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Opioids Inhibit Endothelin-mediated DNA Synthesis, Phosphoinositide Turnover, and Ca^{2+} Mobilization in Rat C6 Glioma Cells

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Opioid agonists inhibit DNA synthesis in C6 rat glioma cells that express opioid receptors, induced by desipramine (DMI). This inhibition was not observed in cells that were not treated with DMI, and thus did not express opioid-binding sites. Endothelin, a known mitogen, increased thymidine incorporation dose dependently (up to 1.7-fold) in DMI-treated C6 cells. This increase was reversed by an anti-idiotypic antibody to opioid receptors, Ab2AOR, which has opioid agonist properties. The opioid antagonist naltrexone blocked the inhibition caused by Ab2AOR. Endothelin also stimulated phosphoinositide (PI) turnover and this effect was inhibited by morphine (50%) or by Ab2AOR (72%) in DMI-treated but not in DMI-untreated C6 cells. These actions of morphine and Ab2AOR were reversed by naltrexone. The inhibition of PI turnover and of thymidine incorporation by Ab2AOR or morphine was insensitive to pertussis toxin (PTX). Since PI turnover is known to induce Ca^{2+} mobilization, it was of interest to examine the effects of the applied opioids on intracellular Ca^{2+} concentrations. Endothelin increased the concentration of cytosolic free Ca^{2+} in the cells while Ab2AOR, morphine, and β-endorphin reversed the endothelin-induced Ca^{2+} mobilization in DMI-treated but not in DMI-untreated C6 cells. The effect of these agonists was also blocked by naltrexone. The results indicate that glial cells can be a target of an opioid receptor–mediated antimitogenic action and that an abatement in PI turnover and Ca^{2+} mobilization may be associated with this mechanism.

[Key words: Ca^{2+} mobilization, DNA synthesis, GTP-binding protein, opioid receptor, phosphoinositide turnover]

Opioid agonists acting via their specific receptors have recently been proposed to function as neurotrophic factors that reduce neural cell proliferation (Zagon and McLaughlin, 1987; Hauser and Stiene-Martin, 1991; Barg et al., 1992, 1993; Leslie, 1993). Relatively little is known about the mechanism of neurotrophic action of the opioids in glial cells. We have previously demonstrated that primary mixed glial cultures prepared from embryonic rat brain contain predominantly κ-opioid binding sites (Barg et al., 1991). In addition, the rat C6 glioma cell line has proven to be a useful model for investigating the properties of opioid receptors on glial cells. Although C6 cells do not display opioid binding under standard tissue culture conditions, they express opioid receptors in the presence of the tricyclic antidepressant drug DMI (Albouz et al., 1982; Tocque et al., 1984; Reggiani et al., 1987). The DMI-induced C6 glioma opioid receptor binds β-endorphin, morphine, etorphine, naloxone, as well as the anti-idiotypic antibody Ab2AOR, which has been shown to have opioid agonist activity (Coscia et al., 1991; Barg et al., 1993b). The DMI-induced opioid binding sites are functional and morphine inhibits both isoproterenol-stimulated and basal cAMP levels in the cells (Tocque et al., 1984; Reggiani et al., 1987).

Stiene-Martin et al. (1991) have recently demonstrated that opioid agonists reduce the cell number and thymidine labeling of a specific type of "flat" (type 1) neonatal mouse brain astrocyte. Thymidine labeling of cells and its incorporation into DNA were also inhibited by opioid agonists in primary mixed glial cultures from embryonic rodent brain (Zagon and McLaughlin, 1987; Hauser and Stiene-Martin, 1991; Stiene-Martin et al., 1991). Similarly, opioid agonists inhibit DNA synthesis in fetal brain cell aggregates during a stage of development in which they contain predominantly proliferating glial cells (Barg et al., 1992, 1993a).

The potent endothelial cell vasoconstrictor endothelin has been shown to exhibit mitogenic activity in glial cells (Lin et al., 1990; MacCumber et al., 1990). It has been suggested that the mitogenic action of endothelin is related to stimulation of PI turnover and Ca^{2+} mobilization (Lin et al., 1990, 1992; MacCumber et al., 1990; Chuang et al., 1991; Yada et al., 1991; Lin and Chuang, 1993). Binding of endothelin to its high-affinity receptor (Et_A) in C6 cells also leads to inhibition of isoproterenol-induced accumulation of cAMP via the inhibitory GTP-binding protein G_i (Couraud et al., 1991). ATP also utilizes G_i and Ca^{2+} to inhibit cAMP formation in C6 cells (Lin and Chuang, 1993). These authors have also suggested that endothelin and ATP can attenuate cAMP production indirectly via PI systems. Although acute treatment by various opioid agonists can directly

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inhibit isoproterenol-stimulated cAMP production, opioids antagonize PI turnover, possibly indirectly, in brain and primary cultures of embryonic brain cells (Reggiani et al., 1987; Mangoura and Dawson, 1991; Periyasamy and Hoss, 1991; Barg et al., 1992, 1993). Therefore, it was of interest to test whether activation of opioid binding sites in C6 cells may be involved in the regulation of PI turnover and cell proliferation. This would afford a valuable system to explore the mechanism of the antimitogenic action of opioids. The results show that opioid agonists inhibit endothelin-stimulated thymidine incorporation, PI turnover, and Ca^{2+} mobilization in DMI-treated C6 cells.

