Muscarinic receptor-mediated activation of p70 S6 kinase 1 (S6K1) in 1321N1 astrocytoma cells: permissive role of phosphoinositide 3-kinase

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In 1321N1 astrocytoma cells, carbachol stimulation of M_3 muscarinic cholinergic receptors, coupled to phospholipase C, evoked a persistent 10–20-fold activation of p70 S6 kinase (S6K1). This response was abolished by chelation of cytosolic Ca^{2+} and reproduced by the Ca^{2+} ionophore ionomycin, but was not prevented by down-regulation or inhibition of protein kinase C. Carbachol-stimulated activation and phosphorylation of S6K1 at Thr^{389} were prevented by rapamycin, an inhibitor of mTOR (mammalian target of rapamycin), or by wortmannin, a phosphoinositide 3-kinase (PI3K) inhibitor. Carbachol also stimulated the phosphorylation of eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), a second mTOR-dependent event, with similar potency to its effect on S6K1. This response was blocked by rapamycin, but was not markedly affected by 100 nM wortmannin, implying separate roles for mTOR and PI3K in S6K1 activation. Wortmannin abolished the carbachol-stimulated rise in PtdIns(3,4,5)P_3 and greatly reduced unstimulated levels of this lipid. By contrast, an inhibitor of epidermal growth factor receptor kinase, AG1478, which prevents carbachol-stimulated ErbB3 transactivation, PI3K recruitment and protein kinase B activation in 1321N1 cells, reduced activation of S6K1 by no more than 30 %. This effect was overcome by 10 nM insulin, which on its own did not stimulate S6K1, but increased cellular PtdIns(3,4,5)P_3 concentrations comparably with carbachol alone. These observations distinguish obligatory roles for mTOR and PI3K in regulating S6K1, but imply that minimal PI3K activity is sufficient to permit stimulation of S6K1 by other activating factors such as increased cytosolic Ca^{2+} concentrations, which are essential to the muscarinic receptor-mediated response. Moreover, 4E-BP1 and hence, presumably, mTOR can be regulated independently of PI3K activation through these mechanisms.

Key words: cytosolic Ca^{2+}, eukaryotic initiation factor 4E-binding protein (4E-BP1), mammalian target of rapamycin (mTOR), phosphoinositide 3-kinase (PI3K), phospholipase C (PLC).

INTRODUCTION

p70 S6 kinase 1 (S6K1) phosphorylates the 40 S ribosomal protein S6 [1]. This is widely believed to enhance translation of mRNAs with 5′-terminal oligopyrimidine tracts, which generally encode ribosomal proteins and elongation factors [1,2]. Through this mechanism, S6K1 activation increases ribosomal biogenesis and thus enhances cellular translation capacity. Inhibition of S6K1 with either specific antibodies or the immunosuppressant drug rapamycin blocks cell-cycle progression through G_1 and can inhibit cell growth [3–5]. In support of the latter, S6K1-deficient *Drosophila* or knockout mice have significantly reduced body sizes [6,7].

The α and β isoforms of p70 S6 kinase are the products of two different genes and are also called S6K1/S6K2 [8–11], but only the former has been the subject of detailed investigations aimed at understanding its regulation. Not surprisingly, a diverse range of hormones and growth factors acting via receptor and non-receptor tyrosine kinases or G-protein-coupled receptors (GPCRs) are capable of activating S6K1 [12,13]. The mechanism of activation is incompletely understood and involves phosphorylation on multiple serine and threonine residues. Five proline-directed sites have been identified in the C-terminal autoinhibitory domain at Ser^{404}, Ser^{411}, Ser^{418}, Ser^{421} and Ser^{424}. They are rapidly phosphorylated in response to various stimuli and this contributes to, but does not fully account for, activation. However, the kinase(s) acting at these sites remain to be identified [14,15]. Mammalian target of rapamycin (mTOR) has been reported to phosphorylate Thr^{389} and possibly some of the C-terminal sites noted above [16]. The immunosuppressive actions of rapamycin are the result of its binding to the immunophilin FK506-binding protein 12 to form a complex that binds and inhibits mTOR. Phosphorylation of Thr^{389} is rapamycin-sensitive and changes in its phosphorylation state correlate well with S6K1 activity [17,18]. Phosphoinositide-dependent kinase 1 (PDK1) also plays a pivotal part in the activation of S6K1 [19]. PDK1 appears to bind S6K1 directly through an interaction of the kinase domain with phosphorylated Thr^{389} of the substrate. This interaction enhances PDK1-dependent phosphorylation of Thr^{229} in S6K1 [20]. Type I phosphoinositide 3-kinases (PI3Ks), which generate the lipid second messenger PtdIns(3,4,5)P_3, are believed to play a role in agonist-stimulated S6K1 activation, since such responses are generally blocked by PI3K inhibitors such as wortmannin. The target for PtdIns(3,4,5)P_3 did not appear to be PDK1, since the latter's ability to phosphorylate Thr^{229} is not modulated by phosphoinositides [19]. Recent data [21–23], however, support a model involving PtdIns(3,4,5)P_3-dependent phosphorylation and activation of protein kinase B (PKB) by PDK1. In this model, activated PKB phosphorylates the tumour suppressor protein tuberous sclerosis complex 2 (TSC2), which is complexed with

