Endogenous Regulator of G Protein Signaling Proteins Reduce μ-Opioid Receptor Desensitization and Down-Regulation and Adenylyl Cyclase Tolerance in C6 Cells

Mary J. Clark and John R. Traynor
Pharmacology Department, University of Michigan Medical Center, Ann Arbor, Michigan
Received July 21, 2004; accepted September 21, 2004

ABSTRACT
Chronic exposure of cells to μ-opioid agonists leads to tolerance which can be measured by a reduced ability to activate signaling pathways in the cell. Cell signaling through inhibitory G proteins is negatively regulated by RGS (regulator of G protein signaling) proteins. Here we examine the hypothesis that the GTPase accelerating activity of RGS proteins, by altering the lifetime of $G_{\alpha}$ and $G_{\beta\gamma}$, plays a role in the development of cellular tolerance to μ-opioids. C6 glioma cells were stably transfected with μ-opioid receptor and pertussis toxin (PTX)-insensitive $G_{\alpha}$ that was either sensitive or insensitive to endogenous RGS proteins. Cells were treated with PTX to uncouple endogenous $G_{\alpha}$ proteins followed by exposure to the μ-opioid agonists [d-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin (DAMGO) or morphine. Receptor desensitization as measured by agonist-stimulated $[^{35}S]$GTP$\gamma$S binding and receptor down-regulation as measured by $[^{3}H]$diprenorphine binding were increased in cells expressing RGS-insensitive $G_{\alpha}$. Exposure to high concentrations of morphine or the peptidic μ-opioid agonist DAMGO led to a tolerance to inhibit adenylyl cyclase activity in both cell types with a rapid (30 min) and a slower component. Using a submaximal concentration of DAMGO to induce a reduced level of tolerance, a shift in the concentration-effect curve for DAMGO to inhibit adenylyl cyclase activity was seen in the cells expressing RGS-insensitive $G_{\alpha}$, but not in the cells expressing RGS-sensitive $G_{\alpha}$, which can be partly explained by an increased supersensitization of the adenylyl cyclase response. The results show that RGS proteins endogenously expressed in C6 cells reduce agonist-induced μ-opioid receptor desensitization, down-regulation, and sensitivity to tolerance to inhibit adenylyl cyclase activity.

The long-term use of μ-opioid agonists for the treatment of pain is hampered by tolerance, requiring escalating doses of drug to achieve the same level of pain relief. At the cellular level, drug exposure leads to changes in receptor-effector signaling including receptor desensitization, receptor down-regulation, up-regulation of adenylyl cyclase activity (super-sensitization), and other chronic changes that involve activation of transcription factors leading to alterations in protein expression (Borgland, 2001; Chao and Nestler, 2004).

The μ-opioid receptor is a member of the G protein-coupled receptor (GPCR) family that couples to heterotrimeric, pertussis toxin (PTX)-sensitive proteins of the $G_{\alpha}$ family. Agonist activation of the receptor leads to exchange of GDP bound to the $G_{\alpha}$ subunit in the resting state for GTP and dissociation of the $G_{\alpha}$-GTP and $G_{\beta\gamma}$ subunits which function as signaling molecules. One function of $G_{\beta\gamma}$ subunits is to recruit GPCR kinases (GRK) to the plasma membrane. These kinases phosphorylate the receptor and allow the subsequent binding of β-arrestin which prevents activation of G protein giving desensitization (Kupnick and Benovic, 1998). The binding of β-arrestin plays an important role in opioid tolerance in vivo (Bohn et al., 2000, 2002). Active $G_{\alpha}$-GTP and $G_{\beta\gamma}$ signaling molecules are returned to their inactive state by the intrinsic GTPase activity of $G_{\alpha}$, causing $G_{\alpha}$-bound GTP to be hydrolyzed to GDP with subsequent reassociation of the $G_{\alpha}$-GDP and $G_{\beta\gamma}$ subunits. This process is accelerated by RGS (regulator of G protein signaling) proteins, which bind to the $G_{\alpha}$ subunit and speed up hydrolysis of $G_{\alpha}$-bound GTP. RGS proteins function as negative regulators of GPCR signaling (for review see Hollinger and Hepler, 2002). RGS proteins comprise a family of more than 30 members. All have a conserved RGS domain that is responsible for the GTPase accelerating properties (GAP) of the proteins. However, there is some degree of selectivity for $G_{\alpha}$ subunits, and the many members of the family have a wide variety of other protein-protein interacting domains which provide for selec-

