Muscarinic Receptor Stimulation Increases Regulators of G-protein Signaling 2 mRNA Levels through a Protein Kinase C-dependent Mechanism*

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RGS2, a member of the Regulators of G-protein Signaling family, modulates the activity of G-proteins coupled to the phosphoinositide signal transduction system, but little is known about what regulates RGS2. In human neuroblastoma SH-SY5Y cells stimulation of muscarinic receptors by carbachol activates phosphoinositide signaling and also caused a rapid, large, and long lasting increase in RGS2 mRNA levels. Direct activation of protein kinase C also rapidly increased RGS2 mRNA levels. Inhibition of protein kinase C with Ro31-8220, GF109203x, or Go6976, or down-regulation of protein kinase C inhibited increases in RGS2 mRNA levels induced by carbachol or by the activation of protein kinase C. Blockade of calcium signaling did not alter carbachol-induced increases in RGS2 mRNA levels. Neither activation of epidermal growth factor receptors nor stimulation of cyclic AMP production with forskolin increased RGS2 mRNA levels. Pretreatment with actinomycin D blocked increases in RGS2 mRNA levels but caused a surprisingly small, although statistically significant, partial blockade of protein kinase C-mediated feedback inhibition of carbachol-induced phosphoinositide hydrolysis. Thus, RGS2 mRNA levels are increased by activation of muscarinic receptors coupled to the phosphoinositide signal transduction system through a protein kinase C-dependent mechanism. This action may contribute to negative feedback control of this signaling cascade, but because the small contribution to negative feedback contrasts with the large and prolonged elevations in RGS2 mRNA levels, we speculate that its primary role may be in modulating other signaling components.

The recent discovery of a family of Regulators of G-protein Signaling (RGS) proteins has provided new insights into mechanisms regulating the signaling cascades initiated by activation of G-protein-coupled receptors (1–3). RGS proteins contribute to the control and cross-talking of signaling cascades by interacting with activated G-protein α-subunits to facilitate their intrinsic inactivating GTPase reaction. By this, and likely other mechanisms (4–7), RGS proteins participate in limiting the activities of G-protein α-subunits and regulating the activities of signaling cascades. How RGS proteins themselves are regulated has begun to be examined in recent investigations, which have generated evidence that one important site of control is at the level of gene expression (2).

RGS2 is of particular interest to investigators studying the phosphoinositide signal transduction system in the brain (8). RGS2 has been linked with regulation of Gq/11, the G-proteins mediating receptor-coupled phosphoinositide hydrolysis, where it functions as a potent inhibitor of Gq/11-mediated activation of phospholipase C (5, 7, 9). Furthermore, RGS2 is rapidly regulated by neuronal activity, as the mRNA level for RGS2 but not seven other RGS subtypes was rapidly and transiently increased by treatments that increase neuronal activity, such as electroshock, in a manner reminiscent of immediate early genes, such as c-fos (9). RGS2 mRNA levels also have been reported to be increased in selective brain regions after treatment of rats with haloperidol (9), amphetamine, cocaine, methamphetamine, or raclopride (9–11). Initial studies of RGS2 mRNA levels in cultured cells have identified several regulatory agents. Concanavalin A stimulated the expression of RGS2 in blood mononuclear cells (12, 13), elevation of cyclic AMP increased RGS2 mRNA levels in PC12 cells and T cells (14, 15), and the calcium ionophore ionomycin increased RGS2 mRNA in blood mononuclear cells (13) but not in PC12 cells (14). Overall, these studies indicate that RGS2 modulates the activity of the phosphoinositide signal transduction system and is itself subject to rapid regulation through modulation of its expression.

Muscarinic receptor-coupled activation of the phosphoinositide signal transduction system has been studied extensively in human neuroblastoma SH-SY5Y, which endogenously express muscarinic M3 receptors linked to phosphoinositide signaling (16–18). Therefore, these cells were used in the present study to determine if RGS2 mRNA levels are subject to modulation by activation of the phosphoinositide signaling system. The results show that in SH-SY5Y cells muscarinic receptor activation leads to rapid, large, and long lasting increases in RGS2 mRNA levels through a protein kinase C-dependent mechanism, and inhibition of transcription when protein kinase C is stimulated reduces feedback inhibition of phosphoinositide hydrolysis.

