Differential Regulation of mTOR-Dependent S6 Phosphorylation by Muscarinic Acetylcholine Receptor Subtypes

Barbara E. Slack* and Jan K. Blusztajn

Department of Pathology and Laboratory Medicine, Boston University School of Medicine, Boston, Massachusetts 02118

Abstract   Muscarinic receptors subserve many functions in both peripheral and central nervous systems. Some of these processes depend on increases in protein synthesis, which may be achieved by activation of mammalian target of rapamycin (mTOR), a kinase that regulates protein translation capacity. Here, we examined the regulation of mTOR-dependent signaling pathways by muscarinic receptors in SK-N-SH human neuroblastoma cells, and in human embryonic kidney (HEK) cell lines transfected with individual muscarinic receptor subtypes. In SK-N-SH cells, the acetylcholine analog carbachol stimulated phosphorylation of the ribosomal S6 protein, a downstream target of mTOR. The sensitivity of the response to subtype-selective muscarinic receptor antagonists indicated that it was mediated by M3 receptors. Carbachol-evoked S6 phosphorylation was blocked by the mTOR inhibitor rapamycin, but was independent of phosphoinositide 3-kinase activation. The response was significantly reduced by the mitogen-activated protein kinase kinase (MEK) inhibitor U0126, which also inhibited carbachol-evoked S6 phosphorylation in HEK cells expressing M2 receptors, but was ineffective in M3 receptor-expressing HEK cells, although carbachol activated MAPK in both transfected lines. The p90 ribosomal S6 kinase has been implicated in mTOR regulation by phorbol esters, but was not activated by carbachol in any of the cell lines tested. The protein kinase C inhibitor bisindolylmaleimide I reduced carbachol-stimulated S6 phosphorylation in SK-N-SH cells, and in HEK cells expressing M3 receptors, but not in HEK cells expressing M2 receptors. The results demonstrate that multiple muscarinic receptor subtypes regulate mTOR, and that both MAPK-dependent and -independent mechanisms may mediate the response in a cell context-specific manner. J. Cell. Biochem. 104: 1818–1831, 2008.   © 2008 Wiley-Liss, Inc.

Key words: mammalian target of rapamycin; mitogen-activated protein kinase; ribosomal S6 protein

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates cell growth and proliferation by increasing protein translation. Its downstream targets include ribosomal S6 kinase 1 (S6K1), the eukaryotic initiation factor 4E binding proteins (4E-BPs), and eukaryotic elongation factor 2 kinase. mTOR is inhibited by reductions in the availability of glucose or amino acids, and by decreases in cellular ATP levels, and is activated by growth factors, hormones, and mitogens [Fingar and Blenis, 2004; Tee and Blenis, 2005]. Thus, mTOR functions to ensure that cell growth occurs at times when nutrient supply and energy levels are adequate. In the brain, mTOR regulates protein translation in response to neuronal activity, thereby modulating synaptic plasticity and long term memory formation [Kelleher et al., 2004a,b].

mTOR activity is tonically inhibited by the tumor suppressor proteins TSC1 (hamartin) and TSC2 (tuberin). These two proteins form a heterodimer that inhibits the small GTPase Rheb (Ras homolog enriched in brain) via the action of a GTPase activating protein (GAP) domain in the C-terminus of TSC2. Phosphorylation of TSC2 by the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) pathway relieves the inhibition of Rheb, which results in activation of mTOR by a mechanism that is not fully understood [Tee and Blenis, 2005].

Activation of mTOR by growth factors is well documented, and typically is PI3K-dependent [Hay and Sonenberg, 2004]. However, mTOR may also be regulated by G protein-coupled receptors (GPCRs), or by direct activators of protein kinase C (PKC), via PI3K-independent pathways [Wang et al., 2001; Wang and Proud, 2002; Tee et al., 2003; Arvisais et al., 2006]. In astrocytoma cells, stimulation of endogenous M3 muscarinic acetylcholine receptors by carbachol activated S6K1 and phosphorylated 4E-BP1 in a rapamycin-sensitive fashion. However, activation of S6K by carbachol was not prevented by the MEK inhibitor U0126, or by the PKC inhibitor Ro-318220 [Tang et al., 2003]. Moreover, although S6K1 activation by carbachol was sensitive to the PI3K inhibitor wortmannin, 4E-BP1 phosphorylation was not.

Because muscarinic receptors modulate a variety of processes that are believed to be dependent on protein synthesis, including long-term synaptic plasticity in hippocampus and visual cortex [Wess, 2004; Origlia et al., 2006], it was of interest to determine which of the subtypes might be coupled directly to the mTOR pathway. In the present study, the regulation of mTOR by muscarinic receptors was examined in a neuronal model, the SK-N-SH neuroblastoma cell line. Although these cells express all five muscarinic subtypes, pharmacological and immunological analyses indicate that the M3 and M1 isoforms predominate [Fisher and Heacock, 1988; Wall et al., 1991; Baumgartner et al., 1993]. Additional experiments were carried out in human embryonic kidney (HEK) cells transfected with individual muscarinic receptor subtypes. Our results suggest that multiple muscarinic receptor subtypes activate the mTOR-regulated translational machinery, but via distinct mechanisms that may be additionally influenced by the cellular context.

MATERIALS AND METHODS

Materials

Antibodies were obtained from the following sources: anti-S6 and anti-phospho-S6 (Ser 235/236), anti-MAPK and anti-phospho-MAPK (Thr202/Tyr204), anti-PKB and anti-phospho PKB (Ser473), anti-phospho-RSK1 (Ser380), anti-MEK1/2, and antibodies to the RXRXXpS/T consensus motif (used to detect TSC2 phosphorylated on Ser1798 [Roux et al., 2004]) were from Cell Signaling Technology (Beverly, MA); anti-tuberin (C-20) and anti-RSK1 (C-21) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and goat peroxidase-linked anti-rabbit IgG from Bio-Rad Laboratories (Hercules, CA). U0126 and rapamycin were obtained from Cell Signaling Technology. Carbamylcholine chloride (carbachol), wortmannin and atropine sulfate were purchased from Sigma, and AF-DX 384 and pirenzepine hydrochloride were obtained from Tocris (Ellisville, MO). Bisindolylmaleimide I (BIM I) was purchased from Calbiochem (La Jolla CA). Reagents, equipment and minigels for electrophoresis were supplied by Bio-Rad Laboratories. Other reagents and supplies were obtained from Sigma or Fisher Scientific (Pittsburgh, PA). The plasmid encoding constitutively active MEK1 was obtained from Stratagene (La Jolla, CA).

Cell Culture

SK-N-SH cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HEK cell lines transfected with M2 or M3 muscarinic receptor subtypes, designated HEK-M2 and HEK-M3 cells, respectively, have been described previously [Peralta et al., 1988; Sandmann et al., 1991; Slack, 2000]. Cells were maintained in Dulbecco's modified Eagle medium (DMEM)/F12 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). For experiments, the cells were seeded onto 60 or 100 mm tissue culture dishes and grown for 3 or 4 days until nearly confluent. Cultures were routinely incubated overnight in serum-free DMEM prior to an experiment. All subsequent pharmacological treatments were carried out in serum-free DMEM. Transient transfections were carried out using Lipofectamine Plus reagent

(Invitrogen) following the manufacturer's instructions.

