Differential effects of opioid and adrenergic agonists on proliferation in a cultured cell line

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Abstract. A novel clonal cell line transfected with the δ -opioid receptor (δ -OR) encoding gene was used to study agonist-activated regulation of cell proliferation. In this cell line, endogenous β_2 -adrenergic receptors (β_2 -ARs) are coexpressed with the exogenous δ -ORs. Upon individual acute treatments with morphine and procaterol (a selective β_2 -AR agonist), both the δ -OR and β_2 -AR are coupled to differential modulation of cyclic AMP (cAMP) levels in accord with the classical second messenger response patterns to these agonists in the normal cellular settings of the receptors. But chronic morphine activation of the δ -OR *inhibits* cellular proliferation, while chronic procaterol activation of the β_2 -AR *stimulates* it. Chronic treatment with the individual agonists is accompanied by differential activation of the mitogen-activated protein kinase (MAPK) isozymes, extracellular-regulated kinase (ERK) and c-Jun *N*-terminal kinase (JNK). The findings suggest that chronic β_2 -AR activation stimulates proliferation by interacting with the ERK signalling cascade independent of a cAMP-mediated pathway. In contrast to treatment with individual agonists, chronic dual agonist treatment suppresses procaterol-induced stimulation of ERK activity and stimulation of proliferation indicating that a cross-regulatory interaction occurs between the δ -OR and β_2 -AR signalling systems in the cells under these conditions.

INTRODUCTION

The molecular events associated with acute opioid receptor (OR) activation, such as the inhibition of adenylate cyclase (AdC), an increase in K^+ conductance, and the inactivation of Ca^{2+} channels, are well characterized in neural cells. The molecular events associated with *chronic* opioid administration, however, are just beginning to be understood, and are thought to involve long-term changes in gene expression (Widnell *et al.* 1996). In addition to neural cells, ORs have also been identified in non-neural cells, raising the question of how chronic opioid exposure affects molecular signalling events in these cells.

Activation of classical opioid receptors by the endogenous enkephalins affects proliferation of neural and non-neural cells both *in vivo* and *in vitro* (Leslie, Chen & Winzer-Serhan 1998, Zhu & Pintar 1998). However, using a knockout mouse model, it has been conclusively shown that the classical effects of activation of ORs by exogenous opioids (e.g. morphine), including analgesia, respiratory depression, reward effect, and withdrawal symptoms, are

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entirely a result of the activation of the μ -receptor subtype (Kieffer 1999). Both the enkephalins and morphine are relatively nonselective between the δ - and μ -receptor subtypes, and only recently have endogenous opioids the endomorphin peptides been identified that are highly selective for the μ -OR (Zadina *et al.* 1997).

The poor selectivities of the enkephalins, presents the challenge of understanding their broader physiological roles (if these peptides activate *both* δ - and μ -ORs, and the classical effects are due only to μ -OR activation, what nonclassical effects are mediated by δ -OR activation?) and a clinically relevant question also arises: does chronic exposure of cells to high levels of morphine cause δ -OR-mediated cellular effects that supersede or interfere with those of the endogenous enkephalins?

We have been investigating the cellular effects of chronic morphine treatment using an *in vitro* cell line (' δ -DA') expressing both δ -ORs and β_2 -ARs (Glasel & Agarwal 1997a, Glasel & Agarwal 1997b), which has at least one advantage in proliferation studies: the cells are cultured in a defined medium that requires only one exogenous growth factor (insulin) to sustain growth. This paper reports work centred on the pathways for nuclear signalling in δ -DA cells following single or dual chronic treatments with morphine and procaterol.

In the δ -DA cell line, it was recently demonstrated that chronic activation of δ -ORs by morphine inhibits proliferation while maintaining anchorage dependence (Glasel & Agarwal 1997a). The cells rebound with a temporary increase in proliferation following withdrawal of chronic morphine (Glasel & Agarwal 1997b). Here, procaterol treatment is shown to stimulate proliferation of the cells (while maintaining anchorage dependence). To begin identifying signalling mechanisms associated with these differential agonist-activated effects, the effects of agonist treatments were examined on cAMP levels and activities of MAPK isozymes known to be involved in regulating cell growth and proliferation. Evidence is presented that the differential proliferation responses observed during individual treatments with morphine and procaterol are accompanied by selective and differential activation of the MAPK isozymes ERK and JNK.