EXPERIMENTAL PROCEDURES

Cell Culture—Human neuroblastoma SH-SY5Y cells were grown in RPMI medium (Cellgro, Herndon, VA) supplemented with 10% horse serum (Life Technologies, Inc.), 5% fetal clone II (Hyclone, Logan, UT), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in humidified 37 °C chambers with 5% CO2. Cells were plated at a density of approximately 105 cells/100-mm dish and were treated with experimental agents approximately 48 h later. Agents used include carbachol, phorbol 12-myristate 13-acetate (PMA), nickel chloride, epidermal growth factor, and actinomycin D from Sig-

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† The abbreviations used are: RGS, regulators of G-protein signaling; PMA, phorbol 12-myristate 13-acetate.
Muscarinic Receptors Induce RGS2 Expression

Ma; Ro31-8220, GF109203x, Go6976, and forskolin from Alexis Biochemicals (San Diego, CA); KN62 from Seikagaku America (Rockville, MD); and 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (tetrakis(acetoxymethyl ester)) from Calbiochem.

Northern Blots—RGS2 cDNA was generously provided by Dr. D. R. Forseydye (Queen’s University, Kingston, Ontario, Canada). Total mRNA was extracted using Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. RNA (10 μg) was separated by electrophoresis in 1.2% agarose gels containing formaldehyde and transferred to nitrocellulose membranes. cDNA was random prime-labeled with [32P]dCTP (Amersham Pharmacia Biotech). Blots were hybridized with labeled probes at 42 °C for 18 h and then washed in two changes of 2× saline-sodium citrate and 0.1% SDS at 20 °C for 20 min and once in 1× saline-sodium citrate and 0.1% SDS at 55 °C for 10 min. Results were obtained using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and all experiments were repeated 2–4 times.

Phosphoinositide Hydrolysis—Cells were prelabeled with 7.5 μCi/ml nystatin (American Radiolabeled Chemicals, St. Louis, MO) for 48 h. Where indicated, cells were treated with 1 μM actinomycin D to inhibit transcription or an equivalent amount of dimethyl sulfoxide (Me2SO) for 15 min and 0.2 μM PMA for a subsequent 2 h to induce feedback inhibition of phosphoinositide hydrolysis. Cells were harvested, resuspended in buffer (30 mM HEPES, pH 7.4, 122 mM NaCl, 3.6 mM NaHCO3, 1.2 mM KH2PO4, 1.2 mM MgCl2, 5 mM KCl, 1.3 mM CaCl2, 10 mM LiCl, 11 mM glucose), and washed two times, as described previously (16). Suspended cells were incubated for 15 or 30 min with or without 1 μM carbachol at 37 °C, and radioactivity was measured in samples after fractionation of lipids, inositol monophosphate, and inositol as described previously (16). Measurements were made in triplicate for each treatment in at least three separate experiments. Statistical significance was determined using a paired Student’s t test.

RESULTS

RGS2 mRNA levels were measured in human neuroblastoma SH-SY5Y cells treated with 1 μM carbachol for 15 min to 24 h. Fig. 1A shows results from a representative time-course experiment demonstrating that RGS2 mRNA levels rapidly increased upon stimulation of muscarinic receptors with carbachol, whereas actin mRNA levels remained unchanged. Maximal levels of RGS2 mRNA were attained between 90 and 120 min of exposure to carbachol, and pretreatment with the muscarinic receptor antagonist atropine (1 μM) completely blocked carbachol-induced increases in RGS2 mRNA. Thus, stimulation of endogenous muscarinic receptors that are coupled to the phosphoinositide signal transduction system causes a rapid, large, and prolonged increase in RGS2 mRNA levels.

Phosphoinositide signaling activates protein kinase C, which is known to cause feedback inhibition of phosphoinositide signaling activity. Therefore protein kinase C was examined as a potential regulator of RGS2 expression. Activation of protein kinase C with 0.2 μM PMA caused a time-dependent increase in RGS2, but not actin, mRNA levels (Fig. 1B). If protein kinase C participates in muscarinic receptor-induced increases in RGS2 mRNA levels, then inhibitors of protein kinase C should cause similar reductions in the increases in RGS2 mRNA levels induced by the activation of protein kinase C and stimulation of muscarinic receptors. To test this, several protein kinase C inhibitors were used. Two bisindolylmaleimide derivatives, GF109203x and Ro31-8220, increased levels in RGS2 mRNA levels induced by treatment with carbachol or PMA with 10 μM GF109203x causing inhibitions of 50–60% and 10 μM Ro31-8220 causing almost 90% inhibition with each stimulant (Fig. 2). Increases induced by PMA and carbachol also were inhibited similarly by an inhibitor of classical protein kinase C subtypes, 10 μM Go6976 (68 ± 2 and 58 ± 11% inhibition, respectively), and by down-regulation of protein kinase C attained by a 24-h pretreatment with 1 μM PMA (48 ± 6 and 47 ± 7% inhibition, respectively). In contrast, carbachol-stimulated RGS2 mRNA levels were unaffected by several agents affecting calcium signaling (Fig. 3A) including 20 μM 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (tetrakis(acetoxymethyl ester)) (an intracellular calcium chelator), 2 mM nickel chloride (an inhibitor of the plateau phase of carbachol-stimulated increases in intracellular calcium), and 30 μM KN62 (an inhibitor of calcium/calmodulin-dependent kinase II). This lack of effects contrasts with previous studies using each of these agents affecting calcium signaling that demonstrated effects on other signaling systems activated by carbachol in SH-SY5Y cells (19).