Cell Lysis and Immunoblot Analysis

Following treatment, the medium was removed, the cells were rinsed once with phosphate-buffered saline, and collected in 400 µl of a lysis buffer containing 1% Nonidet P-40, 0.05% SDS, 0.5% deoxycholate, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate, 25 mM NaF, 2 mM 4-(2-amino-ethyl)benzenesulfonylfluoride (AEBSF), 1 µg/ml leupeptin, 2 µg/ml aprotinin, and either 1 µg/ml okadaic acid or 0.1 µg/ml calyculin A. Lysates were centrifuged and assayed for protein content using the bicinchoninic acid method. Aliquots were mixed with sample loading buffer, boiled, and size-fractionated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes, and the membranes blocked with 5% milk in Tris-buffered saline containing 0.15% Tween-20 (TBST). Membranes were incubated successively with primary and secondary antibodies, and immunoreactive bands were visualized by enhanced chemiluminescence (Pierce Biotechnology, Inc.). Bands were visualized on a Kodak Image-Station 440CF and quantitated using Kodak 1D software.

Statistical Analysis

Comparisons based on immunoblotting data were derived from samples processed on the same blot. The statistical significance of differences was estimated by t-test, or by analysis of variance followed by Fisher's Least-Significant-Difference test, where appropriate. Differences were taken to be significant at $P < 0.05$. Apparent affinity constants (pA$_2$ values) for pirenzepine and AF-DX 384 were calculated by Schild regression analysis [Arunlakshana and Schild, 1959]. Dose response curves to carbachol were obtained in the presence of increasing concentrations of antagonist, and the dose ratio at each concentration obtained by dividing the EC$_{50}$ value observed in the presence of antagonist by the control value. The log of the dose ratio-1 (DR-1) was plotted versus the negative log of the antagonist concentration, and the apparent pA2 value calculated from the Schild regression x-intercept.

Muscarinic M3 Receptor Stimulation Evokes mTOR-Dependent Phosphorylation of Ribosomal S6 Protein in SK-N-SH Cells

Treatment of SK-N-SH neuroblastoma cells with the cholinergic agonist carbachol evoked a robust, dose-dependent increase in S6 phosphorylation. The magnitude of this response increased relatively slowly over a 30-min period (Fig. 1A), was concentration-dependent, and was inhibited by atropine (Fig. 1B). Rapamycin inhibited the response at concentrations as low as 10 nM (Fig. 1C), implicating mTOR as an upstream mediator in this pathway. Although activation of the mTOR pathway in response to growth factors and some GPCR ligands is dependent on the PI3K signaling cascade, the PI3K inhibitor wortmannin did not significantly reduce the phosphorylation of S6 induced by carbachol (Fig. 1D). Moreover, PKB, which mediates PI3K-dependent activation of mTOR via phosphorylation of TSC2 [Tee and Blenis, 2005], was not activated in response to carbachol, as shown by the absence of activated PKB (phosphorylated on Ser473) in carbachol-treated cells, although insulin caused a marked increase in phospho-PKB in these cells (Fig. 1E).

In order to identify the receptor subtype mediating mTOR activation by carbachol, the effects of the M1/M4-selective antagonist pirenzepine and the M2/M4-selective inhibitor AF-DX 384 were assessed. Pirenzepine inhibited carbachol-evoked S6 phosphorylation in SK-N-SH cells with a mean apparent pA$_2$ value of 6.8 ± 0.1 and a slope of 1.08 ± 0.17 (n = 3), excluding M1 receptors as likely mediators of the effect (Fig. 2A). In contrast, the pA$_2$ for pirenzepine against carbachol in HEK-M1 cells was 7.6 and 7.8 in two experiments, respectively (not shown). AF-DX 384 inhibited the response in SK-N-SH cells with pA$_2$ values of 6.7 and 6.8 in two experiments (Fig. 2B), and in HEK-M3 cells with a pA$_2$ of 6.8 (one experiment, not shown). Taken together, the pA2 values derived from our experiments on SK-N-SH cells correspond most closely with those associated with M3 receptors in both binding and functional studies [Caulfield and Birdsall, 1998], and therefore indicate that carbachol-evoked S6 phosphorylation in these cells is mediated principally via activation of M3 receptors.
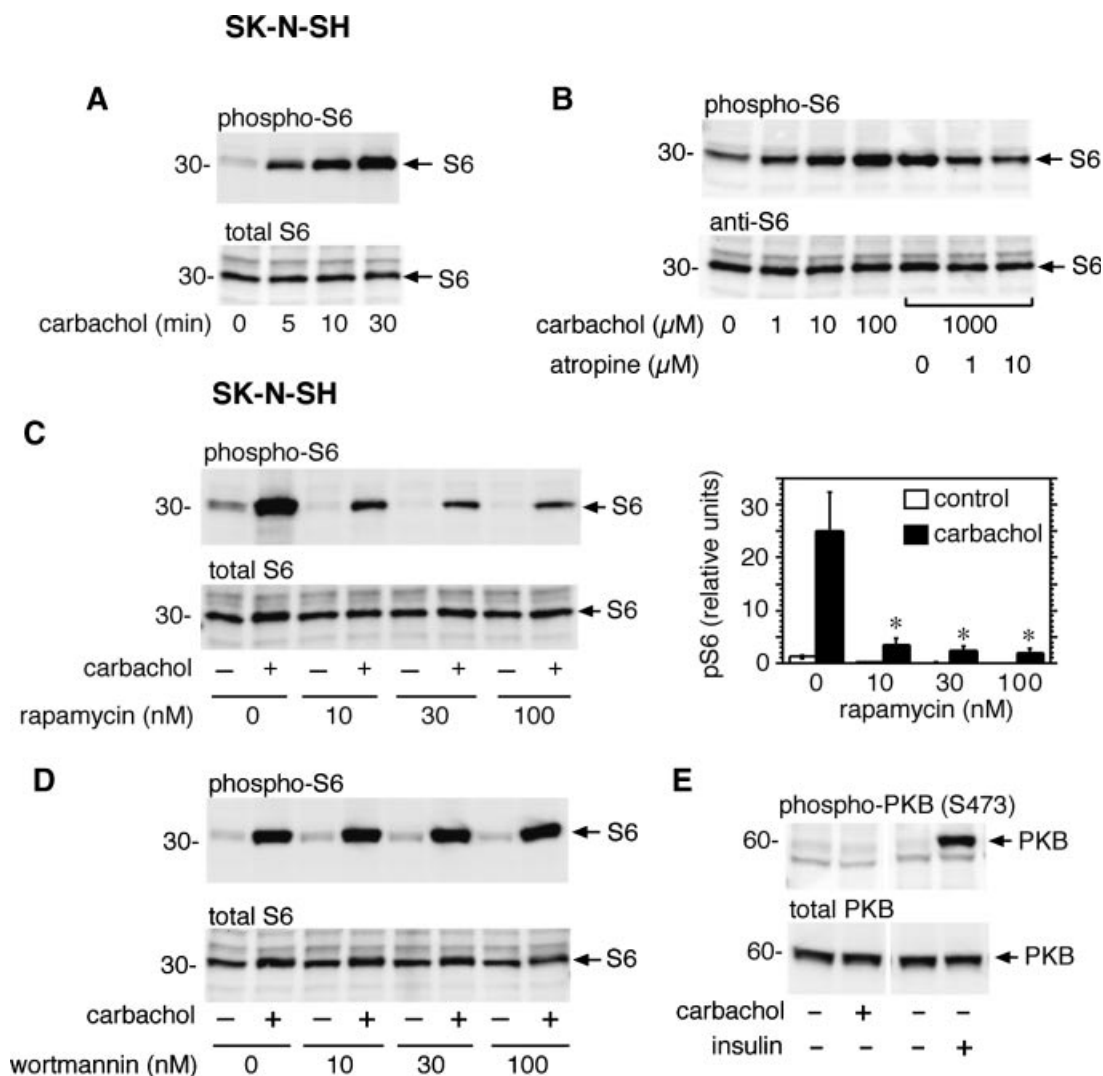
SK-N-SH

