Negative regulation of α_2 -adrenergic receptor-mediated G_i signalling by a novel pathway

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Chinese hamster ovary (CHO) cells stably expressing α_2 adrenergic receptor (α_2 AR) were pretreated with cholera toxin (CTX) and then treated with or without PMA. The α_2 AR-mediated inhibition of forskolin-stimulated cAMP accumulation was completely ablated by CTX pretreatment only after additional treatment with PMA. Although the addition of cycloheximide (protein synthesis inhibitor) and H-89 (cAMP dependent protein kinase inhibitor) did not completely counteract the negative regulation, the elevation of cAMP was a primary factor for negative regulation by treatment with CTX and PMA. In contrast with the cAMP response, the inhibition of membrane adenylate cyclase activity and the agonist competition curve were not influenced by treatment with CTX or PMA, suggesting that a cytosolic factor was involved in this negative regulation. The m2-muscarinic-acetylcholine-receptor-mediated inhibition of the forskolin-stimulated accumulation of cAMP was also attenuated

by treatment with CTX and PMA. The ablation of α_2 AR-mediated inhibition was not observed when α_{2A} AR was expressed in Rat2 fibroblast cells, suggesting that this negative regulation is not dependent on the receptor type but is instead a phenomenon common to G_i -coupled receptors in CHO cells. Reverse-transcriptase-mediated PCR and Northern blot analysis showed that the expression of GOS8/RGS2 mRNA, which is a member of the regulator of G-protein signalling (RGS) group of proteins, was considerably increased by pretreatment with CTX. These results indicate a novel regulatory pathway, whereby a cytosolic factor induced by the elevation of cellular cAMP levels negatively regulates G_i signalling in a protein-kinase-C-dependent manner.

Key words: adenylate cyclase, cholera toxin, protein kinase C, regulators of G-protein signalling.

INTRODUCTION

The signal transduction system mediated by G-protein-coupled receptors consists of three different components: receptors, G-proteins and effectors [1]. To avoid producing excessive signalling within this system, the amplitude of receptor-mediated signalling seems to be regulated by multiple processes in a manner dependent on the receptor and the cell type. Desensitization, in which receptors decrease their original sensitivity in response to prolonged stimulation, is one such level of regulation [2]. Desensitization consists of three processes: the functional uncoupling of receptors from G-proteins, the sequestration of receptors away from the plasma membrane and the down-regulation of receptor expression at the cell surface. The functional uncoupling of receptors from G-proteins results from receptor phosphorylation by G-protein-coupled receptor kinases (GRKs) and/or by second-messenger-activated kinases such as cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). Furthermore, the phosphorylation of receptor by GRKs leads to binding of the accessory protein β -arrestin, which disrupts the coupling of receptor with G-proteins [3,4]. β -Arrestin not only blocks the association of G_s to the phosphorylated β_2 adrenergic receptor (β_2 AR) but also promotes receptor sequestration [3,5]. In cells transfected with β -arrestin, stimulation with agonist results in the association of β -arrestin with clathrin [3,6]. This implies a model in which β -arrestin mediates receptor endocytosis through clathrin-coated pits.

Effector molecules such as adenylate cyclase are also regulated by several mechanisms. At least nine isoforms of mammalian adenylate cyclase have been identified by molecular cloning studies [7]. However, these isoforms are differentially regulated by $G_{\beta\gamma}$ subunits, Ca^{2+} /calmodulin, calcineurin or phosphorylation by PKC or Ca^{2+} /calmodulin kinase. These observations suggest that effector molecules can be regulated not only by receptor signals but also by multiple output from other signalling cascades.

G-proteins can also be regulated by a variety of mechanisms. The receptor-catalysed exchange of GDP for GTP and the hydrolysis of bound GTP to GDP on the G_x subunit are critical events in the G-protein-mediated signalling pathway. In addition to these events, there might be other factors that regulate G_x subunit activity, such as GTPase-activating proteins ('GAPs'), guanine nucleotide exchange factors ('GEFs'), and guanine-nucleotide-dissociation inhibitor proteins ('GDIs').

A family of proteins that negatively regulate G-protein signalling has recently been identified and designated regulators of G-protein signalling (RGSs) [8,9]. At least 19 mammalian genes that share an approx. 125-residue conserved domain (RGS box) have been identified. RGS4, G_x -interacting protein (GAIP), RGS1 and RGS10 have been shown to stimulate the intrinsic GTPase activity of $G_i\alpha$ subfamily members ($G_{i1}\alpha$, $G_{i2}\alpha$ and $G_{i3}\alpha$) but are ineffective against $G_{12}\alpha$ [10–14]. Additionally, RGS2 and RGS4 have been shown to bind to $G_{12}\alpha$ and negatively regulate phospholipase $C\beta$ signalling in a reconstituted membrane assay

Abbreviations used: AR, adrenergic receptor; CHO, Chinese hamster ovary; CTX, cholera toxin; DMEM, Dulbecco's modified Eagle's medium; GAIP, G_x -interacting protein; GRK, G-protein-coupled receptor kinase; IBMX, 3-isobutyl-1-methylxanthine; m2AChR, m2 muscarinic acetylcholine receptor; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PTX, pertussis toxin; RGS, regulator of G-protein signalling; RT-PCR, reverse-transcriptase-mediated PCR.

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[15]. Thus RGS proteins seem to act as potential GTPase-activating proteins specifically for the $G_i\alpha$ and $G_q\alpha$ subfamilies and to deactivate the G_i - and G_q -signalling pathways. It is likely that the patterns of signal regulation in a particular cell depend not only on specific types of receptors, G-proteins and effectors but also on other regulatory proteins within the cells.