ABBREVIATIONS: GPCR, G protein-coupled receptor; PTX, pertussis toxin; GRK, G protein-coupled receptor kinase; RGS, regulator of G protein signaling; GAP, GTPase-accelerating protein; RGSi, RGS-insensitive; PTXi, PTX-insensitive; DAMGO, [d-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin; IBMX, 3-isobutyl-1-methylxanthine.
tivity and allow binding to different intracellular proteins to provide a scaffolding role (Hollinger and Hepler, 2002; Neubig and Siderovski, 2002).

RGS proteins have been shown to decrease specific aspects of μ-opioid signaling in vitro (Chuang et al., 1998; Potenza et al., 1999; Clark et al., 2003; Gold et al., 2003). In vivo, inhibition of RGS9 levels in mice using antisense oligonucleotides leads to an increase in the potency and duration of morphine antinociception with delayed development of antinociceptive tolerance (Garción et al., 2001). Similarly, RGS9 knockout mice show enhanced responses to morphine with delayed antinociceptive tolerance (Zachariou et al., 2003). Following chronic morphine treatment of rats, RGS4 protein levels are increased in the locus coeruleus (Gold et al., 2003), and RGS9 levels are increased on acute morphine treatment in the mouse striatum, spinal cord, and periaqueductal gray but decreased on chronic treatment (Zachariou et al., 2003). Consequently, RGS proteins and more specifically RGS9 appear to be important in the acute and chronic pharmacology of opioids.

In this study, we examined the hypothesis that by shortening the lifetime of Ga-GTP and Gβγ RGS proteins function to reduce μ-opioid receptor-mediated responses and so regulate opioid tolerance. This hypothesis was tested by determining agonist-induced changes in μ-opioid receptor desensitization and down-regulation and in the ability of μ-opioid agonists to inhibit adenyl cyclase activity. For the studies, we used rat C6 glioma cells expressing a μ-opioid receptor (C6μ) and Gαo, that is either sensitive or insensitive to the GAP activity of all RGS proteins (Clark et al., 2003). Gαo is rendered RGS-insensitive (RGSI) by a G184S mutation at the interface where it binds RGS protein (Lan et al., 1998; Clark et al., 2003). Gαo is also made insensitive to PTX (PTXi) by mutation of the sensitive cysteine in the C-terminal tail (C351G; Milligan, 1988). When expressed in C6μ cells, the effects of the RGS-insensitive mutation on cell signaling can be studied following treatment of the cells with PTX to inactivate endogenous Ga subunits.

The results show that agonist-induced μ-opioid receptor desensitization and down-regulation is increased in cells expressing RGS-insensitive Gαo compared with cells expressing RGS-sensitive Gαo. There is also a loss of μ-opioid potency to inhibit adenyl cyclase activity along with a concomitant increase in supersensitization of adenyl cyclase. This implies that at the cellular level endogenous RGS protein action works to prevent agonist-induced desensitization and down-regulation of the μ-opioid receptor and so contributes to the maintenance of signaling on chronic agonist exposure.