In contrast to stimulation of muscarinic receptors and activation of protein kinase C, two other signaling agents failed to cause increases in RGS2 mRNA levels. Treatment with epidermal growth factor, which activates signaling through increased protein tyrosine phosphorylation in SH-SY5Y cells (20), caused little change in RGS2 mRNA levels (Fig. 3B). Forskolin, which causes increases in cyclic AMP and has been reported to increase RGS2 mRNA levels in PC12 cells (14), did not increase but actually decreased RGS2 mRNA levels (Fig. 3C).

To test if RGS2 might contribute to the well known feedback inhibition of phosphoinositide signaling induced by activation of protein kinase C, actinomycin D was used to inhibit transcription. Pretreatment with 1 μM actinomycin D completely blocked increases in RGS2 mRNA (Fig. 4A). In nonpretreated SH-SY5Y cells, carbachol induced a rapid and robust activation of phosphoinositide hydrolysis, attaining at 15 min of incubation an 8-fold stimulation over the basal rate of phosphoinositide hydrolysis (Fig. 4B). Activation of protein kinase C using a 2-h pretreatment with 0.2 μM PMA caused an approximately 70% inhibition of the subsequent carbachol-stimulated phosphoinositide hydrolysis (Fig. 4C). Inhibition of transcription with actinomycin D during the pretreatment with PMA significantly reduced the inhibitory effect on carbachol-stimulated phosphoinositide hydrolysis, with responses attained that were 139 and 124% of those obtained in PMA-treated cells without transcription blockade. Treatment with actinomycin D without PMA treatment did not alter carbachol-stimulated phosphoinositide hydrolysis (data not shown). These results indicate that a portion of the PMA-induced inhibition of phosphoinositide signaling requires activation of gene expression, a finding consistent with the hypothesis that the expression of RGS2 following stimulation of protein kinase C makes a significant contribution to this feedback inhibitory action.

DISCUSSION

This study adds to a small but growing body of research revealing the exquisite responsiveness of RGS2 mRNA levels to extracellular stimuli, as reviewed in the Introduction. The rate at which RGS2 mRNA levels increased after stimulation of muscarinic receptors is comparable to that of the classical c-fos and c-jun immediate early genes (21, 22), a similarity noted previously in a study of increases in RGS2 mRNA levels in rat brain in response to electroshock (9). However, increases in RGS2 mRNA levels in SH-SY5Y cells after muscarinic receptor stimulation were relatively long lasting, as the elevation was still evident after 24 h of stimulation with carbachol. Thus, muscarinic receptor activation results in rapid and large, but also prolonged, elevations in RGS2 mRNA levels.

Protein kinase C appears to mediate a significant portion of the muscarinic receptor-induced increase in RGS2 mRNA levels. Both stimulation of muscarinic receptors coupled to phosphoinositide signaling and activation of protein kinase C, a second messenger-linked outcome of phosphoinositide signaling, increased RGS2 mRNA levels, and these responses were inhibited equivalently by four treatments that inhibit the activity of protein kinase C. Ro31-8220 was the most effective inhibitor of the induction of RGS2 mRNA, but recent studies have discovered several actions of this agent independent of its inhibition of protein kinase C, some of which may contribute to
the practically complete inhibition caused by Ro31-8220 (23–25). The other three treatments, GF109203x, Go6976, and protein kinase C down-regulation, caused 50–60% inhibitions of carbachol- or PMA-induced increases in RGS2 mRNA levels, suggesting that protein kinase C subtypes in the classical family may mediate this portion of the response. Phorbol ester-induced activation of protein kinase C previously was reported to increase the mRNA level of RGS7, but not RGS4, in primary cortical neurons after 12 h of treatment (26) but did not increase RGS2 mRNA levels in blood mononuclear cells (13). Although this is still a limited amount of information, it indicates that, not unexpectedly, there are differences in the mechanisms that regulate mRNA levels for different members of the RGS family of proteins and there are cell-specific differences in the regulation of RGS2 mRNA levels.

