Published in final edited form as:

Cell Signal. 2011 January ; 23(1): 239–248. doi:10.1016/j.cellsig.2010.09.008.

Crosstalk between VEGFR2 and muscarinic receptors regulates the mTOR pathway in serum-starved SK-N-SH human neuroblastoma cells

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Abstract

Muscarinic acetylcholine receptors (mAChRs) are guanosine nucleotide-binding protein (G protein) coupled receptors that crosstalk with receptor tyrosine kinases (RTKs) to signal mitogenic pathways. In particular, mAChRs are known to couple with RTKs for several growth factors to activate the mammalian target of rapamycin (mTOR)/Akt pathway, a regulator of protein synthesis. The RTK for the vascular endothelial growth factor (VEGF), VEGFR2, can signal protein synthesis but whether it cooperates with mAChRs to mediate mTOR activation has not been demonstrated. Using serum starved SK-N-SH neuroblastoma cells, we show that the muscarinic receptor agonists carbachol and pilocarpine enhance the activation of mTOR substrates p70 S6 Kinase (S6K) and its target ribosomal protein S6 (S6) in a VEGFR2 dependent manner. Treatments with carbachol increased VEGFR2 phosphorylation, suggesting that mAChRs stimulate VEGFR2 transactivation to enhance mTOR signaling. Inhibitor studies revealed that phosphoinositide 3 kinase resides upstream from S6K, S6 and Akt phosphorylation while protein kinase C (PKC) functions in an opposing fashion by positively regulating S6K and S6 phosphorylation and suppressing Akt activation. Treatments with the phosphatase inhibitors sodium orthovanadate and okadaic acid increase S6, Akt and to a lesser extent S6K phosphorylation, indicating that tyrosine and serine/threonine dephosphorylation also regulates their activity. However, okadaic acid elicited a far greater increase in phosphorylation, implicating phosphatase 2A as a critical determinant of their function. Finally, pilocarpine but not carbachol induced a time and dose dependent cell death that was associated with caspase activation and oxidative stress but independent of S6K and S6 activation through VEGFR2. Accordingly, our findings suggest that mAChRs crosstalk with VEGFR2 to enhance mTOR activity but signal divergent effects on survival through alternate mechanisms.

1. Introduction

Increasing evidence suggests that guanosine nucleotide binding protein (G protein) coupled receptors (GPCRs) crosstalk with receptor tyrosine kinases (RTKs) to signal mitogenic pathways in response to various stimuli. This crosstalk can involve mechanisms where activated GPCRs induce transactivation of RTKs or, conversely, activated RTKs enhance GPCR signaling to signal downstream events [1]. Muscarinic acetylcholine receptors (mAChRs) are GPCRs that mediate the actions of the neurotransmitter acetylcholine in the

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peripheral and central nervous system [2]. In the brain, they mediate various functions that include learning and memory as well as neuronal processes that depend on protein synthesis such as synaptic plasticity [3,4].

mAChRs exist as five molecularly distinct subtypes (M1-M5) with M1 and M3 as the predominant isoforms [5,6,7]. These receptors interact with distinct G protein subunits to signal various cellular functions through specific downstream effector proteins. For example, the M1, M3 and M5 receptors couple with $G_{q/11}$ proteins to activate phospholipase C (PLC) while M2 and M4 receptors interact with $G_{i/o}$ proteins to inhibit adenylyl cyclase. Subtypes of mAChRs that are coupled to $G_{q/11}$ proteins can protect cells from apoptosis following DNA damage, oxidative stress, and mitochondrial dysfunction [8,9]. In addition, PLC signaling induced by M1, M3 and M5 receptors activates protein kinase C (PKC) and elevates intracellular calcium [10] and these events can be further enhanced through coupling with activated RTKs [11]. Moreover, mAChRs stimulate mitogenic pathways linked to RTKs, such as epidermal growth factor (EGFR) and platelet-derived growth factor (PDGFR) to mediate cell proliferation, differentiation and survival [12].