α_2 ARs are classified into three subtypes (α_{2A} , α_{2B} and α_{2C}) [16]. It has been shown that each subtype differentially undergoes heterologous and homologous regulation. Stimulation of PKC blunted the α_{2B} -mediated and α_{2C} -mediated but not the α_{2A} -AR-mediated inhibition of adenylate cyclase [17,18]. In contrast with regulation by PKC, agonist treatment induced homologous desensitization of α_{2A} AR and α_{2B} AR but not α_{2C} AR, in which α_{2A} AR and α_{2B} AR were good substrates of GRK2 and GRK3 [19,20]. Here we show that persistent activation of $G_s\alpha$ negatively regulates G_i signalling in a PKC-dependent manner. These results suggest that G_i signalling can be modulated by a new regulatory process under a particular cellular context and condition.

EXPERIMENTAL

Materials

[3 H]Adenine (24.0–27.0 Ci/mmol), [14 C]cAMP (20.0–50.1 mCi/mmol), [α - 32 P]ATP (10–25 Ci/mmol) were obtained from DuPont-New England Nuclear Research (Boston, MA, U.S.A.) or American Radiolabelled Chemicals (St. Louis, MO, U.S.A.). PMA, 3-isobutyl-1-methylxanthine (IBMX), cycloheximide, H-89, cholera toxin (CTX), forskolin, adrenaline, phentolamine, carbachol, okadaic acid and calyculin A were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cell culture media [F-12 medium and Dulbecco's modified Eagle's medium (DMEM)], G-418 sulphate, gentamicin, Trizol reagent, SUPERSCRIPT II reverse transcriptase and *Taq* DNA polymerase were obtained from Life Technologies (Rockville, MD, U.S.A.). RNase-free DNase I and RNasin were from Promega Co. (Madison, WI, U.S.A.). Fetal bovine serum was obtained from JRH Bioscience (Lenexa, KS, U.S.A.). UK14304 was from Research Biochemicals International (Natick, MA, U.S.A.). The plasmid encoding for human α_{2A} AR (pBC- α_2 -C10) was kindly provided by Dr. R. J. Lefkowitz (Duke University, Durham, NC, U.S.A.).

Cell culture and cell transfection

Chinese hamster ovary (CHO) cells and Rat2 fibroblasts were co-transfected with 3.0 μ g of pRc/CMV and 30 μ g of pBC- α_2 -C10 by precipitation with calcium phosphate, as described by others. The CHO cells expressing α_{2A} AR were selected with 1.0 mg/ml of G-418 and maintained in 0.5 mg/ml of G-418. Expression of α_{2A} AR was determined by incubating 10 nM [3 H]yohimbine in the presence or absence of 10 μ M phentolamine at 25 °C for 1 h. The incubation reaction was terminated by dilution with ice-cold 25 mM Tris/HCl (pH 7.4)/1 mM $MgCl_2$ buffer followed by rapid filtration over Whatman GF/C glass fibre filters. Protein concentrations were measured by the Lowry method with BSA as a standard [21]. Specific binding was defined as the difference between total and non-specific binding, and was normalized for protein concentration. We used two different kinds of clone: one clone expressed α_{2A} AR at 1.6 pmol/mg of protein (used for the experiment shown in Figure 1) and the other expressed α_{2A} AR at 8.9 pmol/mg of protein (used for the rest of the experiments). CHO cells stably expressing m2 muscarinic acetylcholine receptor (m2AChR) (330 fmol/mg of protein) were kindly provided by Dr. H. Tsuga and Dr. T. Haga (University of Tokyo, Tokyo, Japan) [22]. These cells were maintained in monolayers in F-12

media (for CHO cells) and DMEM (for Rat2 cells) respectively, containing 10% fetal bovine serum, 10 μ g/ml gentamicin and 0.1 mg/ml G-418 (Rat2 cells) or 0.5 mg/ml G-418 (CHO cells), at 37 °C in air/CO₂ (19:1).

Treatment of cells with various reagents

The CHO or Rat2 cells expressing α_{2A} AR or m2AChR were pretreated with 1 μ g/ml CTX or 0.1 μ g/ml pertussis toxin (PTX) for 4 h at 37 °C in air/CO₂ (19:1), then treated with 100 nM PMA for 10 min. To inhibit protein synthesis or PKA, CHO cells were pretreated with 50 nM cycloheximide or 3 μ M H-89 for 5 h (starting from 1 h before the CTX pretreatment).

Adrenaline competition curves of the [3 H]yohimbine binding assay

CHO cells expressing α_{2A} AR were pretreated with or without CTX and then treated with or without PMA as described above. These cells were washed three times with ice-cold PBS and scraped in lysis buffer [10 mM Tris/HCl (pH 7.4)/5 mM EDTA/5 mM EGTA/10 μ g/ml benzamidine/10 μ g/ml soybean trypsin inhibitor/5 μ g/ml leupeptin], then centrifuged at 35000 *g* at 4 °C for 10 min. The membrane pellets were resuspended in binding buffer [75 mM Tris/HCl (pH 7.5)/2 mM EDTA/12.5 mM $MgCl_2$] and homogenized with a Potter-type homogenizer. When the effect of phosphatase inhibitors was examined, okadaic acid or calyculin A were included in all buffers. Membranes (approx. 30 μ g of protein per tube) were incubated with 0–10 μ M of unlabelled adrenaline and 10 nM [3 H]yohimbine in the presence or absence of 1 mM GTP for 1 h at 25 °C. High-affinity and low-affinity dissociation constants (K_{iH} and K_{iL}) and the percentages of receptors in the high-affinity and low-affinity binding states (R_H and R_L) were calculated with the GraphPad Prism program.

