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Regulation of RGS mRNAs by cAMP in PC12 Cells

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The RGS (regulators of G protein signaling) proteins represent a novel family of proteins which attenuate G protein mediated signaling. Using antisense riboprobes selective for rat RGS4, RGS7, and RGS2, we examined the regulation of these RGS mRNAs in PC12 cells in response to agents which elevate intracellular cAMP. Treatment of the PC12 cells with forskolin, dibutryl cAMP, or 8-CPT-cAMP for three hours decreased RGS4 message by nearly 50%. Actinomycin D, a potent inhibitor of transcription, did not affect the forskolin-induced decrease in RGS4 message, suggesting that forskolin does not alter RGS4 message half-life. RGS7 message is also present in these cells, but was not affected by forskolin. In contrast, RGS2 message is not evident in unstimulated cells but is strongly induced by one hour of treatment with forskolin. Taken together, these data suggest that mRNA levels of different RGS family members respond in an idiosynchratic fashion to cAMP challenge.

G proteins transduce signals from membrane receptors to a variety of intracellular effectors. A new family of proteins, termed RGS (*R*egulators of *G* protein *S*ignaling) proteins has recently been shown to antagonize G protein function by serving as GTPase activating proteins (GAPs) for G_α subunits of heterotrimeric G proteins (1). Further, RGS proteins might also act by blocking G_α-effector interactions (2). The first RGS protein was characterized in yeast as the product of the SST2 gene (1). Presently, at least 18 mammalian RGS proteins have been identified (3). Like the G proteins which they modulate, the RGS proteins are expressed in various tissues, with several, including RGS4 and RGS7 expressed principally in the brain (4). To date, all RGS proteins tested as GAPs have proven to increase the GTPase activity of $G_{i\alpha}$, and $G_{q\alpha}$, but not G_s proteins (5).

Although much is being learned of their GAP activity and tissue localization, little is known of their regulation. The yeast RGS protein Sst2p is induced by pheremone (1), and the mammalian RGS protein, RGS2/GOS8 can be induced in lymphocytes by cyclohexamide (6), and in the rat striatum by amphetamine (7). One of the major pathways influenced by G_i proteins in most cells is the adenylyl cyclase-cAMP cascade. To test if RGS mRNAs may be regulated by cAMP, we examined the effect of increasing cAMP or cAMP analogs on mRNA levels of several RGS family members in PC12 cells. In PC12 cells, cAMP induces $G_{i\alpha 1}$ expression, perhaps as a compensatory response to inhibit further cAMP production by adenylyl cyclase (8). A coordinate decrease in the expression of RGS protein could potentiate a compensatory response of $G_{i\alpha}$ upregulation to cAMP overstimulation. Since several RGS proteins, including RGS4, have been shown to negatively regulate Gi_α subunits, we hypothesized that cAMP might downregulate expression of RGS4 message in these cells.

We have also examined expression of other RGS messages in PC12 cells. Northern blot analysis demonstrated that RGS7, like RGS4, was expressed principally in the brain (4), and we wished to determine, by ribonuclease protection assay, whether RGS7 was also expressed in the PC12 cells, and if its expression was affected by cAMP. With regard to RGS2, recent evidence suggests that this gene may be positively regulated by cAMP in the brain. Injection of amphetamine into rats resulted in an increase in RGS2 levels in the striatum (7). Moreover, this effect was likely mediated by the dopamine D1 receptor, which has typically been described as coupling to both stimulation of adenylyl

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Abbreviations used: **ActD,** actinomycin D; **cAMP,** adenosine 3′:5′-cyclic monophosphate; **8-CPT-cAMP,** 8-(4-chlorophenylthio)-adenosine-3′:5′-cyclic monophosphate; **dbcAMP,** dibutryl cAMP; **IBMX,** 3-isobutyl-1-methylxanthine; **RGS,** regulator of G protein signaling; **GAP,** GTPase-activating protein; **NECA,** 5′-N-ethylcarboxamidoadenosine; **PD98059,** 2-(2-amino-3-methoxyphenyl)-4H-benzopyran-4-one.

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cyclase and phospholipase C activity in rat striatum (9). Thus, we examined RGS2 expression in PC12 cells in response to forskolin, a direct activator of adenylyl cyclase, and in response to the calcium ionophore, ionomycin. Using this approach, we have demonstrated that the three RGS transcripts RGS4, RGS2 and RGS7 are differentially regulated in response to cAMP.

MATERIALS AND METHODS

PC12 cell culture. DMEM and serum were purchased from Gibco. Rat pheochromocytoma (PC12) cells were cultured in DMEM + 5% dialyzed fetal bovine serum, 10% horse serum, 100 ug/ml streptomycin, 100u/ml penicillin in primaria tissue culture dishes coated with 1% rat tail collagen. Cells were plated typically two days before treatment.