Abbreviations used: BAPTA/AM, acetoxymethyl ester of bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid; eIF4E, eukaryotic initiation factor 4E; 4E-BP1, eIF4E-binding protein-1; GPCR, G-protein-coupled receptor; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; m^7GTP, 7-methyl-GTP; mTOR, mammalian target of rapamycin; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; Ro-318220, bisindolylmaleimide IX; S6K1, p70 S6 kinase 1; TSC, tuberous sclerosis complex.

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TSC1 and binds and inactivates mTOR. Upon phosphorylation by PKB, the complex appears to dissociate, releasing active mTOR [21–23]. Such a model might account for the PI3K-dependency of S6K1 responses, but it is important to note that mTOR remains active in PDK1-knockout cells [24]. Additionally, contradictory studies on the role of PKB [25] perhaps imply that both PKB-dependent and -independent pathways operate in different cell types and organisms.

Several GPCR agonists, including μ-opioid, gastrin/cholecystokinin type B, prostaglandin $F_{2\alpha}$ and angiotensin II type I [26–29], have been shown to activate S6K1 in a wortmannin-sensitive manner and concomitantly to stimulate PKB activity. By contrast, α-adrenergic receptors stimulate S6K1 in a wortmannin- or LY294002-sensitive manner, which was not, however, accompanied by enhanced PKB activity [11,30,31], implying dependence on PI3K without PI3K activation. An important difficulty in distinguishing the roles of PI3K and mTOR in S6K1 activation results from the fact that the latter is homologous with members of the PI3K family and can itself be inhibited by relatively high levels of wortmannin or by LY294002 [32]. Thus it is necessary to distinguish carefully between effects of PI3K inhibitors on PI3K and mTOR respectively. Our previous work [33] has extensively characterized the signalling pathways that are initiated upon activation of phospholipase C (PLC)-coupled M_3 muscarinic GPCRs in 1321N1 astrocytoma cells. Surprisingly, muscarinic agonists cause a small activation of PI3K via Ca^{2+}-dependent transactivation of ErbB3. We show in the present study that these receptors evoke a large activation of S6K1 via a Ca^{2+}-dependent pathway. The activation of S6K1 is driven primarily via stimulation of mTOR that does not itself require either PI3K or activation of PKB. Interestingly, PI3K provides an essential permissive signal for S6K1 activation requiring only basal or slightly elevated levels of PtdIns(3,4,5)P_3. The likely significance of a signalling role for basal PtdIns(3,4,5)P_3 is discussed.

EXPERIMENTAL

Materials

1321N1 cells were obtained from the European Tissue Culture Collection. 7-Methyl-GTP (m^7GTP)–Sepharose™ 4B and *myo*-[2-^{3}H]inositol (approx. 20 Ci/mmol) were from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Antibodies to the following were obtained from the sources indicated: PKB and S6K1 (MRC Protein Phosphorylation Unit, University of Dundee, Dundee, U.K.), and phospho-Thr^{389} S6K1 (Cell Signalling, Hitchin, Herts., U.K.). Antibodies specific for eukaryotic initiation factor 4E (eIF4E) and eIF4E-binding protein-1 (4E-BP1) were from sources described previously [11]. Other materials were from sources described previously [33–35].

Cell culture

1321N1 cells were cultured in Dulbecco's modified Eagle's medium containing or lacking *myo*-inositol and supplemented with foetal calf serum and antibiotics as described previously [35].

Measurement of cellular [^{3}H]phosphoinositides

Cells grown to confluence in 6-well plates under inositol-depleting conditions were labelled with [^{3}H]inositol (10 μCi/ml) for 2 days, washed twice with 4 ml of serum- and inositol-free Dulbecco's modified Eagle's medium and then serum starved overnight by incubation in the same medium supplemented with 0.5 % (w/v) BSA, as described previously [34,35]. These cells were then incubated under culture conditions with drugs without further change of medium. Cells were fixed and ^{3}H-labelled metabolites were extracted and analysed as described previously [34,35].

