Wortmannin-sensitive Activation of p70s6k by Endogenous and Heterologously Expressed G1-coupled Receptors*

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Moira Wilson‡§, Andrew R. Burt†§, Graeme Milligan†§, and Neil G. Andersont¶
From the ‡Hannah Research Institute, Ayr KA6 9HL, Scotland, United Kingdom and the §Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom

In order to study the regulation of the ribosomal protein S6 kinase, p70s6k, by G protein-coupled receptors, Rat-1 fibroblasts were stably transfected with two versions of the α2 adrenergic receptor, Stimulation of clone 1C cells, which express 3.5 pmol/mg of protein of the human α2C10 receptor, with the α2 agonist UK 14304 led to a transient increase in p70s6k activity. UK 14304 also activated p70s6k in a clone expressing the porcine α2A receptor (400 fmol/mg of protein). Lysophosphatidic acid (LPA), acting through endogenous G protein-coupled receptors, also activated p70s6k in α2 receptors-transfected and in nontransfected cells. Activation of p70s6k by both UK 14304 and LPA was accompanied by increased phosphorylation of the protein. Rapamycin completely blocked the activation of p70s6k by both agents. Activation of p70s6k by UK 14304 and by LPA, but not by platelet-derived growth factor (PDGF), was blocked by preincubation of cells with pertussis toxin. Wortmannin, a selective inhibitor of phosphoinositide (PI) 3-OH kinase, prevented activation of p70s6k by UK 14304, LPA, and PDGF. These data indicate that p70s6k is regulatable by G1-coupled receptor agonists in a pertussis toxin-sensitive fashion in Rat-1 fibroblasts and that activation of p70s6k by such agents appears to involve an isoform of PI 3-kinase.

Mitogenic stimulation of cells results in increased phosphorylation of the ribosomal protein S6. Increased S6 phosphorylation correlates with elevated rates of translation and may be partly responsible for the increase in protein synthesis triggered by mitogens (5). Phosphorylation of S6 occurs on five closely positioned sites and is catalyzed by a protein kinase that is activated by mitogens (5). Phosphorylation of S6 occurs on five multiple sites by more than one protein kinase (13, 14). Various lines of evidence indicate that p70s6k activation is independent of the p21ras/MAP1 kinase pathway (15, 16). For example, p21ras and p74ras mutants block activation of MAP kinases but not p70s6k and certain PDGF receptor mutants, which activate p21ras normally, fail to activate p70s6k (16). The immunosuppressive macrolide rapamycin completely blocks the activation of p70s6k but has no effect on MAP kinase activation (17, 18) and as such has become a useful tool for delineating p70s6k-specific signaling events. Other studies have shown that the PI3-kinase inhibitor wortmannin prevents activation of p70s6k by a range of agonists (19–23), suggesting that PI 3-kinase lies upstream of p70s6k.

Mitogens can be subdivided into those which either are or couple to a tyrosine kinase (the tyrosine kinase class) and those which couple to heterotrimeric G proteins (the GPCRs). Although it was considered that these two classes signalled via distinct and mutually exclusive mechanisms, recent evidence has challenged this view. For example, the p21ras/MAP1 kinase pathway, originally thought only to be activated by tyrosine kinase receptors, is now known to be activated by several GPCRs (24–29). These include the α2 adrenergic, m7 muscarinic, and LPA receptors which couple to the pertussis toxin-sensitive Gi subfamily. The mechanisms linking Gi-linked receptors with the MAP kinase and other mitogenic signaling pathways are still unclear but accumulating evidence from several laboratories indicates an involvement of G protein βγ subunit complexes (30–32).

