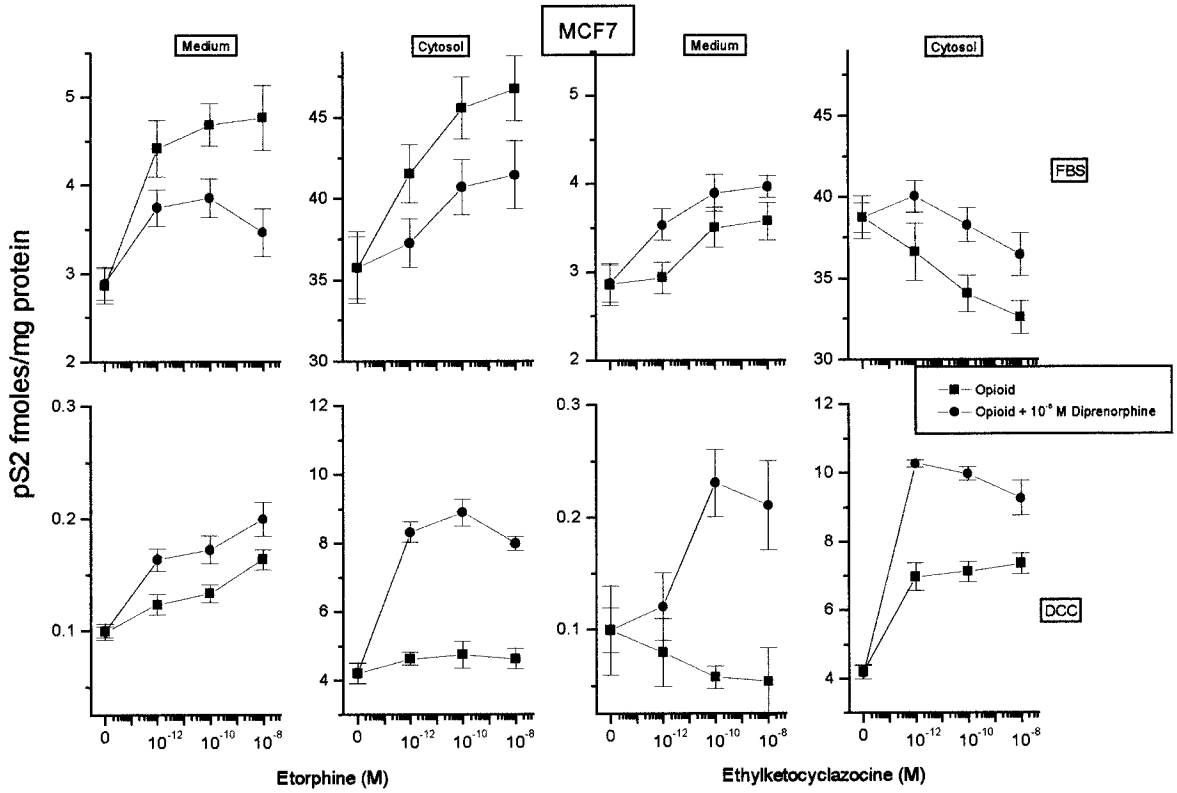

Fig. 3. Effect of opioid agonists ethylketocyclazocine and etorphine on the concentrations of pS2 in the culture medium and the cytosol of MCF7 cells cultured in 10% fetal bovine serum (FBS) or charcoal-stripped serum (DCC). See the legend of Fig. 2 for experimental details. Mean ± SE of three experiments in duplicate.