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that controls protein synthesis related to cell growth and proliferation [13,14] and plays a major role in neuronal development, synaptic plasticity, survival and long term memory formation [15,16,17,18]. Under pathological conditions, aberrant mTOR activity is associated with seizure induction in neurological disorders such as tuberous sclerosis, cortical dysplasia and temporal lobe epilepsy [19,20]. mTOR functions in cells as the catalytic subunit of two distinct molecular complexes, mTOR Complex 1 (TORC1) and mTOR Complex 2 (TORC2). Whereas TORC1 is stimulated by growth factors and inhibited by the immunosuppressant rapamycin [21], the upstream activators of TORC2 remain unclear. TORC2 is known to phosphorylate the Ser473 site on the serine/threonine kinase Akt [22] while phosphatidylinositol 3 kinase (PI3K) mediates Akt activation on Thr308. Akt can potentiate TORC1 signaling by inhibiting the tuberous sclerosis complex (TSC), allowing TORC1 signaling of protein translation via activation of its downstream targets p70 S6 Kinase (S6K) and its target ribosomal protein S6 (S6). In neuronal models, agonists for mAChRs were shown to stimulate activation of S6K in 1321 astrocytoma cells [23], SH-SY5Y [24] and PC12 cells [25] and S6 ribosomal protein via PKC in serum-deprived SK-N-SH neuroblastoma cells [26].

Vascular endothelial growth factor (VEGF) is a well established angiogenic factor that can also confer neuroprotection through its cognate receptor VEGFR2 [27]. We showed previously that serum starvation induced an upregulation in VEGF and VEGFR2 in serum starved SK-N-SH neuroblastoma cells which together function as an autocrine pathway to protect against a caspase-dependent cell death [28,29]. In hippocampal neurons, VEGF was shown to elevate intracellular calcium and the activation of mTOR, S6K and Akt [30]. Thus, VEGF signaling or the activation of mAChRs can influence effectors of translational control in neuronal cells. Evidence shows that EGFR transactivation by GPCRs was required for TOR target activation and protein synthesis [1,31]. Furthermore, recent studies show that insulin receptor activation in pancreatic cancer cells potentiates GPCR signaling to the mTOR pathway to mediate survival [32]. However, the possibility that VEGFR2 cooperates with mAChRs in signaling mTOR activation has not been explored.

In this study, we used SK-N-SH neuroblastoma cells to investigate whether mAChRs couple with VEGFR2 to signal mTOR activation in response to serum deprivation. This inquiry is based on studies showing that SK-N-SH cells express distinct isotypes of mAChRs (M1, M2 and M3) and evidence that M1 and M3 activation [8] and VEGFR2 require $G_{q/11}$ proteins to signal specific physiological events [33,34]. In addition, VEGFR2 and mAChRs activate similar downstream signaling intermediates such as PI3K, Akt, PKC, c-Src and extracellular

signal regulated kinases 1/2 (ERK1/2) [2,27]. We provide evidence that VEGF and the mAChR agonists carbachol and pilocarpine activate mTOR targets in a VEGFR2 dependent mechanism. Our results suggest that PKC and phosphatase 2A (PP2A) are critical regulators of mTOR signaling in a mechanism where PKC exerts opposing regulatory effects on TORC1 and TORC2 targets. Moreover, we show that muscarinic agonists elicit different effects on cell viability in a time and dose dependent fashion.

2. Materials and Methods

2.1 Materials

Recombinant human VEGF 165 was obtained from PeproTech (Rocky Hill, NJ). SU1498, LY294002, GF-109203X, rapamycin and okadaic acid were obtained from LC Labs (Woburn, MA). Pilocarpine was from MP Biomedicals (Solon, OH), carbachol was from Calbiochem (La Jolla, CA), N-acetylcysteine and sodium orthovanadate were from Sigma (St. Louis, MO).

2.2 Cell Culture

Human neuroblastoma SK-N-SH cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained at 37°C in MEM supplemented with 5% fetal bovine serum, non-essential amino acids, sodium pyruvate, L-glutamine and vitamins (Invitrogen; Carlsbad, CA). For each experiment, cells were grown to 80% confluence followed by treatments under serum starvation for a period of 48 hours as described in Gomes et al. [28]. For inhibitor studies, serum starved cells were pretreated for 1 hour with predetermined concentrations of okadaic acid (OA) or sodium orthovanadate (Na_3VO_4), 48 hours with rapamycin, or for 2 hours with SU1498, LY294002 and GF-109203X (GFX). Unless indicated otherwise, these treatments were followed by 15-minute incubations with predetermined concentrations of VEGF or the agonists for mAChRs, carbachol and pilocarpine. In the cell viability experiments, all agonists and inhibitors were incubated for 24 hours.

2.3 siRNA

Cells were transfected with 20 μM of KDR/Flk-1/VEGFR2 SMARTpool siRNA duplexes or non-targeting (control) siRNA duplexes (Dharmacon RNA Technologies; Lafayette, CO) according to the manufacturer's directions. Cells were then incubated without serum for 48 hours before lysis.