Materials and Methods

Materials. [35S]GTPγS and [3H]diprenorphine were from PerkinElmer Life and Analytical Sciences (Boston, MA), and cAMP radioimmunoassay kits were from Diagnostic Products (Los Angeles, CA). Morphine sulfate was obtained through the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). PTX was from List Biological Laboratories Inc. (Campbell, CA). DAMGO, IBMX, forskolin, and all other biochemicals were from Sigma-Aldrich (St. Louis, MO). Tissue culture media, LipofectAMINE Plus reagent, Geneticin, Zeocin, fetal bovine serum, and trypsin were from Invitrogen (Carlsbad, CA). Mouse, PTXi Gαo, (C351G), and RGS and pertussis toxin-insensitive Gαo (RGS/PTXi; G184S/C351G) DNAs were a kind gift from Steve Ikeda (National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD) (Jeong and Ikeda, 2000).

Expression of PTXi or RGS/PTXi Gαo in C6μ Cells and Cell Culture. PTXi or RGS/PTXi Gαo DNA was inserted into Zeo resistance vector pcDNA3.1Zeo then transfected into C6 glioma cells stably expressing rat μ-opioid receptor (C6μ) (Lee et al., 1999) using LipofectAMINE Plus reagent as described previously (Clark et al., 2003). Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum under 5% CO2 in the presence of 0.25 mg/ml Geneticin (to maintain expression of the μ-opioid receptor in a Geneticin-resistant plasmid) and 0.4 mg/ml Zeocin. Clones were typically subcultured at a ratio of 1:20 to 1:30 with partial replacement of the media on day 4 and again on day 6 before subculturing or harvesting at day 7.

Receptor Number and Down-Regulation. C6μ cells expressing PTXi Gαo, or RGS/PTXi Gαo grown as above were treated with PTX (100 ng/ml) 2 days before harvesting followed by the addition of 1 μM DAMGO 18 h before harvesting. After collection, cells were homogenized and membranes prepared as described in Clark et al. (2003) with a 15-min incubation at 37°C between the two centrifugation steps. Membranes (10–20 μg) were incubated in 50 mM Tris-HCl, pH 7.4, with a supermaximal concentration (4 nM) of the opioid antagonist [3H]diprenorphine with or without 50 μM naloxone to define nonspecific binding in a total volume of 0.2 ml for 60 min at 25°C. Samples were filtered through glass fiber filters (no. 32; Schleicher & Schuell, Keene, NH), mounted in a Brandel cell harvester (Brandel Inc., Gaithersburg, MD), and rinsed three times with ice-cold 50 mM Tris-HCl, pH 7.4. Radioactivity retained on each filter was counted by liquid scintillation counting in a 1-ml EcolLume scintillation mixture (ICN Pharmaceuticals Biochemicals Division, Aurora, OH). Data from three experiments, each carried out in triplicate, were expressed as mean ± standard error of the mean and differences analyzed by student’s t test.

Stimulation of [35S]GTPγS Binding. Membranes (14–20 μg) were incubated for 60 min in a shaking water bath at 25°C in 20 mM Tris-HCl, pH 7.4 containing 5 mM MgCl2, 100 mM NaCl, 0.1 mM dithiothreitol, 30 μM GDP, 0.1 nM [35S]GTPγS, and 0.01 to 10 μM DAMGO or distilled water as described by Traynor and Nahorski (1995). Samples were filtered through no. 32 glass fiber filters (Schleicher & Schuell), mounted in a Brandel cell harvester, and rinsed three times with an ice-cold solution containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, and 100 mM NaCl. Each filter was removed and counted by liquid scintillation counting as above.

Inhibition of cAMP Accumulation. Cells were plated to 80 to 90% confluency in 24-well plates 2 days before the assay and treated overnight with 100 ng/ml PTX. Varying concentrations of DAMGO or morphine were added without removing the PTX for varying times from 0.5 to 24 h before the start of the assay. To assay for opioid inhibition of cAMP accumulation, media were replaced with serum-free media containing 30 μM forskolin, 1 mM IBMX, and varying concentrations of DAMGO, morphine, or vehicle (distilled water) as control for 5 min (for single, high concentrations of agonist) or 15 min (for concentration-response curves) at 37°C. The reaction was stopped by replacing the media with ice-cold 3% perchloric acid. Accumulated cAMP was measured by radioimmunoassay as previously described (Clark et al., 2003). Inhibition of cAMP formation was determined as a percentage of forskolin-stimulated cAMP accumulation in the absence of opioid agonist.