Preparation of cell lysates

For experiments measuring phosphorylation of 4E-BP1, cells were incubated with drugs under culture conditions after prior serum starvation, as described above, but without inositol depletion or radiolabelling. Alternatively, confluent cell cultures were washed three times with incubation buffer {Hepes-buffered modified Krebs–Henseleit buffer [118 mM NaCl, 4.69 mM KCl, 1.18 mM $MgSO_4$, 1.29 mM $CaCl_2$, 1.18 mM KH_2PO_4, 11.67 mM glucose, 25 mM Hepes (pH 7.4) at 37 °C with NaOH]} and preincubated for 30 min at 37 °C in the same buffer prior to exposure to stimuli or appropriate vehicle. Where indicated, antagonists or inhibitors were present for all or part of the 30 min preincubation period as specified in the Figure legends. Incubations were stopped by rapid removal of the incubation buffer and addition of ice-cold lysis buffer as described previously [33].

S6K1 assay

S6K1 was immunoprecipitated from cell lysates (approx. 1 mg of protein) and the immunoprecipitates were assayed as described previously [33], except that assays were continued for 45 min at 30 °C.

SDS/PAGE and immunoblotting

SDS/PAGE and Western blotting were performed as described previously [11,36]. For 4E-BP1 dissociation, cell lysates were treated with m^7GTP–Sepharose and bound proteins were analysed as described previously [11].

RESULTS

Muscarinic receptor stimulation activates S6K1

The cholinergic agonist carbachol evoked a large and persistent activation of S6K1 in 1321N1 cells. This response is illustrated in Figure 1(A) in which S6K1 activity was analysed in immunoprecipitates from cells treated for different times with carbachol. Carbachol induced a time-dependent stimulation of S6K1 activity, which showed a delay in onset of approx. 2 min and reached a maximum of 15-fold above basal after 20 min. This response was potently inhibited by atropine (results not shown), showing that it was due to activation of muscarinic receptors (presumably M_3 receptors, since they predominate in this cell line [37]). The effects of carbachol occurred with an EC_{50} of approx. 20 μM (Figure 1B), a concentration similar to that required for half-maximal activation of PLC in these cells [33].

Ca^{2+} signals are necessary and sufficient for carbachol-stimulated S6K1 activity

Muscarinic receptors couple, most probably via G_q, to the stimulation of PLC in 1321N1 cells [37]. The signalling pathways downstream of PLCs involve Ins(1,4,5)P_3-mediated Ca^{2+} release

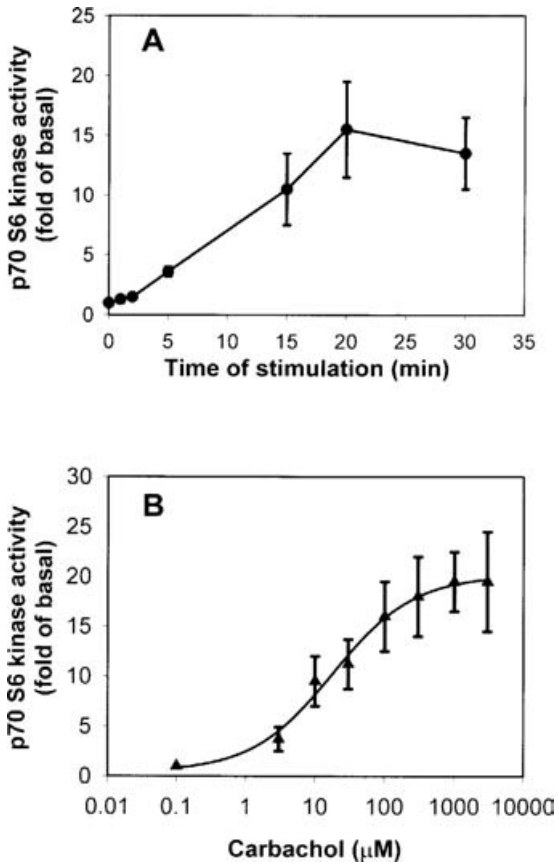

Figure 1 Muscarinic receptor activation stimulates S6K1

1321N1 cells were incubated with carbachol (1 mM) for the times indicated (**A**) or at the concentrations indicated for 20 min (**B**). Lysates were then prepared, S6K1 was immunoprecipitated and its activity was assayed. The results are expressed as fold-stimulation over control values and represent the means ± S.E.M. of three separate experiments.

and diacylglycerol-stimulated PKC activity. To investigate the role of PKCs in the activation of S6K1 by carbachol, these enzymes were either down-regulated by treating the cells for 24 h with the phorbol ester PMA or acutely inhibited using the PKC (protein kinase C) inhibitor bisindolylmaleimide IX (Ro-318220). Similar treatment of 1321N1 cells with PMA has been shown to reduce levels of the predominant PKC α, ε and ζ isoforms expressed in these cells by at least 95%, 80% and 40% respectively [38]. Neither down-regulation of PKCs nor acute Ro-318220 treatment reduced the ability of carbachol to stimulate S6K1 activity (Table 1). On the contrary, Ro-318220 actually enhanced both basal and carbachol-stimulated S6K1 activity, suggesting that either PKC or another Ro-318220-sensitive target [39] exerts a tonic inhibitory influence on S6K1 activity in these cells. The latter effect could also be due to the activation of stress pathways by Ro-318220, but this does not detract from the fact that two different PKC inhibitory strategies failed to prevent carbachol-stimulated S6K1 activation.