2.4 Protein extraction and immunoblotting

Cells were harvested in lysis buffer and quantified for protein content using a previously described protocol [35]. Equal amounts of protein from each total cell lysate were resolved by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were blocked and then incubated overnight at 4°C with primary antibody. All primary and secondary antibodies were from Cell Signaling (Danvers, MA) except for the antibodies against actin (Sigma) and phosphorylated and total VEGFR2 (Santa Cruz Biotechnology; Santa Cruz, CA). Immunoblots were visualized with the SuperSignal West Pico chemiluminescent Substrate (Pierce; Rockford, IL) and quantified where indicated using ImageJ (NIH, <http://rsb.info.nih.gov/ij/>). Phosphorylated proteins were normalized to their respective total protein levels and expressed as the fold difference of the appropriate control.

2.5 Protein Immunoprecipitation

VEGFR2 was immunoprecipitated from equal concentrations (500 μg) of total cell lysates using an anti VEGFR2 antibody (Millipore; Billerica, MA). Antibody antigen complexes were selectively removed by binding to protein A magnetic beads (Millipore) according to the

manufacturer's directions and then incubated overnight at 4°C in a final volume of 200µl. Precipitates were eluted in 2X SDS loading buffer and analyzed by immunoblotting.

2.6 Cell viability

Cells were plated in 96 well plates at a concentration of 5×10^4 cells/well and treated as indicated under serum deprivation for 48 hours at 37°C. Cell viability was determined using a colorimetric MTS assay (Promega Corp; Madison, WI) and quantified according to manufacturer's instruction. Survival measurements are expressed as the percent of the untreated or vehicle control.

2.7 Caspase 3/7 activity

Caspase activity was measured in cells plated in a 96 well format using the fluorescence cell-based Apo-ONE Homogeneous Caspase 3/7 Assay (Promega) according to manufacturer's directions. Caspase activity was quantified using the Molecular Dynamics Typhoon 9410 Imaging System with ImageQuant software (Amersham; Piscataway, NJ). Data were normalized as fluorescent units/µg protein.

2.8 Statistical analyses

Data are expressed as the mean±SEM of cell treatments that were replicated at least three times using GraphPad Software; San Diego, CA. Statistical significance was assessed with GraphPad Prism using a one way (Figure 7) or two way (Figure 6) analysis of variance (ANOVA) with Bonferroni's post test. $P < 0.05$ was considered significant.

3. Results

3.1 Muscarinic and VEGF receptors signal to mTOR

In these studies we investigated whether the VEGF/VEGFR2 pathway would couple with mAChRs in SK-N-SH cells to activate the PI3K/Akt/mTOR pathway as a response to serum deprivation. Since mAChRs signal to the TORC1 target S6 in SK-N-SH cells [26], serum starved cells were treated with the mAChR agonist carbachol and examined over a 30 minute time frame for the phosphorylation of S6K at the TORC1 site Thr389 and Akt activation at Thr308 (Figure 1A). Carbachol elicited opposing effects on S6K and Akt in that an increase in S6K phosphorylation, which peaked at 15 minutes, was accompanied by a concomitant decrease in Akt activation (lane 8). Experiments were then performed to evaluate the phosphorylation of mTORC1 targets and Akt in serum starved cells incubated with VEGF for 15 minutes (Figure 1B). VEGF also increased the phosphorylation of S6K and S6 at the S6K activation sites Ser235/236 while mediating a slight increase in Akt phosphorylation at positions Thr308 and Ser473.

3.2 G protein coupled mAChRs stimulate VEGFR2 activation upstream of mTOR

To address whether mAChRs cooperate with VEGFR2 to signal S6K and S6 activation, serum starved SK-N-SH cells were treated for 10 or 15 minutes with VEGF or carbachol in the presence and absence of SU1498, a pharmacological inhibitor of VEGFR2 (Figure 2A). Treatments with SU1498 (Figure 2A, lanes 4, 5, 8 and 9) or VEGFR2 siRNA (Figure 2B) attenuated the phosphorylation of S6K and S6 induced by VEGF or carbachol, suggesting that mAChRs crosstalk with VEGFR2 in serum-deprived SK-N-SH cells to activate mTOR. Immunoprecipitation of VEGFR2 from VEGF and carbachol treated cells indicated that both agonists stimulated an increase in the autocrine signaling of VEGFR2 phosphorylation by VEGF (Figure 2C) [28]. These results suggest that mAChRs transactivate VEGFR2 to mediate mTOR activation in serum deprived SK-N-SH cells.