Materials and Methods

Chemicals. Human endothelin-2 was purchased from Sigma (St. Louis, MO). ^{3}H-thymidine (specific activity, 29 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK). Myo-^{3}H-inositol (specific activity, 20 Ci/mmol) was purchased from DuPont–New England Nuclear (Boston, MA). Fura-2AM was purchased from Molecular Probes (Eugene, OR). Media and sera were obtained from GIBCO (Grand Island, NY). PTX was purchased from List Biological Laboratories (Campbell, CA). Morphine and naltrexone were provided by NIDA, NIH (Bethesda, MD), and β-endorphin was obtained from Multiple Peptide Systems (San Diego, CA).

Cell cultures. Rat C6BU-1 glioma cells (provided by Dr. Michael Engel, Washington University) were grown in 35 mm culture dishes (5 × 10^5 cells/dish) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) in a humidified atmosphere containing 6% CO_2 at 37°C. Cells were maintained in culture for a total of 3 d in the presence or absence of 5 μM DMI. C6 cells were treated with Ab2AOR-containing ascites fluid, morphine, β-endorphin, naltrexone, or PTX as described in the figure legends. In all experiments two types of ascites fluids were used (3% final v/v): the mouse monoclonal anti-idiotypic antibody to opioid receptors Ab2AOR (Coscia et al., 1991), and an unrelated ascites fluid (unrelated Ab) obtained from CD-1 mice injected with Krebs II carcinoma cells (Shabo et al., 1988).

^{3}H-thymidine incorporation. After 48 hr of culture, the serum-containing medium was replaced and the C6 cells were grown for 24 hr in chemically defined medium (Guentert-Lauber et al., 1985) in the presence or absence of 5 μM DMI and/or opioids. ^{3}H-thymidine was present for the final 23 hr. Labeled cells were then removed from Petri dishes with phosphate-buffered saline (PBS) containing 0.5 mM ethylenediaminetetraacetic acid. Thymidine incorporation into DNA was measured as described previously (Barg et al., 1994).

PI turnover. PI turnover was measured as the accumulation of inositol phosphates (IPs) in the presence of LiCl as described (Xu and Chuang, 1987). Briefly, cells were prelabeled with myo-^{3}H-inositol (specific activity, 20 Ci/mmol, 1 μCi/ml, for 24 hr; DuPont–New England Nuclear), incubated with opioids in the presence of 10 mM LiCl, washed, and scraped off the dishes. Following methanol/chloroform extraction, the aqueous phase was applied to Bio-Rad AG 1 × 8 columns and the ^{3}H-IPs including IP_1, IP_2, and IP_3 were eluted with a mixture of 1 M ammonium formate and 0.1 M formic acid directly into scintillation vials.

Intracellular calcium mobilization. C6 cells were plated on 25 mm coverslips (at a density of 8 × 10^4 cells/coverslip) and cultured for 48 hr in DMEM containing 5% FCS. The cells were then kept in DMEM (without phenol red) for 24 hr and loaded with 2 μM Fura-2AM for 60 min. Excess Fura-2AM was removed by washing the cells with DMEM (without phenol red) (2 ml × 3). The coverslips were mounted in a 25 mm chamber and the fluorescence at 510 nm determined at 22°C using dual excitation at 340 and 380 nm (MacCumber et al., 1990; Lin et al., 1992) with a computerized video imaging system (Biological Detection Systems, Pittsburgh, PA). Relative amount of free intracellular Ca^{2+} concentration was determined according to the increase of the fluorescent signal (ratio of 340:380 nm) in endothelin-stimulated cells compared with unstimulated cells.

Results

Previously we reported that an anti-idiotypic antibody to the opioid receptor, Ab2AOR, inhibits thymidine incorporation into DNA in DMI-treated C6 cells (Barg et al., 1994). As shown in Figure 1, endothelin increased thymidine incorporation in DMI-treated C6 cells in a dose-dependent manner. Maximum increases in thymidine incorporation of 70% were observed with concentrations of >30 nM. Exposure of DMI-treated C6 cells to Ab2AOR in the absence of endothelin resulted in attenuation of thymidine incorporation by 50%. Moreover, at endothelin concentrations of <30 nM, Ab2AOR completely blocked its mitogenic activity. At higher concentrations, endothelin had mitogenic activity despite the presence of Ab2AOR in the culture medium, increasing thymidine incorporation by 1.8- and 2.8-fold compared with DMI/Ab2AOR-treated, endothelin-free cultures. As shown in Figure 2, the inhibitory effect of Ab2AOR on thymidine incorporation was blocked by the opioid antagonist naltrexone, indicating that the process is opioid receptor–mediated. The inhibitory effect of Ab2AOR on thymidine incorporation was PTX insensitive. PTX affected neither the endothelin nor the opioid action.