To investigate possible signal cross-regulation (Selbie & Hill 1998) between activated δ -ORs and β_2 -ARs, proliferation, intracellular second messenger levels, and JNK/ERK activities under dual agonist treatment were measured. These results show that chronic dual agonist treatment suppresses β_2 -AR-stimulated ERK activity and suppresses the proliferation increase associated with β_2 -AR stimulation alone indicating that δ -OR/ β_2 -AR cross-regulation does occur in these cells.

MATERIALS AND METHODS

Materials

AIM-V serum-free culture medium and enzyme-free cell dissociation buffer were purchased from Gibco BRL (Middletown, CT, USA). Morphine sulphate was a gift from the State of Connecticut Department of Drug Control. Other materials used include: naloxone-HCl (DuPont Pharmaceuticals, Wilmington, DE, USA); procaterol-HCl, 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium bromide (MTT), 3-isobutyl-1-methyl-xanthine (IBMX, Sigma, St Louis, MO, USA); 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium (MTS, Promega, Madison, WI, USA); ICI 118 551 HCl, forskolin, ($\pm$)CGP-12177 HCl, PD 098 059 (Research Biochemicals International, Natick, MA, USA); TRI Reagent, oligo-dT columns (Molecular Research Center, Inc., Cincinnati, OH, USA); AmpliWax PCR Gems used for hot start PCR (Perkin Elmer, Norwalk, CT, USA); [α - 32 P]dATP (3000 Ci/mmol), [γ - 32 P]ATP (3000 Ci/mmol), [3 H]($-$)CGP-12177 (New England Nuclear, Boston, MA, USA); the TRK 432 cAMP [3 H] assay system (Amersham, Arlington

Heights, IL, USA); epidermal growth factor receptor (EGFR) (663673) peptide (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA); and agarose-conjugated glutathione-S-transferase (GST)-c-Jun (1-169) (Upstate Biotechnology, Inc., Lake Placid, NY, USA).

Cell culture and drug treatment

Frozen seed stocks (passage number 80) of δ -DA and vector-only transfected control cells (v-DA) were grown and passaged in 75 cm² culture flasks as previously described (Glasel & Agarwal 1997a). Cells were seeded at 1×10^5 cells/flask in AIM-V medium, subcultures were maintained at 80% confluence, fed every 3 days and passaged approximately every 6 days. Cell numbers were monitored by counting viable cells in a haemocytometer using the trypan blue dye exclusion method. In all cases involving chronic drug treatment, growth medium and drug supplements were replenished every 48 h.

Preparation of mRNA

Polyadenylated [poly(A⁺)] RNA was prepared from cells using TRI Reagent LS and oligo-dT columns according to the manufacturer's instructions. Cells were grown to a density of approximately 1×10^6 cells/75 cm² culture flask, as determined by counting viable cells in a haemocytometer. Eight flasks of cells yielded a total of 2–5 μ g of polyA⁺ mRNA.

RT-PCR

Reverse transcribed, DNAase I treated mRNA was amplified by hot start polymerase chain reaction (PCR) in the presence of [α^{32} P]dATP and a set of degenerate primers described elsewhere (Libert *et al.* 1989) using a Cetus DNA thermal cycler (95°C, 1.5 min; 94°C, 30 s; 55°C, 1.5 min; 72°C, 1.5 min; 35 cyclers followed by a 5-min incubation at 72°C). Mouse actin-specific primers were used in the β -actin control reactions. The reaction products were visualized by autoradiography after electrophoresis on a high-resolution polyacrylamide sequencing gel and, as required, were isolated from the gels by cutting out the bands, re-amplified by PCR, and then ligated into a T-tailed pBluescript SK vector for bacterial amplification. The complete nucleotide sequences of the cloned and amplified PCR products were obtained by sequencing the double-stranded insert.

