0026-895X/97/010152-09$3.00/0

MOLECULAR PHARMACOLOGY, **51:**152–160 (1997).

Agonist Activation of δ-Opioid Receptor but not μ-Opioid Receptor Potentiates Fetal Calf Serum or Tyrosine Kinase Receptor-Mediated Cell Proliferation in a Cell-Line-Specific Manner

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Received September 13, 1995; Accepted September 23, 1996

SUMMARY

Activation by opioid receptors of cell proliferation was examined with fibroblast cell lines stably expressing either δ-opioid or μ-opioid receptors. Addition of [D-Ala^2,D-Leu^5]-enkephalin or [D-Pen^2,D-Pen^5]-enkephalin to Chinese hamster ovary (CHO) cells transfected with δ-opioid receptor cDNA resulted in an agonist concentration-dependent potentiation of fetal calf serum (FCS)-stimulated cell proliferation. This potentiation by δ-opioid agonists was antagonized by naloxone and was not observed with the κ-opioid receptor selective agonist U50,488 or the μ-opioid receptor selective agonist [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin. This δ-opioid agonist effect was not observed at FCS concentrations >0.1% and could be blocked by pretreating cells with pertussis toxin, indicating that G_i/G_o were involved in this action. In addition, δ-opioid agonists could potentiate CHO cell proliferation stimulated by those growth factors that are mediated by tyrosine kinase receptors (i.e., insulin, insulin-like growth factor 1, and fibroblast-derived growth factor b). This δ-opioid agonist potentiation of growth apparently was dependent on the level of δ-opioid receptors that were expressed and had cell-line selectivity. Activation of δ-opioid receptors expressed in Rat-1 or NIH3T3 fibroblast did not result in a modulation of the cell growth induced by FCS or by growth factors. Interestingly, in CHO cells transfected with μ-opioid receptor cDNA, activation with agonists did not produce a potentiation of FCS-stimulated proliferation. This lack of μ-opioid receptor effect was not due to the differences among CHO clones. In a CHO cell line transfected with both δ-opioid receptor cDNA and μ-opioid receptor cDNA, activation of δ- but not μ-opioid receptors resulted in a potentiation of growth. These data suggest that δ- and μ-opioid receptors in CHO cells activate similar but divergent second messenger pathways, resulting in the differential regulation of cell growth.

Numerous studies have indicated that endogenous opioid peptides, products of proenkephalin or pro-opiomelanocortin, inhibit DNA synthesis in *in vitro* neuronal or glial cultures or in neuroblastoma cells (1–4). These studies were supported by the observation that opioid antagonists could stimulate DNA synthesis and proliferation in some neuroblastoma cell lines. The opioid antagonist, naltrexone, stereoselectively stimulates cell proliferation in murine neuroblastoma NS20Y and N1E-115 cells, human neuroblastoma SK-N-MC cells, and human fibrosarcoma cells (5). This antagonistic effect on NS20Y cells was demonstrated further by the ability of *in vivo* administration of naltrexone to stimulate the growth of these cells when they were implanted in rodents (6).

This research is supported in part by National Institute on Drug Abuse Grants DA07339 and DA05695.

The suppression effects exhibited by opioid peptide agonists and the stimulatory effects exhibited by naltrexone on neuroblastoma proliferation were rather surprising. By contrast, a substantial volume of evidence suggests that activation of GPCRs could lead to the enhancement of cell proliferation, mitogenesis, and tumorigenicity. Activation of bombesin receptors in Swiss 3T3 fibroblasts and small-cell lung carcinomas resulted in a stimulation of the mitogenic response (7, 8). Activation of transfected serotonin receptors (5-HT_{1B}, 5-HT_{1C}, 5-HT_2), muscarinic receptors, or α_2-adrenergic receptors in Chinese hamster lung fibroblasts, Rat-1 fibroblasts, or NIH3T3 fibroblasts resulted in increases in DNA synthesis, which are incidents of foci formation and growth rates (9–13). These positive effects on growth exhibited by GPCRs were demonstrated further by the ability of constitutively active receptor mutants (14) or constitutively

ABBREVIATIONS: GPCR, G-protein coupled receptor; DADLE, D-Ala^2,D-Leu^5-enkephalin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; DAMGO, [D-Ala^2-*N*-methyl-Phe^4-Gly-ol]-enkephalin; CHO, Chinese hamster ovary; PTX, pertussis toxin; IGF, insulin-like growth factor; FGF, fibroblast-derived growth factor; FCS, fetal calf serum; MAPK, mitogen-activated protein kinases; DOR-1, δ-opioid receptor cDNA; MOR-1, μ-opioid receptor cDNA; 5-HT, 5-hydroxytryptamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

active G protein α-subunit mutants, *gip* (G_{i2} mutant), to stimulate growth (15). These studies and others indicate clearly that activation of GPCRs by agonists and the concomitant activation of heterotrimeric G proteins result in a positive regulation of cell growth or in a potentiation of cell growth stimulated by endogenous growth factors.

The observed positive regulation of neuroblastoma growth by naltrexone implies that activation of opioid receptors, which are members of the GPCR super family, would inhibit cell growth. These observations also suggest that the neuroblastoma cells used in previous studies must release endogenous opioids and that the opioid receptors are therefore under tonic negative control by these endogenous opioid ligands. In support of this suggestion was the demonstration of the presence of Met^5-enkephalin immunoactivity in NS20Y cells as reported by Zagon (16). However, our recent studies with naloxone suggested that blockade of δ-opioid receptors in neuroblastoma NS20Y and N1E-115 attenuated FCS-induced cell proliferation (17). This effect of naloxone was mimicked by pretreating these cells with PTX. In those studies, a positive effect of agonists such as Met^5-enkephalin on growth was not demonstrated. One probable explanation is that the endogenous ligands have maximally activated the opioid receptors in these cell lines with respect to growth control. As such, a positive response to opioid agonists would be observed only if such influences by endogenous ligands were removed. With the cloning of δ-opioid receptors by Evans *et al.* (18) and Kieffer *et al.* (19), followed by cloning of μ- and κ-opioid receptors by others (20–25), it is now possible to examine the role of opioid receptors on cell growth directly without the interference of endogenous ligands. Therefore, in the present communication, stable expression of δ- and μ-opioid receptors in CHO cells, NIH3T3 fibroblasts, and Rat-1 fibroblasts was established. Activation of these receptors and the subsequent effects on cell growth were examined. It could be demonstrated that δ-opioid agonists but not μ-opioid agonists can potentiate FCS or tyrosine kinase receptor-mediated cell proliferation in both a dose-dependent and cell-line-specific manner.

