Mitogenic signalling by δ opioid receptors expressed in Rat-1 fibroblasts involves activation of the p70^{s6k}/p85^{s6k} S6 kinase

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The regulation of mitogenic signalling pathways by G-protein-coupled receptors has been studied in Rat-1 fibroblasts stably transfected with the murine δ opioid receptor. We showed recently that stimulation of this receptor led to the activation of the p42 and p44 isoforms of mitogen-activated protein (MAP) kinase [Burt, Carr, Mullaney, Anderson and Milligan (1996) *Biochem. J.* **320**, 227–235]. The present study has examined the role of the ribosomal S6 kinase p70^{s6k} in mitogenic signalling by the δ opioid receptor. Treatment of Rat-1 fibroblasts expressing this receptor with the synthetic enkephalin [D-Ala,D-Leu]-enkephalin (DADLE) led to a dose-dependent increase in p70^{s6k} enzyme activity. Activation of p70^{s6k} was dependent on the level of δ opioid receptor expressed and was sustained above basal levels for several hours. Immunoblotting revealed that p70^{s6k} was subject to increased phosphorylation, the extent of which co-

incided temporally with enzyme activation. Activation of p70^{s6k} by DADLE, but not by platelet-derived growth factor, was blocked by pretreatment of cells with pertussis toxin. Activation of p70^{s6k} was also partly blocked by wortmannin, indicating that phosphoinositide 3-OH kinase is required for full activation of p70^{s6k} by opioid receptor agonists. Activation of the δ opioid receptor in transfected cells led to increased DNA synthesis. This increase was prevented by rapamycin, which also completely blocked activation of p70^{s6k} by DADLE. In addition, prevention of the activation of p42 and p44 MAP kinases also blocked the induction of DNA synthesis by DADLE. These results suggest that the activation of both MAP kinases and p70^{s6k} might be crucial to the induction of mitogenic responses by G_i-linked receptors such as the δ opioid receptor.

INTRODUCTION

Mitogenic receptors can be subdivided into those that either are, or couple to, a tyrosine kinase (the tyrosine kinase class) and those that couple to heterotrimeric G-proteins [the G-protein-coupled receptors (GPCRs)] (reviewed in [1]). Although it was considered that these two classes employed distinct and mutually exclusive mechanisms to induce mitogenic signals, recent evidence has challenged this view. For example, the p21^{ras}/mitogen-activated protein (MAP) kinase pathway, originally thought to be activated solely by tyrosine kinase receptors, is now known to be activated by several GPCRs [2–8], including the α_2 adrenergic, m₂ muscarinic and lysophosphatidic acid receptors, which couple to the pertussis-toxin-sensitive G_i subfamily. The mechanisms linking G_i-linked receptors with the MAP kinase and other mitogenic signalling pathways are still unclear but accumulating evidence indicates an involvement of G-protein $\beta\gamma$ subunit complexes [9–12].

A common early event in cells stimulated with mitogenic agents is an increase in the phosphorylation of the ribosomal protein S6. Increased S6 phosphorylation is correlated with elevated rates of translation and might be partly responsible for the increase in protein synthesis triggered by mitogens [13]. Phosphorylation of S6 occurs on five closely positioned sites and is catalysed by the S6 kinase p70^{s6k} [14–16]. A common gene encodes two isoforms of the protein: p70^{s6k}, which seems to be localized predominantly in the cytoplasm, and p85^{s6k}, which possesses a 23-residue N-terminal extension containing a nuclear localization sequence. The activation of p70^{s6k} requires its phosphorylation on multiple sites, perhaps by more than one

protein kinase [17,18]. In addition, a variety of evidence indicates that p70^{s6k} lies on a pathway that is independent of the p21^{ras}/MAP kinase pathway [19–22].

Recent studies suggest that p70^{s6k} might regulate a wider range of cellular responses than originally expected. For example, p70^{s6k} phosphorylates the transcription factor CREM τ [23]. In addition, rapamycin, an immunosuppressive macrolide drug that completely blocks the activation of p70^{s6k} by all known agents, has been shown to block a diverse array of responses, collectively involved in cell cycle progression [16,24–26].