Data Analysis. For [35S]GTPγS and cAMP accumulation assays, concentration-response data were fitted to sigmoidal dose response curves using GraphPad Prism (GraphPad Software Inc., San Diego, CA) to determine EC50 and maximal levels. Data from at least three separate experiments, each carried out in duplicate, were presented as mean ± standard error of the mean. Significance differences were determined by Student’s t test.
Results

C6μ cells expressing similar levels of PTX-insensitive Goα without (PTXi) or with (RGS/PTXi) the mutation for RGS insensitivity (Clark et al., 2003) were treated overnight with PTX to inactivate endogenously expressed G proteins in all experiments.

μ-Opioid Receptor Desensitization. The potency (EC50) and maximal effect (E_max) of DAMGO, a full μ-opioid agonist, to cause stimulation of [35S]GTPγS binding were similar in membranes from cells expressing Goα with only the PTX mutation (EC50 = 330 ± 40 nM, E_max = 333 ± 45% of basal) or with the RGS/PTXi mutation (EC50 = 440 ± 40 nM, E_max = 368 ± 44% of basal). The potency of DAMGO is lower than in wild-type C6μ cells but is consistent with our previous findings (Clark et al., 2003). Pretreatment of cells with 1 μM DAMGO for varying times before membrane preparation reduced the maximal DAMGO stimulation of [35S]GTPγS binding in a time-dependent manner but without any consistent change in the EC50 for DAMGO (Table 1). The reduction in maximal [35S]GTPγS stimulation was evident within 30 min and was significantly greater in cells expressing Goα that was insensitive to the action of RGS proteins (RGS/PTXi; Table 1).

μ-Opioid Receptor Down-Regulation. μ-Opioid receptor number, determined with a single saturating concentration of the nonselective antagonist [3H]diprenorphine, was the same (6.4 pmol/mg protein; Table 2) in membranes from PTXi Goα-expressing cells and cells expressing RGS/PTXi Goα. The μ-opioid receptor expression level did vary when cells were grown in different batches of fetal bovine serum, but the effects were the same across clones (data not shown). After a 60-min treatment of the cells with 1 μM DAMGO, there was a similar small decrease in μ-opioid receptor number in membranes prepared from cells expressing PTXi Goα or RGS/PTXi Goα (Table 2). After overnight treatment with 1 μM DAMGO, receptor number was further reduced; however, the reduction in receptor level was significantly greater in cells expressing RGS-insensitive Goα.

Tolerance of the Adenylyl Cyclase Response. The effect of the observed agonist-induced μ-opioid receptor desensitization and down-regulation on tolerance, measured as a reduction in μ-opioid inhibition of adenylyl cyclase, was examined. Cells were treated with varying concentrations of DAMGO or morphine, and the ability of a challenge concentration (10 μM) of DAMGO or morphine to inhibit forskolin-stimulated adenylyl cyclase was determined. This high challenge concentration was used to ensure that maximal inhibition of adenylyl cyclase was obtained, since cells expressing Goα that is only PTXi or both RGS/PTXi differ by more than 10-fold in their sensitivity to acute DAMGO (Clark et al., 2003). The challenge with DAMGO or morphine was added without washout of the pretreatment drug to avoid the overshoot in forskolin-stimulated cAMP levels due to adenylyl cyclase supersensitization caused by treatment with agonist that would be triggered by agonist removal (Watts, 2002; Clark et al., 2004). Following pretreatment with high (1 μM) concentrations of DAMGO or morphine for 18 h, the ability of 10 μM DAMGO or 10 μM morphine to cause inhibition of forskolin-stimulated adenylyl cyclase was completely lost in cells expressing RGS/PTXi or PTXi Goα (Fig. 1) indicating tolerance. The pretreatment concentration of DAMGO or morphine necessary to cause a 50% reduction in the adenylyl cyclase inhibition by an acute challenge with 10 μM DAMGO or 10 μM morphine, respectively, was not significantly different between cells expressing Goα that was RGS-sensitive or RGS-insensitive (Fig. 1).