Assay of membrane adenylate cyclase

CHO cells were pretreated with or without CTX and then treated with or without PMA as described above. These cells were washed three times with ice-cold PBS and scraped in solution containing 5 mM Tris/HCl, pH 7.4, and 2 mM EDTA, then centrifuged at 35000 *g* at 4 °C for 10 min. Membranes were resuspended in 2 mM Hepes, pH 7.4. When the effect of phosphorylation was being examined, one of the phosphatase inhibitors okadaic acid (5 nM) or calyculin A (10 nM) was included in all buffers. Adenylate cyclase activities were determined by the methods of Salomon et al. [23]. In brief, assays were performed at 37 °C for 30 min in a final volume of 50 μ l containing assay mix [0.12 mM ATP/50 μ M GTP/2.8 mM phosphoenolpyruvate/0.1 mM cAMP/1 unit per tube of myokinase/0.1 unit per tube of pyruvate kinase/40 mM Hepes (pH 7.4)/0.8 mM EDTA/1.6 mM $MgCl_2$], 1.0 μ Ci per tube of [α - 32 P]ATP and 0–10 μ M agonist UK14304, with or without 5 μ M forskolin. Incubation reactions were started by the addition of membranes (approx. 20 μ g per tube) and terminated by the addition of 1.0 ml of ice-cold stop solution containing 0.5 mM ATP, 0.5 mM cAMP and 5×10^4 c.p.m./ml [3 H]cAMP, which was used to calculate the recoveries from individual columns. [32 P]cAMP and [3 H]cAMP were isolated on Dowex and Alumina columns as described by Salomon et al. [23].

Forskolin-stimulated accumulation of cAMP

Intracellular cAMP contents were determined as described in [24]. At 2 days before the assay, the cells expressing α_{2A} AR or m2AChR were seeded in 12-well plates (approx. 3×10^5 cells per

well). The next day, approx. 90% confluent cells were then labelled overnight with 1 μ Ci/ml of [3 H]adenine at 37 °C in air/CO₂ (19:1). These cells were pretreated with or without CTX or PTX and then treated with or without PMA as described above. Assays were performed by incubating cells in 0.5 ml of assay medium (F-12 medium or DMEM containing 20 mM Hepes, pH 7.4, and 1 mM IBMX, with or without 5 μ M forskolin) containing various concentrations of agonists, as indicated in each figure, at 37 °C for 10 min. The reactions were terminated by the rapid aspiration of assay medium and the immediate addition of 1 ml of ice-cold stop solution containing 2.5% (w/v) perchloric acid, 100 μ M cAMP and 8000 c.p.m. of [3 H]cAMP. The cells were kept at 4 °C for 1–16 h to elute the [3 H]cAMP produced into the stop solution. A portion of each stop solution was transferred to a new tube and neutralized with 4.2 M KOH. After centrifugation at 1200 g at 4 °C for 5 min, a portion of each supernatant was applied to Dowex and Alumina columns to isolate [3 H]cAMP and [3 H]cAMP, as described by Salomon et al. [23]. Intracellular [3 H]cAMP production was calculated by determining the ratio of [3 H]cAMP to total [3 H]nucleotides.

Analysis of mRNA of RGS proteins

CHO cells expressing α_{2A} AR were pretreated with or without CTX and then treated with or without PMA, as described above. Cells were then washed once with ice-cold PBS and scraped in 1 ml of Trizol Reagent. Cells were passed through a pipette and total RNA was isolated in accordance with the manufacturer's instructions for RNA isolation. RNA was resuspended in diethyl pyrocarbonate-treated water and treated with RNase-free DNase I for 10 min at 37 °C. DNase was removed by extraction with phenol/chloroform and then precipitated with ethanol. RNA was dissolved in diethyl pyrocarbonate-treated water; concentration and purity were determined by measurement of A_{260} and A_{280} . For reverse-transcriptase-mediated PCR (RT-PCR) analysis, 1 μ g of total RNA was reverse-transcribed in a reaction volume of 50 μ l in RT buffer [50 mM Tris/HCl (pH 8.3)/75 mM KCl/3 mM MgCl₂/1 mM each NTP/10 mM dithiothreitol] containing 100 units of Superscript II reverse transcriptase and 20 units RNasin in a cocktail of oligonucleotides (RT primers), each at 0.4 μ M. The RT primers were as follows: GAIP, 5'-ACACGGGAGTCCAGGCT-3'; RGS16, 5'-GATCTTCTTGAACTCCTC-3'; RGS4, 5'-CATGTTCCGGCTTGCTC-3'; RGS3, 5'-CTCGGTGCGAAGGAAGGC-3'; RGS2, 5'-CCTTGCTTTGAGGACAG-3'; RGS1, 5'-GGAAGTCAATATTGATTTGTTT-3'; β -actin, 5'-CCTGCTTGCTGATCCACATCTG-3'. Reverse transcription was performed at 42 °C for 90 min, as described by Heximer et al. [25]; 25 cycles of PCR were performed on 1 μ l of RT product in a total volume of 20 μ l, with 0.5 unit of *Taq* DNA polymerase. The following cycling parameters were used: 94 °C for 45 s, 55 °C for 30 s, 72 °C for 1 min. The PCR oligonucleotide primer sets were as follows: GAIP, 5'-ACCTCCTTGGGGGACAGGATGGA-3', 5'-GCTGTAGCTGCTCCTGGAACCAAGA-3'; RGS16, 5'-CCAGAACTCCAGGTTCTCCTCACTG-3', 5'-CACCTGCCTGGAGAGAGCAAAAGAG-3'; RGS4, 5'-CTGGTGCAAGAAATCCAGGTTCACTC-3', 5'-ATGGGCTGAATCGCTGGAAACCTGATTA-3'; RGS3, 5'-GGACTCGCTCCACTTGAGGGCTTCTC-3', 5'-GGCTCCATGCACCACTTTCCC-3'; RGS2, 5'-GGGGTGATTTGGTTTTTTGAAGTCTTC-3', 5'-GATTGGAAGACCCGTTTGAGCTAC-3'; RGS1, 5'-CAGCATCTGAATGCACAAATGCTTT-3', 5'-ATGATCCACATCTGGAATCTGG-3'; β -actin, 5'-GATGGTGGGAATGGGTCAGAG-3', 5'-CCTGCTTGCTGATCCACATCTG-3'.