The mitogenic signalling mechanisms of GPCRs have recently received much attention. Despite this, little is known about the regulation of p70^{s6k} by these receptors. As a means to study this we have expressed GPCRs stably in Rat-1 fibroblasts and showed recently that stimulation of the α_2 adrenoceptor resulted in the activation of p70^{s6k} [27]. In the present report we have examined the coupling of another GPCR, the murine δ -opioid receptor, to this pathway. We show that ligand stimulation of this receptor, stably expressed in Rat-1 fibroblasts, also activates p70^{s6k} by a mechanism involving phosphatidylinositol 3-kinase (PI 3-kinase). Furthermore, stimulation of the δ opioid receptor in this system leads to increased DNA synthesis. Blockage of this effect by rapamycin indicates a requirement for activation of p70^{s6k} in δ opioid receptor-induced mitogenic responses.

MATERIALS AND METHODS

Materials

Cell culture media, calf serum, platelet-derived growth factor (PDGF), hygromycin B and geneticin were purchased from Life

Abbreviations used: DADLE, [D-Ala,D-Leu]-enkephalin; GPCR, G-protein-coupled receptor; MAP kinase, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PI 3-kinase, phosphatidylinositol 3-kinase.

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Technologies (Paisley, Strathclyde, U.K.). Pertussis toxin was supplied by Porton Products (Salisbury, Wiltshire, U.K.). Protein A-agarose was purchased from Pierce-Warriner (Chester, Cheshire, U.K.). [γ - 32 P]ATP, enhanced chemiluminescence reagents and nitrocellulose were supplied by Amersham International (Little Chalfont, Bucks., U.K.). All other reagents were from Sigma (Poole, Dorset, U.K.). PD 098059 was purchased from Alexis Corporation (Nottingham, Notts., U.K.). Peptides were synthesized at the Hannah Research Institute core facility. The p70^{s6k} antiserum was raised against a synthetic peptide representing amino acid residues 1–30 of rat p70^{s6k} [27]. The cDNA encoding the murine δ opioid receptor [28] was a gift from Dr. Graeme Bell (Howard Hughes Medical Institute, Chicago, IL, U.S.A.).

Generation and isolation of clones of Rat-1 fibroblasts expressing the murine δ opioid receptor

A cDNA encoding the murine δ opioid receptor was ligated into the plasmid pCMV-ms12. Rat-1 fibroblasts were co-transfected with this cDNA (5 μ g) and with the plasmid pBABEhygro (0.5 μ g), which is able to direct expression of the hygromycin B resistance marker, with the use of Lipofectin reagent (Life Technologies) in accordance with the manufacturer's instructions. Clones that demonstrated resistance to hygromycin B (200 μ g/ml) were selected and expanded. Expression of the δ opioid receptor polypeptide in membranes from these clones was assessed initially by the specific binding of the opioid receptor ligand [3 H]diprenorphine and detection of the relevant mRNA by using reverse transcriptase-PCR [29].

Cell culture and treatment

Cells were grown to confluency in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum in 100 mm cell culture dishes. Cells were switched to serum-free medium for 16–20 h before use. In some experiments pertussis toxin was included in the serum-starving medium at a final concentration of 25 ng/ml. Wortmannin was stored as a 10 mM solution in DMSO at -20°C and was diluted into water just before use. Rapamycin [a gift from Dr. Sehgal (Wyeth-Ayerst, Princeton, MA, U.S.A.)] was stored in ethanol at -20°C and diluted with water just before use.