Experimental Procedures

Transfection of fibroblasts with plasmids containing DOR-1 or MOR-1. Fibroblasts were transfected stably with DOR-1 in pCDNA1neo or MOR-1 in pRC/CMV plasmids using the $CaPO_4$ precipitation method as described by Chen and Okayama (26). The construction of these plasmids was carried out as described previously (20, 27). Cells that stably incorporate these plasmids were selected by culturing cells in the presence of 1 mg/ml geneticin, and the level of expression of the opioid receptors was determined by whole-cell binding using [^{3}H]diprenorphine (50,000 cpm; specific activity, 39 Ci/mmol) in 25 mM HEPES buffer at pH 7.6. Nonspecific binding was determined in the presence of 10 μM naloxone. To establish a CHO cell line coexpressing both DOR-1 and MOR-1, MOR-1 was subcloned into pREP4 plasmid at the *Hin*dIII site, with correct orientation of the insert checked by restriction enzymes digestion. This plasmid was then introduced into CHO cells stably expressing DOR-1 (CHODORX1-15) by the calcium phosphate precipitation method. CHO cells expressing both opioid receptors were selected using hygromycin (1 mg/ml) in the presence of 0.25 mg/ml geneticin. The presence of both μ- and δ-opioid receptors in the selected clones was determined by carrying out [^{3}H]diprenorphine binding in the presence of 1 μM DAMGO and in the presence of 1 μM Met^5-enkephalin. Because Met^5-enkephalin has similar affinity for μ- and δ-opioid receptors, the difference in specific binding determined in the presence of DAMGO and in the presence of Met^5-enkephalin should reflect the δ-opioid receptor binding in these cells.

Opioid receptor activities in transfected cells. Opioid receptor binding in various fibroblasts was carried out using membrane preparations devoid of nuclei in 25 mM HEPES buffer, pH 7.6, containing 10 mM $MgCl_2$ at 24° for 90 min as described previously (28). In each assay, 150–300 μg of protein was used, depending on the level of receptor being expressed in each clone. [^{3}H]Diprenorphine concentrations, from 0.1 to 10 nM, were used to carry out saturation binding studies. The K_d and B_{max} values for each clone were determined by computer analysis of the binding data using the LIGAND program (provided by Dr. Peter J. Munson, National Institutes of Health, Bethesda, MD). In each case, 10 μM DADLE was used to estimate nonspecific binding. The ability of opioid agonist to inhibit 10 μM forskolin-stimulated intracellular [^{3}H]cAMP production in the transfected cell lines was determined as described previously (28) using [^{3}H]adenine to label intracellular ATP pools. Radioactive cAMP generated in the presence of forskolin and various concentrations of agonists were separated from other nucleotides using Dowex and alumina column chromatography as described by White and Karr (29). The IC_{50} values of agonists and maximal inhibitory levels were obtained by computer curve-fitting of dose-response curves using Sigma Plot (Jandel Scientific, San Rafael, CA).

Effects of opioid ligands on cell growth. Opioid agonist effects on cell proliferation were determined using CellTiter 96 nonradioactive cell proliferation/cytotoxicity assay kits supplied by Promega (Madison, WI). This kit determines the number of viable cells in each assay by the reduction of a 3-(4,5-dimethylthiazol-2-yl)2-5-diphenyl tetrazolium salt into blue 3-(4,5-dimethylthiazol-2-yl)2-5-diphenyl tetrazolium-formazan (30). In a typical assay, 2×10^6 cells were seeded into T-75 cm^2 flasks and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS for 48 hr. The serum was then removed and cells were cultured in serum-free medium for an additional 48 hr to arrest growth and maintain cells in the quiescent G_0 phase of the cell cycle. Subsequently, cells were detached with phosphate-buffered saline containing 0.4% EGTA, and 5,000–20,000 cells were seeded into each well of 96-well culture plates. Wells contained 10 μg/ml transferrin, 100 units/0.1 mg/ml penicillin, 100 units/0.1 mg/ml streptomycin, various concentrations of opioid ligands, and variable amounts of FCS or other growth factors. At various time points, 15 μl of tetrazolium dye was added to each well and cells were incubated with dye for 4 hr at 37°. A volume of 100 μl of solubilization solution was added and the resulting blue color allowed to develop overnight at room temperature. Plates were read at 570 nm/630 nm with an enzyme-linked immunosorbent assay plate reader. The number of viable cells in each well was then calculated from standard curves constructed for individual cell types. Values from four separate wells were averaged in each experiment. For studies that involved concentration-dependent measurements, tetrazolium dye was added to each well 72 hr after the addition of the growth factors and/or opioid ligands.

Materials. [^{3}H]Diprenorphine (36 Ci/mmol) and [^{3}H]adenine (23 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). All opioid peptides were supplied by Peninsula Laboratory (Belmont, CA). Naloxone was the generous gift of Endo Laboratory (Garden City, NY). CellTiter 96 nonradioactive cell proliferation/cytotoxicity assay kits were supplied by Promega. All growth factors were purchased from Gibco/BRL (Grand Island, NY). All other reagents and buffers were purchased from Sigma Chemical (St. Louis, MO).

Results

To examine the effect of opioid receptor activation on cell proliferation, DOR-1 was transfected and expressed stably in fibroblast cell lines CHO, NIH3T3 and Rat-1. In earlier studies, we have demonstrated that δ-opioid receptors expressed

in CHO cells could couple to multiple G proteins simultaneously and could regulate intracellular cAMP level in a receptor concentration-dependent manner (27, 31). Whether these δ-opioid receptors, when activated, could regulate fibroblast cell proliferation was then investigated. In our previous studies with NS20Y cells and other neuroblastoma cell lines, opioid antagonists exert their effects only after the cells were growth arrested at G_0/G_1 phase of the cell cycle (17). Hence, the CHO-transfected cell line, DORX1-15, expressing 1.42 pmol receptor/mg protein or 854,000 δ-opioid receptors per cell (27), were growth arrested by serum removal. As shown in Fig. 1, upon addition of 0.05% FCS, there was an immediate increase in the number of viable cells in each well.