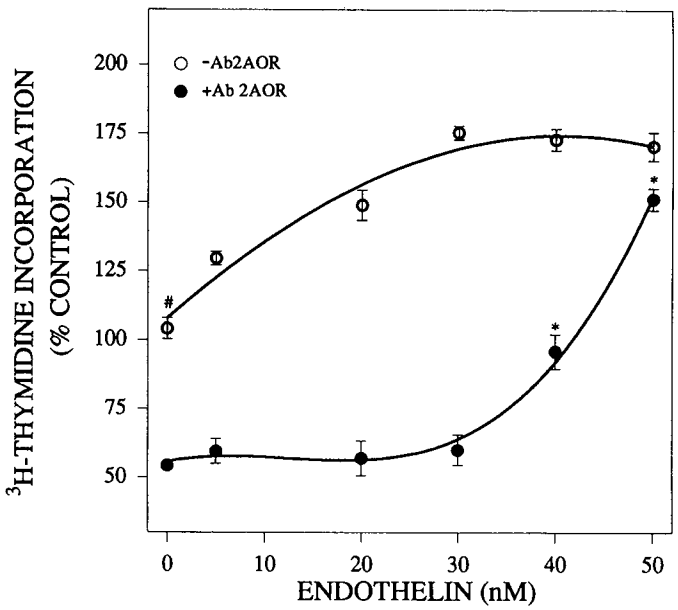


Figure 1. Effect of Ab2AOR and endothelin on ^{3}H-thymidine incorporation into DNA in DMI-treated C6 glioma cells. Cultures were treated for 24 hr with endothelin at the indicated concentrations and/or Ab2AOR. ^{3}H-thymidine was included for the final 23 hr. Data are the mean ± SEM of three to five experiments. Thymidine incorporation in control cells (*−Ab2AOR*, no endothelin) is defined as 100%. All Ab2AOR-treated C6 cells are significantly different from the corresponding Ab2AOR-untreated C6 cells, $P < 0.01$. #, significantly different from the incorporation measured in the presence of endothelin and absence of Ab2AOR, $P < 0.01$; *, significantly different from the incorporation measured for cultures treated with Ab2AOR and exposed to lower concentrations of endothelin, $P < 0.01$.

Since endothelin stimulates PI turnover in C6 glioma cells (Chuang et al., 1991; Lin et al., 1992), the question of whether opioids can affect this signal transduction pathway was addressed. We have previously shown that application of opioid agonists leads to inhibition of the basal, as well as the glutamate-stimulated formation of IPs (Barg et al., 1992). Here we demonstrate that morphine and Ab2AOR inhibit endothelin-mediated ^{3}H-IPs formation by 50% and 72%, respectively, in DMI-treated C6 glioma cells (Figs. 3, 4). Opioid effects were not obtained in control cells untreated with DMI (which do not contain opioid receptors). Since previous studies with Ab2AOR indicated a PTX insensitivity of the opioid action, it is possible

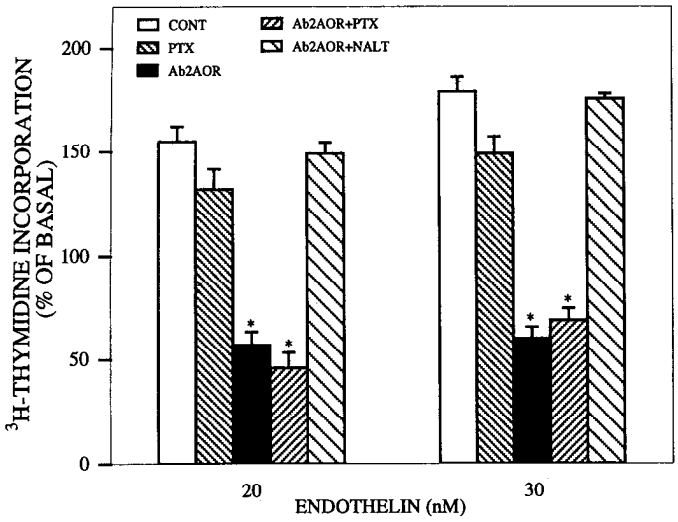


Figure 2. ^{3}H-thymidine incorporation into DNA in DMI-treated C6 glioma cells exposed to endothelin, PTX, and opioids. Cultures were treated for 24 hr with endothelin (20 or 30 nM), Ab2AOR, PTX (100 ng/ml), and/or naltrexone (1 μM), and with ^{3}H-thymidine for the final 23 hr. Data are the mean ± SEM of three to five experiments. *, significantly different from controls, cells exposed to PTX alone or naltrexone-treated cells, $P < 0.001$.

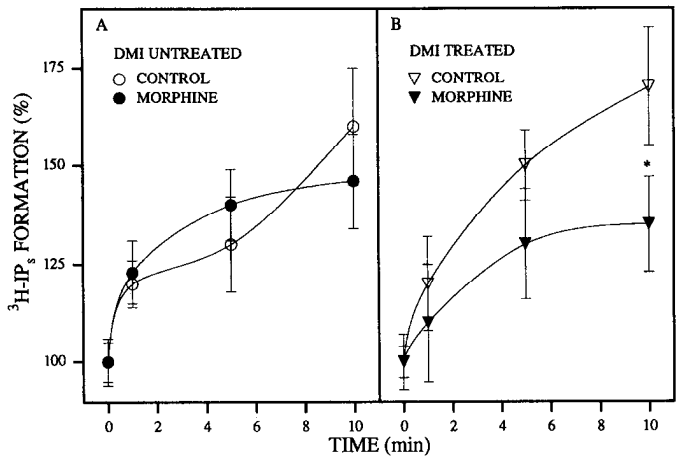


Figure 3. The effect of morphine on endothelin-induced formation of ^{3}H-IPs in C6 glioma cells. Control or DMI-treated cultures were labeled for 24 hr with myo-^{3}H-inositol (1 μCi/ml) in the presence or absence of 1 μM morphine. Endothelin (30 nM) was added for 10 min, and the accumulation of IPs determined at the times indicated. Data are the mean ± SEM of three experiments. *, significantly different from the corresponding DMI-treated C6 cells, $P < 0.05$.