Fig. 1. Muscarinic receptor-mediated stimulation of S6 phosphorylation in SK-N-SH cells is mTOR-dependent and PI3K-independent. **A**: Cells were treated with carbachol (1 mM) for varying periods of time. Cell lysates were immunoblotted for S6 and phosopho-Ser235/236 S6. **B**: Cells were pretreated for 15 min with atropine or vehicle in DMEM. Carbachol or vehicle in DMEM was added and the cells were incubated for an additional 30 min. **C**: Cells were pretreated with DMSO or rapamycin at the indicated concentrations for 15 min, then treated with carbachol (1 mM) or vehicle for 30 min. Responses were quantitated and expressed as means $\pm$ SE, n = 3 (**right panel**). *Significantly different from carbachol/DMSO-treated cultures. **D**: Cells were pretreated with DMSO or wortmannin at the indicated concentrations for 15 min, then treated with carbachol or vehicle for 30 min. **E**: Cells were treated with carbachol (1 mM), insulin (100 nM) or control DMEM for 30 min. Lysates were prepared and analyzed by immunoblotting with antibodies to phospho- and total PKB. All bands in each image are derived from a single blot. Lanes not relevant to the present study were excised.

M3 Receptor-Mediated S6 Phosphorylation in SK-N-SH Cells Is Dependent on MAPK and PKC, and Independent of p90 Ribosomal S6 Kinase (RSK1)

Muscarinic receptors activate the MAPK signaling cascade, which has been implicated in the regulation of mTOR by $\alpha 1$ adrenergic receptor ligands [Wang and Proud, 2002]. In order to determine if MAPK plays a role in S6 phosphorylation elicited by muscarinic receptor stimulation, SK-N-SH cells were pretreated with the MEK inhibitor U0126, and challenged with carbachol. S6 phosphorylation was significantly inhibited in cells pretreated with U0126, implicating MAPK in the response (Fig. 3A). As expected, this concentration of U0126 abolished activation of MAPK, determined using antibodies to diphosphorylated MAPK (Fig. 3A). The MEK1 inhibitor PD98059 was also effective, reducing carbachol-evoked S6 phosphorylation by $66 \pm 6\%$ (n = 3). To determine
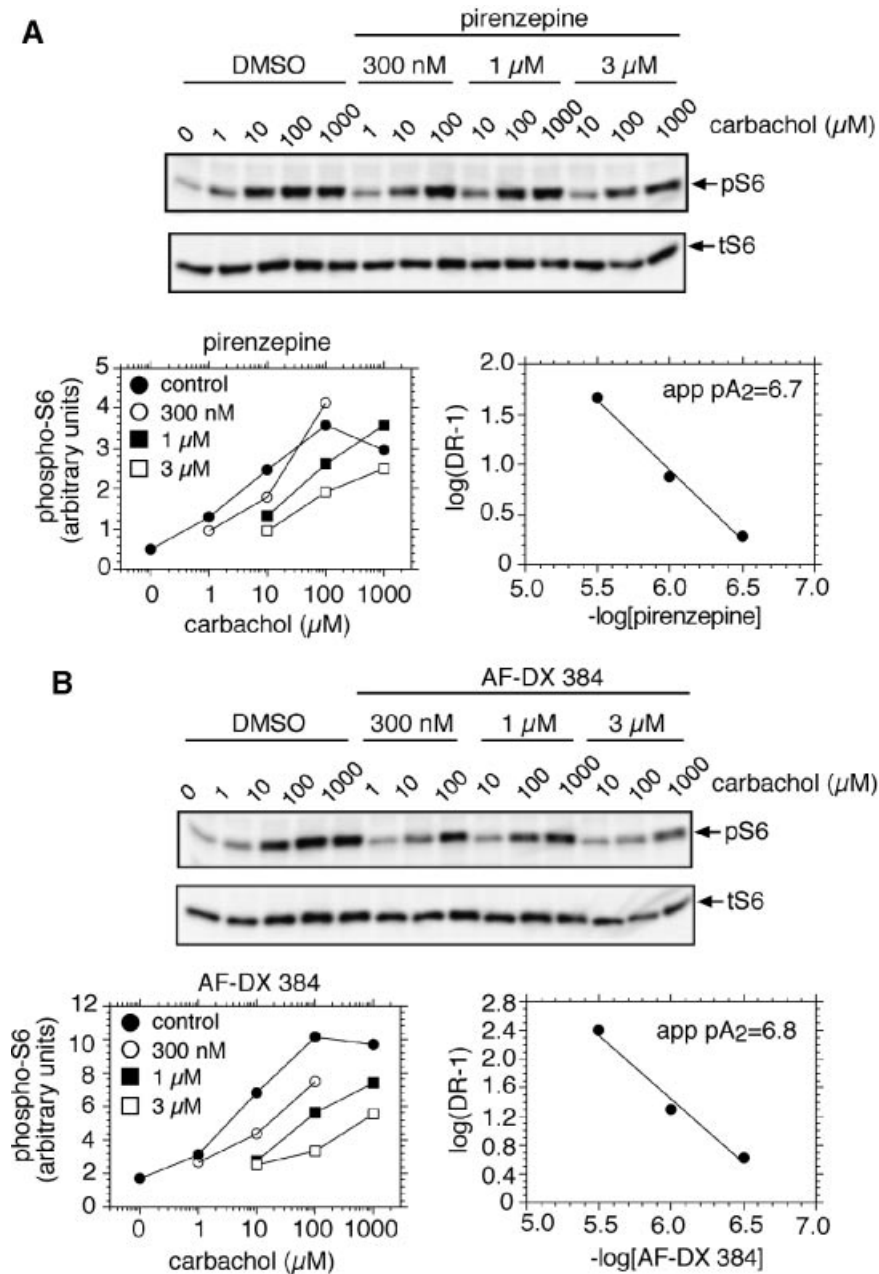
Fig. 2. Carbachol-evoked S6 phosphorylation in SK-N-SH cells is mediated by M3 receptors. **A**: Cells were pretreated with vehicle or pirenzepine for 30 min, then stimulated with carbachol for an additional 30 min. Cell lysates were immunoblotted for S6 and phospho-S6. **B**: Cells were pretreated with DMSO or AF-DX 384 for 30 min, then stimulated with carbachol for an additional 30 min. The apparent pA2 values for each experiment were derived from the x-intercepts of the Schild plots as described in the Materials and Methods Section.

whether MAPK regulates S6 phosphorylation at a point upstream of mTOR, SK-N-SH cells were transiently transfected with a plasmid encoding constitutively active MEK1, or a control vector. After 2 days, the cells were treated for 2 h with rapamycin or DMSO, and lysed. Transient overexpression of constitu-

tively active MEK1 (MEK1-CA) increased levels of phospho-S6 and phospho-MAPK (Fig. 3B). The increase in S6 phosphorylation was blocked by 100 nM rapamycin, whereas levels of activated MAPK were unaffected (Fig. 3B).