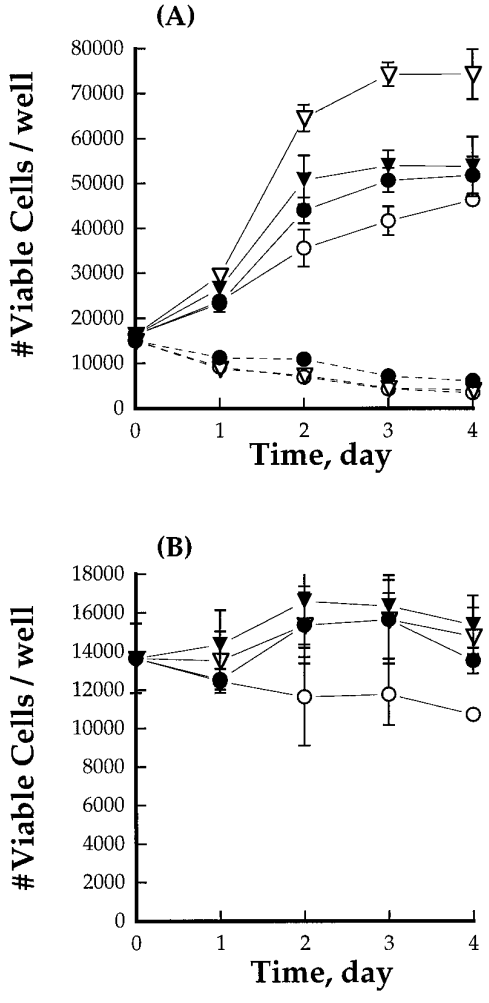

Fig. 1. Effect of δ-opioid receptor activation on the time-dependent increase in viable CHO cells cultured in the presence or absence of 0.05% FCS. Culturing and growth arrest of CHODORX1-15 cells were carried out as described in Experimental Procedures. A, Cell proliferation was initiated by the addition of 0.05% FCS in absence (○), in the presence of 10 nM DADLE (△), in the presence of 10 μM naloxone (●) or in the presence of 10 nM DADLE and 10 μM naloxone (▲) (connected by *solid lines*). Total number of viable cells were determined once every 24 hr. Similar experiments were carried out without the addition of 0.05% FCS in the absence (○) or in the presence of 10 nM DADLE (●), or in the presence of 10 μM naloxone (△) (connected by *broken lines*). B, CHODORX1-15 cells were treated with 100 ng/ml of PTX 24 hr before addition of FCS. The effects of absence (○) of DADLE, in the presence of DADLE (△), in the presence of naloxone (●), or in the presence of DADLE and naloxone (▲) on the proliferation of PTX-treated cells also were determined.

After 96 hr, the number of viable cells was determined to be 52,900 ± 3,500, a 3.2-fold increase in cell number from day 0. When 10 nM DADLE was included in the assay, there was apparent potentiation of the FCS effect. This potentiation was not observed during the first 24 hr. After 96 hr, the number of viable cells in each well was determined to be 75,900 ± 1,300, a 4.6-fold increase (Fig. 1A). The opioid effect could be blocked by the presence of 10 μM naloxone. This antagonism did not reach its maximal level until the fourth day. Ninety-six hours after the addition of 0.05% FCS, in the presence of DADLE and naloxone, the number of viable cells in each well was determined to be 54,100 ± 380, a 3.3-fold increase in cell number, which was not different from the effect of FCS alone. Naloxone alone had no significant effect on the growth increase mediated by FCS. In comparison, when no serum was added back after growth arrest, DADLE did not promote DORX1-15 proliferation (Fig. 1A). The viability of these CHO cells actually decreased with time. During the day tested, DADLE only decreased the rate of cell death during the assay and did not increase the number of viable cells per well from day 0.

The number of viable CHO cells per well was dependent on the concentration of serum added. As shown in Fig. 2, the number of CHO DORX1-15 cells increased from 11,000 ± 4,500 cells/well in the absence of FCS to 132,000 ± 3,000 cells/well in the presence of 10% FCS. Similar to the time course studies, 10 nM DADLE increased the number of viable cells per well as compared with those with FCS alone. This opioid agonist effect was observed only at FCS concentrations less than 0.1%. In the presence of DADLE, the number of cells per well at maximal concentration of FCS was calculated to be 128,000 ± 3,300, which was similar to the control

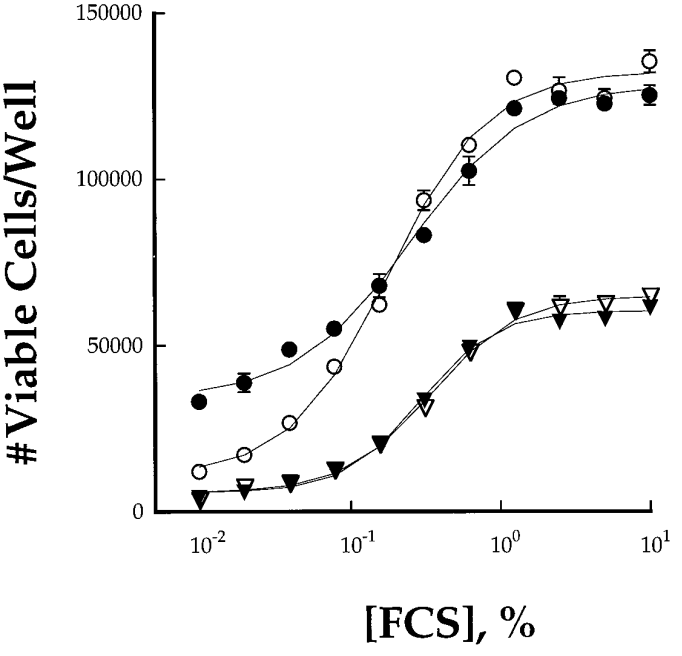

Fig. 2. Effect of DADLE on FCS-concentration-dependent stimulation of cell growth in control (○, ●) and PTX-treated (△, ▲) CHODORX1-15. After growth arrest, CHODORX1-15 proliferation was stimulated by addition of various concentrations of FCS in the absence (○) and in the presence (●) of 10 nM DADLE. The number of viable cells was determined 72 hr later. Similar concentrations of FCS were used to stimulate PTX-treated cells in absence (△) or in presence (▲) of DADLE.

value. Furthermore, DADLE did not alter the EC_{50} values of FCS to stimulate the proliferation of CHO cells: 0.18 ± 0.02% in the presence of DADLE as compared with 0.25 ± 0.03% in the absence of DADLE. Again, this potentiation of FCS activity exhibited by DADLE could be blocked by the presence of naloxone. The FCS concentration-dependent curves in the presence of 10 nM DADLE and 10 μM naloxone or 10 μM naloxone alone were indistinguishable from that of controls (data not shown).