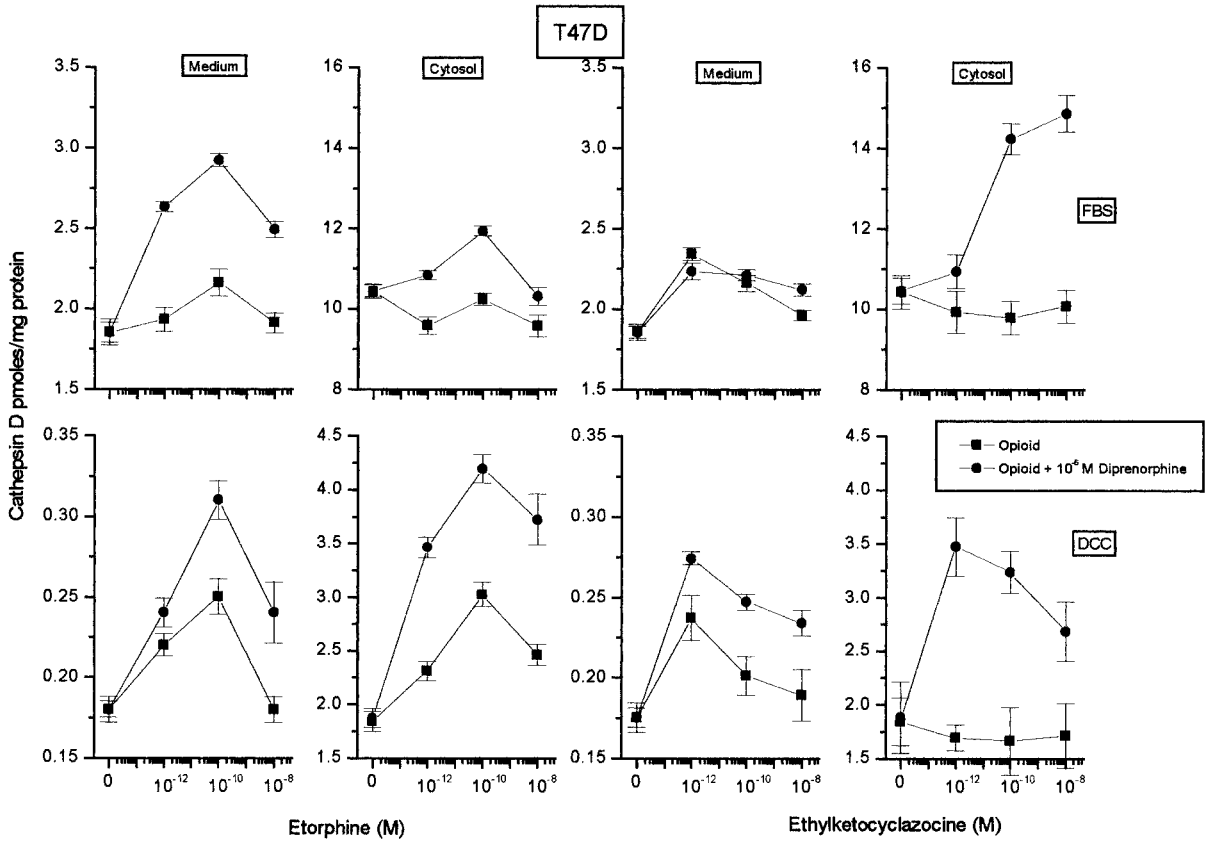

Fig. 4. Effect of opioid agonists ethylketocyclazocine and etorphine on the concentrations of cathepsin D in the culture medium and the cytosol of T47D cells cultured in 10% fetal bovine serum (FBS) or charcoal stripped serum (DCC). See the legend of Fig. 2 for experimental details. Mean ± SE of three experiments in duplicate.

lular levels of the enzyme were not modified. The addition of diprenorphine increased significantly the secreted amount of the enzyme when cells were cultured in FBS or in DCC. Similar results were found when etorphine was replaced by EKC.

MCF7 cells (Fig. 5). In the case of MCF7 cells, etorphine produced a dose-dependent increase of production and release of cathepsin D in cells cultured in the presence of FBS. This was partially reversed by diprenorphine. On the contrary, the increased release of cathepsin D in cells cultured in the presence of DCC was enhanced by diprenorphine. EKC, on the other hand, produced a small increase in the release of cathepsin D but a marked decrease of the production of the protein, partially reversed by diprenorphine, in cells cultured in FBS. No significant modifications of the released cathepsin D but an enhancement of its production was found in cells cultured in DCC. In this case, diprenorphine significantly increased the released and produced protein.