For Northern blot analysis, equal amounts of RNA from each sample were loaded on a formaldehyde/agarose gel. After transfer to nylon membrane, the blots were probed at high stringency with PCR-generated probes representing the entire open reading frames of the RGS cDNAs. cDNAs were obtained through the I. M. A. G.E. Consortium.

RESULTS

Effect of CTX and PMA on α_{2A} AR-mediated inhibition

Figure 1 shows α_{2A} AR-mediated changes in the forskolin-stimulated production of cellular cAMP. In CHO cells, stimulation by α_{2A} AR caused a complex biphasic cAMP response, in which inhibition was obtained at low concentrations of the agonist adrenaline (0–1 μ M) and stimulation of the cellular cAMP level was noted at high concentrations of adrenaline (more than 1 μ M). As previously shown by others, the biphasic (inhibitory at low agonist concentration and stimulatory at high agonist concentration) response was ascribed to functional coupling of α_{2A} AR to both G_i-protein and G_s-protein, because treatment with PTX or CTX specifically ablated inhibitory or stimulatory phases without affecting other phases [26]. To eliminate the G_i or G_s coupling, we incubated cells with PTX or CTX to inactivate or activate each of the respective G-proteins. The α_{2A} AR-mediated G_i- or G_s-signalings in control cells untreated with CTX or PTX were not modified by the activation of PKC with 100 nM PMA for 10 min (Figure 1A), suggesting that heterologous desensitization by PKC was not observed in α_{2A} AR. This is consistent with the previous report [17]. The dose–response curve of inhibitory phases of cAMP accumulation did not seem to fit a Michaelis–Menten type curve. The biphasic cAMP response consisted of an inhibitory phase at low agonist concentration and a stimulatory phase at high agonist concentration, meaning that the inhibition of cAMP accumulation was impaired in part by cAMP production in the stimulatory phase. Thus the dose–response curve might not fit a Michaelis–Menten type curve. It also suggests that the dose–response curve of the inhibitory phase comes close to a typical Michaelis–Menten type when the stimulatory phase is ablated by treatment with CTX. This is consistent with our subsequent findings (see below). In contrast, treatment of cells with 100 nM PMA completely ablated the α_{2A} AR-mediated inhibition of forskolin-stimulated cAMP accumulation, under the persistent activation of G_s by pretreatment with 1 μ g/ml CTX for 4 h (Figure 1B). Treatment with PTX ablated the α_{2A} AR-mediated inhibitory response of cAMP production and revealed an α_{2A} AR-mediated stimulatory response induced by a high concentration of agonist (Figure 1C). Treatment with PTX slightly increased the basal accumulation of cAMP. α_{2A} AR might pre-couple with G_i; treatment with PTX would ablate pre-coupled G_i signalling, leading to increased cAMP accumulation. Figure 1(C) also shows that treatment of PTX-treated cells with PMA did not significantly affect the stimulatory cAMP response. These results suggest that the persistent activation of G_s (probably a continuous high concentration of cellular cAMP) in conjunction with activation of PKC specifically ablates the negative regulation of α_{2A} AR-mediated G_i signalling in CHO cells. It should be noted that treatment with CTX markedly increased the cellular cAMP contents.

Effect of CTX and PMA on coupling of α_{2A} AR to G_i

To observe the inhibition of membrane adenylate cyclase activity by α_{2A} AR stimulation and to obtain a clear agonist competition curve, we used a clone, different from that used for Figure 1, that

Figure 1 Pretreatment with CTX, but not PTX, followed by treatment with PMA abolishes the α_{2A} AR-mediated inhibition of forskolin-stimulated cAMP accumulation in CHO cells

The accumulation of [3 H]cAMP was determined in α_{2A} AR-CHO cells that were labelled with [3 H]adenine (1 μ Ci/ml) for 12–16 h at 37 °C in air/CO₂ (19:1). α_{2A} AR-CHO cells were pretreated without CTX or PTX (**A**), with CTX (1 μ g/ml) (**B**) or with PTX (0.1 μ g/ml) (**C**) for 4 h, then treated with (●) or without (○) 100 nM PMA for 10 min under the same conditions. After that, the cells were incubated in 0.5 ml of assay medium [F-12 medium/20 mM Hepes (pH 7.4)] containing 1 mM IBMX, 5 μ M forskolin and various concentrations of adrenaline as indicated for 10 min at 37 °C. The intracellular accumulation of [3 H]cAMP was estimated by determining the ratio of [3 H]cAMP to total 3 H-labelled nucleotide. The results in (**A**) and (**B**) are means $\pm$ S.E.M. for three separate experiments; the results in (**C**) are means for two independent experiments.

expressed α_{2A} AR at a higher level (8.9 pmol/mg of protein), because a higher level of expression allowed us to detect binding activities more easily and provided a more pronounced inhibition of adenylyl cyclase activity as previously reported [26]. One possible mechanism of this negative regulation as shown in Figure 1 was that pretreatment with CTX followed by PMA treatment caused the uncoupling of α_{2A} AR from G_i, leading to the functional interruption of G_i signalling. To examine whether pretreatment with CTX in conjunction with the activation of PKC influenced the coupling ability of α_{2A} AR to G_i, we studied the agonist competition curves of [3 H]yohimbine binding. The

Figure 2 Effect of treatment with CTX and PMA on the coupling of α_{2A} AR to G_i-protein

Adrenaline competition curves for the binding of [3 H]yohimbine were determined with α_{2A} AR-CHO cell membranes in the presence (●,▲) or absence (○,△) of 1 mM GTP. Before the preparation of membranes, the cells were pretreated with (lower panel) or without (upper panel) CTX and treated with (△,▲) or without (○,●) 100 nM PMA. The amount of [3 H]yohimbine binding obtained for each point was normalized as a percentage of the total [3 H]yohimbine binding calculated by the differences between the maximum and minimum binding, simulated by Prism software. Results are means $\pm$ S.E.M. for three separate experiments. Some of the error bars are within the symbols.