3.3 PKC exerts opposing effects on the signaling of mTORC1 and Akt by VEGF and mAChR agonists

Agonists that stimulate G_q protein coupled receptors (like VEGF and carbachol) typically activate PKC as a downstream effector of signaling events [36,37]. In addition, prior evidence shows that PKC modulates the carbachol mediated phosphorylation of S6 and Akt in different cell lines [26,38,39]. Therefore, we addressed a role for PKC in agonist mediated signaling to mTORC1 targets and Akt by preincubating serum starved cells for 2 hours with GFX, an inhibitor of conventional PKC isoforms (α , β , and γ), followed by a 15 minute stimulation with VEGF or carbachol alone or in combination (Figure 3A). Treatments with SU1498 were included for comparison. Western blot analyses showed that carbachol induced S6K and S6 phosphorylation to levels that were 2 and 3 fold greater than that induced by VEGF (compare lanes 4 and 7). In combination, both agonists further augmented S6 activation by VEGF and carbachol to levels that were 5 fold and 2 fold greater, respectively (compare lanes 4 and 7 with 10). However, Akt phosphorylation on Ser473 was increased only slightly by VEGF and suppressed by carbachol. PKC inhibition mimicked SU1498 in preventing S6K and S6 in all cell treatments while increasing Akt phosphorylation in untreated, VEGF and carbachol treated cells (lanes 3, 6 and 9). These experiments also showed that SU1498 (lane 2) and GFX (lane 3) also blocked an endogenous phosphorylation of S6K and S6. This detection of endogenous signaling depended upon the amount of protein analyzed and length of film exposure. Together, these data suggest that PKC modulates the activation of TORC1 targets and Akt in an opposing fashion in serum starved SK-N-SH cells. To assess whether these results are specific for carbachol, the phosphorylation levels of S6K, S6 and Akt were evaluated in serum starved cells treated with pilocarpine, another agonist for mAChRs. Like carbachol, pilocarpine stimulated S6K and S6 in a PKC and VEGFR2 dependent fashion (Figure 3B) while PKC inhibition augmented Akt phosphorylation (Figure 3C). Together, these data suggest that mAChR stimulation can signal mTOR activation through VEGFR2 and that PKC is an important downstream mediator of this event in serum deprived SK-N-SH cells.

3.4 S6 and Akt are downstream from PI3K

Since PI3K is a well established mediator of TORC1 activation, we examined the relationship among PI3K, mTOR and PKC in the endogenous signaling to S6 and Akt. To this end, serum starved cells were treated with inhibitors of PKC (GFX), PI3K (LY294002) and mTOR (rapamycin) either alone or as combined treatments (Figure 4). Whereas S6 activation was inhibited by all three inhibitor treatments, Akt phosphorylation was increased at Thr308 and Ser473 by GFX (lane 2) and only at Thr308 following a prolonged (48 hour) treatment with rapamycin alone or with GFX (lane 4 and 6). However, PI3K inhibition attenuated the GFX mediated increase in Akt phosphorylation (lanes 5 and 7), placing PI3K as an upstream activator of these events. The rapamycin induced loss of the Akt Ser473 phosphorylation is likely due to an attenuation of TORC2 assembly [40].

3.5 Phosphatase activity modulates S6 and Akt activation

In addition to kinase activity, dephosphorylation also plays an essential role in the signaling cascades mediated by cell surface receptors. In particular, serine/threonine as well as tyrosine phosphatases can affect receptor signaling upstream of mTOR and directly dephosphorylate the pathway members S6K, S6 and Akt. To address these mechanisms, SK-N-SH cells were treated with either the serine/threonine phosphatase inhibitor OA at a concentration that is selective for PP2A activity [41] or Na_3VO_4 , a general inhibitor of tyrosine phosphatases (Figure 5A). Whereas both inhibitors increased S6K, S6 and Akt phosphorylation, OA alone elicited a 2 fold or greater increase in phosphorylation than Na_3VO_4 . Moreover, OA induced S6 phosphorylation was enhanced by VEGF and carbachol by 5 and 8 fold, respectively, while Akt activation increased by 7 and 12 fold (compare lanes 2 with 5 and 8). A similar pattern of

increased activation was induced by Na_3VO_4 but to a lesser extent (compare lanes 3 with 6 and 9). In contrast to these data, S6K activation was enhanced no greater than 3-fold. Given these findings, we reexamined the dependency of S6K, S6 and Akt activation on mTOR and PI3K when PP2A was inhibited in the absence and presence of rapamycin or LY294002 (Figure 5B). Consistent with PI3K as an upstream regulator, LY294002 abrogated the activation of S6K and Akt in OA treated cells (lane 3). Rapamycin only blocked S6K phosphorylation in the presence of OA (lane 2), supporting the notion that TORC1 is a critical regulator of S6K function [42]. In contrast to the data in Figure 4, Akt phosphorylation on Ser473 in OA treated cells was not eliminated by rapamycin while S6 activation at Ser235/236 was both PI3K and mTOR independent.