This opioid agonist stimulation of CHO DORX1-15 proliferation at low FCS concentrations was mediated via the G proteins, G_i/G_o. Pretreatment of CHO cells with 100 ng/ml PTX during the last 24 hr of serum withdrawal and the inclusion of the toxin during the readdition of serum could block the opioid stimulation completely. This amount of PTX was chosen because pretreatment of CHO cells with this concentration could inhibit completely opioid regulation of adenylyl cyclase and could abolish ADP-ribosylation of G_i/G_o by ^{32}P-NAD$^+$ and PTX (31). As shown in both Figs. 1 and 2, PTX greatly reduced the proliferation of cells in each well. PTX treatment prevented the ability of 0.05% FCS to stimulate CHODORX1-15 to proliferate. The toxin itself did not reduce the viability of the cells during the 4-day assay, either in presence or absence of DADLE and/or naloxone (Fig. 1B). Instead, the toxin effect was observed to be inhibiting FCS-stimulated proliferation; the maximal level of cells in each well after 4 days was drastically lower: 65,000 ± 1,300 cells/well compared with 132,000 ± 3000 cells/well in control cells (Fig. 2). After PTX treatment, the concentration of FCS needed to elicit 50% of maximal stimulation also was increased to 0.34 ± 0.025%, compared with 0.18 ± 0.02% in control cells. At low FCS concentrations, DADLE added to PTX-pretreated CHO cells did not enhance cell proliferation (Figs. 1B and 2). The number of viable cells at the maximal level of FCS in the presence of 10 nM DADLE was calculated to be 61,000 ± 1,500 cells/well, although the EC_{50} value of FCS was calculated to be 0.29 ± 0.026%, which is similar to the value obtained from cells treated with PTX alone.

The ability of DADLE to potentiate FCS-stimulated CHODORX1-15 proliferation was agonist concentration-dependent (Fig. 3). The EC_{50} value of DADLE was determined to be 1.2 ± 0.25 nM, which was similar to the IC_{50} value of DADLE to inhibit intracellular cAMP production in this cell line (31). DPDPE, a more selective δ-opioid receptor agonist, has an EC_{50} value of 2.0 ± 0.55 nM, whereas the μ-opioid receptor selective agonist DAMGO and the κ-opioid selective agonist U50,488 have minimal or no effect on FCS-stimulated CHODORX1-15 proliferation (Fig. 3). However, morphine, another μ-opioid selective agonist, could potentiate FCS-stimulated proliferation (Fig. 3). The EC_{50} value of morphine was calculated to be 96 ± 9.8 nM. This EC_{50} value of morphine was drastically lower than the IC_{50} value of morphine to inhibit adenylyl cyclase in this cell line, which was determined to be 600 nM (31). Again, inclusion of naloxone could antagonize this opioid effect, as demonstrated by the ability of 500 nM naloxone to decrease the potency and the maximal stimulatory level of DADLE (Fig. 3). Using the Cheng-Prusoff equation (32), the K_i value of naloxone was determined to be 10 nM, which was in agreement with the naloxone's affinity for δ-opioid receptor.

This δ-opioid effect was not limited to one particular CHO clone. Three other CHO clones expressing different levels of

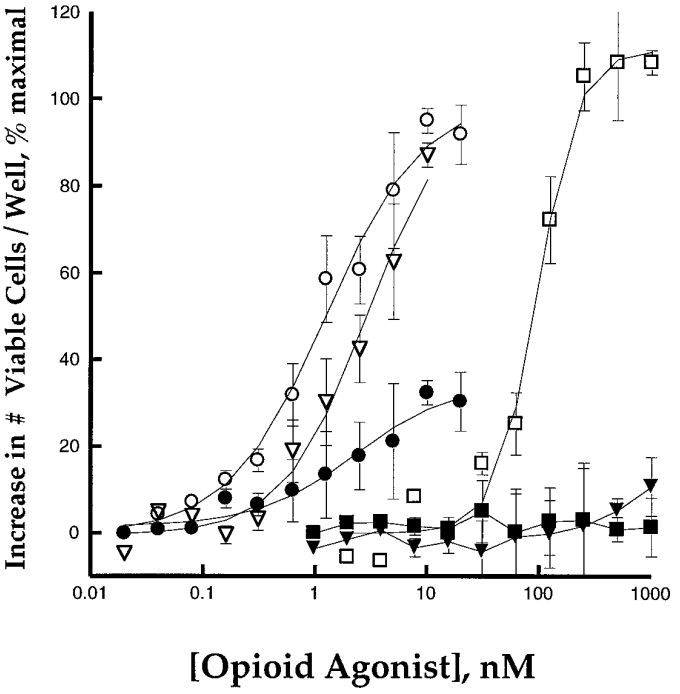

Fig. 3. Agonist dose-dependent potentiation of 0.05% FCS-stimulated CHODORX1-15 proliferation. The ability of 0.05% FCS to stimulate CHODORX1-15 growth 72 hr after the addition of serum was determined with various concentrations of DADLE (○), DPDPE (△), DAMGO (▲), U50,488 (□), morphine (■), and DADLE in the presence of 500 nM naloxone (●). The maximal level of cells was calculated to be 16,400 ± 1040 cells/well from the DADLE dose-response curve.

δ-opioid receptor were examined. As shown in Fig. 4, there was a DPDPE concentration-dependent potentiation of FCS-stimulated CHO cell proliferation in three of the four clones tested. In addition to CHODORX1-15, both CHODORBH3-2 and CHODORX1-2 exhibited DPDPE concentration-dependent potentiation of growth. These two cell lines expressed

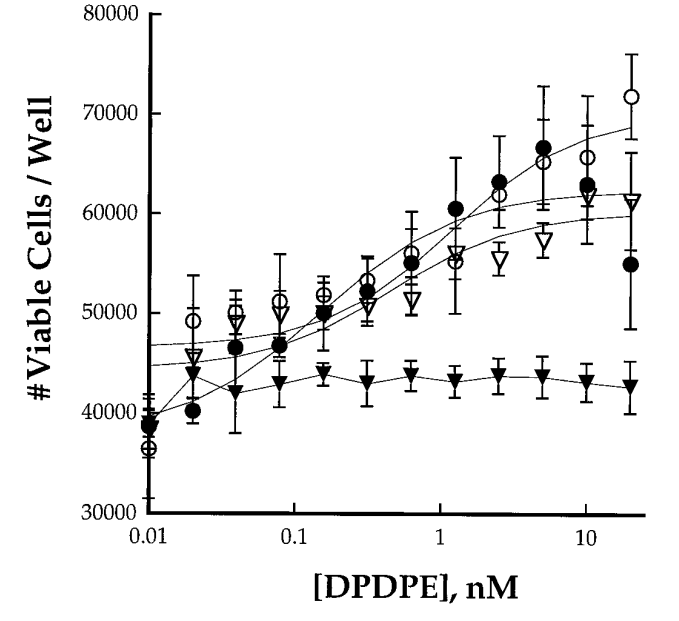

Fig. 4. Ability of various concentrations of DPDPE to potentiate 0.05% FCS stimulated growth in CHODORX1-15 (○), CHODORBH3-2 (●), CHODORX1-2 (△), and CHODORX1-18 (▲). The amount of viable cells in each well was determined 72 hr after addition of serum.