either that PTX-resistant G proteins are involved in regulating thymidine incorporation by endothelins and opioids or that the process is not G protein mediated. The inhibition of PI turnover by the opioids also proved to be unaffected by PTX but was blocked by naltrexone (Fig. 4). Interestingly, endothelin stimulation of PI formation was found to be partially PTX sensitive. PTX inhibited about 40% of the endothelin-stimulated formation of IPs in the DMI-treated cells. This is consistent with previous reports that endothelin stimulates PI turnover and Ca^{2+} mobilization by PTX-sensitive and PTX-insensitive processes in C6 cells (Lin and Chuang, 1993).

Since the DMI-treated C6 cells were exposed to the opioid agonist for a period of 24 hr, a time in which maximal attenuation of cell proliferation was realized in fetal brain cell aggregates (Barg et al., 1992), the decrease in ^{3}H-IPs formation could be due to the smaller number of cells. This possibility was excluded by calculating ^{3}H-IPs formation per mg protein, under the various experimental conditions.

We next investigated the effects of opioids on mobilization of intracellular Ca^{2+}. The concentration of cytosolic free Ca^{2+} was determined with the Ca^{2+} fluorophor Fura-2AM. Application of endothelin (30 nM) rapidly increased the concentration of cytosolic free Ca^{2+}. The kinetics of the process are shown in Figure 5. A representative field containing five cells is shown in Figure 6*A*. Of the total number of C6 cells imaged, >99% exhibited the endothelin-induced increase in Ca^{2+} concentration. Interestingly, Ca^{2+} accumulation was heterogeneously distributed within each cell. A gradual increase of Ca^{2+} concentration from the periphery of the cell to the center was evident (Fig. 6*A*). The mobilization of Ca^{2+} by endothelin reached its maximum level after 5–10 sec (Figs. 5, 6*A*). Following this interval the concentration of cytosolic free Ca^{2+} slowly decreased reaching a plateau of 25–40% above background after 60–250 sec.

The increase by endothelin of Ca^{2+} levels was not influenced by pretreatment of cells with DMI or with Ab2AOR alone. However, application of Ab2AOR to DMI-treated cells resulted in a marked attenuation of the endothelin-induced increase of intracellular Ca^{2+} concentration. We found that Ab2AOR lowered the cytosolic Ca^{2+} concentration in almost all the cells studied (>90%). This finding suggests that opioid receptors are present on almost all of the DMI-treated cells as previously shown by immunostaining procedures (Barg et al., 1994). Interestingly, Ab2AOR did not prevent the initial rise of Ca^{2+} concentration induced by endothelin but had a strong effect on both the maximal level achieved and the rate of decline in Ca^{2+} concentration (Figs. 5*B*, 6*A*). As a consequence, the maximum Ca^{2+} concentration in the presence of Ab2AOR was reached within 3 sec, compared with 6–9 sec for the other treatments. Moreover, in the presence of Ab2AOR, Ca^{2+} concentrations reached background levels following prolonged incubation (>100 sec) with endothelin.

To quantify the effects of Ab2AOR on Ca^{2+} concentration, levels of Ca^{2+} in the presence or absence of Ab2AOR were calculated for the integrated period of 1000 sec. Using this method of calculation we found that incubation of DMI-treated cells with Ab2AOR inhibited Ca^{2+} mobilization by more than 9.5–13.3-fold, compared with the other conditions tested (Fig. 5). An unrelated antibody used as control had no effect on Ca^{2+} concentrations in the tested cells. As shown in Figures 5 and 6, the inhibitory effect of Ab2AOR was evident only in DMI-treated cells. As expected for an opioid receptor–mediated phenomenon, treatment with a combination of Ab2AOR and naltrexone resulted in a Ca^{2+} mobilization profile, which was identical to that observed with endothelin alone. Moreover, the same maximal peak height was obtained with endothelin in untreated and DMI-, Ab2AOR-, unrelated Ab-, and naltrexone-treated cells (Fig. 5).