β_2 -AR radioreceptor binding assay

δ -OR radiobinding assay methods and results for δ -DA cells have been previously reported (Glasel & Agarwal 1997a) and the protocols used in the radioreceptor assay and protein determination reported here for specific β -adrenergic agonist binding were the same.

For the β -adrenergic radioreceptor binding assay, a highly selective radiolabeled ligand for β -ARs was used, [3 H](–)CGP-12177 (48.6 Ci/mmol), in concentrations ranging from 0.1 to 2 nM (Staehelin *et al.* 1983). Nonspecific binding was determined in the presence of 10 mM ($\pm$) CGP-12177. Reactions were performed in triplicate. Derived ligand binding parameters (K_D and B_{max}) were determined from nonlinear regression analysis of the specific binding data.

Cell proliferation measurements

Cells were seeded in flat 96-well tissue culture plates at 1000–2000 cells per well in 100 μ l culture medium. Cell proliferation was quantified using a modified colorimetric MTT or MTS assay (Mosmann 1983) as previously described (Glasel & Agarwal 1997a). δ -DA cells were cloned in soft agar in the presence of 100 μ M procaterol and AIM-V as previously described for other cell lines using *Ras*-transformed 3T3 cells growing in 10% serum-containing medium as a positive control (Glasel & Agarwal 1997a).

Measurement of cAMP levels

Cells were plated in 96-well culture plates at 4×10^4 cells/well and allowed to grow for 24 h in AIM-V before agonist was added to the culture medium for either 15 min or 4 days as specified. As a positive control, untreated cells were stimulated with 100 μ l phosphate-buffered saline (PBS) containing 0.5 mM IBMX plus 10 μ M forskolin for 15 min. After incubation, the culture medium was aspirated, reactions were terminated and cAMP was extracted by replacing the PBS with 0.1 M HCl followed by scraping the cells from the wells and homogenizing them. The plates were then allowed to stand overnight at room temperature. Each well was diluted fivefold in Tris/EDTA buffer and used for measurements of cAMP levels using the Amersham competition binding assay system according to the manufacturer's protocol.

ERK activity

Log phase cells seeded at 5×10^5 cells/75 cm² culture flask were incubated with or without agonist for either 15 min or 4 days as specified. As a positive control for ERK activation, cells were incubated in 20% calf serum for 15 min (Fukuda *et al.* 1996). Cell lysates were prepared and ERK activity isolated as described (Mitchell, Russell & Johnson 1995). The ERK extracts were normalized for protein content using the Bradford method (Bradford 1976). ERK activity was measured using EGFR 663–673 peptide as substrate according to the method outlined by Gardner *et al.* 1994.

JNK activity

Log phase cells seeded at 5×10^5 cells/75 cm² culture flask were incubated with or without agonist for either 15 min or 4 days as specified. As a positive control for JNK activation, cells were irradiated using UV-C at 1400 μ W/cm² for 10 min and then incubated at 37°C for 30 min. Cell lysates were prepared and JNK activity isolated as described (Prasad *et al.* 1995), with the exception that the Bradford protein assay was used to normalize for protein content. GST-c-Jun (1–169)-glutathione-agarose beads were used as a substrate for JNK (Minden *et al.* 1994) according to the protocol provided by the supplier.

Statistics

Cell proliferation measurements represent data collected from eight independent samples. Measurements of binding cAMP levels, and kinase activity levels represent data collected from three independent samples. Values shown represent the mean $\pm$ standard deviation values. Statistical significance of the collected data was tested by ANOVA using a two-tailed Student's *t*-test.

RESULTS

Analysis of GPCR mRNA expression patterns in the 3T3DA cell lines

RT-PCR analysis with degenerate primers was used to confirm the presence of the correct GPCR genes in transfected cell lines. The primers used here are designed to prime highly conserved approximately 30-mer nucleotide sequences found in the transcripts encoding the III and VI transmembrane segments of a large number of members of the GPCR superfamily (Libert *et al.* 1989). In this case, results of RT-PCR are 500–600 bp products characteristic of each different primed GPCR cDNA. Cloning and sequencing of the PCR-amplified cDNA from transfected cells confirmed the identity of the δ -OR (Evans *et al.* 1992). However, among the cells tested, an additional PCR-amplified product appears only in δ -opioid