coupling efficiency of α_{2A} AR with G_i was analysed by the existence of a high-affinity binding state for the agonist adrenaline. In control membranes (no pretreatment with CTX), the agonist competition curve of [3 H]yohimbine binding was biphasic and could be resolved into high-affinity and low-affinity states (Figure 2). The high-affinity binding state of α_{2A} AR for adrenaline (R_H , $27.4 \pm 3.5\%$; K_{IH} , 100 ± 50 nM) was converted into the low-affinity state by the addition of GTP (K_{IL} , 10.5 ± 1.2 μ M). The high-affinity and low-affinity states of α_{2A} AR for adrenaline were not significantly modified by treatment with PMA [R_H , $35.7 \pm 1.4\%$, $P = 0.0925$ compared with control/no PMA ($n = 3$); K_{IH} , 90 ± 4 nM] (Figure 2, upper panel). Similar results were observed even in CTX-pretreated membranes [R_H without PMA, $26.4 \pm 0.3\%$; R_H with PMA, $33.1 \pm 3.1\%$; $P = 0.0978$ compared with treatment with CTX/no PMA ($n = 3$); K_{IH} without PMA, 40 ± 9 nM; K_{IH} with PMA, 100 ± 30 nM] (Figure 2, lower panel). These results suggest that treatment with CTX and PMA did not significantly alter the coupling efficiency of α_{2A} AR to G_i.

Possible involvement of a cytosolic factor in the negative regulation of G_i signalling

We changed the endogenous agonist adrenaline to the synthetic agonist UK14304 as the agonist for receptor stimulation owing to the greater efficacy of UK14304 in inhibiting and stimulating cAMP responses in CHO cells [26]. Forskolin-stimulated adenylyl cyclase activities were inhibited by stimulation with

Figure 3 Membrane adenylate cyclase activity is not modified by treatment with CTX and PMA

α_{2A} AR-CHO cells were pretreated with (B) or without (A) CTX and then treated with (●) or without (○) 100 nM PMA, as described in the legend to Figure 1. Adenylate cyclase activities were measured in the presence of 5 μ M forskolin and various concentrations of UK14304, as described in the Experimental section. Results are means $\pm$ S.E.M. for three separate experiments.

UK14304 in a concentration-dependent manner, and were not significantly affected by treatment with PMA (Figure 3A). Although the stimulatory phase of adenylate cyclase was also observed at a high concentration of agonist, it was ablated by treatment with CTX, which is consistent with Figure 1 and the previous paper [26]. The adenylate cyclase activities of membranes prepared from CTX-treated cells were also inhibited by stimulation with UK14304 even when the membranes were prepared from PMA-treated cells (Figure 3B). Figure 3 is consistent with the result of the membrane binding assay that treatment with CTX and PMA did not affect the receptor- G_i coupling. Forskolin-stimulated adenylate cyclase activities were increased slightly by treatment with PMA for control cells or decreased for CTX-treated cells. In general, the differences between cellular cAMP responses and membrane adenylate cyclase activities can be explained by the involvement of various factors such as cytoskeletal proteins, cytosolic proteins and membrane lipids. The possibility of rapid dephosphorylation during membrane preparation is unlikely because the inclusion of phosphatase inhibitors such as okadaic acid and calyculin A did not reverse the α_{2A} AR-mediated inhibition of forskolin-stimulated adenylate cyclase activities (results not shown). Thus one hypothesis for this phenomenon was that a cytosolic factor involved in this negative regulation was induced by the treatment of CHO cells with CTX. To address whether a negative regulatory factor could be induced by pretreatment with CTX, CHO cells expressing α_{2A} AR were pretreated with cycloheximide to block new protein synthesis. Treatment with cycloheximide enhanced basal cAMP production approx. 2-fold for an unknown reason

Figure 4 Cycloheximide counteracts the negative regulation of G_i signalling caused by treatment with CTX and PMA

The accumulation of [3 H]cAMP was determined in α_{2A} AR-CHO cells. The cells were labelled with [3 H]adenine and pretreated with (B) or without (A) CTX, then treated with (●) or without (○) 100 nM PMA as described in the legend to Figure 1, except that 50 μ M cycloheximide was added to cells 5 h before the assay, to inhibit protein synthesis. The assays were performed with 0.5 ml of assay medium [F-12 medium/20 mM Hepes (pH 7.4)] containing 1 mM IBMX, 5 μ M forskolin and various concentrations of UK14304 as indicated for 10 min at 37 $^{\circ}$ C. Intracellular [3 H]cAMP accumulation was estimated by determining the ratio of [3 H]cAMP to total 3 H-labelled nucleotide. Results are means $\pm$ S.E.M. for three separate experiments. Some of the error bars are within the symbols.

(Figure 4A). Although treatment with PMA did not affect the inhibitory coupling as judged from the magnitudes of inhibition (control, 34.7 %; PMA, 30.9 %), it seemed to ablate the α_{2A} AR-mediated enhancement of cAMP accumulation at high agonist concentrations. When cells were pretreated with cycloheximide, treatment with PMA resulted in the inhibition of cAMP production by stimulation with UK14304 (Figure 4B). However, the inhibition by stimulation with UK14304 was not completely the same as that of treatment with PMA without cycloheximide. Because cycloheximide at the concentration used here can inhibit protein synthesis almost completely, the incomplete restoration of the α_{2A} AR-mediated inhibition of cAMP production by cycloheximide indicated that ablation of the α_{2A} AR-mediated inhibition of cAMP accumulation by treatment with CTX and PMA is partly independent of protein synthesis. This result is consistent with the hypothesis that a newly synthesized protein is a primary factor in this negative regulation of α_{2A} AR-mediated signalling in CHO cells. Although the treatment of control cells with PMA enhanced basal (forskolin-stimulated) cAMP accumulation, it did not increase basal cAMP accumulation in CTX-treated cells. One of the reasons was that adenylate cyclase is already maximally activated by ADP-ribosylation of $G_{s\alpha}$. Because CTX ADP-ribosylates $G_{s\alpha}$ subunit and ablates the intrinsic GTPase activity that leads to an increase in intracellular cAMP levels, a putative factor would be induced by the elevation