Drug treatments and RNA harvest. Forskolin and ionomycin were obtained from Calbiochem. Actinomycin D, 8-CPT-cAMP, and dibutryl-cAMP were purchased from Sigma and added to cells in either DMSO or water. Following treatment, media was aspirated, and total RNA was prepared using Trizol reagent (Gibco).

Ribonuclease protection assay. An expressed sequence tag for rat RGS4 was generously provided by The Institute for Genome Research (TIGR). PCR was performed on the sequence tag using standard conditions with primers corresponding to the ends of the coding sequence (5′-ATG TGC AAA GGA CTC GCT GGT C-3′), and (5′-TTA GGC ACA CTG AGG GAC TAG-3′). The PCR product was cloned into pCR2.0 (Invitrogen) and digested with either HindIII or BglII. Antisense [^{32}P]labeled riboprobes for RGS4 were prepared by transcription from the T7 promoter using a maxiscript T7 kit (Ambion). A partial rat RGS7 cDNA was obtained by PCR on rat total brain cDNA (Clontech) using primers based on the human sequence (5′ ATG GCT GCC CAC GGC TAC 3′) and (5′ GTT AGA AGG GTC AGG TGG CA 3′), giving a fragment of 659 bp. The PCR product was cloned into pCR2.1 (Invitrogen); plasmid linearized by StuI was used to generate an antisense riboprobe with T7 RNA polymerase (Ambion). A partial rat RGS2 clone was obtained by RT-PCR on total RNA from rat caudate. PCR was performed with degenerate primers based on the RGS domain (5′-GAG AGA GAA ATC ATC TIG AGA TTT CTG G-3′) and (5′-AAG CGG GGA GTA GGA AGT CTI TTC TTT CCA T-3′) (4) using standard conditions. Products were cloned into vector pCR2.0 (Invitrogen); the plasmid digested with BamHI was used to generate RGS2 antisense riboprobe using T7 RNA polymerase. A rat [^{32}P]labeled cyclophilin control probe was generated according to Ambion, and ribonuclease protection assays were also performed according to an RPAII kit (Ambion). Briefly, 20 micrograms of total PC12 RNA were precipitated with labeled riboprobes, heated at 85-90°C and hybridized overnight at 42-45°C. RNA-probe samples were digested with a mixture of RNAse A/T1, precipitated and electrophoresed on a 6% acrylamide TBE-urea gel (Novex). Gels were fixed for 30 minutes in 10% ethanol/10% acetic acid, dried, and exposed to Kodak Biomax MR or MS film for 1 hour to overnight.

RESULTS

We have used the ribonuclease protection assay, with [^{32}P]-labeled riboprobes to assess changes in mRNA levels for rat RGS4, RGS7 and RGS2 in PC12 cells. Preliminary studies with both undifferentiated cells and PC12 cells treated for three days with 100 ng/ml NGF indicated that NGF-induced differentiation did not alter RGS message expression (data not shown). For this reason, undifferentiated cells were used for

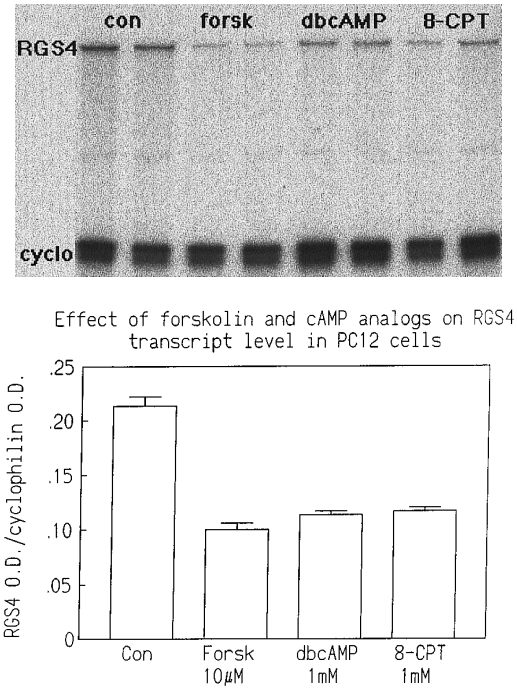

FIG. 1. (Top) Effect of forskolin and cAMP analogs on RGS4 message levels in PC12 cells. Cells were grown in 10 cm dishes and treated for 3 hours with 10 μm forskolin (forsk), 1 mM dibutryl cAMP (dbcAMP), or 1 mM 8-CPT-cAMP (8-CPT). Total RNA was prepared and ribonuclease protection assays for RGS4 mRNA were performed as described under Materials and Methods. (Bottom) Bands were quantified using a Bio-Rad GS-670 densitometer, and normalized for expression of control message cyclophilin.