It was previously reported that activation of mTOR by phorbol 12-myristate 13-acetate
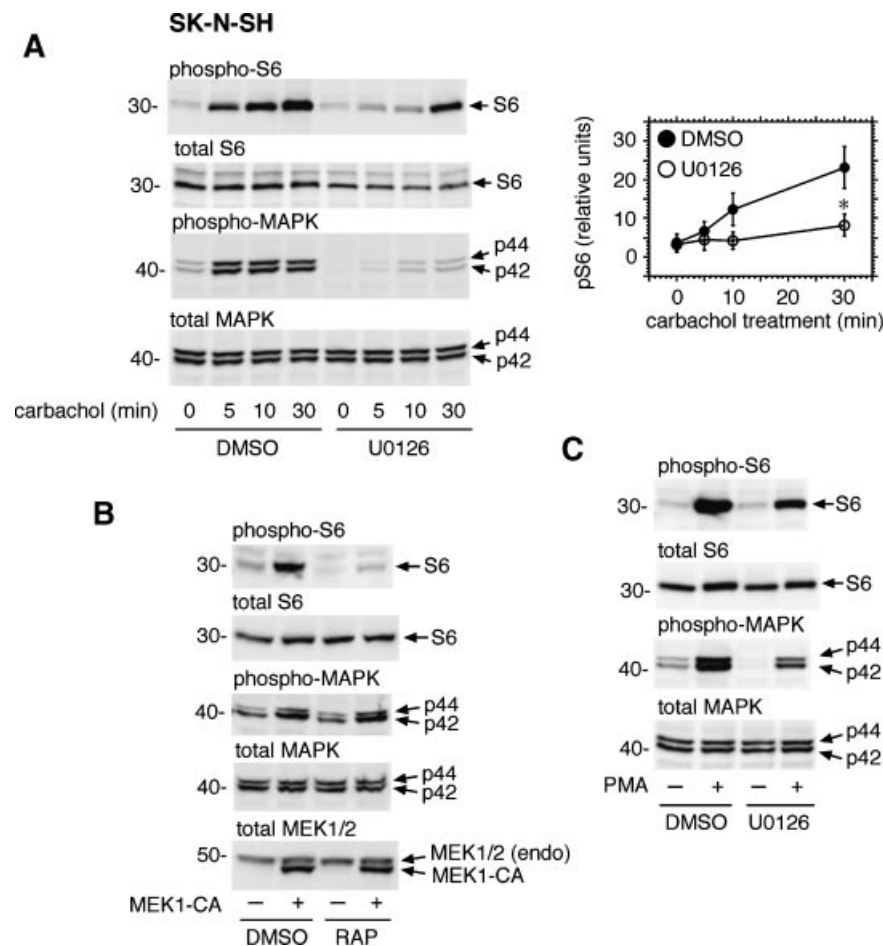
Fig. 3. Carbachol-evoked S6 phosphorylation in SK-N-SH cells is MAPK-dependent. **A**: SK-N-SH cells were pretreated with DMSO or U0126 (10 μM) for 15 min, then treated with carbachol (1 mM) for varying periods of time. S6 and MAPK phosphorylation was determined by immunoblotting (**left panel**). Levels of phospho-S6 were quantitated and expressed as means ± SE, n = 3 (**right panel**). *Significantly different from corresponding DMSO-treated cultures. **B**: SK-N-SH cells were transfected with constitutively activated MEK1 (MEK1-CA) or an empty vector.

After 24 h, the medium was replaced with serum-free DMEM and the cells were incubated overnight. Cells were then incubated for 2 h in DMSO or rapamycin (100 nM), and analyzed by immunoblotting with antibodies to the indicated proteins. MEK1/2 (endo) indicates the endogenous MEK (**C**) SK-N-SH cells were pretreated with U0126 (10 μM) or DMSO for 15 min, then treated with PMA (1 μM) or DMSO for 30 min. Lysates were immunoblotted for total and phosphorylated S6 and MAPK.

(PMA) is due to phosphorylation of TSC2 and suppression of its inhibitory function. This effect of PMA was antagonized by both the PKC inhibitor bisindolylmaleimide I (BIM I), which targets conventional and novel PKC isoforms, and by the MEK inhibitor U0126 [Tee et al., 2003]. In agreement with this study, S6 phosphorylation in SK-N-SH cells was increased by PMA, and this response was inhibited, although not abolished, by U0126 (Fig. 3C). Since PKC is activated by stimulation of muscarinic receptor subtypes (M1, M3, and M5) coupled to phosphoinositide turnover [Sandmann et al., 1991; Felder, 1995], the effect of BIM I on carbachol-evoked S6 phosphoryla-

tion in SK-N-SH cells was next examined. BIM I (1 μM) inhibited the increase in S6 phosphorylation evoked by carbachol without affecting activation of MAPK (Fig. 4). This suggests that in these cells, carbachol-evoked activation of S6 phosphorylation, but not MAPK, is dependent on conventional or novel PKC isoforms, even though direct stimulation of these PKC isoforms by PMA caused a robust increase in MAPK activity (Fig. 3C).

Stimulation of mTOR by the PKC activator PMA is MAPK-dependent [Tee et al., 2003], and was reported to be mediated at least in part via activation of RSK1, a MAPK substrate that phosphorylates TSC2 on Ser-1798, thereby
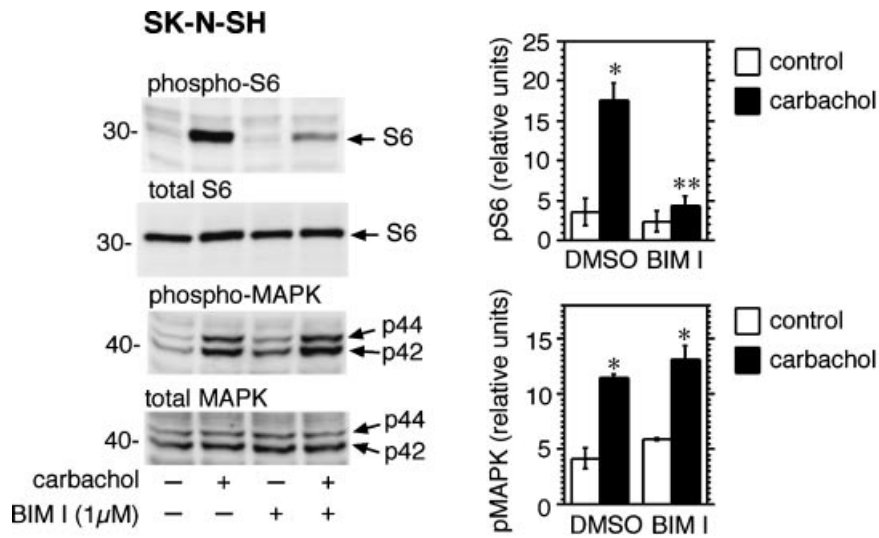
Fig. 4. M3 receptor-evoked S6 phosphorylation in SK-N-SH cells is PKC-dependent. Cells were pretreated with DMSO or BIM I (1 μM) for 15 min, then treated with vehicle or carbachol (1 mM) for 30 min. Lysates were immunoblotted for total and phosphorylated S6 and MAPK (**left panel**). Results were quantitated and expressed as means ± SE from 3 to 5 experiments (**right panel**). *Significantly different from corresponding controls; **significantly different from carbachol-treated cultures.

inhibiting its suppression of the mTOR activator Rheb [Roux et al., 2004; Ballif et al., 2005]. However, although PMA caused a robust, MEK-dependent increase in phosphorylation of RSK1 on Ser-380 in SK-N-SH cells, indicative of activation, carbachol had little effect (Fig. 5A,B). Moreover, whereas PMA strongly stimulated phosphorylation at Ser-1798 of the RSK1 phosphorylation motif in TSC2, carbachol did not (Fig. 5B), suggesting that RSK1 is not a major mediator of mTOR activation initiated by M3 receptors in these cells.