Since RGS proteins accelerate the GTase activity of Goα, it can be hypothesized that RGS proteins should affect the rate of tolerance development rather than the degree of tolerance achieved. After 30 min of treatment with 1 μM DAMGO, there was a reduction in the ability of subsequently added 10 μM DAMGO to inhibit forskolin-stimulated adenylyl cyclase (Fig. 2). This reduction was similar (p = 0.13) in cells expressing PTXi Goα (reduced by 0.29 ± 0.09 pmol/μg protein) and RGS/PTXi Goα (reduced by 0.92 ± 0.07 pmol/μg protein). After 24 h of pretreatment with 1 μM DAMGO, there was a greater loss of the effectiveness of acute 10 μM DAMGO but no significant difference (p = 0.22) between cells expressing PTXi Goα (reduced by 0.95 ± 0.13 pmol/μg protein) or RGS/PTXi Goα (reduced by 0.98 ± 0.11 pmol/μg protein).

Is it possible that differential responses between the PTXi and RGS/PTXi Goα-expressing cells are present at lower pretreatment concentrations but masked by the supramaxi-

TABLE 1

<table>
<thead>
<tr>
<th>Pretreatment Time</th>
<th>DAMGO Stimulation</th>
<th>PTXi</th>
<th>RGS/PTXi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E_max (pmol/mg)</td>
<td>E_max (pmol/mg)</td>
<td></td>
</tr>
<tr>
<td>h</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>79 ± 6</td>
<td>65 ± 6</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>78 ± 4*</td>
<td>56 ± 6</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>61 ± 9*</td>
<td>26 ± 5*</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05, compared with no pretreatment (time 0).
* E_max values at time 0 were 333 ± 45% (PTXi) and 368 ± 44% (RGS/PTXi) of basal binding.

TABLE 2

μ-Opioid receptor numbers (B_max) in membranes from C6μ cells expressing PTX-insensitive and RGS-sensitive (PTXi) or PTX-insensitive and RGS-insensitive (RGS/PTXi) Goα following pretreatment with 1 μM DAMGO

<table>
<thead>
<tr>
<th>Pretreatment Time</th>
<th>B_max (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTXi</td>
<td>RGS/PTXi</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1.0</td>
<td>90.4 ± 0.7</td>
</tr>
<tr>
<td>18</td>
<td>72.5 ± 7.6*</td>
</tr>
</tbody>
</table>

* p < 0.05, compared with no pretreatment (time 0).
* The B_max values in control (untreated cells) were the same in the two clones (PTXi Goα cells = 6.4 ± 0.3 pmol/mg protein and RGS/PTXi Goα cells = 6.4 ± 0.3 pmol/mg protein).

* p < 0.05, compared with cells expressing PTXi Goα.
The aim of this study was to define a role for endogenous RGS proteins in cellular tolerance to \(\mu\)-opioid agonists identified by a reduction in ability to inhibit forskolin-stimulated adenylyl cyclase. Agonist-induced receptor desensitization and receptor down-regulation in C6\(\alpha\) cells expressing RGS-sensitive or insensitive \(\alpha\) resulted in a reduction in the ability of morphine and DAMGO to inhibit adenylyl cyclase activity. There was a greater degree of desensitization and down-regulation and a higher sensitivity to tolerance in RGS-insensitive \(\alpha\)-expressing cells compared with cells expressing RGS-sensitive \(\alpha\). This indicates that RGS proteins in this cell model serve to protect against \(\mu\)-opioid mediated tolerance. In this study, \(\alpha\) was the only functional \(\gamma\) protein confirming the ability of this \(\alpha\) protein to mediate chronic effects of \(\mu\)-opioid agonist exposure in the absence of other functional adenylyl cyclase inhibitory G proteines.