3.6 Treatments with pilocarpine induce cell death in a time and dose dependent manner

Since VEGF and mAChRs mediate neuronal survival [9,43], we examined the effects of increasing concentrations of carbachol or pilocarpine (0.01 to 1.0 mM) on the viability of serum starved SK-N-SH cells over a 24 hour period of exposure (Figure 6A). Interestingly, pilocarpine, unlike carbachol, induced a dose and time dependent loss in viability at 20 and 24 hours that was accompanied by sustained activation levels of S6K and S6 phosphorylation (Figure 6B, compare lanes 2 and 3). To evaluate the contribution of PKC at 24 hours, serum starved cells were also treated with either agonist in the presence of GFX (Figure 6B, lanes 4 and 5) and analyzed for the phosphorylation levels of S6K, S6, Akt at Ser473 and the PKC α / β II isoforms at T638/641 together with the total levels of PKC α . Consistent with the data in Figure 3, PKC inhibition elicited a decrease in the phosphorylation levels of S6K and S6 with a parallel increase in Akt. These GFX mediated events were accompanied by reduced levels of phosphorylated PKC α / β II and total PKC α , suggesting that PKC activation influences the phosphorylation levels of S6K, S6 and Akt.

3.7 Pilocarpine induced cell death is ROS dependent and TORC1 independent

Pilocarpine is used to induce seizures in a rat model of epilepsy and recent evidence shows that this event is associated with a hippocampal neuronal cell death involving oxidative stress and caspase activation [44]. To assess whether pilocarpine mediates a similar cell death mechanism in our neuronal model, we assessed viability, caspase activation and mTOR activation in cells treated for 24 hours with 1 mM pilocarpine without and with the antioxidant N-acetylcysteine (Nac) alone or in combination with SU1498 (Figure 7). Consistent with in vivo studies, the loss in viability induced by pilocarpine was accompanied by caspase 3/7 activation and these events were prevented by treatments with Nac independent of SU1498 (Figure 7A). However, the phosphorylation of S6K and S6 persisted in pilocarpine treated cells in the presence of Nac (Figure 7B, lane 2) but was attenuated by the inclusion of SU1498 (lane 3). These findings suggest that TORC1 activation by VEGFR2 was dispensable for survival in pilocarpine treated cells. Taken together, these results suggest that the duration and magnitude of stimulation by mAChRs can affect cell fate independent of mTOR signaling in serum deprived SK-N-SH cells.

4. Discussion

In this study, we employed serum deprived SK-N-SH cells as a neuronal model to investigate whether mAChRs and the VEGF/VEGFR2 pathway crosstalk to activate mTOR. We based these studies on previous work showing that VEGF and carbachol, an agonist for mAChRs, signal activation of mTOR targets in different neuronal models [26,30]). We reveal a previously unknown link between mAChRs and VEGFR2 in which carbachol and pilocarpine enhance the activation of the TORC1 targets S6K and S6 in a VEGFR2 dependent manner. This interpretation is based on our findings that 1) carbachol treatment increases VEGFR2 phosphorylation (Figure 2), 2) the combined action of VEGF and carbachol augments S6

phosphorylation to levels greater than either agonist alone (Figure 3) and, 3) inhibition of VEGFR2 blocks S6K and S6 activation not only by VEGF, but also by carbachol and pilocarpine (Figures 2 and 3).

Crosstalk between GPCRs and RTKs serves as a mechanism whereby signaling components are shared to presumably potentiate cellular responses. Our findings suggest that mAChRs signal the activation of TORC1 through a transactivation of VEGFR2 to enhance the activation of downstream targets. Activated GPCRs have been shown to couple with RTKs for epidermal growth factor (EGF), platelet derived growth factor (PDGF), nerve growth factor (NGF), and insulin growth factor (IGF) to signal cell proliferation, differentiation, and other cellular functions [12,45]. For example, upregulation of NGF by mAChRs was shown to enhance acetylcholine release from synaptosomes in the brain [46]. In non-neuronal cells, mAChRs were shown to interact with PDGFR or transactivate EGFR to enhance ERK1/2 activation [47,48,49]. In our paradigm of serum deprivation in SK-N-SH cells, carbachol and pilocarpine mediated activation of mAChRs may augment TORC1 signaling through VEGFR2 to enhance the cellular response to VEGF.