Immunocomplex assay of p70^{s6k}

After treatment, cells were washed twice in ice-cold PBS and scraped into 1 ml of lysis buffer A containing 50 mM Tris/HCl, pH 8.0 (4 $^\circ\text{C}$), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 10 mM Na pyrophosphate, 30 mM 4-nitrophenyl phosphate, 1% (v/v) Nonidet P40, 1 mM PMSF and leupeptin, aprotinin and pepstatin A (2 μ g/ml each). After 20 min, lysates were centrifuged for 10 min at 14000 *g*. The supernatant was precleared by addition to 20 μ l (packed volume) of protein A-agarose for 1 h. Pre-cleared lysates (250 μ g of protein) were immunoprecipitated with 2.5 μ l of p70^{s6k} antiserum for 2 h before addition to 20 μ l of protein A-agarose for 1 h. Immunoprecipitates were washed twice with lysis buffer and twice with kinase assay buffer [25 mM Mops (pH 7.2 at 30 $^\circ\text{C}$)/1 mM EDTA/0.05% (v/v) Triton X-100/1 mM dithiothreitol/20 mM 4-nitrophenyl phosphate]. Immunoprecipitates were then resuspended in kinase assay buffer (50 μ l) containing S6 substrate [a 32-residue peptide (KEAKEKRQEIQAKRRRLSSLRAS-SKSGGSQK) from the C-terminal sequence of S6; 20 μ M] and 2 μ M cAMP-dependent protein kinase inhibitor. After a 2 min preincubation at 30 $^\circ\text{C}$, the reaction was initiated by the addition

of ATP/MgCl₂ [25 μ M [γ - 32 P]ATP (5000 c.p.m./pmol)/10 mM MgCl₂]. After 10 min, 30 μ l of the reaction was spotted on P81 paper, which was then washed five times in 75 mM phosphoric acid. Dried papers were placed in 4 ml of scintillant and counted for radioactivity.

Immunoblotting of p70^{s6k}

Cell lysates (25 μ g) were subjected to electrophoresis on 9% (w/v) (30:0.32 acrylamide/bisacrylamide) gels and transferred to nitrocellulose for 2 h at 400 mA. After blocking in 3% (w/v) BSA, immunoblots were incubated with anti-p70^{s6k} (1:1000 dilution) for 3 h followed by horseradish peroxidase-conjugated anti-(rabbit IgG) (1:5000 dilution) for 1 h. Immunoreactive bands were detected by enhanced chemiluminescence in accordance with the manufacturer's instructions.

[3 H]Thymidine incorporation

Cells were grown to approx. 70% confluence in six-well plates and then serum-starved in Dulbecco's modified Eagle's medium for 24 h. Agonists were then added for 24 h, with 1 μ Ci of [3 H]thymidine being present for the last 4 h. When the effects of rapamycin and PD 098059 were being tested, these were added to the cultures 1 h before the agonists. Cells were then washed twice with ice-cold PBS, with 10% (w/v) trichloroacetic acid and finally once with 95% ethanol. The fixed precipitates were then dissolved in 0.5 ml of 1 M NaOH and, after 20 min shaking, neutralized with 0.5 ml of 1 M HCl. Aliquots were then counted for radioactivity.

RESULTS

Of the clones of cells obtained by transfection of the murine δ opioid receptor into Rat-1 fibroblasts, two were selected for the present study. The first, referred to as D2, was found to express approx. 6.4 pmol of the δ opioid receptor per mg of membrane protein, as measured by specific binding of the ligand [3 H]diprenorphine. The second clone, referred to as DOE, expressed approx. 0.17 pmol of receptor/mg of membrane pro-

Figure 1 Dose-dependent activation of p70^{s6k} by DADLE in Rat-1 fibroblasts expressing the δ opioid receptor

Non-transfected Rat-1 cells (○) or transfected cells [DOE (□) and D2 (◆)] were treated with the indicated concentrations of DADLE for 10 min. p70^{s6k} activity was measured with an immunocomplex assay as described in the Materials and methods section. Data are expressed as the activation (fold) above that measured in untreated cells and are averages of duplicate assay determinations from representative experiments done on each cell line at least twice.