Total production of pS2 and cathepsin D. Figure 6 presents the effect of opioid agonists at a concentration of 10^{-8} M on the total production of pS2 and cathepsin D. Furthermore, it shows the effect of opioids on the ratio of secreted over intracellular protein, which is an indication of the opioid effect on mechanisms involved in the secretion of these proteins. As shown, both ethylketocyclazocine and etorphine produce a significant increase of the production of pS2 in both cell lines in FBS conditions. In contrast, in the presence of DCC serum this increase is found only in the MCF7 cell line and in the presence of etorphine. In the T47D cell line, on the contrary, opioids decrease the total production of the protein. Cathepsin D, on the other hand, shows similar changes as for pS2 in the presence of FBS in both cell lines and in MCF7 cells in DCC conditions. In the T47D cell line, on the contrary, the production of cathepsin D is inversely related to that of pS2. Indeed, cathepsin D increased in the presence of opioid agonists, while pS2 decreased.

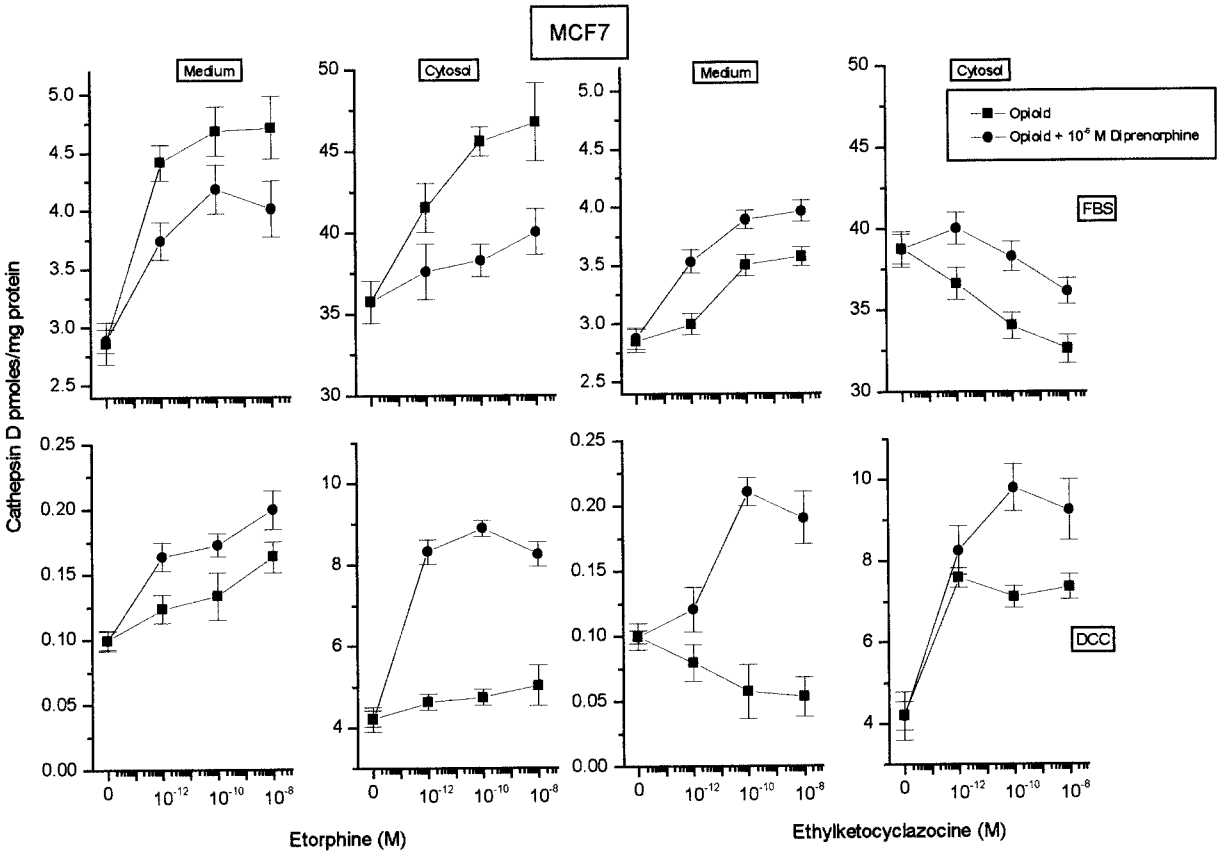

Fig. 5. Effect of opioid agonists ethylketocyclazocine and etorphine on the concentrations of cathepsin D in the culture medium and the cytosol of MCF7 cells cultured in 10% fetal bovine serum (FBS) or charcoal-stripped serum (DCC). See the legend of Fig. 2 for experimental details. Mean ± SE of three experiments in duplicate.