Morphine and β-endorphin also inhibited endothelin-increase of intracellular Ca^{2+} concentration. Figure 6*B* shows the computerized image for intracellular Ca^{2+} following 12 sec of stimulation with endothelin and the effect of opiates on Ca^{2+} levels. The mean values of free Ca^{2+} concentration in the cells under various conditions are given in Figure 7. The opiate agonists reduced the levels of free Ca^{2+} by 80–90% in comparison with

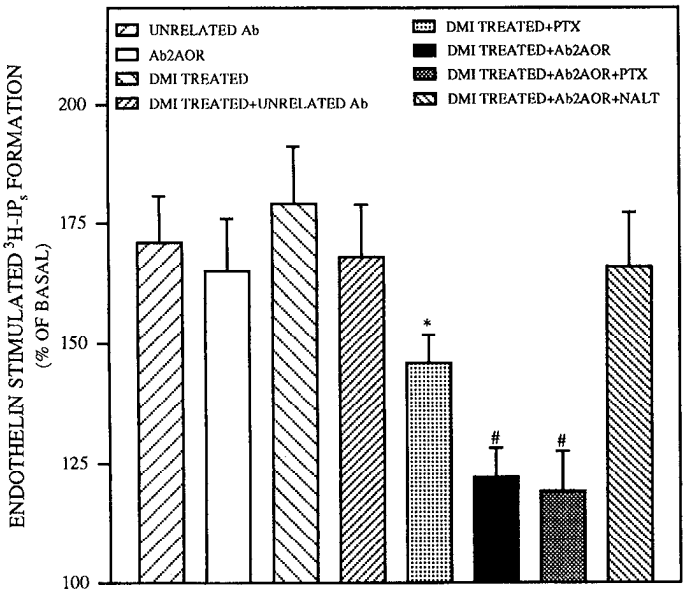


Figure 4. Effect of naltrexone and PTX on Ab2AOR inhibition of ^{3}H-IPs formation in C6 cells. Control and DMI-treated cultures were exposed for 24 hr to Ab2AOR or unrelated Ab, PTX (100 ng/ml), and naltrexone (1 μM), as indicated. Cells were labeled with myo-^{3}H-inositol (1 μCi/ml) during the same 24 hr. Endothelin (30 nM) was added for 10 min and the accumulation of IPs determined as described. Data are the mean ± SEM of three experiments. * and #, significantly different from all others, $P < 0.05$ and $P < 0.01$, respectively.

controls (endothelin alone). The attenuation of Ca^{2+} levels was completely reversed by naltrexone, indicating an opioid receptor–mediated effect. Additional experiments with morphine indicated that this opioid agonist decreased Ca^{2+} entry from the extracellular milieu as well as Ca^{2+} release from intracellular stores (data not shown).

Discussion

Recently the mechanism of opioid regulation of thymidine incorporation into DNA in neural cultures, composed of both neurons and glial cells, has been investigated (Barg et al., 1992, 1993; Mangoura and Dawson, 1993). We have shown that preincubation with opioid agonists for periods as short as 90 min significantly reduced thymidine incorporation in brain cell aggregates. Moreover, the results imply that inhibition of phosphoinositol signal transduction by opioid agonists seems to be responsible for reduction in thymidine incorporation in these cells (Barg et al., 1992). Few such studies have been performed on a single cell type to identify the target cells for opioid action and to eliminate the possibility of interaction between different cell types (MacCumber et al., 1990; Barg et al., 1994). It has been previously shown that C6 glioma cells can be induced to express a population of functional opioid receptors (Tocque et al., 1984; Reggiani et al., 1987). The nature of the opioid receptor in C6 glioma cells has been an enigma given its low affinity for δ ([D-Ala^2,D-Leu^5]enkephalin), μ ([D-Ala^2,mePhe^4,Gly-ol^5]enkephalin), dihydromorphine, and κ (ethylketocyclazocine) ligands. Interestingly the endogenous opioid peptide β-endorphin possesses high affinity for the C6 opioid receptors (K_d = 3 nM), whereas the affinity of met-enkephalin and other naturally occurring peptides tested is lower by an order of magnitude (Barg et al., 1991).

Activation of opioid sites normally leads to inhibition of cAMP formation, and previous data suggest that opioid agonists have such an effect on isoproterenol-stimulated adenylyl cyclase activity in C6 cells (Reggiani et al., 1987). β-Endorphin proved to be most potent in this assay (K_i = 8 nM). Moreover, as shown in this study, opioid agonists also attenuate endothelin-stimulated PI turnover, Ca^{2+} mobilization, and DNA synthesis. All of these actions can be prevented by the opioid antagonist naltrexone, demonstrating the intermediacy of an opioid receptor.

All of the inhibitory cAMP-signaling systems characterized thus far have been reported to be coupled to PTX-sensitive G proteins (Simon et al., 1991). In contrast, our results indicate that the inhibition of PI turnover by activation of C6 opioid receptor is mediated via a PTX-insensitive G protein. The $G_{q/11}$ G protein family, which is PTX insensitive, has been implicated as mediator of the activation of the β-1 isozyme of PI-specific