Figure 1. Radiolabeled RT-PCR of GPCR mRNA expression in the β -DA line and control lines, v-DA, and 5HT-DA (DA cells transfected with the gene encoding the serotonin 5HT_{2C} receptor). As a control for specificity and purity, PCR was also performed with either the GPCR primers and H₂O, or β -actin primers and each of the cell-specific cDNAs. Aliquots of the final PCR products were subjected to 5% denaturing polyacrylamide gel electrophoresis at sequencing resolution and visualized by exposure of the gel to autoradiography film.

receptor gene transfected cells (Figure 1). The sequence of this additional PCR product band was identical to mouse β_2 -AR gene cDNA (Allen *et al.* 1988) over a length of 461 bp (sequencing data not shown), indicating that transcription of the endogenous mouse β_2 -AR gene is fortuitously stimulated in the cells which have been transfected with exogenous mouse δ -OR gene cDNA. (The mouse β -AR gene, whose product in tissues is often found to be co-expressed with that of β_2 -AR gene, has only 60% homology to the β_2 -AR gene in the sequenced region, making the the present identification of the β_2 -AR gene certain.)

Radioligand-receptor binding analysis

The analysis of adrenergic agonist binding (Figure 2) to δ -DA cells revealed that β -ARs are expressed and resulted in derived binding parameters; dissociation constant, $K_D = 0.24 \pm 0.21$ nM; $B_{\max} = 29.1 \pm 7.7$ fmole/mg protein. The $B_{\max}$ corresponds to $\sim 10\,000$ binding sites per cell. For comparison, the published values for binding of the same ligand to β -ARs endogenously expressed on C6 glioma cells is $K_D \sim 0.2$ nM with $B_{\max} \sim 8500$ binding sites per cell (Staehelin *et al.* 1983).

Differential modulation of cAMP levels by δ -OR and β_2 -AR agonists

Inhibition of AdC activity resulting in decreased cAMP production is characteristic of the activation of G_i-initiated signalling by endogenously expressed δ -ORs (Sharma, Nirenberg & Klee 1975). Dose-dependent, antagonist-reversible, decreases in forskolin-stimulated cAMP levels following acute (15 min) morphine treatment of δ -DA cells have been previously shown (Glasel & Agarwal 1997a). Extending this observation, Figure 3a shows that after chronic (4 day) exposure to 100 μ M morphine, forskolin-stimulated cAMP levels remain depressed, indicating that the second messenger response is retained under these conditions.

Figure 2. Saturation (main) and Scatchard (inset) plots of the β_2 -adrenergic radioreceptor assay of specific $[^3\text{H}](+)\text{CGP-12177}$ ($[^3\text{H}]\text{CGP}$) binding to cells. The saturation data was analysed by nonlinear regression and the theoretical fit to the data is shown as thin lines in the saturation and Scatchard plots.

Stimulation of AdC activity resulting in increased cAMP production is characteristic of the activation of G_s -initiated signalling by endogenously expressed β_2 -ARs. To examine whether or not β_2 -AR activation is stimulating a G_s -initiated signalling pathway in the δ -DA cell line, procaterol-induced changes in intracellular cAMP levels were measured. Figure 3b shows that after cells are exposed to 100 μM procaterol for 15 min there is an antagonist-reversible increase in cAMP levels over the basal level, suggesting that the agonist-induced change in cAMP levels measured is a β_2 -AR mediated event. However, if the cells are treated with 100 μM procaterol for 4 days, cAMP levels are no longer elevated above the untreated levels. Thus, in contrast to chronic morphine treatment, sustained treatment with procaterol leads to a desensitized cellular response at the second messenger level in δ -DA cells.