Figure 5 H-89 counteracts the negative regulation of G_i signalling mediated by treatment with CTX and PMA

The accumulation of $[^3\text{H}]\text{cAMP}$ was determined in $\alpha_{2A}\text{AR}$ -CHO cells. The cells were labelled with $[^3\text{H}]\text{adenine}$ and pretreated with (B) or without (A) CTX and treated with ($\bullet$) or without ($\circ$) 100 nM PMA as described in the legend to Figure 1, except that $3\ \mu\text{M}$ H-89 was added to cells 5 h before the assay, to inhibit protein kinase A. The assays were performed with 0.5 ml of assay medium [F-12 medium/20 mM Hepes (pH 7.4)] containing 1 mM IBMX, $5\ \mu\text{M}$ forskolin and various concentrations of UK14304 as indicated for 10 min at 37°C . Intracellular $[^3\text{H}]\text{cAMP}$ accumulation was estimated by determining the ratio of $[^3\text{H}]\text{cAMP}$ to total ^3H -labelled nucleotide. Results are means for two separate experiments.

of intracellular cAMP levels. CHO cells were then pretreated with H-89, which specifically inhibits PKA activity at the concentration used ($3\ \mu\text{M}$), and cellular cAMP levels were determined. Treatment with PMA enhanced the forskolin-stimulated cAMP accumulation approx. 1.5-fold. Although treatment with H-89 did not affect the $\alpha_{2A}\text{AR}$ -mediated inhibition of cAMP production in control cells, it attenuated the $\alpha_{2A}\text{AR}$ -mediated cAMP accumulation observed at high agonist concentration (Figure 5A). PKA activity might be necessary for $\alpha_{2A}\text{AR}$ - G_i coupling. In contrast with $\alpha_{2A}\text{AR}$ -mediated G_i signalling in control cells, treatment with H-89 substantially counteracted the effect of treatment with CTX and PMA on $\alpha_{2A}\text{AR}$ -mediated G_i signalling, indicating that the effect of treatment with CTX and PMA is dependent on PKA (Figure 5B).

Characterization of the negative regulation of G_i signalling

To characterize further the negative regulation of G_i signalling observed in CHO cells, we examined the receptor or cell type specificity of the negative regulation by using two different kinds of cell line: one was CHO cells expressing m2AChR (m2AChR-CHO); the other was Rat2 cells expressing $\alpha_{2A}\text{AR}$ (Rat2- $\alpha_{2A}\text{AR}$). In control m2AChR-CHO cells, forskolin-stimulated cAMP accumulation was inhibited by stimulation with G_i in an agonist (carbachol)-dependent manner and was not altered by treatment with PMA (Figure 6A). In m2AChR-CHO cells there is almost no enhancement of forskolin-stimulated cAMP production by treatment with PMA. In CTX-treated cells, however, the

Figure 6 Pretreatment with CTX followed by treatment with PMA abolishes the m2AChR-mediated inhibition of forskolin-stimulated cAMP accumulation in CHO cells

The accumulation of $[^3\text{H}]\text{cAMP}$ was determined in m2AChR-CHO cells. The cells were labelled with $[^3\text{H}]\text{adenine}$, pretreated with (B) or without (A) CTX and treated with ($\bullet$) or without ($\circ$) 100 nM PMA as described in the legend to Figure 1. The assay was performed with 0.5 ml of assay medium [F-12 medium/20 mM Hepes (pH 7.4)] containing 1 mM IBMX, $5\ \mu\text{M}$ forskolin and various concentrations of carbachol as indicated for 10 min at 37°C . Intracellular $[^3\text{H}]\text{cAMP}$ accumulation was estimated by determining the ratio of $[^3\text{H}]\text{cAMP}$ to total ^3H -labelled nucleotide. Results are means $\pm$ S.E.M. for three separate experiments.

m2AChR-mediated inhibition was decreased markedly by the additional treatment with PMA (Figure 6B). In contrast with $\alpha_{2A}\text{AR}$ -mediated inhibition, m2AChR-mediated inhibition was still observed at high agonist concentration. Considering the expression level of m2AChR (330 fmol/mg of protein) and $\alpha_{2A}\text{AR}$ (1.6 or 8.9 pmol/mg of protein), and the same magnitudes of inhibition of cAMP accumulation, m2AChR might couple with G_i more tightly than $\alpha_{2A}\text{AR}$. It indicates that a greater amount of a factor is required for the inhibition of m2AChR- G_i coupling. It also suggests that the negative regulation by treatment with CTX and PMA is not dependent on receptor types but is a characteristic common to G_i -coupled receptors in CHO cells. When $\alpha_{2A}\text{AR}$ was expressed in Rat2 cells, completely different cAMP responses were obtained. As shown in Figure 7, treatment with CTX and PMA did not reverse the $\alpha_{2A}\text{AR}$ -mediated inhibition of cAMP accumulation. These results indicate that the negative regulation of G_i signalling occurs in a cell-type-specific manner. Therefore the effect of the cytosolic factor is specific to CHO cells and is independent of receptor type.