The mitogen-activated protein kinase (MAP kinase) and/or the c-Jun N-terminal kinase (JNK) pathways [11] have both been implicated in S6K1 responses to stimulation of GPCRs. However, selective inhibitors of MAP kinase activation such as U0126 had no effect at all on the activation of S6K1 by carbachol (Table 1), whereas JNK was not activated by this stimulus in 1321N1 cells.

The data in Figure 2 and Table 1 address the role of Ca^{2+} in the activation of S6K1 by carbachol. Figure 2 shows that the

Table 1 Effects of pharmacological intervention on carbachol-stimulated S6K1 activity

For inhibitor studies, cells were transferred to incubation buffer and preincubated for 30 min before exposure to carbachol (1 mM) for 15 min. Cells were lysed and S6K1 activity was measured. For PKC down-regulation, cells were pretreated overnight with PMA (1 μM) in the absence of serum, but in the presence of 0.5% BSA, or similarly treated with vehicle control (0.01% DMSO) and then transferred into incubation buffer and preincubated for 30 min before exposure to carbachol (1 mM) for 15 min. BAPTA/AM treatment was performed as follows: cells were preloaded for 60 min with BAPTA/AM (40 μM) in the absence of serum or similarly treated with vehicle control (1% DMSO) and then transferred into incubation buffer and preincubated for 30 min before exposure to carbachol (1 mM) for 15 min. Results are the means ± S.E.M. for the number of independent experiments indicated, except where $n = 2$, when results are the means ± the range of the data obtained.

	S6K1 activity (% of carbachol-stimulated activity)	
	− Carbachol	+ Carbachol
Control	9.4 ± 3	100 ($n = 5$)
PMA (1 μM overnight)	15.2 ± 1.3	92 ± 6.5 ($n = 3$)
PMA (1 μM for 10 min)	32 ± 5.5 ($n = 2$)	
Ro-318220 (10 μM)	24.3 ± 1.5	213 ± 18.7 ($n = 3$)
BAPTA/AM (40 μM)	7.2 ± 0.9	13.5 ± 8.7 ($n = 3$)
U0126 (10 μM)	10.2 ± 1.2	103 ± 8.3 ($n = 2$)
Wortmannin (100 μM)	5 ± 0.76	6.6 ± 0.8 ($n = 3$)
Rapamycin (100 μM)	4.2 ± 1.3	3.8 ± 0.9 ($n = 3$)

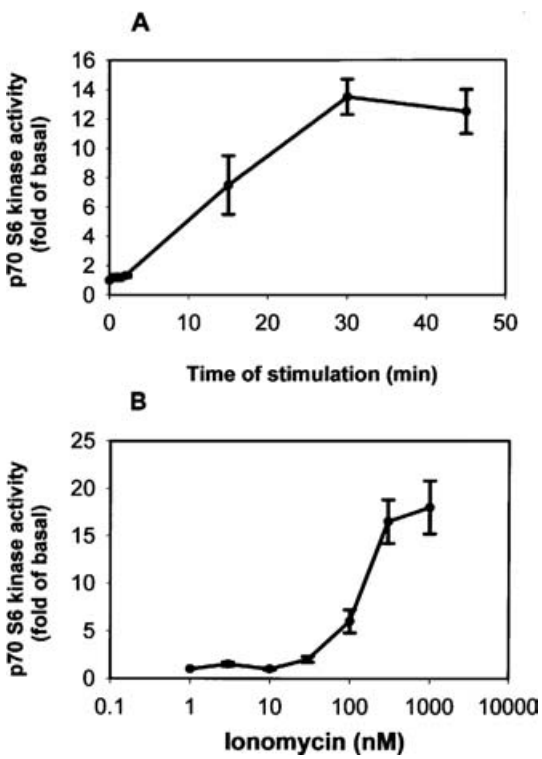

Figure 2 Increased intracellular Ca^{2+} is sufficient for stimulation of S6K1

Cells were incubated with ionomycin (1 μM) for the times indicated (**A**) or at the concentrations indicated for 30 min (**B**). Lysates were then prepared, S6K1 was immunoprecipitated and its activity was assayed. The results are expressed as fold stimulation over control values and are the means ± S.E.M. of three experiments.