As a means to study the mitogenic signaling of GPCRs, we have previously transfected Rat-1 fibroblasts with the human α2C10 adrenergic receptor and shown that the receptor interacts directly with two pertussis toxin-sensitive G proteins, G12 and G13, and thus induces both adenylyl cyclase inhibition and phospholipase D activation (33, 34). Activation of the receptor also results in a mitogenic response characterized by a pertussis toxin-sensitive activation of the p21ras/MAP kinase pathway and DNA synthesis (26, 29). Given the role of p70s6k in mitogenic signaling, we reasoned that this kinase should also be activated following stimulation of α2 receptors in these cells. Our results show that this does occur and thus provide the first demonstration of p70s6k activation by a receptor acting solely through Gi. We further show that wortmannin attenuates activation of p70s6k suggesting that an isoform of PI 3-kinase is involved in the activation mechanism.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media, calf serum, PDGF, hygromycin B, and genetin were purchased from Life Technologies, Paisley, UK. Pertussis toxin was supplied by Speywood. Protein A-agarose was purchased from Pierce-Warriner, Chester, UK, and 32P was from ICN Biomedicals. [γ-32P]ATP, ECL reagents, and nitrocellulose were supplied by Amersham International, UK. All other reagents were from Sigma. Peptides were synthesized at the Hannah Research Institute core facility. The p70s6k antisera was raised against a synthetic peptide representing amino acids 1–30 of rat p70s6k (35).

Cell Culture and Treatment—Rat-1 fibroblasts were transfected with the human α2C10 receptor (clone 1C cells) as described previously (33) or

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† To whom correspondence should be addressed. Tel. 44-1292-476-013; Fax: 44-1292-671-052.
¶ The abbreviations used are: MAP kinase, mitogen-activated protein kinase; PI 3-kinase, phosphoinositide 3-OH kinase; GPCR, G protein-coupled receptor; PDGF, platelet-derived growth factor; LPA, lysophosphatidic acid; HA, hemagglutinin; MOPS, 4-morpholino propane sulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.
with an HA-tagged variant of the porcine α2a adrenergic receptor (36, 37), equivalent to the human α2c10 receptor as follows. Cells were transfected in a 1:10 ratio with the plasmid pBABE hygro, which is able to direct expression of the hygromycin B resistance marker, and an HA-tagged porcine α2a adrenergic receptor in plasmid pCMV4 using Lipofectin reagent according to the manufacturer’s instructions (Life Technolo-
gies, Inc.). Clones which demonstrated resistance to hygromycin B (2000 μg/ml) were selected and expanded. The clone TAG WT3 displayed high affinity binding of [3H]yohimbine (data not shown) and were used in this study. All cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% calf serum and either genetin (500 μg/ml for clone 1C cells) or hygromycin B (50 μg/ml for TAG WT3 cells). Cells were grown to confluency in 100-mm cell culture dishes and serum-starved for 16–20 h prior to 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 dimethyl sulfoxide at −20 °C and was diluted into water just prior to use. Rapam-
ymycin (a generous gift from Dr. Sehgal, Wyeth-Ayerst, Princeton, N.J.) was stored in ethanol at −20 °C and diluted with water just prior to use.

Immunoprecipitation of p70<sup>65k</sup>—After treatment, cells were washed twice in ice-cold phosphate-buffered saline and scraped into 1 ml of lysis buffer containing 50 mM Tris-HCl, pH 8.0 (4 °C), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 10 mM sodium pyrophosphate, 30 mM 4-nitrophenyl phosphate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg each of leupeptin and pepstatin A, and 20 μM 4-nitrophenyl phosphate. After 20 min, lysis were centrifuged for 10 min at 14,000 × g. The supernatant was preadepted by addition to 20 μl (packed volume) of protein A-agarose for 1 h. Preadepted lyses (250 μg of protein) were immunoprecipitated with 2.5 μl of p70<sup>65k</sup> antisem for 2 h before addition to 20 μl 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 (30 °C), 1 mM EDTA, 0.05% (v/v) Triton X-100, 1 mM DTT, and 20 mM 4-nitrophenyl phosphate). Immunoprecipitates were then resuspended in kinase assay buffer (50 μl) containing 56 substrate (a 32-amino acid peptide (KEAEKKRQEIARKRRLSRTAASKS-GGSQK) from the C-terminal sequence of S6; 20 μM) and 2 μM cyclic AMP-dependent protein kinase inhibitor. After a 2-min preincubation at 30 °C, the reaction was initiated by the addition of ATP/Mg (125 μM [γ<sup>32</sup>P]ATP (5000 cpm/pmol), 10 mM MgCl<sub>2</sub>). After 10 min, 30 μl of the reaction was spotted onto P81 paper which was then washed five times in 75 mM phosphoric acid. Dried papers were placed in 4 ml of scintil-
lant and counted.