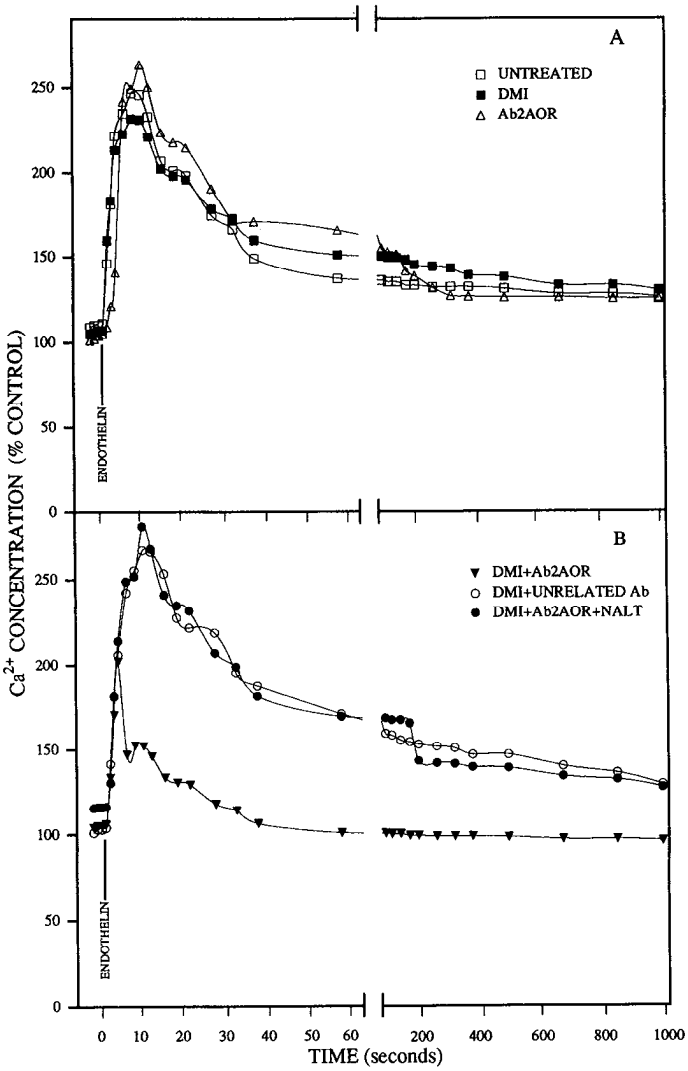
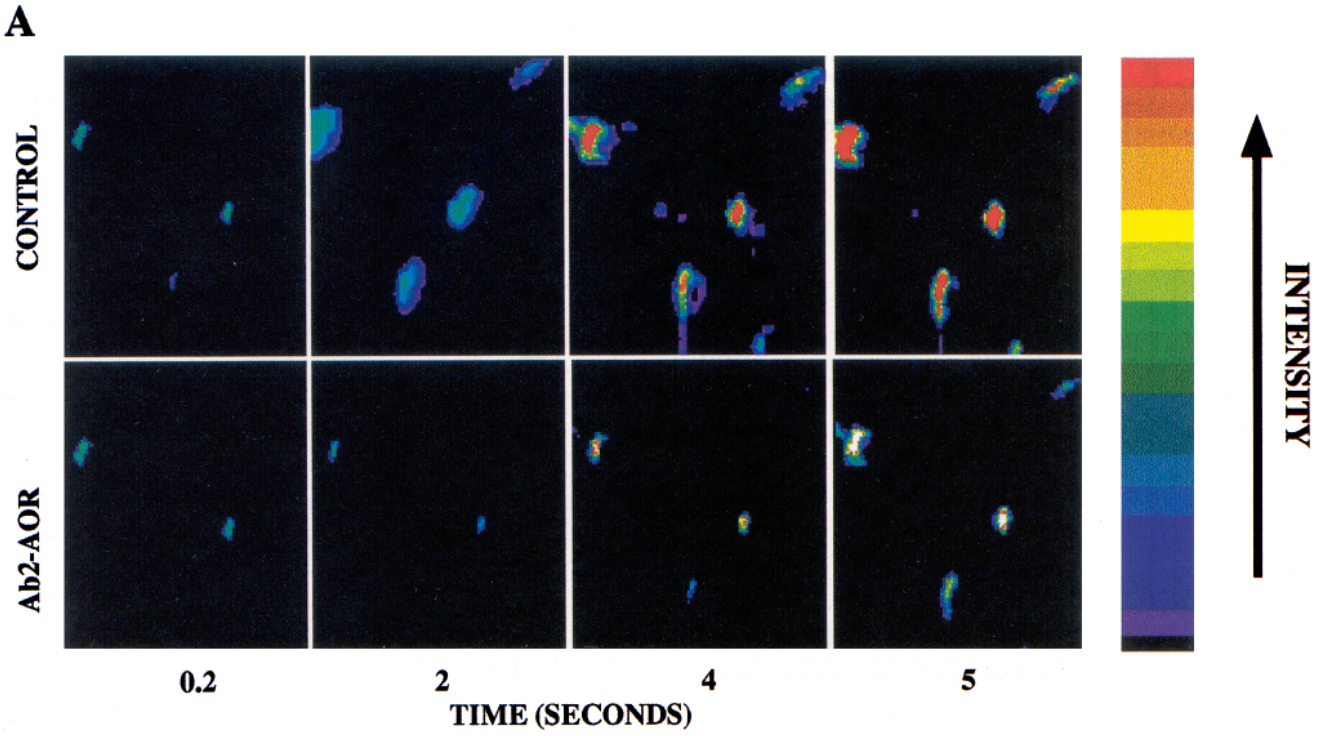