2.61 and 0.84 pmol receptor per mg protein, respectively (27). However, in CHODORX1-18, which expressed a relative low density of δ-opioid receptor, 0.03 pmol/mg protein, DPDPE did not potentiate FCS-stimulated cell proliferation. The EC_{50} values for DPDPE to potentiate this FCS-stimulated CHO cell proliferation in DORBH3-2 and DORX1-12 were determined to be 0.15 ± 0.12 nM and 0.45 ± 0.33 nM, respectively. These EC_{50} values compared favorably with that obtained with CHODORX1-15. Also, these EC_{50} values correlated well with the affinities of this opioid peptide for the δ-opioid receptors in these cell lines (27).

In addition to CHO cells, the ability of δ-opioid receptor activation to modulate the proliferation of other fibroblasts was investigated. Rat-1 and NIH3T3 cells stably expressing δ-opioid receptors were established by the calcium phosphate DNA precipitation method and selected with geneticin. One clone of each of these two fibroblasts was examined. The Rat1DORX1-18 clone stably expressed 5.2 ± 0.34 pmol opioid receptor/mg protein with a K_d value for [^{3}H]diprenorphine determined to be 2.3 ± 0.24 nM. Although the apparent affinity of this receptor for diprenorphine was comparatively lower than those observed with CHO clones, DADLE remained very potent in its inhibition of forskolin-stimulated intracellular [^{3}H]cAMP production in this cell line. Dose-dependent studies revealed that DADLE maximally inhibited [^{3}H]cAMP production by 81.7 ± 1.0% with an IC_{50} value determined to be 0.084 ± 0.01 nM in Rat1DORX1-18. As for the NIH3T3 cells stably expressing the δ-opioid receptor, NIH3T3DORX1-15 expressed 0.93 ± 0.14 pmol/mg protein receptor with a K_d value for [^{3}H]diprenorphine of 0.37 ± 0.08 nM. Again, DADLE was very potent in regulating intracellular [^{3}H]cAMP levels. Dose-dependent studies revealed that DADLE inhibited forskolin-stimulated intracellular [^{3}H]cAMP production by 74.1 ± 3.5% with an IC_{50} value of 0.46 ± 0.11 nM. Therefore, the binding and functional characteristics of the δ-opioid receptor populations expressed in these two fibroblast cell lines were comparable with those expressed in CHO cells, in which activation of the δ-opioid receptors resulted in potentiation of FCS-stimulated cell proliferation.

However, in contrast to the δ-opioid receptors expressed in CHO cells, activation of δ-opioid receptors in these two cell lines did not result in a potentiation of the FCS effect. When Rat1DORX1-18 cells were arrested at G_0/G_1 phase by serum withdrawal for 48 hr, readdition of 0.05% FCS elicited a time-dependent increase in the number of viable cells detected in each well (Fig. 5A). Addition of 10 nM DADLE did not increase the number of viable cells per well. The absence of opioid agonist effect was not caused by the inability of G_i/G_o to modulate FCS-stimulated proliferation in this cell line. Pretreatment of Rat1DORX1-18 cells with PTX during the last 24 hr of serum withdrawal resulted in drastic decrease in the ability of FCS to stimulate proliferation (Fig. 5A). Again, opioid agonist DADLE did not alter FCS effect on Rat1DORX1-18 proliferation after PTX pretreatment. As for NIH3T3DORX1-15 cells, addition of 0.05% FCS after serum withdrawal for 48 hr did not stimulate cell growth further. As a matter of fact, there was a time-dependent decrease in the number of viable cells per well (Fig. 5B). Addition of 10 nM DADLE did not stimulate cell proliferation. Instead, as in the case of culturing CHODORX1-15 in the absence of FCS (Fig. 1A), DADLE attenuated the time-dependent decrease in the number of viable cells per well. Culturing NIH3T3DORX1-15 cells in higher FCS concentrations resulted in cell proliferation and absence of any opioid agonist effects on the number of viable cells per well (data not shown). Again, pretreating this particular NIH3T3 clone with PTX during the last 24 hr of serum withdrawal resulted in an additional decrease in the number of viable cells per well and the absence of any measurable opioid effects on growth (Fig. 5B).

Analysis of the deduced amino acid sequences of the cloned opioid receptors shows a high degree of homology among δ-, μ-, and κ-opioid receptors (18–25). As might be expected, the coupling pattern to various G proteins was similar among these three opioid receptors (27, 33). Therefore, it might be predicted that when these opioid receptors are expressed in the same clonal cell line, μ- and κ-opioid receptor selective agonists might potentiate FCS-stimulated cell growth in the same manner as δ-opioid receptor agonists. Therefore, CHO cells were transfected with MOR-1 subcloned in the expression vectors pRc/CMV. These CHO cells (CHOMORIVA3) stably expressing MOR-1 at a level of 1.68 pmol/mg protein have been characterized in an earlier report (33). Although CHOMORIVA3 expressed a level of receptors similar to that