Agonist-induced differential proliferation regulation of log phase cells

In the absence of agonist, proliferation rates for the nontransfected DA cells, the v-DA cells, and the δ -DA cells are similar but chronic exposure to morphine inhibits δ -DA cell proliferation in an antagonist reversible, dose-dependent, manner (Glasel & Agarwal 1997a). The morphine treatment concentration was chosen on the following basis: plasma concentrations in addicted individuals are $> 10 \mu\text{M}$ (Martin & Jasinski 1969), which is in agreement with the concentration required for the inhibition of AdC via activation of δ -ORs expressed on NG108-15 cells (Sharma *et al.* 1975) where it was shown that the inhibition reached a maximum plateau at approximately 10 μM morphine. However, initial studies for the present work showed that the inhibition continued to occur at doses from 10–100 μM

Figure 3. Intracellular cAMP levels measured in response to agonist exposure. (a) cAMP levels were determined in cells that were untreated; stimulated with 10 μ M forskolin; treated with 100 μ M morphine for 4 days prior to stimulation with 10 μ M forskolin. *** P < 0.001 with respect to untreated control values. ### P < 0.001, forskolin and 4 days morphine + forskolin. (b) cAMP levels were determined in cells that were untreated; treated with 100, 10 or 1 μ M procaterol for 15 min; treated with 100 μ M procaterol + 100 μ M ICI 118 551 for 15 min; treated with 100 μ M procaterol for 4 days. As a negative control v-DA cells were also treated with 100 μ M procaterol for 15 min *** P < 0.001 with respect to untreated control values. # P < 0.05, 100 μ M procaterol and 10 μ M procaterol; 10 μ M procaterol and 100 μ M procaterol. ## P < 0.01, 100 μ M procaterol and 100 μ M procaterol + 100 μ M ICI 118,551; 100 μ M procaterol, 15 min and 100 μ M procaterol, 4 days. %% P < 0.01, cells + 100 μ M procaterol and v-DA cells + 100 μ M procaterol.

morphine (Figure 4a), and that the upper dose level was not lethal to the cells, so signalling effects were examined at 100 μ M morphine (where the receptors are theoretically 96% occupied). Cell viability measurements showed that the decrease in proliferation is not a result of cell death.

Procaterol was chosen as the agonist for the β_2 -AR proliferation studies because it is pharmacologically significant, highly selective, stable in media, and readily available. ICI 118,551 is a highly selective antagonist for the β_2 -AR. In tissues, the K_D s for procaterol and ICI 118,551 binding to the β_2 -AR are $\sim 0.10.3 \mu$ M and 0.01μ M, respectively (Minneman, Hedburg & Molinoff 1979). Since dose-dependent stimulation of proliferation with procaterol also did not plateau at 100 μ M, signalling effects were examined at this concentration. In the presence of 100 μ M procaterol in AIM-V medium, δ -DA cells do not form colonies in soft agar while *ras*-transformed 3T3 cells do. These results are the same as previously reported for all the δ -DA cell lines (Glasel & Agarwal 1997a) and are not described here.

In contrast to morphine-induced inhibition of proliferation, exposure to procaterol, increases proliferation of the cell line compared to untreated cells (Figure 4a): both agonist-induced proliferation effects are dosage-dependent above 10 μ M of each drug. Application of 100 μ M procaterol for 6 days increases cell numbers by approximately 128% compared with untreated cells starting from the same density, whereas application of 100 μ M morphine for 6 days decreases cell numbers to approximately 60% of untreated cells starting from the same density. This implies that both the δ -OR and β_2 -ARs possess proliferation regulatory potential. It has previously been shown that the proliferation effects of morphine are antagonist-reversible (Glasel & Agarwal 1997a). The β_2 -adrenergic antagonist, ICI 118,551, reverses $\sim 75\%$ of the procaterol-induced increase in proliferation at equimolar concentration (Figure 4a). Increasing the ICI 118,551 concentration beyond 100 μ M (to the minimum antagonist/agonist concentration ratio of 10 usually used to demonstrate reversibility) dramatically decreased the viability of the cells. Thus, it can be argued that only that the procaterol-induced increase in proliferation depends on specific agonist-activation of β_2 -ARs. Other results in this paper on signal response to procaterol treatment strengthen this conclusion.

It was previously demonstrated that the removal of insulin from the culture medium of the δ -DA cell line results in proliferation arrest and synchronization of the cell cycle (Glasel & Agarwal 1997a). When insulin is omitted from the culture medium, procaterol treatment does not stimulate proliferation of cells (Figure 4b), indicating that activating the β_2 -adrenergic agonist response pathway alone is not sufficient to induce proliferation.