The ability of DAMGO to stimulate \(^{35}\)S\(\gamma\)TPyS was the same in cells expressing RGS-sensitive or -insensitive \(\alpha\), respectively or RGS/PTXi \(\alpha\)-expressing cells (84.2 ± 2.2% and 84.0 ± 2.4%, respectively).

Increased activity of adenylyl cyclase due to increased supersensitization could contribute to the reduction in potency of DAMGO in the RGS/PTXi \(\alpha\)-expressing cells. To attempt to mimic this, DAMGO inhibition of cAMP accumulation was measured in the presence of increasing concentrations of forskolin (3, 10, or 30 \(\mu\)M) to provide increased levels of adenylyl cyclase stimulation. The EC\(_{50}\) values for DAMGO inhibition increased with the degree of forskolin-stimulated adenylyl cyclase in cells expressing PTXi \(\alpha\), but this was not significant (\(p = 0.11\)). In contrast in cells expressing RGS/PTXi \(\alpha\), the EC\(_{50}\) values for DAMGO inhibition were 2-fold higher (\(p < 0.01\)) at the highest concentration of forskolin (Table 3). This demonstrates that at least part of the loss of effect of DAMGO in the RGS/PTXi \(\alpha\)-expressing cells is due to supersensitization of adenylyl cyclase.

### Discussion

The aim of this study was to define a role for endogenous RGS proteins in cellular tolerance to \(\mu\)-opioid agonists identified by a reduction in ability to inhibit forskolin-stimulated adenylyl cyclase. Agonist-induced receptor desensitization and receptor down-regulation in C6\(\alpha\) cells expressing RGS-sensitive or insensitive \(\alpha\) resulted in a reduction in the ability of morphine and DAMGO to inhibit adenylyl cyclase activity. There was a greater degree of desensitization and down-regulation and a higher sensitivity to tolerance in RGS-insensitive \(\alpha\)-expressing cells compared with cells expressing RGS-sensitive \(\alpha\). This indicates that RGS proteins in this cell model serve to protect against \(\mu\)-opioid mediated tolerance. In this study, \(\alpha\) was the only functional \(\gamma\) protein confirming the ability of this \(\alpha\) protein to mediate chronic effects of \(\mu\)-opioid agonist exposure in the absence of other functional adenylyl cyclase inhibitory G proteins.

The ability of DAMGO to stimulate \(^{35}\)S\(\gamma\)TPyS was the same in cells expressing RGS-sensitive or -insensitive \(\alpha\), respectively or RGS/PTXi \(\alpha\)-expressing cells (84.2 ± 2.2% and 84.0 ± 2.4%, respectively).
The development of adenylyl cyclase tolerance to \(\mu\)-opioid agonists had a fast component apparent within 30 min and a slow component which was not apparent until 6 h of agonist exposure in both the RGS-sensitive and -insensitive \(\mathrm{G}_\alpha_o\)-expressing cells. This fast component is likely due to desensitization of receptor activation of G proteins which was evident after 30 min of treatment with 1 \(\mu\)M DAMGO. The degree of desensitization was greater in the RGS-insensitive \(\mathrm{G}_\alpha_o\)-expressing cells (Fig. 2). C6 cells express GRK2, GRK5, and arrestin (Gray et al., 2001) so this is probably the result of increased recruitment of a GRK leading to increased receptor phosphorylation and \(\beta\)-arrestin binding. This could occur due to the lengthened lifetime of free G\(\gamma\) as a result of the inability of endogenous RGS proteins to accelerate the hydrolysis of G\(\alpha\)-bound GTP in the presence of the RGS-insensitive \(\mathrm{G}_\alpha_o\) mutant. Additionally, activation of the mitogen-activated protein kinase cascade, which is negatively regulated by RGS protein action in C6\(\mu\) cells (Clark et al., 2003), has also been implicated in the rapid phosphorylation and desensitization of the \(\mu\)-opioid receptor (Polakiewicz et al., 1998; Schmidt et al., 2000).