Regulation of S6 Phosphorylation by M3 Receptors Expressed in HEK Cells Is PKC-Dependent, and MAPK-Independent

Muscarinic receptors may be subdivided into two groups with distinct signaling characteristics. The M1, M3, and M5 subtypes are coupled to pertussis toxin-insensitive G proteins and strongly stimulate phosphoinositide hydrolysis, among other actions. The M2 and M4 subtypes signal via pertussis toxin-sensitive G proteins, and are notable for inhibiting adenylyl cyclase [Van Koppen and Kaiser, 2003]. Our results demonstrate that MAPK mediates activation of an mTOR-dependent signaling pathway following stimulation of endogenous M3 receptors in SK-N-SH cells. However, since M2 receptors are also efficiently coupled to MAPK activation

[Winitz et al., 1993], they might similarly be capable of activating mTOR via this mechanism [Crespo et al., 1994; Koch et al., 1994].

In order to compare the regulation of the mTOR pathway by individual muscarinic receptor subtypes, the effect of carbachol on S6 phosphorylation was tested in HEK cell lines transfected with M2 or M3 receptors. Carbachol increased phospho-S6 levels in HEK-M3 cells with a time-course very similar to that observed in SK-N-SH cells (Fig. 6C). The response was inhibited by rapamycin, although less effectively than in SK-N-SH cells (Fig. 6A), and was resistant to wortmannin (Fig. 6B). Unexpectedly, however, carbachol-evoked S6 phosphorylation in HEK-M3 cells was resistant to U0126 (Fig. 6C), even though MAPK was strongly activated by carbachol in these cells, and its activation completely prevented by this inhibitor. To determine if PKC might play a role in regulating S6 phosphorylation in HEK-M3 cells, the effects of the PKC inhibitor BIM I were next assessed. S6 phosphorylation and MAPK activation elicited by carbachol were both reduced by approximately 50% by BIM I (Fig. 7A). Although this concentration (1 μM) of BIM I nearly abolished S6 phosphorylation induced by carbachol (Fig. 7A), it did not inhibit the response evoked by overexpression of MEK1-CA (Fig. 7B). In contrast, a higher concentration of BIM I (5 μM) reduced S6
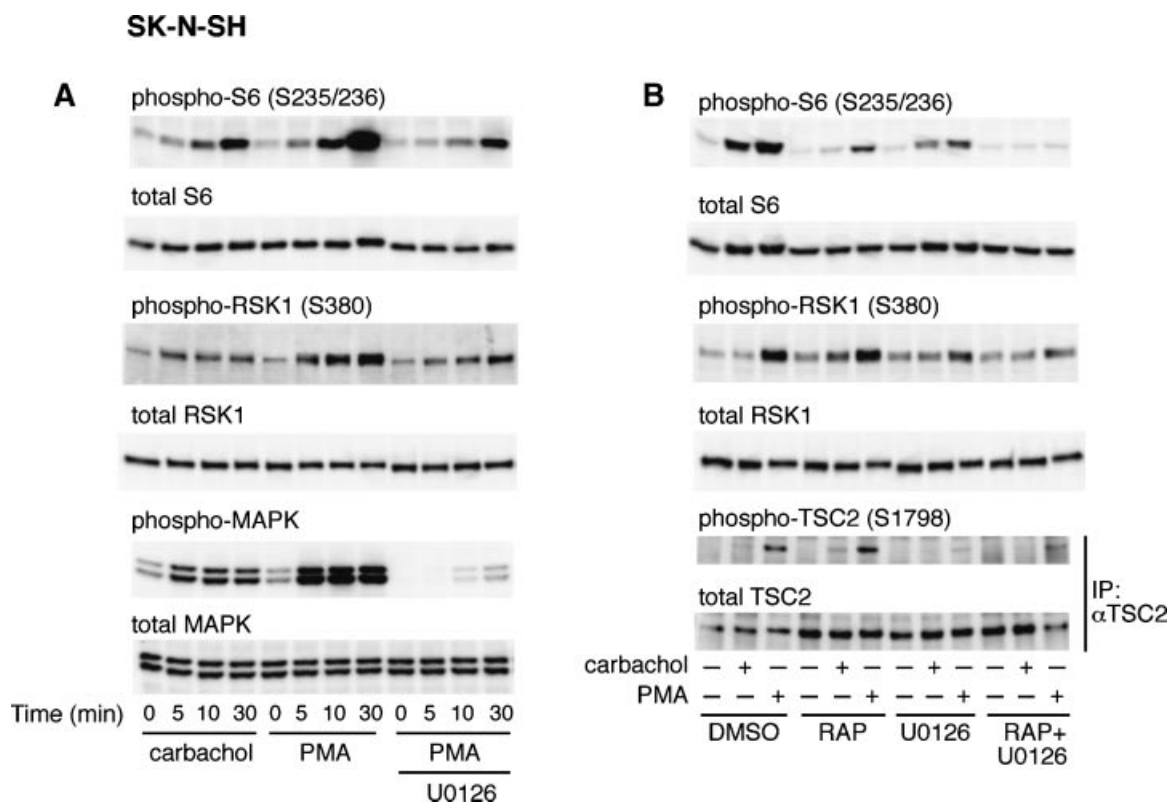
SK-N-SH

Fig. 5. RSK1 phosphorylation is strongly activated by PMA, but not by carbachol, in SK-N-SH cells. **A:** Cells were pretreated with DMSO or U0126 for 15 min, then treated with carbachol (1 mM) or PMA (1 μM) for varying periods of time. Lysates were immunoblotted for total and phosphorylated S6, MAPK, and RSK1. **B:** Cells were pretreated with DMSO, rapamycin (RAP, 100 nM), U0126 (10 μM) or both inhibitors for 15 min, then treated with carbachol (1 mM) or PMA (1 μM) for 30 min. Lysates were immunoblotted for total and phosphorylated S6 and RSK1. Immunoprecipitates were prepared with anti-TSC2 antibodies, and immunoblotted with antibodies that detect TSC2 phosphorylated on Ser1798.

phosphorylation due to MEK1-CA overexpression by 80%, on average (n = 2) (Fig. 7B). Thus, although higher concentrations of BIM I may inhibit RSK1 and S6 kinase, as well as PKC [Alessi, 1997; Roberts et al., 2005], the failure of 1 μM BIM I to inhibit the effect of MEK1-CA, together with the inability of carbachol to activate RSK1 in SK-N-SH (Fig. 5) and HEK-M3 cells (not shown), suggests that the inhibition of S6 phosphorylation by 1 μM BIM I cannot be attributed to non-specific inhibition of either RSK1 or S6 kinase.

The stimulation of S6 phosphorylation by MEK1-CA overexpression was largely inhibited, although not abolished, by rapamycin, indicating that, as in SK-N-SH cells, the response is principally mediated by mTOR activation (Fig. 7C). U0126 reduced phosphorylation of S6 by MEK1-CA, consistent with its ability to directly inhibit MEK [Favata et al., 1998].