The addition of diprenorphine in most cases does not antagonize the effect of opioids.

Ratio of secreted/total proteins. The ratio of secreted over the total production of each protein is equally presented in Figure 6.

In T47D cells, the pS2 ratio was not modified significantly after the application of opioids in the presence of FBS. On the contrary, a significant increase of the secreted protein, not reversed by diprenorphine, was found. In the same cell line, slight modifications of the ratio for cathepsin D were found in FBS conditions, which were more pronounced when cells were cultured in the presence of DCC. Diprenorphine did not antagonize this effect. In the MCF7 cell line, slight differences of the ratio for both pS2 and cathepsin D were found. On the contrary, major modifications were found for both proteins in DCC conditions, which were antagonized by the addition of diprenorphine in almost all cases.

Effects of Cycloheximide

Cycloheximide acts selectively in ribosomes, inhibiting the translation of mRNA to the final protein product. Therefore, the addition of cycloheximide in our culture medium decreases the *de novo* protein synthesis. The addition of this drug in MCF7 and T47D cell cultures with or without the addition of opioids has shown the following (Fig. 7):

T47D cell line. Cycloheximide produced a significant decrease of cytosolic and secreted pS2 and cathepsin D in FBS conditions. Under these conditions, opioids did not produce any notable change. In DCC-treated cells, on the contrary, we have observed an increase of pS2 secretion under basal conditions. This was reversed by the addition of opioids. It is further interesting to note that, in both cell lines, etorphine treatment increased significantly the intracellular concentration of pS2 even after cycloheximide treatment. The same result was equally found in T47D cells after EKC treatment.

MCF7 cells. Similar results as for the T47D cell line were observed in the MCF7 line and the production and secretion of cathepsin D both in FBS and DCC conditions. The effect