Figure 5. Representative time curves of endothelin-induced increase of intracellular Ca^{2+} concentration in C6 cells. *A*, Time curves for untreated and DMI- and Ab2AOR-treated cultures. *B*, Time curves for cultures treated with DMI/Ab2AOR, DMI/unrelated Ab, and DMI/Ab2AOR/naltrexone (1 μM). Cells were preloaded with Fura-2AM, incubated with opioids for 15 min at 22°C, and stimulated with 30 nM endothelin. Ca^{2+} concentrations in various fields of cells were determined at the times indicated. The concentration of Ca^{2+} in untreated C6 cells before adding endothelin (control) was defined as 100%. The experiments were repeated three times.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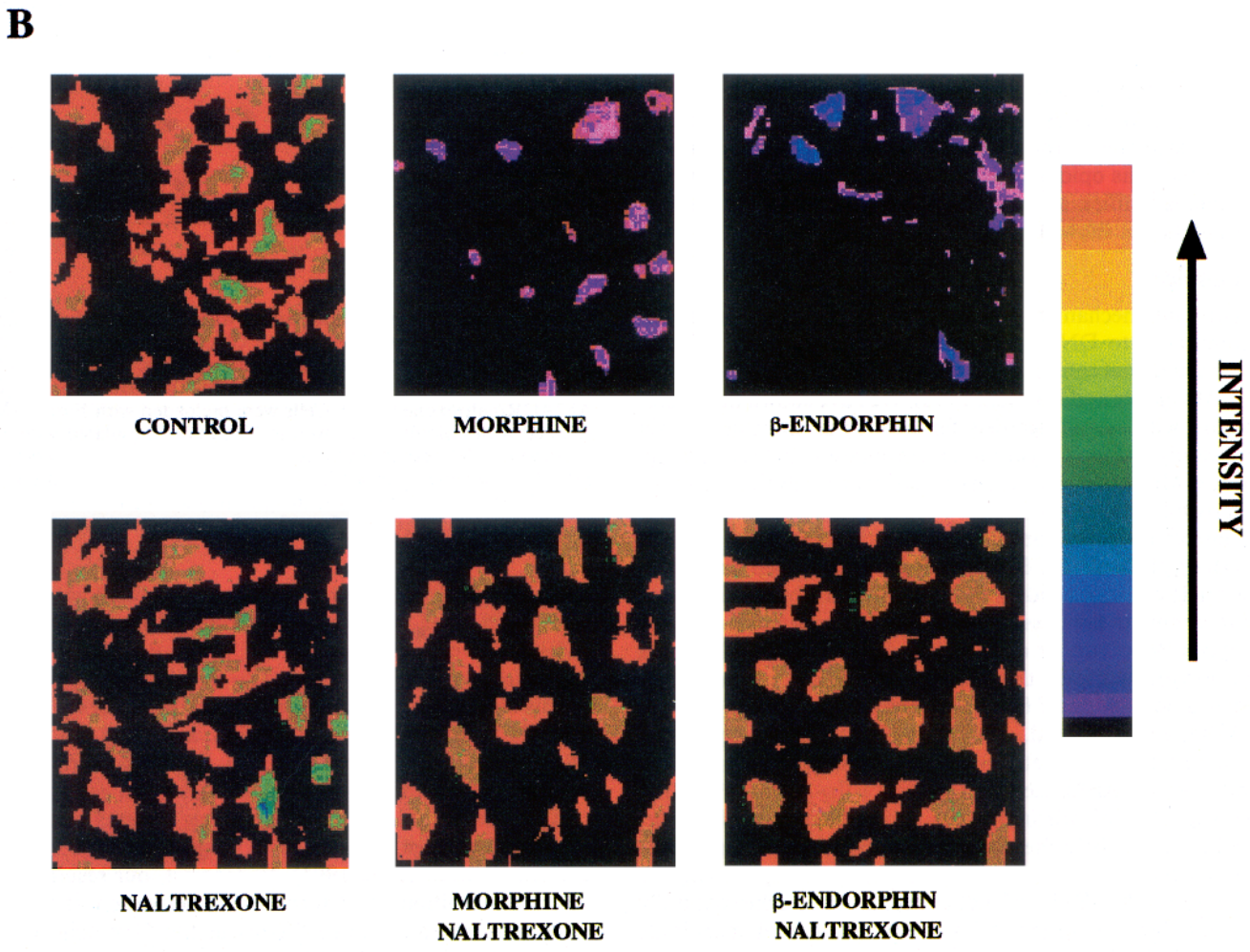
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phospholipase C and the enhanced turnover of PI (Blank et al., 1991). Moreover, the purinergic (P_{2p}) receptor–mediated PI turnover of C6 cells has also been demonstrated to be PTX resistant (Lin and Chuang, 1993). Taken together, this raises the possibility that opioid receptors in C6 cells may interact with two types of G proteins to elicit different second messengers. The phenomenon of one receptor activating two signaling systems in the same cell is now widely known. For example, it has been recently reported that the D_2 dopamine receptor in pituitary cells acts via two different G proteins to modulate four types of ion channels (Piomelli et al., 1991). The thrombin receptor activates the formation of IP_3 and inhibits cAMP synthesis in a leukemic cell line (Brass et al., 1991), while the human muscarinic receptor Hm1 transfected into Chinese hamster ovary cells activates PI turnover, as well as adenylyl cyclase (Heldman et al., 1993). Thus, there may be one receptor that, by interacting with different G proteins, displays cofunctionality. Alternatively, we cannot exclude a second possibility that the DMI treatment induces two different types of opioid receptors in C6 cells, one that is coupled to a PTX-sensitive G protein and the other to a PTX-resistant G protein. For example, there is evidence for the existence in C6 cells of a P_{2i} purinergic receptor, which mediates cAMP inhibition via G_i, as well as the above mentioned P_{2p} type, which activates PI turnover via PTX-resistant G proteins (Lin and Chuang, 1993).