Ca^{2+} ionophore ionomycin stimulated S6K1 activity to a similar extent and over a similar time course to carbachol. Table 1 shows the effects of buffering intracellular [Ca^{2+}] by treating cells with the cell-permeant acetoxymethyl ester of the Ca^{2+}

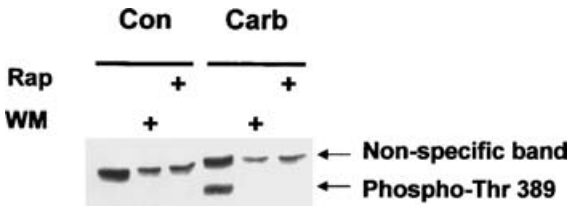

Figure 3 Stimulation of S6K1 activation and phosphorylation of S6K1 at Thr^{389} by carbachol are blocked by wortmannin and rapamycin

Cells were serum-starved overnight and then pretreated in the absence or presence (+) of wortmannin (WM; 100 nM) or rapamycin (Rap; 100 nM) for 30 min. Cells were then incubated further without (as control; Con) or with carbachol (Carb; 1 mM) for 15 min. Lysates were then prepared. Phosphorylation of S6K1 at Thr^{389} was determined by resolving samples (50 μg of protein) by SDS/PAGE and immunoblotting with anti-(phospho-S6K1-Thr^{389})-specific antibodies. Similar results were obtained in three further experiments. The upper band shown is non-specific protein.

chelator bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid (BAPTA/AM). Carbachol-induced S6K1 activity was almost abolished in cells loaded with BAPTA/AM. As the PLC response to carbachol was not similarly affected by the chelator (results not shown), these data clearly imply that the muscarinic receptor-mediated stimulation of S6K1 is dependent upon an increase in intracellular Ca^{2+} distal to PLC activation and that elevated intracellular Ca^{2+} suffices to elicit activation of S6K1.

Carbachol-stimulated S6K1 activation is blocked by either rapamycin or wortmannin

Most current models of regulation of S6K1 involve PI3K acting upstream of mTOR, but the inter-relationships between these components are still poorly understood. It was not surprising, therefore, to observe that carbachol-simulated S6K1 was potently and completely inhibited both by rapamycin, an inhibitor of mTOR, and by the PI3K inhibitor wortmannin (Table 1). Also as expected, carbachol stimulated the phosphorylation of Thr^{389} in S6K1 (the key residue for mTOR-dependent regulation of S6K1 [40]) and this effect was also potently inhibited by either rapamycin or wortmannin (Figure 3). At the same concentration, wortmannin abolished the carbachol-stimulated rise in cellular PtdIns(3,4,5)P_3, reducing its level to approx. 60% below that which was observed in unstimulated cells (Table 2). Moreover, wortmannin also reduced basal levels of PtdIns(3,4,5)P_3 by approx. 75%, implicating significant basal PI3K activity in the maintenance of a steady state concentration of this lipid signal in unstimulated cells (Table 2). These observations are consistent with an obligatory role for PI3K in S6K1 activation.

Table 2 Cellular PtdIns(3,4,5)P_3 level in resting and carbachol-stimulated cells is reduced by wortmannin

Serum-starved cells labelled with [^{3}H]inositol (10 μCi/ml) were incubated for 30 min with or without wortmannin (100 nM) and then incubated further in the absence (control) or presence of carbachol (1 mM) for 15 min before extraction and analysis of the ^{3}H-labelled metabolites. Results are expressed as the means ± S.E.M. of five experiments and reflect radioactivity (d.p.m.) in PtdIns(3,4,5)P_3/well of cells (approx. 1 mg of protein). Control concentrations of [^{3}H]PtdIns(3,4,5)P_3 were approx. 0.1% of those of [^{3}H]PtdIns(4,5)P_2.

	PtdIns(3,4,5)P_3 (d.p.m./well of cells)
Control	1500 ± 160
Control + wortmannin	320 ± 78
Carbachol	3520 ± 450
Carbachol + wortmannin	610 ± 140

Wortmannin discriminates between mTOR and PI3K signalling

To establish whether the wortmannin-sensitive step in the activation of S6K1 lies upstream or downstream of mTOR, we studied the phosphorylation of 4E-BP1 (also known as PHAS-1), another target of mTOR signalling [41]. Phosphorylation of 4E-BP1 leads to its dissociation from eIF4E and both can serve as readouts of mTOR activity [42,43]. Carbachol stimulated the dissociation of 4E-BP1 from eIF4E with a similar time course (Figure 4A) to the S6K1 response. The dose-dependency was also