PKC and MAPK Mediate S6 Phosphorylation by M2 Receptors in Transfected HEK Cells

S6 phosphorylation was next examined in HEK cells stably transfected with M2 receptors. Carbachol caused a time-dependent increase in S6 phosphorylation in these cells, although the response was smaller in magnitude than that observed in the HEK-M3 and SK-N-SH cell lines (Fig. 8C). The response in HEK-M2 cells was inhibited by atropine (not shown) and by rapamycin (Fig. 8A), and was insensitive to wortmannin (Fig. 8B). However, in contrast with HEK-M3 cells, carbachol-evoked S6 phosphorylation in HEK-M2 cells was reduced by approximately 60% by U0126 at a concentration that completely abolished MAPK activation by carbachol (Fig. 8C), whereas BIM I did not affect either S6 phosphorylation or MAPK activation in these cells (Fig. 8D). As in the other cell lines tested, carbachol did not effectively increase
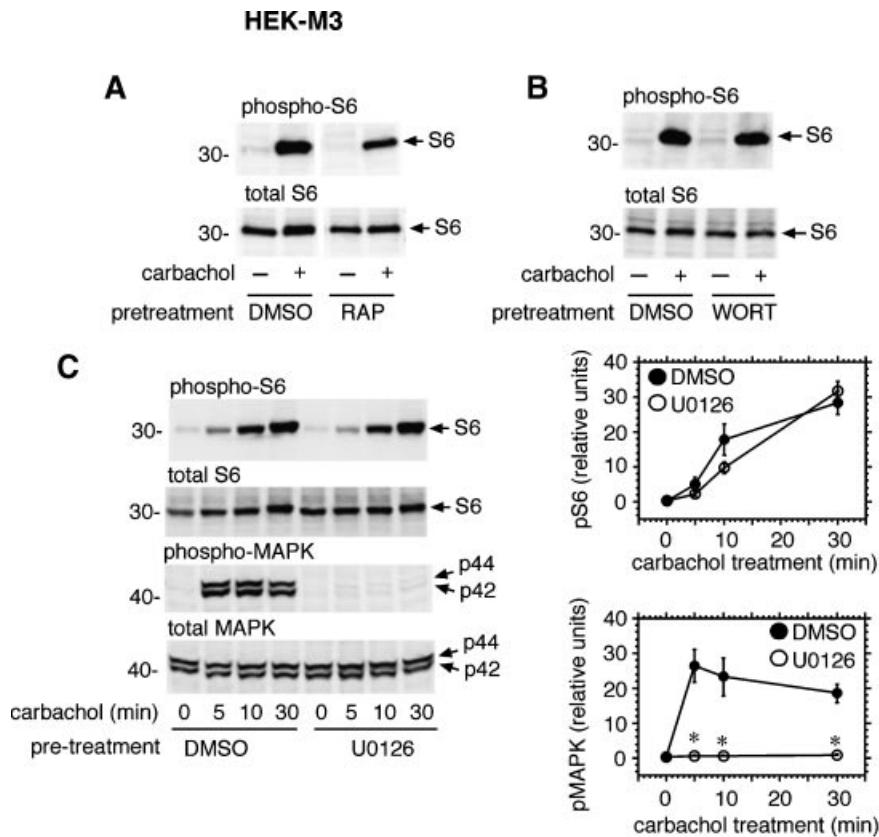
HEK-M3

Fig. 6. Carbachol-evoked S6 phosphorylation in HEK-M3 cells is MAPK-independent. **A**: Cells were pretreated with DMSO or rapamycin (100 nM) for 15 min, then treated with carbachol (100 μM) or vehicle for 30 min. All bands in each image are derived from a single blot. **B**: Cells were pretreated with DMSO or wortmannin (100 nM) for 15 min, then treated with carbachol or vehicle for 30 min. **C**: Cells were pretreated with DMSO or U0126 for 15 min prior to treatment with carbachol (100 μM) for varying periods of time and analyzed for phosphorylated and total S6 and MAPK (**left panel**). Results were quantitated and expressed as means ± SE, n = 3 (**right panels**). *Significantly different from corresponding DMSO-treated cultures.

phosphorylation of RSK1, suggesting that this kinase does not mediate activation of mTOR by M2 receptors (not shown).

DISCUSSION

The activation of the mTOR signaling pathway by growth factors (including insulin and insulin-like growth factors) has been intensively studied in recent years. The canonical pathway involves phosphorylation of the tumor suppressor protein TSC2 by a PI3K/PKB-dependent mechanism. This inhibits the suppression of the GTPase Rheb by TSC2, leading to activation of mTOR. Multiple downstream targets of mTOR, including ribosomal S6K and the 4E-BPs, are important regulators of protein translation and cell growth [Wullschleger et al., 2006]. The mechanisms underlying regulation of mTOR by neurotransmitter receptors have received less attention, although it is now

appreciated that, in addition to its importance for cell growth, mTOR-dependent signaling is important for long-term potentiation and synaptic plasticity in brain [Tang et al., 2002; Cammalleri et al., 2003; Gong et al., 2006].

In the present study, regulation of mTOR-dependent signaling by muscarinic receptors was examined in SK-N-SH neuroblastoma cells, and in HEK cell lines overexpressing M2 or M3 receptors, using phosphorylation of the ribosomal S6 protein, a substrate of S6K, as an indicator of mTOR activation. Results obtained using antagonists with varying selectivity for individual muscarinic subtypes indicated that the response elicited by carbachol in SK-N-SH cells is principally due to activation of endogenous M3 receptors (Fig. 2). Carbachol-evoked S6 phosphorylation on Ser 235/236 in all three lines was inhibited by rapamycin (Figs. 1, 6, and 8), confirming that the response is mediated principally by mTOR, although HEK-M3 cells
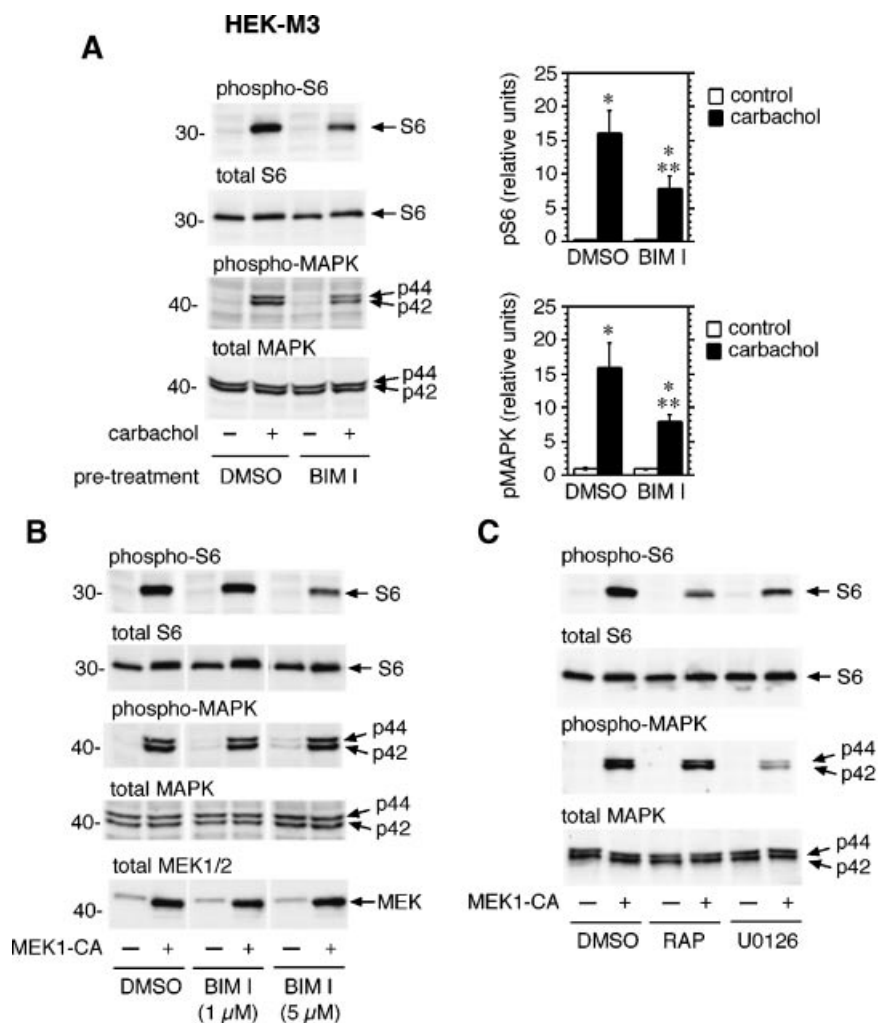
Fig. 7. Carbachol-evoked S6 phosphorylation in HEK-M3 cells is PKC-dependent. **A**: Cells were pretreated with DMSO or BIM I (1 μM) for 15 min prior to treatment with vehicle or carbachol (100 μM) for 30 min, and analyzed for phosphorylated and total S6 and MAPK (**left panel**). Results were quantitated and expressed as means ± SE from three experiments (**right panels**). *Significantly different from corresponding control cultures; **significantly different from carbachol-treated cultures, $P < 0.05$. All bands in each image are derived from a single blot.