the subsequent studies. Initial experiments focused on our ability to stimulate distinct signaling pathways in the PC12 cells. Treatment of PC12 cells for three hours with 10 μM forskolin or 1 mM cAMP analogs 8-CPT-cAMP or dibutryl cAMP decreased RGS4 message by 50% relative to control. Cyclophilin message was unchanged by forskolin (Figure 1). To determine if this effect could be mimicked by an endogenous receptor, we examined the effect of the non-selective adenosine receptor agonist 5′-N-ethylcarboxamidoadenosine (NECA) on RGS4 message expression. PC12 cells express an endogenous $A2_a$ receptor which is positively coupled to adenylyl cyclase (12). In the presence of the phosphodiesterase inhibitor IBMX, 10 μM NECA decreased RGS4 signal (Figure 2), again consistent with elevated cAMP levels decreasing RGS4 expression. In order to show that forskolin did not affect RGS4 mRNA stability, PC12 cells were pre-treated with actinomycin D (ActD), a potent inhibitor of transcription, and RGS4 message was assessed at one and three hours in the presence and absence of forskolin. Following ActD treatment, forskolin did not enhance the rate of RGS4 message decay (Figure 3).

Since the mitogen activated protein (MAP) kinase signaling pathway has been shown to be activated by

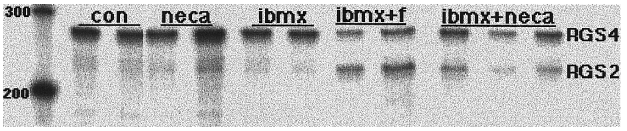

FIG. 2. Effect of adenosine agonist NECA on RGS4 and RGS2 expression in PC12 cells. PC12 cells were treated for three hours in serum-free media with either 10 μM NECA alone, 1 mM IBMX alone, IBMX +10 μM forskolin, or IBMX +NECA. Total RNA was prepared, and ribounuclease protection assay for RGS2 and RGS4 was performed as described under Materials and Methods.

cAMP in PC12 cells (14), we also examined whether stimulators of MAPK signaling could decrease RGS4 message expression. Several agents, including the calcium ionophore ionomycin and bradykinin were shown to be strong activators of MAPK in PC12 cells (data not shown), but had no effect on RGS4 mRNA levels. Moreover, the MEK (MAPK kinase) inhibitor PD98059 did not prevent the forskolin-induced decrease in RGS4 message (data not shown). Of the agents we tested, only the direct stimulators of the adenylyl cyclase-cAMP pathway lowered RGS4 message in PC12 cells.

We then examined expression of RGS7 in the PC12 cells. Like RGS4, RGS7 appears to be expressed principally in the brain, with much smaller amounts in lung (4). Undifferentiated PC12 cells were treated for either one or six hours with 10 μM forskolin and assayed for RGS7 message. RGS7 mRNA levels were not affected by either short (1 h) or longer term (6 h) treatment with forskolin (Figure 4). RGS7 signal was also unaffected by 3-hour treatment with forskolin (data not shown).

Finally, we assessed the expression of RGS2 in response to forskolin in PC12 cells. Unstimulated cells did not express detectable message for RGS2, however treatment with forskolin for one hour strongly induced

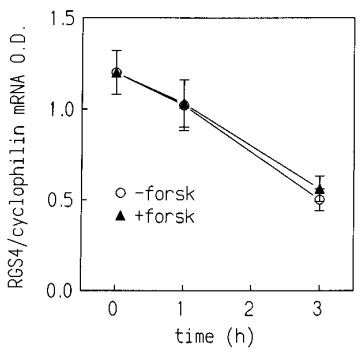

FIG. 3. Determination of RGS4 mRNA stability. PC12 cells were pre-treated for 30 minutes with 5 ug/ml actinomycin D, and incubated for the indicated times in the presence or absence of 10 μM forskolin. Total RNA was prepared and assayed for expression of RGS4 mRNA as described under Materials and Methods. Results are the mean of three individual dishes and are expressed as the ratio of RGS4 to cyclophilin optical density.

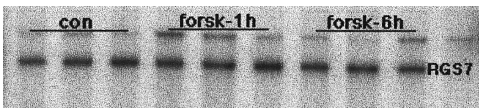

FIG. 4. Effect of forskolin treatment on RGS7 message in PC12 cells. Cells were treated for the indicated times with 10 μM forskolin. Total RNA was prepared and assayed for RGS7 message as described under Materials and Methods.

expression of RGS2 mRNA (Figure 5). RGS2 expression was slightly lower at three hours and had clearly decreased with 6 hours of forskolin treatment (data not shown). This induction of RGS2 was also mimicked by the adenosine agonist NECA. In the presence of the phosphodiesterase inhibitor IBMX, both forskolin and NECA were capable of inducing RGS2 (Figure 2). Treatment of the cells with the calcium ionophore, ionomycin however, did not induce expression of RGS2 (Figure 5).