The similarity in the regulatory pattern of S6K and S6 activation by VEGFR2 and PKC raises the possibility that, in our studies, PKC is a downstream effector of the coupled muscarinic receptor/VEGFR2-mediated signaling to TORC. The observation that PKC inhibition exerts inverse effects on the phosphorylation patterns of TORC1 substrates and Akt in cells treated with VEGF, carbachol or pilocarpine (Figures 3 and 6) as well as untreated cells (Figure 4) suggests that PKC targets these pathways independent of agonist treatment. These opposing effects of PKC on TORC1 and Akt activation are supported by evidence that EGFR signals TORC1 through PKC independently of Akt activation in different cancer cell lines [50]. In addition, the suppression of Akt phosphorylation by PKC is consistent with reports that PKC α negatively regulates VEGFR2 stimulated phosphorylation of Akt at Ser473 and GPCR mediated transactivation of EGFR in response to carbachol in non-neuronal cells [51,52]. However, signaling between PKC and Akt is complex as PKC can also positively regulate Akt activation induced by carbachol in astrocytoma and PC12 cells [53,54]. Also, our results cannot preclude the possibility that carbachol negatively regulates Akt phosphorylation through PKC independent mechanisms [38,39].

Nevertheless, one explanation for our diverse results is that VEGF and muscarinic receptor agonists independently signal PKC through their respective receptors. Both mAChRs and VEGFR2 can signal through PLC and intracellular Ca²⁺ mobilization to activate PKC as a critical effector of downstream signaling events [55,56]. It is also possible that the agonist mediated stimulation of mAChRs and VEGFR2 leads to the activation of several isoforms of PKC that simultaneously modulate mTOR and Akt function in an opposing fashion. For example, PKC isoforms were shown to confer distinct regulatory effects on Akt phosphorylation in keratinocytes [57]. Thus, a given PKC isoform would positively regulate TORC1-mediated S6K phosphorylation and the activation of its downstream target S6 while another isoform would suppress the phosphorylation of Akt on Thr308 via PI3K and its phosphorylation on Ser473 by TORC2. Interestingly, in pilocarpine-treated cells incubated with GFX, the increase in Akt Ser473 phosphorylation and the loss of S6K and S6 activation are coincident with decreased levels of total PKC α and Thr638/664 phosphorylation of the PKC α / β II isoforms (Figure 6). This reduction in activated and total PKC may result from its dephosphorylation and subsequent degradation by the ubiquitin/proteasome pathway [58]. Consequently, the negative and positive effects of PKC on TORC1 and Akt activation may result from a decrease in the functional levels of PKC. Taken together, our studies reveal a novel pathway in SK-N-SH cells in which mAChRs cooperate with VEGFR2 to mediate contrasting effects on mTOR and Akt activation through a PI3K pathway that is further modulated by PKC.

Our studies also revealed that the extent of S6K, S6 and Akt phosphorylation by VEGF and carbachol was enhanced by blocking tyrosine and to a greater extent serine/threonine dephosphorylation (Figure 5). Consequently, these findings suggest that in serum starved SK-N-SH cells, phosphorylation by RTKs and dephosphorylation by PP2A coordinate as mechanisms to control TORC1 and Akt function induced by muscarinic/VEGFR receptor signaling. Interestingly, carbachol was 3 fold more effective than VEGF in augmenting the phosphorylation levels of S6 and Akt when combined with tyrosine or serine/threonine phosphatase inhibition. However, the far greater increase (12 to 15 fold) in S6 and Akt activation by VEGF and carbachol in OA treated cells implicates dephosphorylation by PP2A as a critical regulatory mechanism in the response to serum starvation. This line of reasoning is supported by evidence that PP2A can interact with Akt and mediate dephosphorylation of Ser 473 [59,60]. Furthermore, a regulatory link between PKC and PP2A was provided by evidence that PKC activates PP2A and mediates the association of Akt with the PP2A catalytic subunit for subsequent dephosphorylation [57]. In agreement with our findings, this study also showed that PKC inhibition induced an increase in Akt activation at Ser 473 that was attributed to a blockade of PKC mediated dephosphorylation of Akt. Whether PKC coordinates with PP2A to regulate Akt in serum starved SK-N-SH cells is currently under investigation. Although PP2A can directly interact with S6K [61], S6K was not as responsive as S6 or Akt to the stimulatory effects of phosphatase inhibition on phosphorylation.

Our inhibitor studies also provide insight regarding the capacity of upstream kinases to phosphorylate endogenous S6K, S6 and Akt when dephosphorylation by PP2A is blocked (Figures 4 and 5B). These results place PI3K as an upstream regulator of S6K and Akt phosphorylation while mTOR alone modulates the activation of S6K. In this context, Akt activation is mTOR independent which is consistent with Akt residing upstream from TORC1 [42]. Interestingly, the observation that S6 phosphorylation at the S6K target site Ser235/236 is independent of PI3K and mTOR with OA treatment is supported by reports that kinases such as p90RSK can serve as compensatory regulators of S6 phosphorylation [62,63]. Given these findings, an alternative explanation for the divergent phosphorylation patterns of TORC1 targets and Akt in GFX and rapamycin treated cells is that this response results from a negative feedback loop in which activated S6K inhibits the PI3K mediated Akt phosphorylation at Thr308 [42,64]. Accordingly, the blockade of S6K activation by PKC and mTOR inhibition, as observed in our studies, would inactivate this feedback inhibitory loop, leading to enhanced PI3K activity and an increase in phosphorylated Akt.