B: Cells were transfected with MEK1-CA or control vector. After 24 h, the cells were placed overnight in serum-free DMEM, than treated for 2 h with BIM I (1 or 5 μM) or DMSO. Lysates were prepared and analyzed by immunoblotting. All bands in each image are derived from a single blot. Lanes not relevant to the present study were excised. **C**: Cells were transfected with MEK1-CA or control vector. After 24 h, cells were placed overnight in serum-free DMEM, then treated for 2 h with rapamycin (100 nM) or U0126 (10 μM).

appeared to be less sensitive to rapamycin than the other two lines. Carbachol-evoked S6 phosphorylation in all cell lines was resistant to wortmannin (Figs. 1, 6, and 8), and was not associated with increased phosphorylation of PKB (Fig. 2 and not shown), suggesting that the response is independent of PI3K. However, whereas the response to M2 receptors expressed in HEK cells, and to endogenous M3 receptors in SK-N-SH cells, was substantially reduced by the MEK inhibitor U0126 (Figs. 3 and 8), stimulation of S6 phosphorylation by M3 receptors expressed in HEK cells was MAPK-independent (Fig. 6), even though carbachol strongly activated the MAPK cascade in all three cell lines (Figs. 3, 6, and 8).

These findings contrast with an earlier study, in which activation of S6K by carbachol in 1321N1 astrocytoma cells, although mediated by endogenous M3 receptors, was reduced by PI3K inhibitors but not by U0126 [Tang et al., 2003]. However, other investigators, using the same cell line, reported that carbachol-evoked S6K1 activation was inhibited by U0126 [Guizzetti and Costa, 2002]. The reasons for this discrepancy are not clear, but might be related
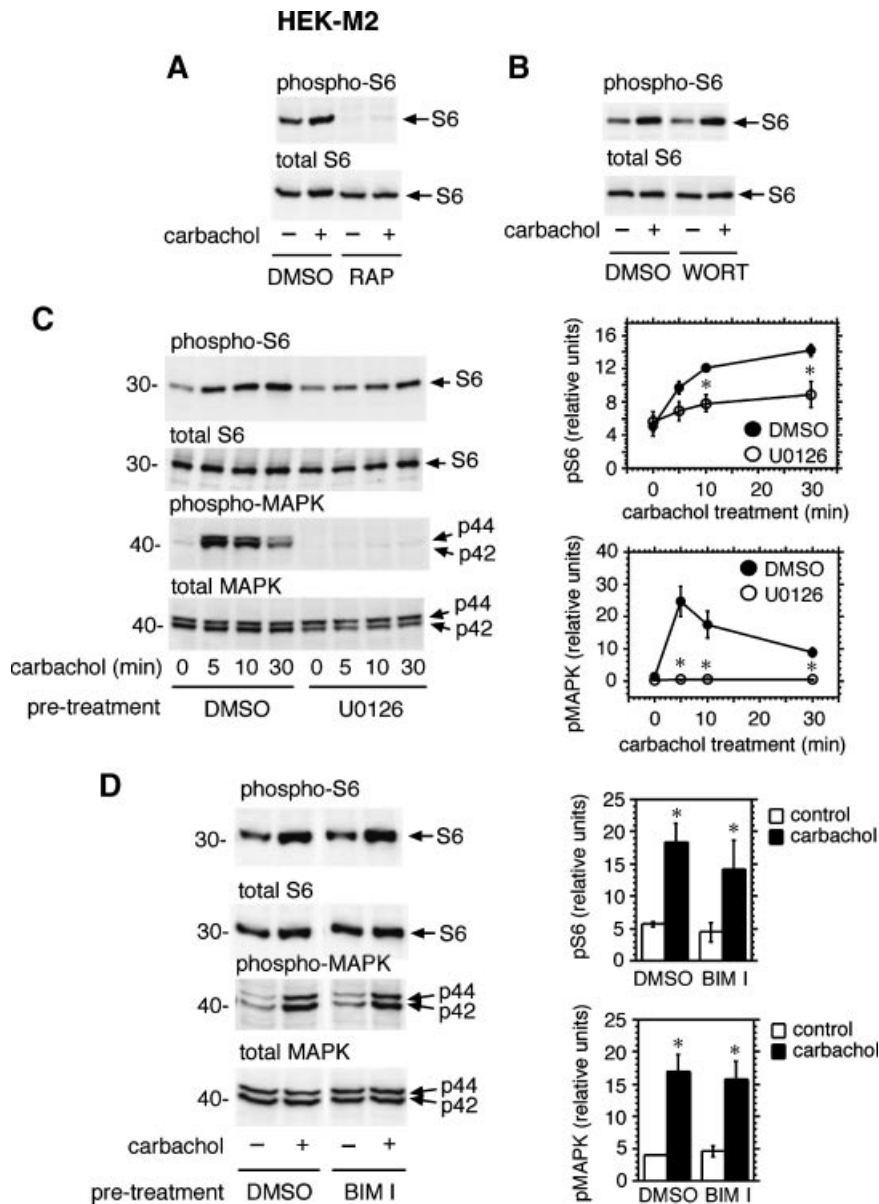
HEK-M2

Fig. 8. Carbachol-evoked S6 phosphorylation in HEK-M2 cells is MAPK-dependent and PKC-independent. **A**: Cells were pretreated with DMSO or rapamycin (100 nM) for 15 min, then treated with carbachol (100 μM) or vehicle for 30 min. **B**: Cells were pretreated with DMSO or wortmannin (100 nM) for 15 min, then treated with carbachol or vehicle for 30 min. **C**: Cells were pretreated with DMSO or U0126 for 15 min prior to treatment with carbachol (100 μM) or vehicle for varying periods of time and analyzed for phosphorylated and total S6 or MAPK (**left panel**). Results were quantitated and expressed as means ± SE, n = 3 (**right panel**). *Significantly different from corresponding DMSO-treated cultures. **D**: HEK-M2 cells were pretreated with BIM I (1 μM) for 15 min prior to treatment with carbachol (100 μM) or vehicle for 30 min, and analyzed for phosphorylated and total S6 and MAPK (**left panel**). Results were quantitated and expressed as means ± SE, n = 3 (**right panel**). *Significantly different from corresponding control cultures, $P < 0.05$.

to the different methods used to measure S6K1 activation, which was determined via in vitro kinase assay in one study [Tang et al., 2003], and by measuring phosphorylation of S6K1 at Thr-389, a rapamycin-sensitive phosphorylation event that correlates with enzyme activity [Dufner and Thomas, 1999; Tee and Blenis,

2005], in the other [Guizzetti and Costa, 2002]. Interestingly, in proliferating SH-SY5Y neuroblastoma cells, which express muscarinic receptors predominantly of the M3 subtype, carbachol increases the activity of the AMP-activated protein kinase (AMPK) [King et al., 2006], which is activated by conditions, such as

energy deprivation, that increase the AMP/ATP ratio. Phosphorylation of TSC2 by AMPK induces it to inhibit mTOR [Inoki et al., 2003], in marked contrast to the mTOR-activating effect evoked by muscarinic receptors in the present study. In order to reconcile these conflicting results, it will be important to identify factors, such as energy status and nutrient availability, that may influence the responses of mTOR to muscarinic receptor stimulation.