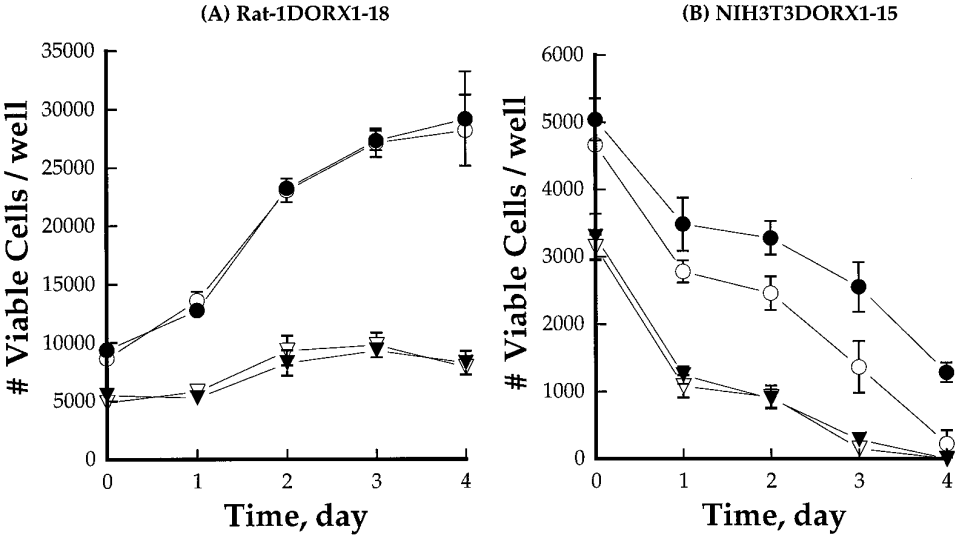

Fig. 5. Effect of DADLE and PTX on 0.05% FCS-stimulated growth in RAT1DORX1-18 (A) and NIH3T3DORX1-15 (B). The ability of 0.05% FCS to stimulate growth in these two cell lines, which had serum removed for 48 hr was determined in the absence (○) and in the presence (●) of 10 nM DADLE. Similar experiments were carried out with cells pretreated with 100 ng/ml of PTX 24 hr before addition of serum. The effect of PTX on serum-stimulated growth in absence (△) or in presence (▲) of 10 nM DADLE was determined in these two cell lines.

of CHODORX1-15, addition of 100 nM of either morphine or DAMGO had minimal effect on FCS-stimulated cell growth after growth arrest at G_0/G_1 phase (Fig. 6). Because the K_d values for morphine and DAMGO in this cell line were determined to be 1.2 nM and 1.6 nM, respectively (33), the absence of agonist effect on FCS-stimulated growth was not caused by the lack of affinity of these two ligands for the receptor. Furthermore, varying the concentration of these two agonists did not produce any potentiation of the FCS effect in CHOMORIV3 cells (data not shown).

One possible explanation for the lack of μ-opioid receptor effect on proliferation could be potential phenotypic differences between the CHOMORIV3 and other CHO cells stably expressing the δ-opioid receptor. To address this issue, CHO cells stably expressing both μ- and δ-opioid receptors were established. Such a cell line is established by transfection of CHODORX1-15 with MOR-1 that was subcloned into the *Hin*dIII site of vector pREP-4. The presence of μ-opioid receptors in CHO cells that survived hygromycin (1 mg/ml) selection was determined by comparison of the specific [^{3}H]diprenorphine binding, either in the presence of 100 nM DAMGO or 5 μM Met^5-enkephalin. Because Met^5-enkephalin has similar affinity for μ- and δ-opioid receptors (34), the differences between the specific binding determined by the μ-opioid receptor selective peptide DAMGO and receptor nonselective peptide Met^5-enkephalin should reflect δ-opioid receptor binding. Using such an approach, several clones were isolated, one of which (designated CHOMOR/DOR11) expresses similar levels of μ- and δ-opioid receptors. When Scatchard analysis was carried out with [^{3}H]diprenorphine and nonspecific binding was determined with 5 μM Met^5-enkephalin, a K_d value of 0.24 ± 0.035 nM and a B_{max} value of 2.4 ± 0.12 pmol/mg protein were obtained. When the same analysis was carried out using 5 μM of DAMGO to eliminate [^{3}H]diprenorphine binding to μ-opioid receptors, K_d and B_{max} values of 0.13 ± 0.055 nM and 0.93 ± 0.11 pmol/mg protein, respectively, were obtained. The same analysis was carried out using 5 μM DPDPE to eliminate binding to δ-opioid receptors. In this case, K_d and B_{max} values of 0.36 ± 0.072 nM and 1.64 ± 0.14 pmol/mg protein, respectively, were obtained. The sum of the number of binding sites determined for μ- and δ-opioid receptors was similar to that obtained using Met^5-enkephalin to determine the total receptor concentration. Also, the episomal expression of μ-opioid receptor did not seem to alter the chromosomal expression of δ-opioid receptor, as reflected in similar levels of δ-opioid receptors in CHOMOR/DOR11 and the parent cell line CHODORX1-15. These results indicated that μ-opioid receptor was expressed stably in CHOMOR/DOR11. Furthermore, the μ-opioid receptors in this cell line were coupled to the adenylyl cyclase, as were the δ-opioid receptor population. As shown in Fig. 7A, both DPDPE and DAMGO inhibited forskolin-stimulated intracellular [^{3}H]cAMP production in a dose-dependent manner. The maximal levels of inhibition for each of these two agonists were similar. Moreover, the IC_{50} values for DPDPE and DAMGO were determined to be 1.5 ± 0.52 nM and 8.7 ± 1.7 nM, respectively, meaning that these two opioid receptor selective agonists regulated adenylyl cyclase activity via activation of their selective receptors (27, 33). Therefore, in CHOMOR/DOR11, both μ- and δ-opioid receptors were present and were coupled to at lease one common effector system.

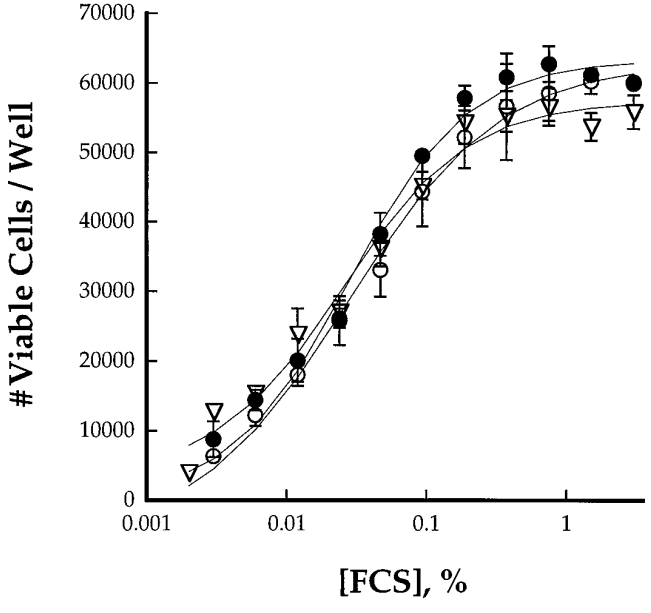

Fig. 6. FCS-concentration-dependent growth in CHOMORIV3. The ability of various concentrations of FCS to stimulate proliferation in CHOMORIV3 after growth arrest was determined in absence (○) or in presence of 100 nM DAMGO (●), or in the presence of 100 nM morphine (△).