Figure 2 Time course for activation of p70^{S6K} by DADLE in D2 cells

D2 cells were treated with 1 μ M DADLE for the indicated times. Upper panel: p70^{S6K} activity was measured with an immunocomplex assay as described in the Materials and methods section. Data are means $\pm$ S.E.M. for triplicate assay determinations from a representative experiment done on two other occasions with similar results. Lower panel: lysates from cells treated as above were immunoblotted with anti-p70^{S6K} antibodies as described in the Materials and methods section. A representative experiment is shown.

tein. We examined whether the synthetic enkephalin [D-Ala,D-Leu]-enkephalin (DADLE) would activate p70^{S6K} in the clones D2 and DOE and in parental non-transfected Rat-1 fibroblasts, by using an immunocomplex kinase assay. Figure 1 shows that DADLE induced a dose-dependent activation of p70^{S6K} in both DOE and D2 cells. As expected, DADLE failed to induce p70^{S6K} activation in parental Rat-1 fibroblasts, which do not express detectable levels of the δ opioid receptor. The maximal activation of p70^{S6K} by DADLE was 3–4-fold higher in D2 than in DOE cells. In addition the EC₅₀ for DADLE was lower in the D2 clone (in D2 approx. 1.1 nM; in DOE approx. 6.3 nM).

The activation of p70^{S6K} by DADLE in D2 cells was rapid (maximal activation at approx. 10–20 min) and, although it declined thereafter, the activity of p70^{S6K} persisted well above basal levels for at least 5 h (Figure 2, upper panel). The activation of p70^{S6K} involves its phosphorylation on multiple sites [17,18]. This results in a retardation in the mobility of the protein in SDS gels [21]. Figure 2 (lower panel) shows that DADLE also induced a marked alteration in the mobility of p70^{S6K}, with the appearance of more slowly migrating forms of the protein. The degree of this 'band-shift' was correlated with the extent of p70^{S6K} activation induced by DADLE.

The δ opioid receptor couples to its known effectors via the G_i and/or G_o class of heterotrimeric G-proteins [30]. To test whether the activation of p70^{S6K} by DADLE also involves this mechanism, cells were pretreated with pertussis toxin, which prevents functional interactions between receptors and G_i/G_o. Table 1 shows that such pretreatment completely blocked the activation of p70^{S6K} by DADLE but failed to affect significantly its activation by PDGF.

A number of studies have suggested that the lipid kinase PI 3-kinase lies upstream of p70^{S6K} in a signalling pathway initiated by the activation of several tyrosine kinase-linked receptors [31–35]. We therefore next tested the involvement of PI 3-kinase in the activation of p70^{S6K} via the δ opioid receptor by using the

Table 1 Pertussis toxin blocks activation of p70^{S6K} by DADLE

D2 cells were pretreated or not with pertussis toxin (25 ng/ml) for 16 h before the addition of DADLE (1 μ M) or PDGF (10 ng/ml) for 10 min or without further addition (basal). p70^{S6K} activity was then measured with an immunocomplex assay as described in the Materials and methods section. Data are means $\pm$ S.E.M. for three independent experiments. Significant effects (Student's *t* test) of pertussis toxin treatment relative to untreated cells are denoted by * ($P < 0.05$) and ** ($P < 0.01$).

Condition	p70 ^{S6K} activity (pmol/min per mg)	
	Without pertussis toxin	With pertussis toxin
Basal	0.22 $\pm$ 0.02	0.10 $\pm$ 0.02*
PDGF	1.42 $\pm$ 0.37	1.47 $\pm$ 0.06
DADLE	0.84 $\pm$ 0.07	0.12 $\pm$ 0.07**

selective inhibitor wortmannin. Table 2 shows that pretreatment of cells with 100 nM wortmannin largely prevented the activation of p70^{S6K} by DADLE and by PDGF. The concentration of wortmannin required to elicit 50 % inhibition of p70^{S6K} activation by a maximally effective concentration of DADLE was approx. 30 nM (results not shown). The inhibitory effects of wortmannin on p70^{S6K} enzyme activation by DADLE and by PDGF were paralleled by increased mobility of the p70^{S6K} on SDS/PAGE (Figure 3, upper panel). Activation of the δ opioid receptor in these cells also results in activation of the p44 and p42 isoform MAP kinases (ERKs 1 and 2) [36]. However, in contrast with its

Table 2 Wortmannin blocks activation of p70^{S6K} by DADLE

D2 cells were pretreated or not with wortmannin (100 nM) for 10 min before the addition of PDGF (10 ng/ml) or DADLE (1 μ M) for 10 min or without further addition (basal). p70^{S6K} activity was then measured with an immunocomplex assay as described in the Materials and methods section. Data are means $\pm$ S.E.M. for three independent experiments. *Significant effect of wortmannin compared with untreated cells (Student's *t* test; $P < 0.05$).