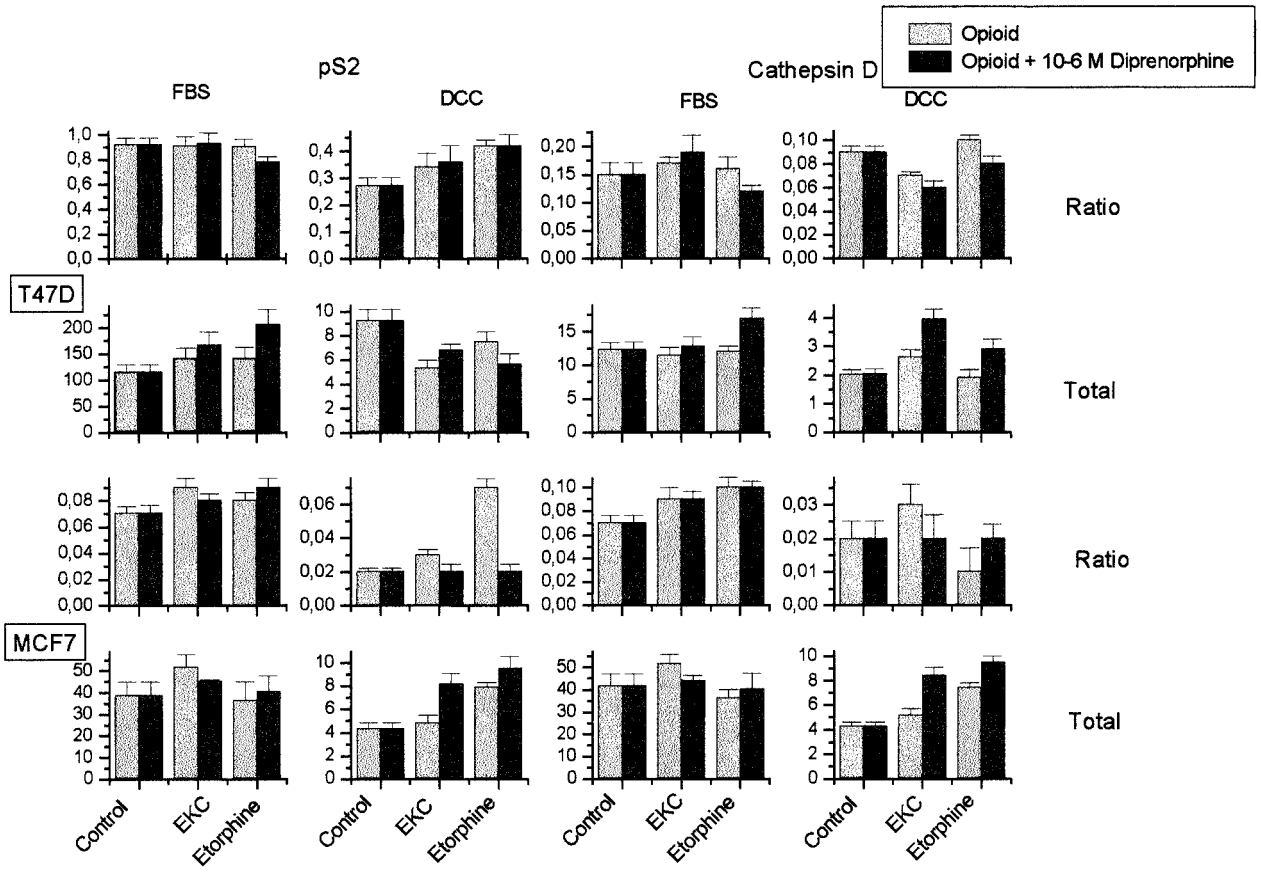

Fig. 6. Effect of opioid agonists ethylketocyclazocine and etorphine on the calculated ratio secreted/total and the total production of pS2 and cathepsin D in T47D and MCF7 cells cultured in 10% fetal bovine serum (FBS) or charcoal-stripped serum (DCC). The effect of 10^{-8} M concentration of ethylketocyclazocine (EKC) and etorphine is presented in T47D (top two rows) or MCF7 cells (bottom two rows) after 3 days of treatment. The figure presents the calculated ratio secreted/(total) (first and third rows) and the total (intracellular plus secreted) (second and fourth rows) concentration of pS2 (first two lines) and cathepsin D (bottom two lines). Results are expressed in femtomoles/milligram protein for pS2 and in picomoles/milligram protein for cathepsin D. Mean ± SE of three experiments in duplicate.