Possible involvement of a specific type of RGS protein

A family of RGS proteins has recently been identified in *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and mammalian cells [8]. At least 19 mammalian genes that share a conserved core domain called the RGS box have been cloned. RGS proteins act as negative regulators of G-protein signalling by stimulating the hydrolysis of GTP bound to the α subunits of

Figure 7 G_i signalling is not modified by treatment with CTX and PMA in Rat2- α_{2A} AR cells

The accumulation of [3 H]cAMP was determined in Rat2- α_{2A} AR cells. The cells were labelled with [3 H]adenine and pretreated with (B) or without (A) CTX and treated with (●) or without (○) 100 nM PMA. The assays were performed with 0.5 ml of assay medium [(DMEM/20 mM Hepes (pH 7.4)) containing 1 mM IBMX, 5 μ M forskolin and various concentrations of UK14304 as indicated for 10 min at 37 °C. Intracellular [3 H]cAMP production was estimated by determining the ratio of [3 H]cAMP to total 3 H-labelled nucleotide. Results are means for two separate experiments.

the G_{12} , G_{13} or G_{12} subfamilies [10,27,28]. The present results led to speculation that one or several RGS proteins might be involved in the negative regulation of α_{2A} AR-mediated G_i signalling. Preliminary RT-PCR experiments with degenerate primers specific for the RGS box (as described in [29]) showed an increased expression of GOS8/RGS2 mRNA in CTX-treated CHO cells (A. Takesono, T. Nagao and H. Kurose, unpublished work). A more deliberate approach was then used to determine which RGS mRNA species are expressed in CHO cells in response to treatment with CTX. Northern blot analysis was performed with probes corresponding to the open reading frames of the rat or mouse versions of RGS1, RGS2, RGS3, RGS4, RGS16 or GAIP. A Northern blot analysis showed that RGS2 mRNA was specifically induced by treatment with CTX but not with PMA (Figure 8A). No messages corresponding to the other RGS proteins were detected by Northern blot analysis. Owing to the difficulty in obtaining sufficient quantities of high-quality RNA for unequivocal Northern blots, RT-PCR analysis was performed by using oligonucleotide primers selected from regions of perfect identity between human and mouse or rat RGS sequences. RT-PCR analysis demonstrated that the RGS2 mRNA was present in greater amounts in cells treated with CTX, whereas treatment with PMA did not affect the level of RGS2 mRNA (Figure 8B). Although RGS1, RGS3, RGS16 and GAIP mRNA species were found at very low to moderate levels, no RGS4 mRNA was found in CHO cells (Figure 8B, and results not shown). However, it is difficult to interpret what this means

Figure 8 Increase in message of RGS2 brought about by treatment with CTX but not with PMA

The levels of RGS2 mRNA present in α_{2A} AR-CHO cells were determined by Northern blot analysis (A) and by RT-PCR analysis (B). β -Actin primers were used as a control for mRNA quantity in the RT-PCR experiments. DNA size markers (sizes indicated in bp at the right) were run on the outermost lanes of the RT-PCR gel. The experiments were repeated at least twice, with similar results.

because the degree of sequence identity is uncertain between the present oligonucleotide primers and the CHO sequences.

DISCUSSION

Receptor-mediated G-protein signalling is regulated in various ways. The receptors, G-proteins and effector molecules are regulated individually by phosphorylation, protein-protein interaction and lipid modification such as reversible palmitoylation. It has been known that PKC is involved in the heterologous regulation of G-protein-coupled receptor-mediated signalling. PKC phosphorylates several receptors including α_2 AR and β_2 AR and attenuates the ability of the receptor to stimulate G_i or G_s proteins [17,30]. The treatment of cells expressing α_{2B} AR and α_{2C} AR but not α_{2A} AR with PMA resulted in the ablation of the receptor-mediated inhibition of adenylate cyclase [17,18]. We confirmed the previous report that the α_{2A} AR-mediated inhibition of adenylate cyclase is insensitive to the activation of PKC and found a new regulatory mechanism of α_{2A} AR by PKC. The ability of α_{2A} AR to inhibit adenylate cyclase was ablated by PKC activation in combination with the pretreatment of CTX. It could be claimed that the effect of CTX on signal transduction is not specific in its action and is not mediated only by the G_s -cAMP-PKA pathway. A recent paper indicated that $G_s\alpha$ overexpressed in heart activates the L-type Ca^{2+} channel independently of the cAMP-PKA pathway [31]. Therefore we cannot conclude that the effect of treatment with CTX is mediated by the cAMP-PKA pathway. We have tried to reproduce the effect of CTX treatment with dibutyryl cAMP. However, the

dose-response curve for treatment with dibutyryl cAMP (200 μ M) was essentially the same as that for control cells (results not shown). The two treatments should result in a difference in cAMP levels and might need a greater amount of dibutyryl cAMP to mimic the effect of CTX. However, it is reasonable to conclude at present that the effect of CTX is mediated by the cAMP-PKA pathway, because treatment with H-89 almost completely ablated the effect of treatment with CTX and PMA.

We also observed that the effect of PMA treatment (activation of PKC) on basal forskolin-stimulated cAMP accumulation was variable between experiments. Although we cannot explain the variation in forskolin-stimulated cAMP production by treatment with PMA at the molecular level, there are several possibilities. Treatment with PMA might disrupt the spontaneous coupling between inhibitory receptors and G_i -protein. The effect of treatment with PMA on basal cAMP production might be explained by the direct phosphorylation of adenylate cyclase or putative associated protein(s), leading to an enhancement of cAMP production. However, treatment with PMA did not significantly enhance basal cAMP production in CTX-treated cells, because G_s protein is already maximally activated by ADP-ribosylation of α subunit. We also noticed that the cAMP response at high agonist concentration was also variable between experiments. As the cAMP responses of inhibitory and stimulatory phases are sensitive to the level of receptor expression [26], cells might lose their receptors during continuous culture. However, it should be noted that the stimulatory phase is ablated by treatment with CTX and that treatment with PMA profoundly affected the inhibitory phase of the cAMP response when cells were treated with CTX. Then the effect of treatment with CTX and PMA was a result of an ablation of inhibitory coupling but not an enhancement of stimulatory coupling.