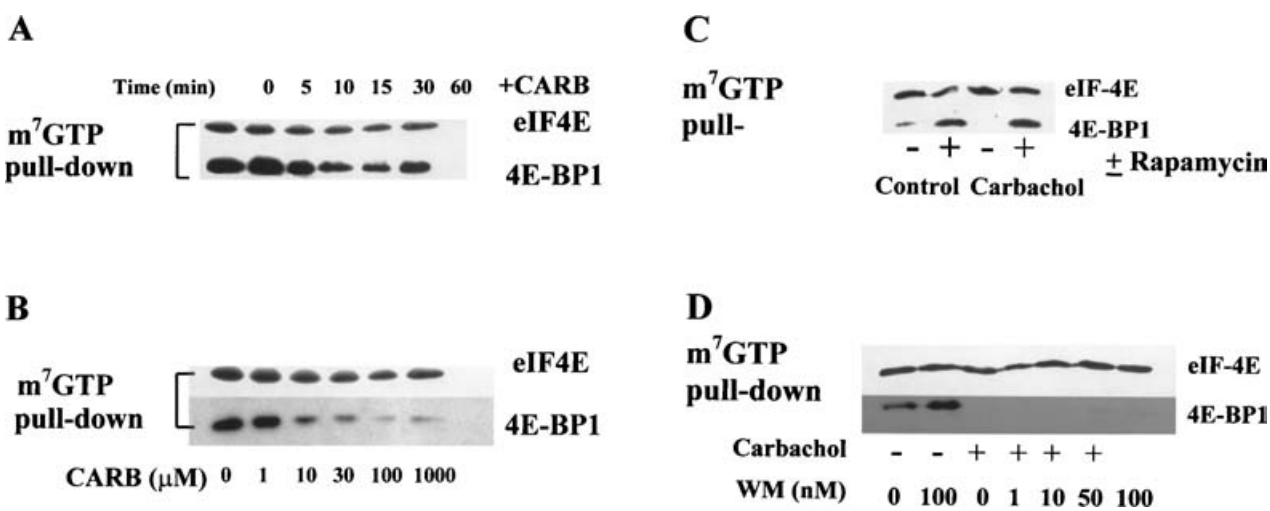

Figure 4 Carbachol stimulation of 4E-BP1 release from eIF4E is inhibited by rapamycin, but not wortmannin

(**A**) Time course: cells were serum-starved overnight and incubated with 1 mM carbachol (+ CARB) for the times indicated and lysed. (**B**) Dose-dependence. (**C**) Effects of rapamycin (100 nM). (**D**) Effects of wortmannin (WM; 1–100 nM). In (**C**) and (**D**), cells were incubated in the absence (−) or presence (+) of inhibitors for 30 min prior to stimulation with carbachol (1 mM) for 15 min before lysis. Cell extracts were applied on to m^{7}GTP–Sepharose and bound proteins were separated by SDS/PAGE. eIF4E and 4E-BP1 were detected by immunoblotting. The upper eIF4E band indicates even sample loading. Similar results were obtained in three further experiments.

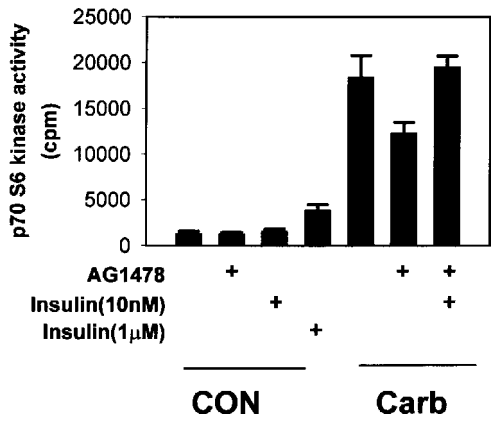

Figure 5 Carbachol-stimulated S6K1 activation is only partially inhibited by AG1478

Cells were preincubated with AG1478 (100 nM) or treated with vehicle control [0.01 % (v/v) DMSO] and then transferred into incubation buffer and preincubated for 30 min before exposure to carbachol (Carb; 1 mM) and/or insulin (10 nM or 1 μM) for 15 min. Cells were then lysed and assayed for S6K1 activity. Data represent the means $\pm$ S.E.M. of triplicate incubations in a single experiment representative of three similar experiments.

similar (EC_{50} approx. 10 μM; Figure 4B) As expected, 4E-BP1 dissociation was ablated by pretreatment of cells with rapamycin (Figure 4C). Surprisingly, however, increasing concentrations of wortmannin, even up to 100 nM, had little effect on the carbachol-induced dissociation of 4E-BP1 from eIF4E (Figure 4D). This behaviour contrasts with the effects of wortmannin on S6K1, activation and phosphorylation (at Thr^{389}) of which were completely blocked at 100 nM (Figure 3 and Table 1). This suggests that the wortmannin-sensitive step in the pathway through which carbachol activates S6K1 lies downstream of mTOR or on a parallel, but essential, pathway. The results also strongly suggest that carbachol-stimulated 4E-BP1 dissociation and hence mTOR activation do not require PI3K. Moreover, the results show that wortmannin can be used as a selective pharmacological tool that inhibits PI3Ks at significantly lower doses than are required to block mTOR directly. This contrasts with LY294002, another commonly used inhibitor of PI3Ks, which does not discriminate between these two targets [44].