Metabolic Labeing of Cells—Serum-starved cells were incubated for 4 h in 4 ml of phosphate-free Dulbecco’s modified Eagle’s medium containing 1 mM of 32P<sub>3</sub>P. Stimulants were then added following which cells were rinsed twice with phosphate-buffered saline, lysed, and im-
munoprecipitated with p70<sup>65k</sup> antibodies as described above. Proteins eluted from the immune complex were electrophoresed on 9% (30:0.8 acrylamide:bisacrylamide) gels. The p70<sup>65k</sup> bands were located by autoradiography, excised from the gel, and counted.

Immunoblotting of p70<sup>65k</sup>—Immunoprecipitates or cell lysate sam-
ples (25 μg) were electrophoresed on 9% (30:0.32; acrylamide:bisacrylamide) gels and transferred to nitrocellulose for 2 h at 400 mA. Following banding in 3% bovine serum albumin, immunoblots were incubated with anti-p70<sup>65k</sup> (1:1000 dilution) for 3 h followed by horseradish per-
oxidase-conjugated goat anti-rabbit IgG (1:5000 dilution) for 1 h. Immu-
noactive bands were detected by enhanced chemiluminescence (ECL, Amersham) according to the manufacturer’s instructions.

Phosphatase Treatment of p70<sup>65k</sup>—p70<sup>65k</sup> was immunoprecipitated from cells as described above. The immunoprecipitates were washed twice with lysis buffer and twice with phosphatase buffer (20 mM PIPES, pH 6.8, 20 mM KCl, 1 mM dithiothreitol, 1 mM MgCl<sub>2</sub>, 0.05% Brij 35). The complexes were then incubated for 30 min at 25 °C in 50 μl of phosphatase buffer containing 0.375 unit of potato acid phosphatase (Calbiochem-Novabiochem). Following incubation with phosphatase, an equal volume of stop buffer (20 mM potassium phosphate, pH 7.2, 0.1 mM sodium vanadate, 20 mM β-glycerophosphate) was added, and the complexes were washed twice with Tris-buffered saline. Proteins were eluted from the complex by boiling in SDS-sample buffer, and the samples were then immunoblotted with p70<sup>65k</sup> antibodies as described above.

RESULTS AND DISCUSSION

To examine possible coupling of GPCRs to p70<sup>65k</sup>, we used Rat-1 fibroblasts stably transfected with either the human α2c10 adrenergic receptor (clone 1C) or with an equivalent HA-tagged version of the porcine receptor (clone TAG WT3).

TABLE I

| p70<sup>65k</sup> activity | Rat-1 | TAG WT3 | Clone 1C |
|--------------------------|------|--------|---------|
| pmol phosphate min<sup>-1</sup> mg protein<sup>-1</sup> |      |        |         |
| Basal                     | 0.19±0.04 | 0.54±0.08 | 1.26±0.05 |
| PDGF (10 ng/ml)           | 1.08±0.16   | 3.61±0.17  | 2.02±0.12  |
| LPA (5 μM)                | 0.93±0.12   | 2.39±0.13  | 1.79±0.07   |
| UK 14304 (1 μM)           | 0.18±0.03   | 0.91±0.02  | 3.07±0.11   |

*Significantly different from basal activity (p < 0.05; Student’s t test).

Stimulation of 1C cells (which express around 3.5 pmol of receptor/mg of membrane protein (33)) with the α2 agonist UK 14304 led to a transient increase in p70<sup>65k</sup> activity measured by an immunocomplex kinase assay (Table I). Maximal activation (approximately 3-fold) was observed around 10–20 min after stimulation and although activity declined thereafter it remained above basal levels for at least 3 h (data not shown). In TAG WT3 cells (which express around 400 fmol of receptor/mg of membrane protein), UK 14304 activated p70<sup>65k</sup> to a lesser extent than in 1C cells (Table I). In parental Rat-1 cells, which do not express detectable levels of α2 receptor (33), UK 14304 did not significantly increase p70<sup>65k</sup> activity. In addition to the effects of UK 14304, the mitogenic glycerophospholipid LPA, acting through endogenously expressed GPCRs, activated p70<sup>65k</sup> in all three cell types (Table I). Thus, activation of p70<sup>65k</sup> by G protein-coupled agonists may be a generalized phenomenon in these cells which does not require heterologous high level expression of the appropriate receptor.