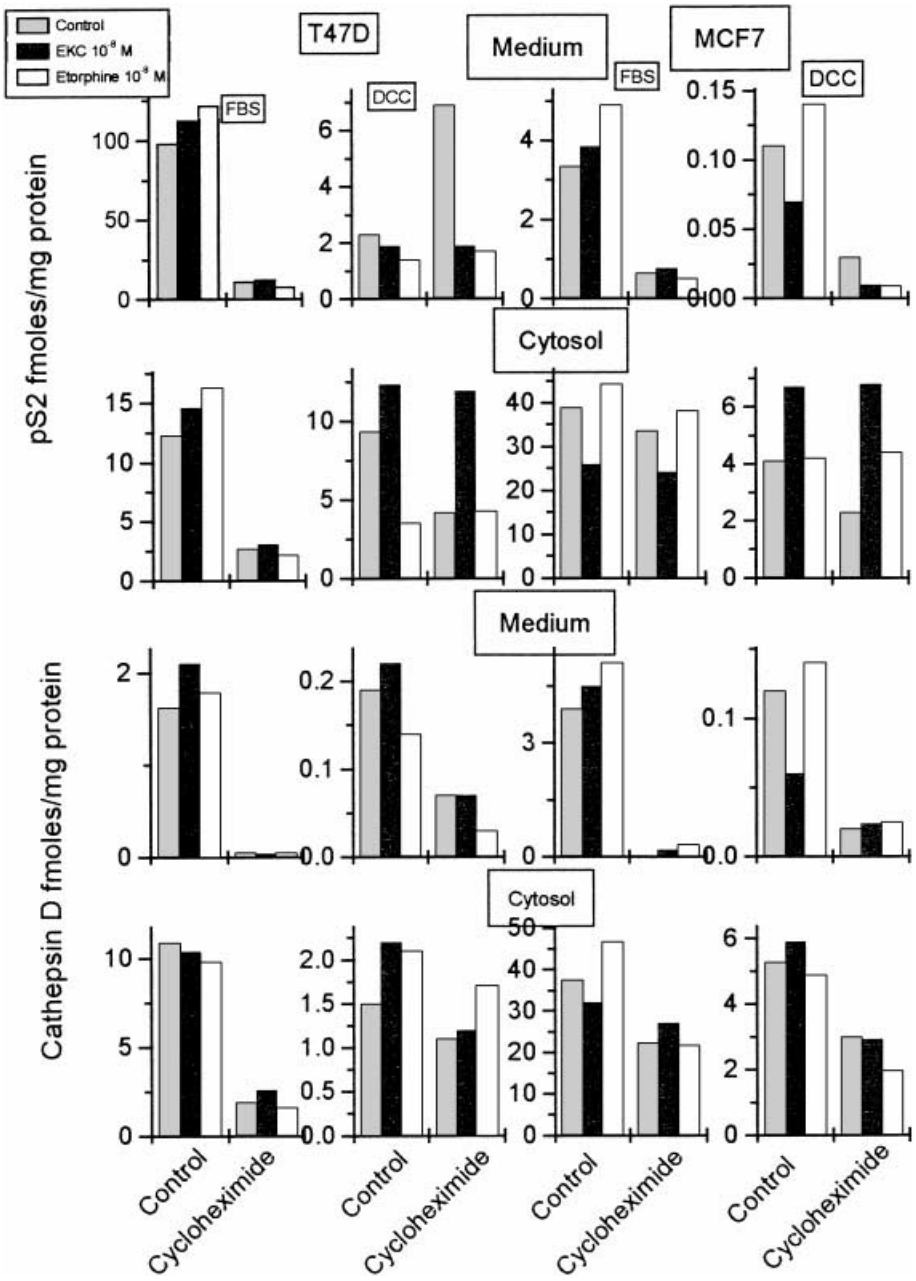

Fig. 7. Effect of cycloheximide on the concentration intracellular and secreted pS2 and cathepsin D. Effect of opioids. Cells were cultured in the presence of 10% fetal bovine serum (FBS) or charcoal-stripped serum with the addition of 2 nM L-glutamine and 6 ng/ml insulin (DCC). Ethylketocyclazocine or etorphine was introduced at a concentration of 10^{-8} M for 4 days. On the third day of culture, 10 μg/ml cycloheximide was also added for 24 h. Thereafter, medium and cytosol were processed for the assay of cathepsin D and pS2 as described in Materials and Methods.

ofopioids was parallel to that of control cells. On the contrary, cycloheximide produced a decrease of pS2 secreted and intracellular levels prevented by the addition of opioids. Indeed, as shown in Figure 7, under both ethylketocyclazocine and etorphine, intracellular pS2 levels remained almost constant.

DISCUSSION

Opioid agonists decrease growth of hormone-dependent breast cancer cell lines in a dose-dependent manner [Hatzoglou et al., 1996b; Maneckjee et al., 1990]. This effect is mediated by opioid and somatostatin receptors [Hatzoglou et al., 1995, 1996b; Maneckjee et al., 1990].

In the former case, opioid antagonists reverse the effect of the opioid agonists, while in the latter no effect is found. In addition, opioids were reported to be active in many other systems, as indicated at the beginning. It has recently been proposed that the opioid system might play a functional role in the control of cancer cells and potentially in the development of tumor growth [Gkonos et al., 1995; Noordzij et al., 1995]. Furthermore, endogenous opioids were identified in a multitude of human cancers [Zagon et al., 1987b].

Both in the breast [Maneckjee et al., 1990] and the prostate [Kampa et al., 1997], the effect of opioid agonists in hormone-sensitive and -independent cell lines is different. Indeed, as reported here, opioids can act only in hormone-sensitive (T47D and MCF7) cell lines, while no effect was observed in the BT20, hormone-independent line [Panagiotou et al., 1997]. Furthermore, this effect is dose-dependent in the absence [Hatzoglou et al., 1996b], or in the presence of steroids (Fig. 1), indicating that these two systems might be functionally coupled at a postreceptor level, or that steroids might be necessary for the accomplishment of opioid action.