DISCUSSION

In this study, we have examined expression of mRNA for several RGS proteins in PC12 cells in response to agents which elevate cAMP. The RGS proteins represent powerful regulators of G protein signaling, which directly stimulate the endogenous GTPase activity of G_i-coupled G proteins (10), and may also directly block G_α-effector interactions (2). Presently, at least sixteen mammalian RGS proteins have been identified, several of which, including RGS4 and RGS7 appear to be selectively expressed in the brain (4).

We were particularly interested if RGS expression could be modulated by specific signaling pathways. To address this question we turned to the rat PC12 cells. RGS4 is expressed endogenously in these cells, and was not affected by NGF-induced differentiation. Initial attempts to modulate RGS4 expression using bradykinin, ionomycin and phorbol ester were unsuccessful, suggesting that RGS4 was not modulated by protein kinase C or the MAPK signaling pathway in these cells. We then asked whether RGS4 expression may be modulated by the cAMP signaling pathway. Earlier studies in the PC12 cells demonstrated that cAMP could induce

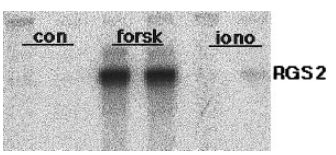

FIG. 5. Effect of forskolin and the calcium ionophore, ionomycin on RGS2 transcript levels in PC12 cells. Cells were treated for one hour with either 10 μM forskolin or 1 μM ionomycin. Total RNA was prepared, and RNAse protection assay was performed as described under Materials and Methods.

expression of $Gi_{\alpha 1}$ (8). Since several RGS proteins have been shown to negatively regulate $G_{i\alpha}$ proteins, we hypothesized that in a compensatory manner, PC12 cells might decrease expression of RGS proteins in response to cAMP. Indeed, RGS4 message was consistently decreased by 50% in three hours with forskolin, cAMP analogs, or treatment with the G_s-coupled adenosine receptor agonist, NECA, suggesting negative regulation by the adenylyl cyclase pathway.

Also, the decrease in RGS4 mRNA was not due to decreased stability of mRNA since RGS4 mRNA declined at the same rate in the presence and absence of forskolin following suppression of transcription by actinomycin D. It is also interesting to note that RGS4 message was decreased to the same extent by forskolin treatment alone as with the transcriptional inactivator actinomycin D. These results would suggest that over this time course (3 h), forskolin completely suppresses RGS4 transcription. Although message for RGS4 is decreased by cAMP, there are not yet suitable tools for detecting changes in endogenous RGS4 protein levels in the PC12 cells. Complete understanding of this regulatory phenomenon will require characterization of the RGS4 promoter elements, as well as determining if levels of RGS4 protein and/or function are also modulated in response to increases in cAMP.

In contrast to the inhibition of expression observed with RGS4 in response to forskolin, our results in the PC12 cells suggest that RGS2 expression is strongly induced by cAMP. This RGS2 is the rat homolog of human RGS2/GOS8 first characterized in lymphocytes, where it was shown to be induced by concanavalin A and cyclohexamide (6). Whereas the downregulation of RGS4 may represent the cell's attempt to compensate for an increase in cAMP, the rapid up-regulation of RGS2 suggests that the target for RGS2 might differ from that of RGS4. In addition, the regulation of RGS2 expression might be tissue specific, since RGS2 is upregulated by ionomycin in human lymphocytes, but not in PC12 cells (Figure 5).

We also examined expression of RGS7 in the PC12 cells. This RGS protein appears to be selectively expressed in the brain, particularly in areas of the brainstem, hypothalamus, thalamus and at high levels in cerebellar granule cells (3 and personal observation). In contrast to both RGS2 and RGS4, RGS7 mRNA expression did not respond to forskolin treatment, although it is possible that changes in RGS7 protein levels or intracellular targeting might be affected in response to cAMP challenge.

Following cAMP challenge then, RGS4 mRNA is decreased by 50%, RGS7 mRNA is unaltered, and RGS2 mRNA is strongly elevated. These effects on RGS4 and RGS2 are the first reports of cAMP-inducible changes in RGS transcript levels, and may represent a complex cellular response to changes in cAMP. The effects of cAMP on RGS expression are particularly interesting in light of the recent cloning of an A-kinase anchoring protein (AKAP) containing an RGS domain (13).

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