Low levels of PI3K activity provide a permissive signal for Ca^{2+}-stimulated S6K1 responses

As the above data show that PLC-mediated Ca^{2+} signals are capable of activating S6K1 in a wortmannin-sensitive manner, we examined in more detail the role of PI3K in this response. Our previous work established that carbachol couples indirectly to PI3K through the Ca^{2+}-dependent transactivation of ErbB3 [33]. This pathway can be blocked by treating cells with the epidermal growth factor receptor kinase inhibitor AG1478. Surprisingly, 100 nM AG1478, which prevents any detectable increase of PtdIns(3,4,5)P_3 or PKB activity in response to carbachol [33], only reduced S6K1 activation by at most 30 % (Figure 5), and increasing the concentration of this inhibitor to 1 μM had no greater effect (results not shown). Carbachol is a relatively weak activator of PI3K causing, at most, only a 1.5–2-fold increase in PtdIns(3,4,5)P_3 levels ([33] and Table 2), but this is amplified to give a substantial activation of PKB [33]. Thus PKB activity is a sensitive indicator of the activity state of PI3K, and the failure of carbachol to activate PKB in the presence of AG1478 provides strong evidence that PtdIns(3,4,5)P_3 remains at the basal level under these conditions. Thus carbachol can stimulate S6K1 by approx. 10-fold without any activation of PI3K. Moreover, the full response to carbachol could be restored to AG1478-treated cells by a low dose of insulin, which caused a 3-fold increase of PtdIns(3,4,5)P_3 levels and a similar enhancement of immunoprecipitable PI3K activity [33] comparable with that induced by carbachol (Figure 5; I. H. Batty and C. P. Downes, unpublished work). On its own, this low dose of insulin was unable to stimulate S6K1 significantly (Figure 5). At 1 μM, insulin elicited a 2-fold increase in S6K1 activity (Figure 5) and a more than 10-fold increase of PtdIns(3,4,5)P_3 [35]. Taken together, these results suggest either that the wortmannin-sensitive step in S6K1 activation is not PI3K or that a very low level of PI3K provides a permissive signal for the Ca^{2+}-dependent activation of S6K1 via mTOR. Indeed, basal levels of PI3K activity appear to be capable of supporting a large Ca^{2+}- and mTOR-dependent activation of S6K1. One possible alternative candidate for the wortmannin-sensitive component is mTOR, but this can be eliminated because concentrations of wortmannin that completely block phosphorylation and activation of S6K1 had little effect on a second target of mTOR, 4E-BP1.

DISCUSSION

We have made use of PLC-coupled M_3 receptors on 1321N1 astrocytoma cells to reveal novel features of the mechanism of activation of S6K1. Initially it was found that carbachol caused only a small increase in PtdIns(3,4,5)P_3 levels, but greatly elevated S6K1 activity, whereas insulin only modestly stimulated S6K1, but was a strong activator of PI3K [35]. The Ca^{2+} signals elicited by carbachol (presumably via activation of PLC) were found to be both essential and sufficient for strong activation of S6K1, but conventional PKCs, MAP kinase and JNK are not involved. As in the majority of reported studies, S6K1 activation was blocked by rapamycin and wortmannin, implicating mTOR and apparently also PI3K in the response to carbachol. We have previously shown [33] that carbachol can stimulate PI3K and hence PKB in these cells via Ca^{2+}-dependent transactivation of ErbB3. Surprisingly, however, inhibition of epidermal growth factor receptor kinase-mediated phosphorylation of ErbB3, using AG1478, only partially (<30 %) blocked the S6K1 response, while completely preventing any carbachol-stimulated activation of PKB. Indeed, in the presence of AG1478, carbachol was still able to elicit a 10-fold increase in S6K1 activity and remained much more effective than was insulin alone. These findings have allowed us to dissect the roles of stimulation of PI3K and PKB in the regulation of both mTOR and S6K1 via M_3 receptors.