Agonist-specific induction of JNK/ERK isozymes

MAPKs act downstream of second messengers and are known to be involved in regulating transcription factors associated with controlling proliferation. Since δ -OR and β_2 -AR agonists differentially modulate cell proliferation, the activity levels of the JNK and ERK MAPK isozymes may also be differentially modulated by them. Whether or not separate agonist activation of δ -opioid and β_2 -ARs results in selective JNK/ERK activation was first investigated.

Acute individual agonist exposure

To assess whether or not the differential proliferation elicited by δ -opioid and β_2 -adrenergic agonists correlates with selective activation of JNK and ERK, cells were either left untreated or treated with the specified agonist as described in the *Materials and methods*. To terminate each experiment, cells were harvested, and either JNK or ERK was isolated and tested for

Figure 4. Proliferation responses to agonist, antagonist, and insulin. (a) Cells were cultured for six days without added agonist (untreated); treated with 1, 10, or 100 μ M morphine; treated with 1, 10, or 100 μ M procaterol; treated with 100 μ M procaterol + 100 μ M ICI 118,551. * P < 0.05; ** P < 0.01; *** P < 0.001 with respect to untreated control values. ## P < 0.01, 100 μ M procaterol and 100 μ M procaterol + 100 μ M ICI 118,551. (b) Growth curves representing the cell line as normally cultured in insulin-containing AIM-V medium; cultured in AIM-V without insulin; cultured in AIM-V without insulin but with 100 μ M procaterol. Note that the small error bars in these experiments are covered by the symbols.

activity as outlined. Although acute serum or UV stimulation significantly elevated ERK and JNK activities, respectively, no activity of either kinase at 15 min after drug treatment was detected (Figure 5). These results may indicate that GPCR activation of the cAMP response pathway does not immediately stimulate MAPK activity or, in accordance with observations of other workers in another cell system, the peak of activity following acute treatment with drugs had subsided within 15 min (Fukuda *et al.* 1996, Li & Chang 1996). Because the kinetics of MAPK activity during acute treatment were not germane to the present study's primary focus on chronic agonist exposure, this point was pursued further. More detailed subsequent studies may show that events within this time frame trigger the later ones we describe in the present paper.

Figure 5. JNK and ERK activation response to agonist exposure expressed as fold increases over untreated cells. JNK and ERK activity levels were determined in cells that were untreated; treated with 100 μ M morphine for 15 min; treated with 100 μ M procaterol for 15 min; treated with 100 μ M morphine for 4 days; and with 100 μ M procaterol for 4 days. As a positive control for direct stimulation of kinase activity, cells were treated with either 20% serum for 15 min to stimulate ERK activation or with UV-C at 1400 μ W/cm² for 10 min to stimulate JNK activation. The culture medium and drug supplements were replenished every 48 h where chronic exposure is indicated. Values shown represent the mean the standard deviations of three determinations. * P < 0.05; ** P < 0.01 with respect to untreated control values. # P < 0.05, JNK activity in response to morphine, 15 min and JNK activity in response to morphine, 4 days. % P < 0.05, JNK activity in response to morphine, 4 days and ERK activity in response to morphine, 4 days. ## P < 0.001, ERK activity in response to procaterol, 15 min and ERK activity in response to procaterol, 4 days. %% = P < 0.001, ERK activity in response to procaterol, 4 days and JNK activity in response to procaterol, 4 days. Cells were cultured for six days without drug treatment (untreated); treated with 50 μ M PD 089,059 alone; treated with 100 μ M procaterol + 50 μ M PD 089,059.

Chronic individual agonist exposure

After 4 days of chronic agonist exposure, MAPK isozyme activities selectively rise depending on which receptor is chronically activated (Figure 5): (i) in response to procaterol, ERK activity is increased threefold, but JNK activity remains the same as in untreated cells and (ii) in response to morphine treatment, JNK activity is increased twofold, but ERK activity remains the same as in the untreated cells.