In order to address the question of a possible interaction between opioids and steroids, we have performed the present study, assaying the effect of opioids in two different estrogen-inducible proteins, namely cathepsin D and pS2. Indeed, these proteins are estrogen-regulated in human breast cancer cell lines [Rochefort, 1995]. Cathepsin D has further been considered as a potential prognostic factor in breast cancer [Bolufer et al., 1996], either independent or not [Ferrandina et al., 1997; Garcia et al., 1996; Gohring et al., 1996; Joensuu et al., 1995; Spyratos et al., 1992]. Nevertheless, its prognostic significance is disputed [Glikman et al., 1997; Nadji et al., 1996; Pelizzola et al., 1996; Ravdin et al., 1997; Razumovic et al., 1997; Schwartz, 1995]. pS2, on the other hand, a member of the small peptide family, with a typical cystein-rich domain (trefoil motif or P domain), was proposed as a predictor of the possible endocrine response of the tumors [Horiguchi et al., 1996; Nichols et al., 1995; Racca et al., 1995; Soubeyran et al., 1996], as pS2 gene is estrogen-inducible [May and Westley, 1997] and dependent also on the methylation of its DNA promoter/enhancer [Martin et al., 1997]. A menstrual cycle–dependent expression of pS2 was observed, while cathepsin D as well as estrogen receptor status was not modified [Khan et al., 1997], indicating a possible differential estrogen regulation of the two proteins [Rajah et al., 1996]. Indeed, previous results indicated that estrogen treatment of hormone-responsive cells (MCF7 and ZR75.1) increased intracellular and secreted pS2 levels in both lines and intracellular (MCF7, ZR75.1) and secreted (ZR 75.1) levels of cathepsin D, indicating further a differential regulation of production and/or secretion [Cappelletti et al., 1996]. This differential regulation was further indicated by the results of the present study. Indeed, in both cell lines, the secreted pS2 protein in DCC fetal bovine serum was about only 2.5% of that secreted in FBS, while cathepsin D was under the same conditions about 10%. The same ratio in the intracellular concentrations was found in cathepsin D, while minor modifications of pS2 were observed (7 over 10 fmoles/mg protein). We are conscious that the replacement of FBS by DCC deprives cells of all small molecules present in FBS (steroid and small polypeptide hormones, growth factors, etc.). Nevertheless, cells grown in DCC with the addition of insulin and glutamine show a satisfactory growth profile and viability, indicating that DCC provides them with all necessary components for their growth. We have preferred this method in the present study, knowing that steroid hormone receptor affinity is about 10^{-11}–10^{-10} M, concentrations achieved in FBS–phenol red conditions.

Opioid agonists produce different effects in the two cell lines and under the two conditions used.

1. When cells are cultured in the presence of FBS, in which case a minimal quantity of estrogens and/or opioids is provided by the serum and phenol red [Berthois et al., 1986], we have observed either no effect of the opioid agonists or an increase of secretion and/or production of pS2 and cathepsin D.
2. On the contrary, when cells are cultured in charcoal-stripped serum, the effect of the two opioids is different: EKC produced a decrease of the production and/or secretion of pS2 and cathepsin D, while etorphine produced an increase of the synthesis and/or secretion.

The differential effect of the two general opioids was equally observed in our previous works,

both in the breast [Hatzoglou et al., 1996b] and in the prostate [Kampa et al., 1997]. As is known, these two opioids have a different receptor profile. Both bind with high affinity to delta and mu opioid sites. In addition, EKC binds with high affinity to the kappa1 and kappa2 subtypes of the kappa receptor, while etorphine binds to the kappa2 and kappa3 subtypes [Castanas et al., 1985a,b]. Taking into account this different receptor selectivity, we can plausibly attribute the effects of EKC to its interaction with kappa1 and the effect of etorphine to its selective binding to the kappa3 opioid site. Both these opioid sites were detected in previous studies in breast cancer cells [Hatzoglou et al., 1996b].