The specific ablation of G_i -mediated signalling is explained by several mechanisms. Pfeifer et al. [32] reported that cGMP-dependent protein kinase phosphorylates and inactivates G_i -proteins. However, two independent experiments with phosphatase inhibitors did not support the involvement of cGMP-dependent protein kinase because the dose-response curves for the inhibition of adenylate cyclase activities in membranes prepared in the presence of okadaic acid or calyculin A were essentially the same as those for control membranes (results not shown). Another mechanism is the overexpression of β -arrestin. De Blasi's group has reported [33,34] that elevated levels of cAMP increase the β -arrestin mRNA and protein levels in rat thyroid FRTL cells and T cells. It is possible that β -arrestin is involved in the ablation of G_i -mediated signalling. β -Arrestin binds to GRK-phosphorylated receptors, including α_{2A} AR, and inhibits the subsequent coupling of the receptors with G-proteins [2,3]. The overexpression of β -arrestin induced by treatment with CTX might impair the coupling of α_{2A} AR with G_i -proteins, because α_{2A} AR can be desensitized in a GRK-dependent manner. However, such an idea does not agree with the present finding that the effect of β -arrestin can be observed in adenylate cyclase activity in membranes. We demonstrated that the effect of treatment with CTX and PMA could not be observed in membrane preparation. Furthermore, overexpressed β -arrestin cannot totally ablate α_{2A} AR-mediated signalling [35]. These considerations exclude the contribution of β -arrestin to the ablation of α_{2A} AR-mediated G_i signalling that occurs with treatment with CTX and PMA. Spence et al. [36] reported that the stimulation of CHO cells with P_2 -purinoceptor, lysophosphatidic acid receptor or thrombin receptor causes a rapid induction of Ca^{2+} /calmodulin-stimulated cAMP phosphodiesterase (PDE) activity. However, the increased PDE activity does not explain our finding because the cAMP responses were

measured in the presence of a PDE inhibitor, IBMX. Therefore the possibility of involvement of PDE is also excluded.

The present results suggest that a cytosolic factor is induced by treatment with CTX and phosphorylated by PKC, which leads to the inhibition of α_{2A} AR- G_i signalling. However, cycloheximide treatment did not completely reverse the effect of treatment with CTX and PMA, suggesting a pathway that is independent of protein synthesis. One possibility is the translocation of a pre-existing cytosolic factor to the membrane in a manner dependent on PKA and PKC. It has been reported that PKA and PKC can phosphorylate and modulate the activities of some isoforms of adenylate cyclases [7,37]. A putative cytosolic factor might therefore be targeted to the phosphorylated adenylate cyclase and impair α_{2A} AR- G_i signalling. Treatment with H-89 seemed to attenuate the α_{2A} AR-mediated enhancement of cAMP accumulation (Figure 5A). It suggests that α_{2A} AR- G_s coupling requires a protein with a rapid turnover. Two groups have reported that the intracellular third loop of α_{2A} AR can interact with eukaryotic initiation factor 2B and the ζ isoform of 14-3-3 proteins [38,39]. Although the physiological significance of the interaction between α_{2A} AR and these proteins is unknown at present, it is possible that an unidentified protein interacts with α_{2A} AR and inhibits α_{2A} AR- G_i coupling when the factor is phosphorylated by PKA.

In contrast with the complete ablation of α_{2A} AR-mediated inhibition by treatment with CTX and PMA, m2AChR-mediated inhibition was still observed even after treatment with CTX and PMA. Although the two receptors are expressed at different levels (α_{2A} AR, 1.6 or 8.9 pmol/mg of protein; m2AChR, 0.33 pmol/mg of protein), they showed nearly the same magnitudes of inhibition of cAMP accumulation. Because a larger amount of receptor can inhibit adenylate cyclase more profoundly in CHO cells [26], the resistance to treatment with CTX and PMA indicates that m2AChR couples more tightly with G_i -protein than with α_{2A} AR. Thus the attenuation of m2AChR-mediated inhibition requires a larger amount of a putative cytosolic factor than does α_{2A} AR; the condition used in the present study might not have been enough to induce a putative factor for the ablation of m2AChR-mediated inhibition.

RGS proteins are a novel family of proteins that are purported to be involved in the desensitization process by functioning as G-protein GTPase-activating proteins [15,40-42]. In the present study we have found that levels of RGS2 mRNA increase selectively with treatment with CTX. We therefore raise the possibility that RGS2 might negatively regulate G_i activity in our assay with CHO cells stably expressing α_{2A} AR (α_{2A} AR-CHO cells). Despite the report that RGS2 interacts specifically with G_q *in vitro* [43], several lines of evidence suggest that RGS2 might negatively regulate G_i activity. For example, it has been shown that RGS2 can partly compensate for the role of SST2, a *S. cerevisiae* RGS protein that is specific for GPA1, a G_i -like α subunit involved in the pheromone mating process [12,38]. Additionally, RGS2 has been shown to partly inhibit mitogen-activated protein kinase activation in 293T cells transfected with interleukin 8 receptor [12]. Ingi et al. [44] recently reported that RGS2 stimulates the GTPase activity of $G_{i\alpha 1}$ when reconstituted in phospholipid vesicles together with m2AChR, and RGS2 also inhibits m2AChR-mediated mitogen-activated protein kinase activation transfected in COS cells. These results suggest a role for RGS2 in G_i signalling. The results presented here raise the intriguing possibility that RGS2 might need some sort of post-translational processing or subcellular localization that is modulated by a PKC-dependent pathway if it is to associate physically with G_i -proteins. For example, Chatterjee et al. reported that expression of a truncated form of RGS3 containing

only the RGS box could inhibit G_q - and G_s -coupled responses in BHK cells, whereas full-length RGS3 could not [45]. Furthermore, Srinivasa et al. [46] have shown that RGS4 must be associated with the plasma membrane in order to inhibit the pheromone response pathway in *S. cerevisiae*. Nevertheless, no conclusion can yet be made that RGS2 activity is regulated in a PKC-dependent manner.

We have demonstrated a new heterologous regulatory mechanism of a G_i -coupled response (i.e. inhibition of adenylate cyclase activity in the CHO cells) that seemed to be dependent on the activation of both PKA and PKC.

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