Activation of δ-opioid receptors in CHOMOR/DOR11 by DPDPE exhibited a greater effect on FCS-stimulated cell proliferation than μ-opioid receptors activated by DAMGO in the same cells (Fig. 7B). At the maximal concentrations of agonists used, the number of viable cells per well in the presence of 100 nM DPDPE was determined to be 16,800 ± 590, which was markedly greater than the number of cells per well in the presence of 100 nM DAMGO (9,330 ± 390). As a matter of fact, the number of viable cells was not significantly different among the various concentrations of DAMGO tested (Fig. 7B). The EC_{50} value for DPDPE from Fig. 7B was determined to be 0.24 ± 0.36 nM, which was in agreement with the potency of DPDPE to regulate intracellular [^{3}H]cAMP level in these cells. Therefore, in the same cell line expressing both μ- and δ-opioid receptors, only the activation of δ-opioid receptors resulted in a potentiation of FCS effect.

In addition to having pronounced effects on FCS-stimulated proliferation, δ-opioid receptors also potentiated the effects of growth factors that activated protein tyrosine kinase receptors. As summarized in Fig. 8, of all the growth factors tested, only FGFa and platelet-derived growth factor ab did not exhibit a concentration-dependent stimulation of CHODORX1-15 proliferation. Insulin, IGF_1, and FGFb all stimulated the CHO cell proliferation in a dose-dependent manner. At the high concentrations tested, $IGF_2 > 100$ ng/ml also exhibited some activity. However, such effect of IGF_2 could be due to its activation of IGF_1 receptors. Nevertheless, in all cases, the presence of 10 nM DADLE drastically increased the number of viable cells per well (Fig. 8). Similar to the effects on FCS-stimulated cell proliferation, the δ-opioid effect was more pronounced at the low concentrations of growth factor. At the high concentrations of growth factors, the ability of DADLE to potentiate proliferation was not observed. The presence of DADLE did not alter the potency of these growth factors. For example, the EC_{50} values of FGFb

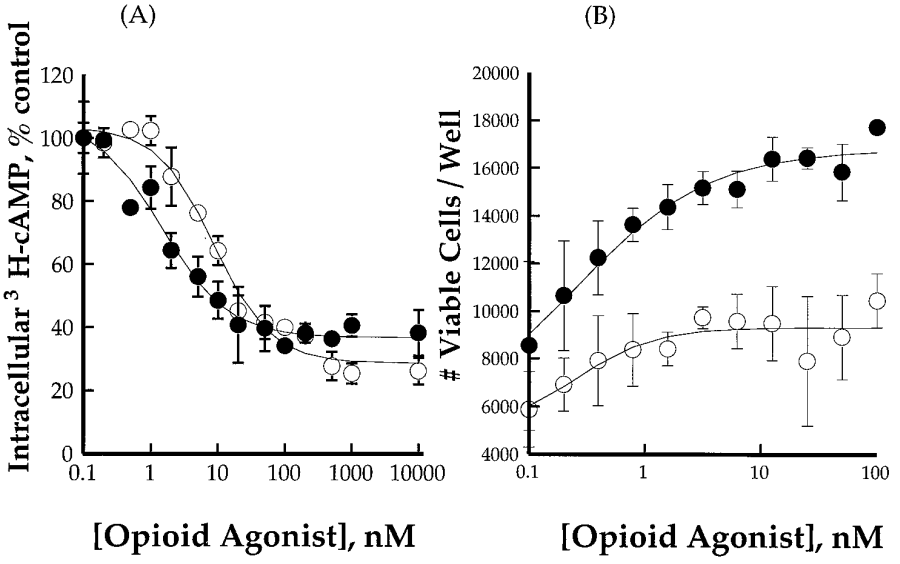

Fig. 7. Ability of various concentrations of DPDPE (●) or DAMGO ([crico]) (A) to inhibit 10 μM forskolin-stimulated intracellular [^{3}H]cAMP accumulation and (B) to potentiate 0.1% FCS-stimulated cell growth in CHOMOR/DOR11.

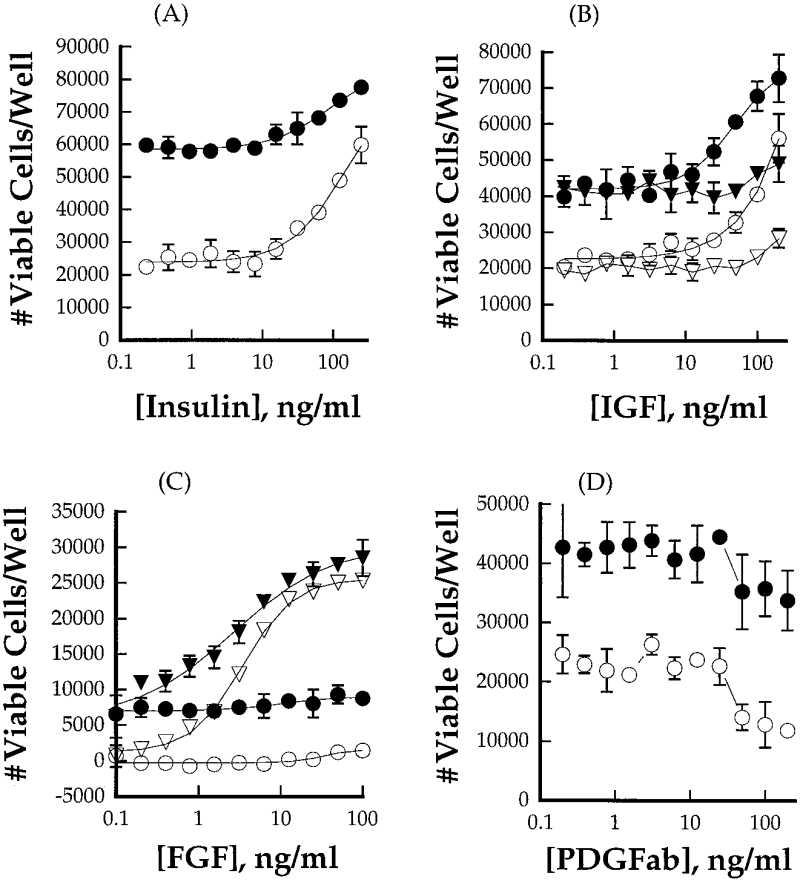

Fig. 8. Effect of the presence (●, ▲) or absence (○, △) of 10 nM DADLE on the ability of various concentrations of (A) insulin (○), (B) IGF1 (○) and IGF2 (△), (C) FGFa (○) and FGFb (△), and (D) platelet-derived growth factor ab (○) to stimulate CHODORX1-15 proliferation after growth arrest induced by serum removal.

to stimulate proliferation in the absence and in the presence of DADLE were calculated to be 3.5 ± 0.2 ng/ml and 2.6 ± 0.8 ng/ml, respectively. Therefore, as in the case of FCS, DADLE did not increase the number of viable cells at the maximal levels of growth factors used. This δ-opioid effect on growth factor action was also cell-line-selective. Similar to the FCS effect on growth, FGFb stimulated NIH3T3DORX1-15 proliferation in a dose-dependent manner. However, again similar to observations with FCS, 10 nM DADLE did not further stimulate the growth of this NIH3T3 cell line at any concentration of FGFb tested (data not shown).