The calculated ratio medium/(medium + cytosolic) concentrations of each protein reflects its secretion/production ratio. Figure 6 presents this ratio in the absence and in the presence of 10^{-8} M of opioid agonists. It is obvious that each opioid in the absence or the presence of the minimal amounts of estrogens provided by the serum and phenol red has a differential effect on pS2 and cathepsin D. In order to investigate whether the effect of opioid agonists was exercised on the new synthesis or the storage of preformed protein, we treated cells by cycloheximide, an agent which blocks the translation of mRNA at the ribosomal level, after 3 days of opioid treatment. As shown in Figure 7, the effects are different in the two cell lines and the different conditions used. Indeed, control and opioid-treated MCF7 and T47D cells show similar trends in the decrease of pS2 and cathepsin D secretion and their intracellular levels in the presence of steroids (FBS conditions). In addition, cathepsin D intracellular and secreted levels were decreased by cycloheximide in the absence of any steroid (DCC conditions). On the contrary, opioids prevented the decrease of cytosolic pS2 decrease produced by cycloheximide, confirming the differential regulation of the control of pS2 and cathepsin D and indicating that a much more complex interaction of opioids with the cellular machinery than a direct effect on the production of one or more proteins might exist. Furthermore, under the absence of any steroid, cycloheximide occurs in an increase of secreted pS2. A possible explanation of these data might be a decrease of the synthesis of different granule and/or membrane proteins under cycloheximide, resulting in a modification of the protein exocytosis process. In that case, as opioid agonists prevent this release, they might have indirect effects on the secretion of proteins, possibly mediated via their interaction with the cytoskeleton, as we have described recently [Papakonstanti et al., 1998].

The above results indicate the following:

1. Cathepsin D and pS2 are regulated by different cellular pathways. Indeed, it seems that estrogens exert a dual regulation of cathepsin D synthesis and secretion [Couissi et al., 1997] and that this regulation involves mainly the Golgi apparatus and insulin-like growth factor (IGF) II/mannose-6-phosphate receptors. In turn, IGF I regulates the release of cathepsin D via alpha-1 antichymotrypsin M6P soluble receptor, which in turn could regulate cell proliferation and/or invasion [Confort et al., 1995]. Furthermore, IGF II overexpression increases cathepsin D production, and endogenous IGF II modulates the routing of the enzyme [De Leon et al., 1996]. On the other hand, pS2 gene transcription is stimulated by estrogen and cAMP via different mechanisms, both requiring the estrogen receptor [el-Tanani and Green, 1996b].
2. Opioids modify selectively each regulatory pathway in a discrete manner according to the affinity of agonists for each opioid binding site. Similar results have been reported by Herman and Katzenellenbogen [1996] in tamoxiphene-resistant MCF7 cells, in which case the regulation of pS2 by estrogen is maintained while other ER pathways were defective.
3. The increased secretion and/or production of the two regulated proteins after the application of diprenorphine indicates either a proper action of the opioid antagonist, as was found in previous studies for naloxone [Hatzoglou et al., 1996a], or that FBS might contain, in addition to a small quantity of estrogens, endogenous opioid peptides, whose action might be reversed by diprenorphine. Alternatively, T47D and/or MCF7 cells could synthesize and secrete opioids, either in the form of endogenous opioid peptides [Scopsi et al., 1989] or in the form of casomorphin molecules, which could have a proper opioid activity [Hatzoglou et al., 1996a; Kampa et al., 1997], reversed by diprenorphine. Nevertheless, the fact that diprenorphine did not reverse, in many cases, the action of opioid agonists indicates that the

opioid modification of pS2 and cathepsin D might not be solely an opioid regulated action but that other membrane receptors might participate in it. This was also presented by our group in the same cell line, implying somatostatin receptors [Hatzoglou et al., 1995].

In conclusion, the results of the present study indicate that opioid agonists, through the interaction with opioid and perhaps with other membrane-receptor sites, modify in a dose-dependent manner the production and the secretion of two estrogen-regulated proteins, namely pS2 and cathepsin D, in a differential manner. This interaction is different according to the presence of estrogens in the culture medium, indicating that opioids can disturb hormonal signals mediated by the estrogen receptor and suggests that these chemicals have potential endocrine-disrupting activities at a postreceptor level which has to be determined.

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