FIG. 1. Carbachol and protein kinase C activation increased RGS2 mRNA levels. A, RGS2 and actin mRNA levels were measured in SH-SY5Y cells treated with 1 mM carbachol (Carb) for 15 min to 24 h. Pretreatment with 1 μM atropine (AT) for 10 min blocked the increase in RGS2 mRNA caused by treatment with 1 mM carbachol for 60 min. B, RGS2 and actin mRNA levels were measured in SH-SY5Y cells treated with 0.2 μM PMA for 15–180 min.

FIG. 2. Inhibitors of protein kinase C reduce increases in RGS2 mRNA levels. SH-SY5Y cells were pretreated with 10 μM Ro31-8220 (Ro), 10 μM GF109203x (GF), or 10 μM Go6976 (Go) for 10 min to inhibit protein kinase C or with 1 μM PMA for 24 h to down-regulate (DR) protein kinase C. RGS2 mRNA levels were measured after a 60-min incubation with 1 mM carbachol (which caused RGS2 mRNA levels to increase to 517 ± 45% of basal) or a 90 min incubation with 0.2 μM PMA (which caused RGS2 mRNA levels to increase to 258 ± 33% of basal). Values shown are percentages of RGS2 mRNA levels obtained by carbachol or PMA treatment without protein kinase C inhibitors. Ctl, control.
Muscarinic Receptors Induce RGS2 Expression

FIG. 3. Modulation of RGS2 mRNA levels by calcium, epidermal growth factor, and forskolin. A, SH-SY5Y cells were preincubated with 2 mM NiCl₂ (Ni) for 10 min to block the plateau phase of the carbachol-stimulated rise in intracellular calcium, 30 μM KN62 (KN) for 10 min to inhibit calcium/calmodulin-dependent protein kinase II, or 20 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetraakis(acetoxymethyl ester) (BAPTA) for 30 min to chelate intracellular calcium. RGS2 mRNA levels were measured after a 60-min incubation with 1 mM carbachol. Ctl, control. B, SH-SY5Y cells were incubated with 50 ng/ml epidermal growth factor (EGF) for 0, 30, 60, or 90 min, followed by measurements of RGS2 mRNA levels. C, SH-SY5Y cells were incubated with 10 μM forskolin (FSK) for 0, 30, 60, 90, or 120 min followed by measurements of RGS2 mRNA levels.

pretreatment with PMA inhibited carbachol-stimulated phosphoinositide hydrolysis by 70%, a regulatory influence that has been known for many years but for which a mechanistic basis has not been delineated. Inhibition of transcription by actinomycin D both blocked increases in RGS2 mRNA levels and attenuated the inhibition of phosphoinositide hydrolysis caused by activation of protein kinase C. Taken together, all of these results are consistent with the hypothesis that activation of protein kinase C increases RGS2 expression, which attenuates the activity of the G-proteins mediating phosphoinositide signaling, although selective knockouts of RGS2 will be necessary to fully test this scheme. However, the minor portion of the protein kinase C-induced inhibition of phosphoinositide signaling that was blocked by actinomycin D treatment indicates that other mechanisms contribute the major share to this feedback inhibition. This raises the intriguing possibility that physiological increases in RGS2 may primarily serve other functions. This speculation will be better tested once antibodies are available to measure RGS2 protein levels and after the diverse actions of RGS2 are more completely identified.

In summary, stimulation of muscarinic receptors increased RGS2 mRNA levels through a mechanism partially dependent on protein kinase C activation, and transcription was necessary for maximal feedback inhibition by protein kinase C of phosphoinositide signaling. It is likely that other receptors coupled to the phosphoinositide signal transduction system also will modulate RGS2 mRNA levels because protein kinase C mediated a portion of this regulatory response. Thus, stimulation of RGS2 expression may contribute to the control of phosphoinositide signaling activity, and considering the multiple functions of RGS proteins that are being identified, RGS2 may constitute a rapid intracellular mediator of cross-talk among signaling systems.

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