Receptor down-regulation was not apparent until more than 1 h after pretreatment with \(\mu\)-opioid agonist and was also seen to a greater degree in cells expressing RGS-insensitive \(\mathrm{G}_\alpha_o\). Down-regulation could be involved in the slower developing tolerance; however, there is little literature support for this idea. Gomes et al. (2002) showed that PTX-treatment can eliminate tolerance without affecting receptor down-regulation. There are also ligand-specific differences in the ability of \(\mu\)-opioids to cause receptor down-regulation that do not correlate with differences in tolerance (Patel et al., 2002). In addition, \(\mu\)-opioid antinociceptive tolerance in mice is reduced by treatment with \(\mathrm{G}_{\alpha_2}\) antisense oligodeoxynucleotides, although \(\mu\)-opioid receptor down-regulation is not affected (Yoburn et al., 2003). These and other studies (Chakrabarti et al., 1997; Yabaluri and Medzihradszky, 1997; Pak et al., 1999) provide evidence for separate mechanisms of receptor down-regulation, namely a G protein-dependent and -independent mechanism (Pak et al., 1999) which may show some redundancy. The increase in receptor down-regulation in the RGS-insensitive \(\mathrm{G}_\alpha_o\)-expressing C6\(\mu\) cells implies at least the presence of a G protein-dependent component.

Although increased levels of \(\mu\)-opioid receptor desensitization and down-regulation in C6\(\mu\) cells contribute to adenylyl cyclase tolerance, there is also an increased adenylyl cyclase supersensitization in cells expressing RGS-insensitive \(\mathrm{G}_\alpha_o\) but the potency to inhibit adenylyl cyclase activity was 10-fold lower in the RGS-sensitive \(\mathrm{G}_\alpha_o\)-expressing cells confirming a role for RGS proteins in inhibiting signaling downstream of G protein (Clark et al., 2003). After overnight treatment with increasing concentrations of DAMGO or morphine, there was a concentration-dependent loss in the ability of the \(\mu\)-agonists to inhibit adenylyl cyclase activity. Despite the 10-fold higher potency of \(\mu\)-agonists to acutely inhibit adenylyl cyclase activity in the RGS-insensitive \(\mathrm{G}_\alpha_o\)-expressing cells, no difference in the potency of morphine or DAMGO to induce tolerance was observed between RGS-insensitive and -sensitive \(\mathrm{G}_\alpha_o\)-expressing cells using a high challenge concentration of agonist to detect tolerance. However, in cells pretreated overnight with 100 nM DAMGO to induce a mild adenylyl cyclase tolerance, there was a 10-fold shift in DAMGO potency to inhibit adenylyl cyclase activity in the RGS-insensitive \(\mathrm{G}_\alpha_o\)-expressing cells but no change in the RGS-sensitive \(\mathrm{G}_\alpha_o\)-expressing cells. This suggests that cells expressing RGS-insensitive \(\mathrm{G}_\alpha_o\) are more susceptible to tolerance development and therefore endogenous RGS proteins in the C6\(\mu\) cells are acting to inhibit tolerance, although tolerance does still develop.

The development of adenylyl cyclase tolerance to \(\mu\)-opioid agonists had a fast component apparent within 30 min and a slow component which was not apparent until 6 h of agonist exposure in both the RGS-sensitive and -insensitive \(\mathrm{G}_\alpha_o\)-expressing cells. This fast component is likely due to desensitization of receptor activation of G proteins which was evident after 30 min of treatment with 1 \(\mu\)M DAMGO. The degree of desensitization was greater in the RGS-insensitive \(\mathrm{G}_\alpha_o\)-expressing cells (Fig. 2). C6 cells express GRK2, GRK5, and arrestin (Gray et al., 2001) so this is probably the result of increased recruitment of a GRK leading to increased receptor phosphorylation and \(\beta\)-arrestin binding. This could occur due to the lengthened lifetime of free G\(\gamma\) as a result of the inability of endogenous RGS proteins to accelerate the hydrolysis of G\(\alpha\)-bound GTP in the presence of the RGS-insensitive \(\mathrm{G}_\alpha_o\) mutant. Additionally, activation of the mitogen-activated protein kinase cascade, which is negatively regulated by RGS protein action in C6\(\mu\) cells (Clark et al., 2003), has also been implicated in the rapid phosphorylation and desensitization of the \(\mu\)-opioid receptor (Polakiewicz et al., 1998; Schmidt et al., 2000).