As expected, the tyrosine kinase receptor ligand PDGF also strongly coupled to p70<sup>65k</sup> activation in both α2 receptor-transfected and nontransfected cells (Table I). The relative activa-
tion of p70<sup>65k</sup> induced by PDGF, and indeed by LPA, was substantially higher in the untransfected cells, but this appears to reflect elevated basal p70<sup>65k</sup> activities in both the TAG WT3 and 1C cells (Table I). Attempts to down-regulate basal p70<sup>65k</sup> activity in these cells by manipulating the conditions used for serum deprivation were unsuccessful (data not shown). Similarly, elevated MAP kinase basal activity in these cells has previously been reported (29).

Activation of p70<sup>65k</sup> correlates strongly with its phosphoryla-
tion on multiple sites (13, 14). Therefore, we next examined whether the α2 receptor-mediated activation of p70<sup>65k</sup> was ac-
companied by enhanced phosphorylation of the protein. Phos-
phorylation of p70<sup>65k</sup> results in a reduction in the relative mobility of the protein on SDS-polyacrylamide gel electro-
phoresis, and this is often used as an indicator of p70<sup>65k</sup> activa-
tion status (17). Fig. 1 shows an immunoblot of p70<sup>65k</sup> fol-
lowing treatment of cells with different agonists. In parental Rat-1 cells, PDGF and, to a lesser extent, LPA induced the appearance of slower migrating forms of p70<sup>65k</sup>, whereas UK 14304 had no such effect. Immunoblotting of clone 1C cells revealed that most of the p70<sup>65k</sup> protein was detected as the slower migrating p70<sup>65k</sup> isoform in both the TAG WT3 and 1C cells (Table I). Attempts to down-regulate basal p70<sup>65k</sup> activity in these cells by manipulating the conditions used for serum deprivation were unsuccessful (data not shown). Similarly, elevated MAP kinase basal activity in these cells has previously been reported (29).

To examine possible coupling of GPCRs to p70<sup>65k</sup>, we used Rat-1 fibroblasts stably transfected with either the human α2c10 adrenergic receptor (clone 1C) or with an equivalent HA-tagged version of the porcine receptor (clone TAG WT3).
Together, these results show that activation of p70S6K by GPCRs results in increased phosphorylation of p70S6K. A, Rat-1 cells (left panel) or clone 1C cells (right panel) were left untreated (C) or treated for 10 min with 10 ng/ml PDGF (P), 10 μM LPA (L), or 1 μM UK 14304 (U). Lysates were prepared, and 25 μg of lysate protein from each sample was immunoblotted with anti-p70S6K. The fastest and slowest of the four immunoreactive bands representing various phosphorylation states of the enzyme are indicated by the arrowheads. B, p70S6K immunoprecipitated from control, LPA, or UK 14304-stimulated clone 1C cells were treated or not with potato acid phosphatase and then subjected to immunoblotting with anti-p70S6K antibodies as described under "Experimental Procedures." C, clone 1C cells were metabolically labeled with 32P-P, and then left untreated (control) or treated with LPA or UK 14304. p70S6K was immunoprecipitated from the cells as described under "Experimental Procedures" and electrophoresed, and the dried gel was exposed to x-ray film for 24 h. The p70S6K bands were excised from the gel and counted for radioactivity. Duplicate immunoprecipitations from a single experiment, performed on one other occasion with similar results, are shown.