However, at this point we cannot rule out the possibility that inhibition of PI turnover by opioids is not mediated through a G protein–dependent process. Many mitogens act through tyrosine kinase to activate protein kinase C via γ- phospholipase (Berridge, 1993). There is evidence that in chick embryo neuronal cultures a μ-opioid receptor ligand mediates such a system (Mangoura and Dawson, 1993). We also have evidence to implicate protein kinase C in μ- opioid inhibition of DNA synthesis (Barg et al., 1992) and this enzyme may play an important role in feedback modulation of the cAMP-generating system in C6 cells (Lin and Chuang, 1993; Gorodinsky and Coscia, unpublished observations).

There are now a number of reports demonstrating that opioids can inhibit PI turnover. For example, various opioid agonists have been shown to inhibit bradykinin-, glutamate-, and carbachol-stimulated PI turnover (Mangoura and Dawson, 1991; Periyasamy and Hoss, 1991; Barg et al., 1992, 1993). Using fetal brain cells in aggregate cultures, we have previously shown that opioid agonists inhibit PI turnover induced by glutamate (Barg et al., 1992, 1993). Here we demonstrate that opioids can also inhibit the endothelin-stimulated PI turnover in C6 glioma cells.

The relationship of PI formation and mobilization of intracellular Ca^{2+} in C6 glioma cells has important consequences in cell proliferation (MacCumber et al., 1990; Couraud et al., 1991; Lin et al., 1992; Berridge, 1993). Moreover, mitogenic effects of growth factors and the action of glutamate in hippocampal neurons involve c-*fos* expression via a Ca^{2+}-mediated process

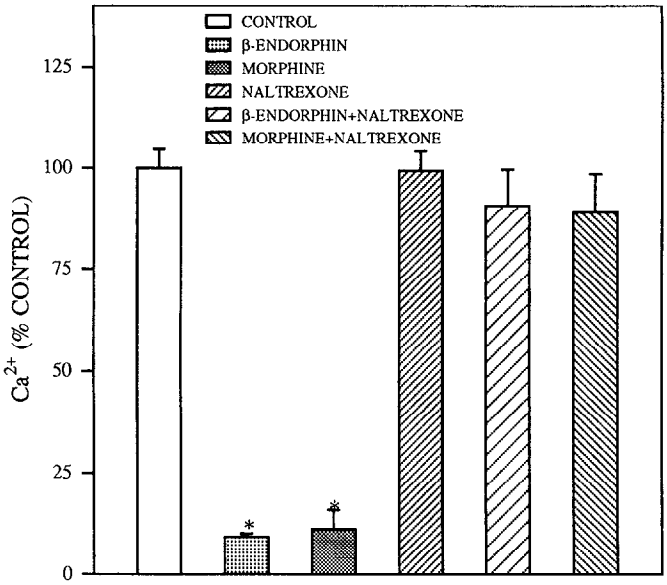

Figure 7. Morphine and β-endorphin inhibit endothelin-induced increase of intracellular Ca^{2+} concentration in C6 glioma cells. DMI-treated cultures were preloaded with Fura-2AM, incubated with opioids for 15 min at 22°C, and stimulated with 30 nM endothelin. Ca^{2+} concentrations of various fields of cells were determined 12 sec after endothelin stimulation. The concentration of Ca^{2+} in DMI-treated C6 cells in the absence of opioids was defined as 100% (control). Data are the mean ± SEM of three experiments. *, significantly different from controls or naltrexone-treated cells, $P < 0.01$.

(Bading et al., 1993). The results of these studies, as well as the endothelin-induced expression of the immediate early gene c-*fos* and of nerve growth factor mRNA in C6 cells (Yin et al., 1992; Ladenheim et al., 1993), support the existence of a similar neurotrophic mechanism. On the basis of the above and other recent findings (Berridge, 1993; Charpentier et al., 1993; Lin and Chuang, 1993), the possibility that opioids inhibit DNA synthesis via two interrelated processes—Ca^{2+} mobilization and PI turnover—is compelling.

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Figure 6. Representative computerized pseudocolor images of endothelin-induced increase of intracellular Ca^{2+} concentration in DMI-treated C6 glioma cells. *A,* Cultures preloaded with Fura-2AM were stimulated with 30 nM endothelin and images were obtained at the indicated times. The same cultures were extensively washed with DMEM (1.5 ml × 5), incubated with Ab2AOR (15 min), and reexposed to endothelin, and a second series of images was then taken. Control is defined as cells that were not treated with Ab2AOR. The experiments were repeated three times with new cultures. *B,* DMI-treated cultures preloaded with Fura-2AM were incubated with morphine, β-endorphin, and/or naltrexone (concentration of all opioids was 1 μM) for 15 min at 22°C, and stimulated with 30 nM endothelin. Ca^{2+} concentrations in various cells were determined 12 sec after endothelin stimulation. The basal level of Ca^{2+} concentration (before the addition of endothelin) was not affected by the various treatments. Control is defined as cells that were not treated with opioids. The experiments were repeated three times.

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