Overexpression of constitutively-active MEK1 increased S6 phosphorylation in SK-N-SH cells (Fig. 3) and in HEK cells (Fig. 7) in a rapamycin-sensitive manner. This suggests that MAPK stimulates S6 phosphorylation by an action upstream of, or at the level of, mTOR. Other GPCRs that have been shown to regulate mTOR in a MEK/MAPK-dependent fashion include the α1-adrenergic receptor in cardiomyocytes [Wang et al., 2001; Wang and Proud, 2002], and the prostaglandin F2α receptor in luteal cells [Arvisais et al., 2006]. Like these GPCRs, the PKC activator PMA activates mTOR in a MAPK-dependent fashion [Tee et al., 2003]. Once activated by PMA, MAPK phosphorylates and activates RSK1, which in turn phosphorylates TSC2 on Ser-1798, inhibiting its suppressor function and activating mTOR [Roux et al., 2004; Ballif et al., 2005]. Two additional sites on TSC2, Ser-540 and Ser-664, are phosphorylated by MAPK in a RSK1-independent fashion [Ma et al., 2005]. In addition to phosphorylating TSC2, RSK1 was recently shown to directly phosphorylate S6 on Ser-235/236 in a MEK-dependent, mTOR-independent fashion [Roux et al., 2007]. However, RSK1 is unlikely to be an important mediator of S6 phosphorylation downstream of muscarinic receptors, given that carbachol only weakly activated RSK1, indicated by phosphorylation on Ser-380, in all of the cell lines tested in the present study, and had no effect on the RSK phosphorylation site Ser-1798 in TSC2. Moreover, rapamycin reduced carbachol-evoked S6 phosphorylation by over 90% in SK-N-SH cells, suggesting that mTOR-independent S6 phosphorylation is not a major component of this pathway.

Phosphorylation of TSC2 on Ser-1364 is also increased by PMA, and occurs independently of MAPK activation [Ballif et al., 2005]. This phosphorylation site, and the sites phosphorylated by MAPK (Ser-540 and Ser-664) and RSK1

(Ser-1798), are distinct from those phosphorylated by the classical PI3K/PKB pathway [Inoki et al., 2002]. It is possible that phosphorylation of all four sites may be necessary for full activation of mTOR by GPCRs or PMA. However, overexpression of MEK1-CA alone effectively increased S6 phosphorylation (Figs. 3 and 7), and the response was insensitive to 1 μM BIM I (Fig. 7), suggesting that prolonged stimulation of MAPK may be sufficient to activate mTOR signaling in the absence of PKC activity. Moreover, mutation of Ser-540 and Ser-664 of TSC2 to alanine was sufficient to abolish the stimulation of S6K by MEK1-CA overexpression [Ma et al., 2005]. Thus phosphorylation of these two sites, at least in some cell types, may be sufficient for complete suppression of TSC2, and concomitant activation of mTOR-dependent signaling.

In agreement with earlier studies [Tee et al., 2003], S6 phosphorylation evoked by PMA in SK-N-SH cells was reduced by U0126 (Fig. 5), and abolished by 1 μM BIM I (not shown), indicating that one or more PMA-responsive PKC isoforms stimulates S6 phosphorylation in part via activation of MAPK. In contrast, BIM I strongly inhibited carbachol-evoked S6 phosphorylation in SK-N-SH cells without altering MAPK activation (Fig. 4). Thus, in these cells MAPK activity in the absence of PKC was insufficient to activate mTOR. Nevertheless, U0126 substantially reduced S6 phosphorylation in response to carbachol (Fig. 3). Taken together, these results suggest that PKC and MAPK activity are both required for full activation of the mTOR pathway by M3 receptors in SK-N-SH cells. In addition, the inability of BIM I to inhibit MAPK activation indicate that MAPK is regulated via a PKC-independent mechanism, or via redundant pathways, in these cells.

In HEK-M3 cells, carbachol-evoked S6 phosphorylation was inhibited by BIM I (1 μM) (Fig. 7), and unaffected by U0126 (Fig. 6C), even though MAPK was robustly activated by this agonist. Thus, in contrast to our findings in SK-N-SH cells, mTOR regulation by M3 receptors expressed in HEK cells appears to be independent of MAPK. Given that S6 phosphorylation was reduced by approximately 50% by BIM I, and not at all by U0126, it is likely that additional signaling intermediates participate in regulating this response. Potential mediators include atypical PKC isoforms, which are highly

resistant to BIM I [Martiny-Baron et al., 1993]. Indeed, in 1321N1 astrocytoma cells, the mTOR-dependent stimulation of S6K by carbachol, although mediated by M3 receptors, was inhibited by a peptide inhibitor of PKCζ, (derived from its pseudosubstrate region), whereas neither BIM I nor a peptide inhibitor of PKCα blocked the response [Guizzetti and Costa, 2002]. Thus, distinct PKC isoforms may mediate the response to M3 receptor activation in different cell types. Although a recent report indicates that BIM I-sensitive PKC isoforms may promote both MAPK-dependent and -independent phosphorylation of TSC2 [Ballif et al., 2005], it is not clear if the latter is a direct effect; nor is it known if atypical PKC isoforms, such as PKCζ, or PKC-activated kinases, such as protein kinase D, are able to directly phosphorylate and inhibit TSC2.

In HEK-M2 cells, mTOR-dependent S6 phosphorylation evoked by carbachol was partially dependent on MAPK (Fig. 8C), and independent of conventional and novel PKC isoforms, as indicated by resistance to BIM I (Fig. 8D). Activation of MAPK by M2 receptors was also resistant to BIM I, consistent with earlier reports that these receptors are coupled to MAPK via PKC-independent pathways [Crespo et al., 1994]. Mutational analysis will be needed to establish the importance of individual phosphorylation events in regulating mTOR activation, but our results reinforce the interpretation that multiple signaling pathways, activated by a variety of receptors, act in a coordinated fashion at the level of TSC2 to regulate the activity of mTOR in an incremental fashion.

The experiments described in this study demonstrate that muscarinic receptors signal via multiple MAPK- and/or PKC-dependent mechanisms to activate mTOR-dependent signaling pathways. Specifically, M2 receptors utilize a MAPK-dependent mechanism to activate this pathway, whereas M3 receptors utilize either MAPK-dependent or independent mechanisms, depending on cellular context. The contributions of individual muscarinic subtypes to the many physiological actions of ACh are just beginning to be understood, largely as a result of recent gene-targeting studies [Wess, 2004; Gautam et al., 2006; Origlia et al., 2006]. Our results suggest that it will be important to consider the role of mTOR activation as a possible mechanism underlying many of these processes.

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