Receptor down-regulation was not apparent until more than 1 h after pretreatment with \(\mu\)-opioid agonist and was also seen to a greater degree in cells expressing RGS-insensitive \(\mathrm{G}_\alpha_o\). Down-regulation could be involved in the slower developing tolerance; however, there is little literature support for this idea. Gomes et al. (2002) showed that PTX-treatment can eliminate tolerance without affecting receptor down-regulation. There are also ligand-specific differences in the ability of \(\mu\)-opioids to cause receptor down-regulation that do not correlate with differences in tolerance (Patel et al., 2002). In addition, \(\mu\)-opioid antinociceptive tolerance in mice is reduced by treatment with \(\mathrm{G}_{\alpha_2}\) antisense oligodeoxynucleotides, although \(\mu\)-opioid receptor down-regulation is not affected (Yoburn et al., 2003). These and other studies (Chakrabarti et al., 1997; Yabaluri and Medzihradszky, 1997; Pak et al., 1999) provide evidence for separate mechanisms of receptor down-regulation, namely a G protein-dependent and -independent mechanism (Pak et al., 1999) which may show some redundancy. The increase in receptor down-regulation in the RGS-insensitive \(\mathrm{G}_\alpha_o\)-expressing C6\(\mu\) cells implies at least the presence of a G protein-dependent component.

Although increased levels of \(\mu\)-opioid receptor desensitization and down-regulation in C6\(\mu\) cells contribute to adenylyl cyclase tolerance, there is also an increased adenylyl cyclase supersensitization in cells expressing RGS-insensitive \(\mathrm{G}_\alpha_o\)
In vitro, RGS proteins serve to limit μ-opioid receptor desensitization by 
utes to the action of RGS proteins, such as G protein gated inwardly rectifier K+ channels (Chuang et al., 1998) and adenylyl cyclase (Clark et al., 2003) but protect signaling to pathways that are not so responsive, for example, signaling to intracellular calcium (Clark et al., 2003).

As summarized, endogenously expressed RGS proteins in C6 glioma cells decrease the chronic opioid effects of desensitization and receptor down-regulation. This results in a reduced sensitivity to tolerance as measured by opioid-agonist-induced inhibition of adenylyl cyclase. The decreased sensitivity to tolerance afforded by endogenous RGS proteins could in part be due to an inhibition of adenylyl cyclase supersensitization. The findings are opposite to experiments in vivo where knockdown or knockout of members of the R7 family of RGS proteins inhibits tolerance to the antinoceptive effects of morphine (Garzón et al., 2001; Sánchez-Blázquez et al., 2003; Zachariou et al., 2003). Taken together, these findings show that endogenous RGS proteins act as a modulators rather than play a critical role in controlling the various signaling processes that contribute to tolerance such that the degree of tolerance observed will depend on the system, the output measure, and the complement of RGS proteins expressed.

Acknowledgments

We thank Dr. Huda Akil for the μ-opioid receptor and Dr. Stephen Ikeda for the RGS- and PTX-insensitive Goα mutants.

References

- Gomes BA, Shen J, Stafford K, Patel M, and Yokurn BC (2002) μ-Opioid receptor...


Address correspondence to: Dr. John R. Traynor, Department of Pharmacology, University of Michigan Medical School, 1301 Medical Science Research Building III, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0632.

E-mail: jitraynor@umich.edu