Condition	p70 ^{S6K} activity (pmol/min per mg)	
	Without wortmannin	With wortmannin
Basal	0.30 $\pm$ 0.07	0.06 $\pm$ 0.01*
PDGF	1.93 $\pm$ 0.42	0.81 $\pm$ 0.20
DADLE	1.67 $\pm$ 0.39	0.40 $\pm$ 0.05*

Figure 3 Wortmannin blocks the phosphorylation of p70^{S6K} but not that of p42 MAP kinase by DADLE

Cells were treated as described in the legend to Table 2 and lysates were immunoblotted for p70^{S6K} as described in the Materials and methods section or for p42 MAP kinase [36]. Abbreviations: C, basal; P, PDGF; L, 10 μ M lysophosphatidic acid; D, DADLE. The result shown is representative of two independent experiments.

Figure 4 Rapamycin prevents stimulation of DNA synthesis and p70^{S6K} by DADLE

Left panel: [³H]thymidine uptake was measured in D2 cells stimulated with 1 μ M DADLE for 24 h in the absence (zero point) or presence of the indicated concentrations of rapamycin as described in the Materials and methods section. Data are means $\pm$ S.E.M. for triplicate wells of cells. Right panel: D2 cells were pretreated with the indicated concentrations of rapamycin before the addition of 1 μ M DADLE. p70^{S6K} activity was then measured with an immunocomplex assay as described in the Materials and methods section. Data are expressed as the activation (percentage of maximal in the absence of rapamycin) and are averages of duplicate assay determinations from a representative experiment done on two other occasions.

Table 3 PD 098059 prevents stimulation of DNA synthesis by DADLE

Cells were pretreated with PD 098059 (50 μ M) for 1 h before the addition of PDGF (10 ng/ml) or DADLE (1 μ M) for 24 h. DNA synthesis was assessed by the uptake of [³H]thymidine as described in the Materials and methods section. The data are means $\pm$ S.E.M. for three independent experiments. **Significant effect of PD 098059 compared with untreated cells (Student's *t* test; *P* < 0.01).

Condition	[³ H]Thymidine incorporation per well	
	Without PD 098059	With PD 098059
Basal	10697 $\pm$ 1595	14564 $\pm$ 1504
PDGF	32222 $\pm$ 2445	9531 $\pm$ 1991**
DADLE	47743 $\pm$ 2815	13902 $\pm$ 3469**

effects on p70^{S6K} activation, wortmannin failed to inhibit the activation of p42 MAP kinase (Figure 3, lower panel) or p44 MAP kinase (results not shown) by DADLE.

We finally determined whether activation of p70^{S6K} might be part of a mitogenic response stimulated by occupancy of the δ opioid receptor by examining the effects of rapamycin on DADLE-induced DNA synthesis. Figure 4 (left panel) shows that DADLE induced an approx. 6-fold increase in [³H]-thymidine uptake. Co-incubation of the cells with rapamycin potentially inhibited this effect of DADLE, with rapamycin concentrations above 1 nM causing complete blockage of the response (Figure 4, left panel). Parallel experiments confirmed that rapamycin blocked the activation of p70^{S6K} by DADLE with a similar dose-dependence (IC₅₀ approx. 2 nM; Figure 4, right panel).

We showed recently that stimulation of δ opioid receptors in D2 cells also results in the activation of p42 and p44 MAP kinases [36]. As the Ras/MAP kinase pathway is heavily implicated in transducing mitogenic signals, we examined its involvement in mitogenic signalling by the δ opioid receptor, by using PD 098059, a highly specific inhibitor of MAP kinase activation [37]. These experiments revealed that pretreatment of cells with 50 μ M PD 098059, a concentration that completely blocked the ability of DADLE to activate p42 MAP

kinase, as judged by mobility shift (results not shown), prevented the increase in DNA synthesis induced by DADLE (Table 3). These results therefore indicate that both the Ras/MAP kinase and p70^{S6K} pathways are necessary for δ -opioid receptor-stimulated DNA synthesis.