Response to dual agonist exposure: cAMP levels, JNK/ERK activities, and proliferation rates

To begin investigation of possible cross-regulation between signalling pathways we measured AdC, MAPK isoform, and proliferation responses to dual agonist treatments. Acute dual agonist treatment initially increases the level of cAMP (Figure 6a), as it does under procaterol-alone treatment. This suggests that under acute dual agonist treatment, δ -OR activation does not significantly interfere with AdC stimulation by the β_2 -AR.

In contrast, when cells are chronically treated for 4 days with both agonists, cAMP levels return to near basal indicating that the procaterol-induced increase in cAMP level is abolished in agreement with the observation that β_2 -AR second messenger signalling is desensitized by chronic procaterol treatment. However, second messenger inhibition by chronic morphine treatment of the δ -OR is not desensitized when both agonists are present in the culture medium.

To determine whether downstream nuclear signalling is altered by chronic dual agonist treatment, JNK/ERK activity levels were measured after 4 days of agonist treatment. Two aspects of the JNK/ERK response to chronic dual agonist treatment were found to be noteworthy (Figure 6b,c): (i) the presence of procaterol is no longer associated with an increase in ERK activity and (ii) JNK activity is near the level measured in the presence of δ -OR agonist alone. As shown in Figure 6b, when both agonists are present JNK activity is approximately 2.5-fold above the kinase activity level of untreated cells. ERK activity, on the other hand, drops to 0.7-fold of the activity measured in untreated cells. The fact that ERK activation is inhibited in the presence of dual agonist, while JNK activity remains stimulated, indicates that the sensitivity of one GPCR-activated signalling pathway is being altered by the other GPCR-activated signalling pathway.

The rate of cell proliferation in response to dual agonist exposure initially increases (2 days; Figure 6c), but by 4 days the rate of cell proliferation drops below the level measured in procaterol-alone treated cells showing that chronic dual agonist treatment nullifies the proliferation increase induced by procaterol. In summary, simultaneous chronic exposure to both agonists is accompanied by a decrease in cAMP, an increase in JNK activity, and a decrease in proliferation: results similar to the responses elicited by chronic morphine treatment alone (Figure 4a).

DISCUSSION

The δ -DA cell line is unique in that the intracellular signalling events associated with δ -OR activation can be studied in the absence of signal input from multiple different GPCRs usually found on opioid receptor-expressing cell types. Another useful aspect of the cell line is the presence of endogenous β_2 -AR gene expression, and the dependence of the cell-line on insulin as the sole exogenously applied growth factor. There appears to be no explanation at this time for the induction of endogenous adrenergic receptor expression by transfection with the δ -OR gene. This interesting phenomenon will require a separate investigation. Figure 1 shows that transfection of DA cells with the serotonin-5HT_{1C} receptor gene (5HT-DA cells)

Figure 6. Effects of dual agonist treatment on intracellular cAMP levels, ERK/JNK activity, and proliferation in cells. (a) cAMP levels were determined in cells that were untreated; treated with 100 μ M morphine + 100 μ M procaterol for 15 min, and then assayed without forskolin stimulation; treated with 100 μ M morphine + 100 μ M procaterol for 4 days, and then assayed without forskolin stimulation; stimulated with 10 μ M forskolin alone; treated with 100 μ M morphine + 100 μ M procaterol prior to stimulation with 10 μ M forskolin. ** P < 0.01 with respect to untreated control values. ### P < 0.01, morphine + procaterol, 15 min and morphine + procaterol, 4 days. %% P < 0.01, morphine + procaterol for 4 days prior to forskolin stimulation and forskolin stimulation alone. (b) JNK and ERK activity levels were determined in cells that were untreated, or treated with 100 μ M morphine + 100 μ M procaterol for 4 days. ** P < 0.01 with respect to untreated control values. #### P < 0.001, JNK activity in response to morphine + procaterol and ERK activity in response to morphine + procaterol. (c) For proliferation studies, cells were cultured for 6 days without added agonist (untreated); treated with 100 μ M procaterol; treated with 100 μ M morphine + 100 μ M procaterol. * P < 0.05; ** P < 0.01; *** P < 0.001 with respect to untreated control values. ## P < 0.01 with respect to cells treated with procaterol alone.

in the same vector does not induce expression of the β_2 -AR and preliminary data indicates that agonist-activation of the δ -OR stimulates expression of the β_2 -AR.