Since VEGF elicits neuroprotection under stress conditions [28,65,66], it seemed reasonable to assume that mAChRs would couple with VEGFR2 to protect against the apoptotic cell death induced by serum starvation in SK-N-SH cells [29]. Unexpectedly, pilocarpine, but not carbachol, induced a concentration and time dependent loss in viability that was manifested by caspase activation and oxidative stress. Interestingly, this mode of cell death is also associated with the neuronal cell loss mediated by pilocarpine induced seizures in a rat model of epilepsy [44,67]. Similarly, the sustained activation of S6K and S6 induced by pilocarpine in SK-N-SH cells is consistent with evidence that aberrant mTOR expression contributes to pilocarpine induced seizures [20]. However, neither the presence of S6K and S6 activation nor its suppression by VEGFR2 inhibition affected the survival mediated by the antioxidant in our model, suggesting that the pilocarpine stimulation of mTOR activation through VEGFR2 is dispensable for survival. This finding suggests that pilocarpine mediates cell death and mTORC1 activation through different mAChRs. Nonetheless, muscarinic receptors may vary in their stimulatory effects depending on the cell type, the receptor subtype activated as well as the agonist and extent of stimulation [1]. For example, pilocarpine was shown to promote neuroprotection against glutamate-induced apoptosis in retinal neurons through the M1 mAChR [66]. Furthermore, divergent pilocarpine and carbachol signaling to ERK1/2 has been documented in salivary cells, with receptor subtype M3 and EGFR downstream of pilocarpine

while both M1/M3 subtypes and PKC acted downstream of carbachol [47]. Our results suggest that both agonists stimulate VEGFR2 to signal mTOR activation but use different mechanisms to regulate survival.

5. Conclusions

In summary, we present evidence for a novel signaling mechanism in serum starved SK-N-SH neuroblastoma cells in which mAChRs crosstalk with the VEGF/VEGFR2 pathway to regulate the activation of the PI3/Akt/mTOR pathway. A model of this crosstalk (Figure 8) shows that muscarinic receptor agonists share a common mechanism to stimulate protein synthesis in response to serum starvation. In this context, activated mAChRs enhance the phosphorylation of S6K and S6 activation by VEGF through a signaling pathway that is PI3K dependent and further modulated by PKC and PP2A. PKC positively regulates TORC1 targets S6K and S6 and negatively modulates the TORC2 substrate Akt while the magnitude and duration of this response is determined by dephosphorylation via PP2A. However, our finding also revealed that that length and extent of agonist mediated stimulation confer divergent effects on survival.

Acknowledgments

This investigation was supported by Grant Number RR003037 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH) and a NINDS/NIH SC1NS066033 grant. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

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Figure 1.

Carbachol and VEGF induce activation of mTORC1 targets with different effects on Akt phosphorylation in serum-deprived SK-N-SH cells. (A) SK-N-SH cells were serum deprived for 48 hours as described in *Materials and Methods*. Cells were treated without and with 1mM carbachol during the final 30 minutes of serum deprivation as indicated. Cell lysates were analyzed by immunoblotting as described in *Materials and Methods*. Blots were probed with antibodies that specifically recognize the phosphorylation of S6K on Thr389 and Akt on Thr308. (B) Cells were treated with 10ng/ml VEGF for 15 minutes and analyzed for phosphorylation of S6K on Thr389, S6 on Ser235/236 and Akt on Thr308 and Ser473. Blots in (A) and (B) were stripped and reprobed with antigen specific antibodies to detect total protein levels for use as loading controls. Data for each time frame were quantified as the fold difference of normalized phosphorylation relative to the untreated control. Results are typical of the phosphorylation levels observed in 3 independent experiments.

Figure 2.