DISCUSSION

Recently it has become clear that the activation of p70^{S6K} leads to a multitude of cellular responses including, but not restricted to, phosphorylation of ribosomal protein S6. Although a number of mitogenic and other agents that signal via tyrosine kinase receptors have been shown to activate p70^{S6K}, little information is available on the coupling of GPCRs to this signalling pathway. The present report shows that ligand-activation of the δ opioid receptor, heterologously expressed in Rat-1 fibroblasts, results in sustained activation of p70^{S6K}. The degree of p70^{S6K} activation induced was dependent on the levels of δ opioid receptor expressed in the cells. Stimulation of the D2 clone of cells, which expresses approx. 6.4 pmol of receptor/mg of membrane protein, with a maximally activating concentration of DADLE induced a 6–7-fold maximal activation of p70^{S6K}. In comparison DADLE induced only a 2–3-fold activation in the DOE clone, which expresses approx. 0.17 pmol/mg. The half-maximally effective dose of DADLE in the D2 cells was one-sixth of that in the DOE cells.

The molecular mechanisms involved in the activation of p70^{S6K} have not yet been fully elucidated. It is known that p70^{S6K} must be phosphorylated on several sites [17,18] for activation but the kinase(s) involved has/have not been identified. In common with all agents tested so far, activation of p70^{S6K} by DADLE was completely blocked by rapamycin. Rapamycin prevents or reverses the phosphorylation of p70^{S6K} at a distinct set of sites required for activity [18,38]. It is not yet clear how this occurs but a specific target for rapamycin, the protein kinase known as FRAP/RAFT1, has been identified [39–41].

Other studies have provided evidence that the lipid kinase PI 3-kinase is necessary for the activation of p70^{S6K} by various tyrosine-kinase-linked receptors [31–35]. The present study indicates that PI 3-kinase is also involved in the activation of p70^{S6K} by the G-protein-coupled δ opioid receptor. We showed

recently [27] that the activation of p70^{s6k} by α_2 agonists and by lysophosphatidic acid, both of whose receptors are coupled to G_i, was also dependent on the activation of PI 3-kinase. This mechanism might therefore be common to the activation of p70^{s6k} by GPCRs. At least four isoforms of PI 3-kinase have now been isolated and it is worth noting that specific G-protein-activated forms of PI 3-kinase have been identified [42–45], including PI 3-kinase γ [45], which is inhibited by wortmannin. It will be of interest to determine whether PI 3-kinase γ is stimulated by opioid receptor agonists and other G-protein-coupled ligands, and whether it is required for the activation of p70^{s6k}.

The involvement of both PI 3-kinase and FRAP/RAFT in the activation of p70^{s6k} by many extracellular agents has now been demonstrated and it has been suggested that these are components of a linear pathway, with PI 3-kinase lying upstream of FRAP/RAFT. If this is so, then the intermediate steps remain to be identified. Recently, a serine/threonine kinase known as PKB (or RAC/AKT), which is activated by several tyrosine kinase receptors, was shown to occur downstream of PI 3-kinase [46–49]. In another report, expression of an activated form of PKB in fibroblasts led to the activation of p70^{s6k} [49], suggesting that PKB might represent an additional component of the p70^{s6k} signalling pathway. Preliminary experiments have revealed a small stimulation of the kinase activity of PKB in response to DADLE in D2 cells (M. A. Wilson, G. Milligan and N. G. Anderson, unpublished work). Work is currently in progress to confirm this and to test whether PKB mediates the activation of p70^{s6k} by GPCRs.