To confirm that activation of p70S6K by GPCRs results in increased phosphorylation of the protein, the cells were metabolically labeled with 32P-P, and the levels of phosphate incorporated into p70S6K were measured. Fig. 1C shows that both UK 14304 and LPA induce an increase in the phosphorylation of p70S6K. Counting of the 32P incorporated into the p70S6K bands revealed that LPA induced a 43.0 ± 4.0% increase and UK 14304 induced a 65.9 ± 4.3% increase in p70S6K phosphorylation. For comparison, PDGF, the most potent activator of p70S6K used in this study, increased the phosphorylation of p70S6K by around 2-fold in Rat-1 cells (data not shown). Taken together, these results show that activation of p70S6K by GPCRs correlates with enhanced phosphorylation of the protein. Whether G1-mediated signals result in phosphorylation of the same sites on p70S6K induced by other effectors remains to be determined.

The immunosuppressive drug rapamycin blocks the activation of p70S6K by preventing or reversing its phosphorylation at key sites necessary for activity (11–14, 17, 18). The mechanism underlying this effect has not been characterized completely, but recent reports have identified the protein kinase FRAP/RAFT (38–40) as a specific target for rapamycin in mammalian cells. The activation of p70S6K by UK 14304, LPA, and PDGF in the present study was completely blocked by pretreatment of cells with rapamycin (Fig. 2) indicating that there are commonalities in the mechanisms employed by both G protein-coupled agonists and other agents to elicit activation of p70S6K. In contrast, only the activation of p70S6K by UK 14304 and LPA was blocked by pretreatment of cells with pertussis toxin (Fig. 3), consistent with their effects being mediated via the G1 family of heterotrimeric G proteins. Although pertussis toxin pretreatment also led to a significant reduction in the p70S6K activity precipitated from PDGF-stimulated cells (Fig. 4), this is entirely accounted for by pertussis toxin-mediated inhibition of basal p70S6K activity. Thus, the relative increases in p70S6K activity induced by PDGF in control and pertussis toxin-treated cells were 42 ± 4% and 45 ± 3% (n = 3), respectively.

Although the precise mechanisms of activation of p70S6K are unknown, a number of studies using the selective inhibitor wortmannin indicate that PI 3-kinase lies upstream of p70S6K in a signaling cascade induced by a number of tyrosine kinase receptors (19–23). More recent work suggests that p70S6K contains a set of wortmannin-sensitive phosphorylation sites (13, 14, 41). We therefore sought evidence for PI 3-kinase involvement in the activation of p70S6K by GPCRs. Pretreatment of clone 1C cells with wortmannin led to a dose-dependent attenuation of p70S6K activation by UK 14304 (Fig. 4). Approximately 50% inhibition occurred with concentrations of wortmannin around 30 nM with complete inhibition evident at 50–100 nM. These concentrations are similar to those reported to inhibit p70S6K activation by other growth factors (19–23). Wortmannin also prevented the full activation of p70S6K by LPA (Table I). We also tested the effects of LY 294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), another PI 3-kinase inhibitor, structurally unrelated to wortmannin (20). Prèncubation of
3-kinase-identified (1–3) and, very recently, a novel G protein-activated clone 1C cells with 50\(^{-}\text{triplicate assays and are representative of experiments carried out on hyperphosphorylation of the S6 kinase p70s6k. The activation is results in a pertussis toxin-sensitive activation and coincident agonists in Rat-1 cells. Whether this is the same PI 3-kinase 3-kinase is required for the activation of p70s6k by Gi-linked kinase receptor ligands is not known. It is worth noting that Lysates were prepared, and p70s6k activity was measured on the immune complex as a percentage of the maximal stimulatory effect of UK 14304 for 10 min. Prior to stimulation with the agonists as given in Table I. Lysates were prepared and p70s6k activity was measured on the immune complex as a percentage of the maximal stimulatory effect of UK 14304 for 10 min. Wortmannin blocks activation of p70s6k by UK14304. In conclusion, we have shown that stimulation of both endogenous and heterologously expressed G\(_i\)-coupled receptors on a pertussis toxin-sensitive activation and coincident hyperphosphorylation of the S6 kinase p70\(^{\text{S6K}}\). The activation is sensitive to both rapamycin and wortmannin and as such may be similar mechanistically to that stimulated by ligands signaling via tyrosine kinase receptors. Future work will be directed toward understanding the precise mechanisms underlying the effects of G\(_i\)-coupled agonists on p70\(^{\text{S6K}}\). particularly the potential role of \(\beta y\) signaling complexes.

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