The activity of mTOR signalling was also monitored by measuring 4E-BP1 release from eIF4E, which showed a similar dose-dependent relationship to the activation of S6K1 by carbachol. However, these responses were barely affected by 100 nM wortmannin. This result is compatible with *in vitro* data suggesting that mTOR is inhibited less potently by wortmannin than PI3K and establishes that PI3K is not involved in the stimulation of 4E-BP1 phosphorylation by muscarinic agonists [32]. The discriminating ability of wortmannin contrasts with the effects of LY294002, a frequently used alternative to wortmannin as an inhibitor of PI3K, but which inhibits mTOR at very similar concentrations. The wortmannin-sensitive step in the activation of S6K1 must therefore lie either downstream of mTOR and/or on an independent and parallel, but essential, pathway. The limited effects of wortmannin on mTOR signalling and of AG1478 on the overall activation of S6K1 together suggest that PKB stimulation plays little part in the activation of S6K1 by carbachol.

Although 100 nM wortmannin had little effect on mTOR signalling to 4E-BP1, it profoundly blocked activation of S6K1 and phosphorylation of Thr^{389}. This result implies distinct requirements for mTOR to phosphorylate and/or activate S6K1 and 4E-BP1 respectively. As the majority of the muscarinic receptor-mediated activation of S6K1 occurs without any stimulated increase in PtdIns(3,4,5)P_3 levels, this result implies either that wortmannin acts on a novel target (neither mTOR nor PI3K) or that basal PtdIns(3,4,5)P_3 provides a permissive signal for the activation of S6K1, but not 4E-BP1, via mTOR. The ability of 100 nM wortmannin to reduce basal PtdIns(3,4,5)P_3 levels by approx. 75 % is compatible with the latter possibility, which is the simplest interpretation of our results. In the presence of AG1478, a low dose of insulin that had no significant effect on its own restored the maximum activation of S6K1. This suggests that basal PtdIns(3,4,5)P_3 is sufficient to support 70 % of the response to carbachol and that only a modest stimulation of PI3K is needed to achieve the maximal activation.

One explanation for the apparent signalling role of basal PtdIns(3,4,5)P_3 could be that it is unusually high in these cells. This does not appear to be the case, however, since the activity state and degree of phosphorylation of PKB is very low in these cells [33]. Also, PtdIns(3,4,5)P_3 levels in several cell lines are very similar to those in 1321N1 cells, at approx. 0.1 % of those of PtdIns(4,5)P_2 [45,46]. Our results imply that stimulated PI3K activity is not a prerequisite for a cellular response to be sensitive to PI3K inhibitors. Moreover, the basal level of PtdIns(3,4,5)P_3 represents a set point that may be sufficient for some responses, but not others, and which, like other second messengers such as cAMP, may be subject to both positive and negative regulation. The potential for the resting concentration of cellular PtdIns(3,4,5)P_3 to contribute permissively to responses regulated by other stimulatory inputs has also been considered previously with respect to both Ras [46,47] and PKC [48].

How do our results contribute to the current understanding of the regulation of S6K1? The permissive role of basal PtdIns(3,4,5)P_3 suggests that the wortmannin-sensitive component in the pathway from M_3 receptors might have a relatively high affinity for this lipid signal. One such component, which is proposed to have a key role in regulating S6K1, is PDK1, whose pleckstrin homology ('PH') domain binds PtdIns(3,4,5)P_3 with an affinity approx. 10-fold greater than that of PKB [49]. PDK1 has been identified biochemically and genetically as an essential regulator of S6K1 [19,24] via the direct phosphorylation of Thr^{229} and via its role in activation of PKB. Neither mechanism, however, seems likely to account fully for the responses we have observed. In the first case, phosphorylation of Thr^{229} appears to be independent of PI3K/PtdIns(3,4,5)P_3 *in vitro* and requires the prior phosphorylation of Thr^{389}, which primes the site for PDK1 to interact directly with S6K1 [19,20]. As noted in the Introduction, recent data support a model in which PKB plays a critical role in the activation of mTOR through the phosphorylation and dissociation of TSC2/TSC1 [21–23]. This cannot explain the present data, since activation of mTOR itself was found to be relatively insensitive to wortmannin and because most of the response was still observed in AG1478-treated cells where carbachol-stimulated PKB activation was eliminated. Moreover, an obligatory role here for PKB is disputed in the literature [25], suggesting that alternative pathways also exist. Our data also suggest that either TSC2 is not involved in the carbachol-stimulated activation of S6K1 or TSC2 can be regulated independently of PKB. These possibilities are currently under investigation.

The large activation of S6K1 in response to PLC-coupled M_3 receptor activation in 1321N1 astrocytoma cells has allowed a detailed examination of this response under circumstances in which a role for stimulated PKB can be eliminated. Moreover, although mTOR is required for the response, its activation does not require PI3K. Finally, we have shown that, although PI3K is required for S6K1 activation, basal or minimally activated levels of PtdIns(3,4,5)P_3 are sufficient to fulfil this important signalling role.

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