VEGFR2 signals VEGF and carbachol mediated S6K and S6 phosphorylation. (A) Serum deprived cells were pretreated for 2 hours with 10 μ M SU1498 followed by stimulation with either 10 ng/ml VEGF or 1mM carbachol as indicated. Immunoblotting was carried out as described in Figure 1 for detection of total and phosphorylated S6K and S6. (B) Cells were transfected with either scrambled (nontargeting) or VEGFR2 siRNA and then serum deprived for 48 hours. During the final 15 minutes of serum deprivation, cells were treated with 1mM carbachol for 15 minutes. Immunoblotting and the quantification of phosphorylation were carried out as described for Figure 1. (C) VEGFR2 was immunoprecipitated from lysates of serum starved cells treated with either 10ng/ml VEGF or 1mM carbachol for 15 minutes. Immunoprecipitates were analyzed by immunoblotting and probed with antigen specific antibodies that detect VEGFR2 phosphorylation on TYR951 and total protein.

Fig 3.

PKC positively regulates S6K and S6 phosphorylation and negatively regulates Akt activation. (A) Serum deprived cells were treated for 2 hours with 10 μ M SU1498 or 1 μ M GFX followed by 15 minute incubations with either 10 ng/ml VEGF (VE) or 1 mM carbachol (Carb) alone or in combination as indicated. Immunoblotting and data quantification for total and phosphorylated S6K, S6 and Akt were performed as described for Figure 1. (B, C) Cells were treated with inhibitors as described in (A) followed by a 15 minute exposure to 1 mM pilocarpine. Immunoblots were probed for total and phosphorylated S6K and S6 (B) and Akt (C).

Fig 4.

PI3K is upstream from TORC1 and Akt. Serum starved cells were treated with either 1 μ M GFX or 20 μ M LY294002 (LY) for 2 hours or 1 μ M rapamycin (Rap) for 48 hours either alone or in combination. Lysates were then analyzed by immunoblotting for detection of total and phosphorylated S6 and Akt as described for Figure 1.

Fig 5.

PP2A regulates the phosphorylation levels of S6K, S6 and Akt. (A) Serum starved cells were treated with either 400 nM OA or 50 μ M Na₃VO₄ for 1 hour followed by a 15 minute exposure to 10ng/ml VEGF or 1mM carbachol as indicated. Cell lysates were analyzed by immunoblotting for detection of total and phosphorylated S6K, S6 and Akt followed by data quantification as described for Figure 1. (B) Cells were treated with 400 nM OA in combination with 1 μ M rapamycin (Rap) or 20 μ M LY294002 (LY) as indicated and analyzed by immunoblotting for phosphorylated S6K, S6 and Akt as in (A).

Fig 6.

Pilocarpine, unlike carbachol, induces a dose and time dependent cell death that correlates with elevated TORC1 target activation. (A) Serum starved cells were treated with increasing concentrations of carbachol or pilocarpine as indicated for the final 8, 20 or 24 hours of the 48 hour incubation without serum. (A) Cells were assayed for viability as described in *Materials and Methods*. Survival was expressed as the percent viability relative to the vehicle treated control (100%) $\pm$ S.E.M from at least three independent experiments. Asterisks indicate significant differences between pilocarpine treatments at 20 and 24 hours versus the carbachol treatments at 8, 20 and 24 hours (*** $P < 0.001$). (B) Cells were incubated for 24 hour with 1 mM carbachol or pilocarpine as in (A) and analyzed by immunoblotting for detection of phosphorylated S6K, S6 and Akt, phosphorylation of PKC α /βII on Thr638/641 and the total protein levels of S6K, S6 and Akt and PKC α . Total PKC α levels were quantified and plotted relative to the untreated control.

Fig 7.

The cell death induced by pilocarpine involves oxidative stress and caspase cleavage. Serum deprived cells were treated for 24 hour with 1 mM pilocarpine alone or with 5 mM N-acetyl cysteine (Nac) or Nac in combination with 10 μ M SU1498. (A) Cell viability and caspase 3/7 activation was measured as described in *Materials and Methods*. Results represent the percent cell viability or caspase activity (units/ μ g protein) relative to the vehicle treated control $\pm$ S.E.M from at least three independent experiments. The asterisk indicates a significant difference between pilocarpine treatments with Nac versus pilocarpine alone (* $P < 0.05$; *** $P < 0.001$). (B) Immunoblots from cells treated as in (A) were analyzed for detection of total and phosphorylated S6K and S6 as described for Figure 1.

Fig 8.

Model of crosstalk between mAChR and VEGFR2 signaling. Agonists for mAChRs carbachol (carb) and pilocarpine (pilo) stimulate VEGFR2 activation by VEGF to enhance the phosphorylation of the TORC1 targets S6K and the ribosomal protein S6. PKC influences phosphorylation by positively regulating TORC1 targets and negatively regulating Akt activation at the PI3K site Thr308 and the TORC2 site, Ser473. Dephosphorylation by the phosphatase PP2A serves as an additional mode of regulation for S6K, S6 and Akt activation.