Discussion

The current studies demonstrated that opioid agonists could potentiate the effects of growth factors in CHO cells that were in G_0/G_1 phase. However, similar to observations with other GPCR neuropeptides such as bradykinin (35),

δ-opioid peptides *per se* could not sustain growth themselves (Fig. 1A). Furthermore, as with growth-factor-stimulated fibroblast cell division (36), this opioid effect could be inhibited by PTX pretreatment, indicative of the involvement of the G proteins G_i/G_o in this opioid receptor activity. Although CHO cells might not serve as an ideal model for proliferating neurons or brain cells in G_0/G_1 phase, the data presented make the point that opioid receptors, similar to other receptors in the GPCR family, could regulate cell viability and growth.

It is apparent that there is receptor selectivity for the ability of opioid agonists to potentiate CHO cell proliferation induced by either FCS or growth factors. Activation of δ- but not μ-opioid receptors in CHO cells resulted in a potentiation of FCS- or growth-factor-stimulated growth that was PTX sensitive. Our preliminary studies with CHO cells transfected with κ-opioid receptor cDNA also demonstrated a potentiation of FCS-stimulated growth upon κ-opioid receptor activation (data not shown). Although the lack of a μ-opioid effect could have been caused by phenotypic differences among cell lines, this possibility was eliminated by our studies using CHO cells expressing both μ- and δ-opioid receptors. In this cell line, both μ- and δ-opioid receptors inhibited intracellular cAMP production. However, activation of δ- but not μ-opioid receptors resulted in the potentiation of FCS effect on growth (Fig. 7). The absence of μ-opioid receptor modulation of cell growth in this particular cell line distinguishes the growth promoting effect from other opioid receptor activities.

The disparate effects of μ- and δ-opioid receptors on FCS-stimulated proliferation are surprising in light of observations that these opioid receptors activate the same complement of G proteins (27, 33). The similarity in G proteins being activated could be predicted by the sequence homology among these opioid receptors, especially in the intracellular loops (18–25). To compound this finding, all three types of opioid receptors activate common effector systems. In transfected CHO cells, all three opioid receptors regulate adenylyl cyclase activity (27, 33). When coinjected with G protein-coupled, inward-rectifying potassium channels, these opioid receptors could open the K^+ channels when activated (37, 38). Regulation of voltage-dependent Ca^{2+} channels by μ-opioid agonists also was observed in GH3 cells transfected with MOR-1 (39). These multiple responses to opioid receptor activation were PTX sensitive, indicative of involvement of G_i/G_o. Because the δ-opioid receptor modulation of CHO proliferation was PTX-sensitive (Figs. 1 and 2) and the potencies of δ-opioid agonists to induce such response paralleled the potencies of agonists in regulating adenylyl cyclase activity (Fig. 3), the absence of μ-opioid receptor modulation of CHO proliferation suggests that this particular opioid receptor activity must lie downstream of the receptor/G protein interaction. It would seem that the effect of δ-opioid receptor activation is not mediated via opioid regulation of intracellular cAMP. This conclusion is supported by observations that (a) both μ- and δ-opioid receptors could regulate cAMP level in CHO cells (27, 33); (b) the potency of morphine to alter FCS-stimulated proliferation in CHODORX1-15 was different from that to regulate adenylyl cyclase activity; and (c) δ-opioid receptors could regulate cAMP levels in NIH3T3 and Rat-1 cells, whereas δ-opioid receptor activation in these cell lines did not result in the potentiation of cell growth. Such cell line specificity in growth potentiation is analogous to that observed previously with *gip2*, the oncogenic form of $Gi_2\alpha$ (15). Therefore, absence of the ability of μ-opioid receptors to potentiate CHO proliferation suggests a divergence in the effector systems regulated by these opioid receptors.

Alternatively, the difference in the ability of μ- and δ-opioid receptor to potentiate growth could be due to the differences in pathways rather than a difference in the effectors being regulated by these opioid receptors. Studies with other GPCRs have suggested that activation of MAPK involved in cellular proliferation could be mediated via different cellular components. For example, α_{1B}- and α_{2A}-adrenergic could both activate MAPK via phospholipase C. However, α_{1B}-adrenergic receptor activated MAPK via the $p21^{ras}$ independent pathway mediated by protein kinase C and $p74^{raf}$ (40). Meanwhile, α_{2A}-adrenergic receptor activated MAPK via $p21^{ras}$ and $p74^{raf}$ independent protein kinase C pathway (40). However, activation of phospholipase C by GPCRs might not be able to account for all the GPCR effects on cell proliferation. An excellent example is the M_1-muscarinic receptor modulation of the growth of NIH3T3 cells. Although M_1-muscarinic receptors can activate phospholipase C_β, which then leads to elevation of IP_3 and intracellular Ca^{2+} level, activation of phospholipase C_β alone is not sufficient to activate MAPK. As well, mutation of Glu209 of G_q resulted in a constitutively activated G_q, which subsequently caused an elevated level of IP_3 without activation of MAPK (41). It is possible that the difference between the ability of δ- and μ-opioid receptors to potentiate growth-factor-stimulated cell proliferation is a reflection of an intrinsic difference in their ability to active the MAPK or a similar network of protein kinases that promote proliferation. The mechanism behind these observations remains to be elucidated.

In addition to the potentiation of FCS-stimulated CHO proliferation, activation of δ-opioid receptors also potentiated growth factors that mediate their actions via tyrosine kinase receptors. As summarized in Fig. 8, DADLE could potentiate the effects of insulin, IGF1, and FGFb. These data clearly indicated interaction or "cross-talk" between opioid receptors and tyrosine kinase receptors. Interaction between tyrosine kinase receptors and GPCRs have been well documented. In fact, it has been proposed that GPCRs modulate the tyrosine kinase receptors activities at Raf-1 protein kinase (42). Recent studies using the yeast two hybridization system suggested that there is a direct interaction between Raf-1 protein kinase and βγ subunits of G proteins (43). Again, whether opioid receptor potentiation of CHO cell proliferation is mediated by a similar mechanism remains to be demonstrated.

Acknowledgments

We thank Drs. Anna Marie Babey and Derek Strassheim for reading the manuscript.

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