It was found that, in contrast to δ -OR agonist-induced inhibition of proliferation, β_2 -AR agonist stimulates proliferation in the same cells. However, under prolonged dual agonist

treatment, the initial stimulation of proliferation after 2 days exposure to the drugs was followed by inhibition after 6 days exposure (Figure 6b). This leads to an hypothesis that, under chronic treatment with these drugs, cross-regulation between the δ -OR and β_2 -AR signalling systems may modulate nuclear events controlling rates of δ -DA cell proliferation.

MAPKs have been implicated in mediating cellular responsiveness to a variety of different extracellular stimuli. Individual MAPK isozyme activities have been best characterized in association with cell proliferation control. ERK activity is commonly associated with induction of proliferation and JNK activity has been associated with cellular stress responses. Since chronic morphine treatment and procaterol treatment differentially influence the rate of proliferation of δ -DA cells, second messenger levels and JNK/ERK activity levels were compared in the presence of each agonist separately.

Chronic procaterol-alone activation of the β_2 -AR results in a long-term increase in ERK activity without a long-term elevation in cAMP level. Chronic morphine-alone activation of the δ -OR results in a long-term increase in JNK activity with a long-term depression of cAMP level. Chronic dual agonist treatment does not produce the same JNK/ERK activation patterns as the chronic single agonist treatments: a dual agonist suppression of ERK activity was observed, but not JNK activity, indicating cross-regulation between the δ -OR and the β_2 -AR activated signalling pathways. If cross-regulation were not occurring between these receptor activated signalling pathways, simultaneous activation of both receptors to increase the activity of both kinases would be expected. Since both single and dual agonist treatments elicit the same cAMP responses, but different JNK/ERK responses, either the two signalling pathways intersect downstream of cAMP or via a pathway other than the cAMP response pathway. The delayed JNK/ERK response patterns observed in the dual treatment with morphine and procaterol suggest that regulation of gene expression may precede the selective activation of MAPK isozymes. For example, it is possible that increasing cAMP levels leads to transcriptional activation affecting ERK, and decreasing cAMP levels relieves transcriptional repression affecting JNK.

Recently, it was found that insulin can activate both ERK (Lazar *et al.* 1995) and JNK via a *ras*-dependent pathway(s) (Miller *et al.* 1996). The present study shows that in the absence of insulin, β_2 -AR activation is not sufficient to stimulate proliferation. This indicates that an acute increase in cAMP level alone cannot initiate transcription from genes required to implement cell division. However, the simultaneous addition of insulin and procaterol to the culture medium causes the cell proliferation rate to increase above that of cells treated with insulin alone demonstrating an interesting proliferation-promoting synergism between the insulin-stimulated pathway and the procaterol-stimulated AdC response pathway in δ -DA cells.

In conclusion, it has been shown that JNK and ERK activities are differentially up-regulated in response to chronic individual morphine and procaterol treatments although the proliferative effects of the two different drug treatments are opposite. This suggests that JNK/ERK activities can be selectively regulated by δ -OR and β_2 -AR initiated signalling but does not necessarily connect JNK and ERK activities directly to proliferation. Agonist effects on JNK and ERK activities are delayed for several days (as opposed to the rapid effects of serum and UV stimulation), indicating that GPCR signalling may involve transcriptional regulation affecting MAPK cascade functioning. Furthermore, neither JNK/ERK activities nor proliferative effects are additive when cells are simultaneously treated with morphine and procaterol. Under morphine-alone or dual agonist treatments, JNK is activated at the expense of ERK, showing that morphine's effects dominate.

The physiological consequences of chronic morphine exposure are known to involve long-term cellular adaptations. Transcriptional adaptation of CREB has been identified as one

cellular consequence of chronic morphine exposure (Widnell *et al.* 1996). The present work shows that another cellular consequence of chronic morphine exposure is the modulation of MAPK isozyme activities. Using subtractive cDNA libraries, attempts to identify transcripts implicated in proliferation inhibition and stimulation under chronic drug treatments are being made.

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