The results of our experiments with rapamycin and wortmannin suggest that very similar, or perhaps identical, mechanisms operate immediately upstream of p70^{s6k} when the enzyme is activated by either GPCRs or tyrosine kinase-linked receptors. The signals must therefore diverge at a point closer to the respective receptor subtypes. In this respect, activation of p70^{s6k} by DADLE, but not by PDGF, was completely blocked by pretreatment of cells with pertussis toxin. The δ opioid receptor couples to its known effectors, adenylate cyclase and ion channels for Ca²⁺ and K⁺, via the pertussis toxin-sensitive G_i and G_o classes of G proteins [30]. However, because Rat-1 cells do not express detectable levels of G_o [50], the activation of p70^{s6k} by DADLE must be mediated via G_i. An involvement of G_i in mediating mitogenic signalling has been inferred from several studies employing different experimental approaches (reviewed in [51]), not least of which is the demonstration that G_i mediates the activation of the Ras/MAP kinase cascade by GPCRs. Although not fully characterized, the activation of this pathway by GPCRs is mediated by G $\beta\gamma$ subunits released from the heterotrimeric G_i complex [9–12]. Whether a similar mechanism operates in the stimulation of p70^{s6k} through G_i remains to be determined. Certainly, the subset of G-protein-activated PI 3-kinases are activated *in vitro* by G $\beta\gamma$ [45].

Although not considered to be a classical mitogenic receptor, activation of the δ opioid receptor, heterologously expressed in Rat-1 fibroblasts, resulted in increased DNA synthesis (Figure 4). We showed recently that stimulation of these cells by δ opioid agonists led to a strong activation of p42 and p44 MAP kinases [36]. A wealth of evidence now indicates that these enzymes are involved in mitogenic signalling by a wide range of growth factors, including those that signal via GPCRs. Our experiments with PD 098059, which prevents the activation of MAP kinase kinase, the upstream activator of MAP kinases, further show that activation of the MAP kinase pathway is essential for the induction of DNA synthesis by DADLE in D2 cells. In contrast with the well-established role of the Ras/MAP kinase pathway in

mitogenesis, the participation of the p70^{s6k} cascade might be less universal. Two lines of evidence support the idea that p70^{s6k} is essential for cell cycle progression. The first is that by micro-injecting neutralizing antibodies, Reinhard et al. [52] showed that the p85^{s6k} isoform is required for the entry of rat embryo fibroblasts into S-phase. The precise role of the p85^{s6k} isoform has not been clarified although it is apparently localized in the nucleus, where it may be responsible for the increased phosphorylation of nuclear S6 induced by mitogens [53]. The second line of evidence implicating p70^{s6k} in mitogenesis comes from studies showing that rapamycin causes G1 arrest in some cell types and delays entry into S-phase in others. Thus the role of the p70^{s6k} pathway in mitogenesis might be cell context-dependent. The present paper shows that rapamycin blocks the stimulation of DNA synthesis by DADLE in D2 cells, suggesting that the p70^{s6k} pathway is essential for mitogenic signalling by the δ opioid receptor in this cellular context. However, because other effects of rapamycin have been reported [16], we cannot yet state unequivocally that p70^{s6k} itself is an essential component of the mitogenic response induced by δ opioid receptors. Nevertheless, our results show that, in addition to the Ras/MAP kinase cascade, a distinct, rapamycin-sensitive pathway is also critical to mitogenesis by GPCRs. The potent and sustained activation of p70^{s6k} induced by DADLE (this study), and by other agonists that signal via GPCRs [36], and the inhibition of this response by concentrations of rapamycin that block DNA synthesis, provide strong correlative evidence that the two events are linked.

In conclusion, we have shown that stimulation of the δ opioid receptor, heterologously expressed in fibroblasts, leads to the activation of p70^{s6k} via a mechanism involving PI 3-kinase. This, together with our previous report showing p70^{s6k} activation via a similar mechanism by lysophosphatidic acid and α_2 -adrenergic agents [27], firmly establishes this signalling pathway, in addition to the Ras/MAP kinase pathway, as a target for GPCRs. Our results also show that both of these pathways appear to be necessary for GPCRs to induce a mitogenic response, implying that neither is itself sufficient.

We thank Dr. Graeme Bell for the murine δ opioid receptor cDNA, and Sandra Woodburn for expert technical assistance. This work was supported by the Biotechnology and Biological Sciences Research Council.

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