Purification of A₁ adenosine receptor–G-protein complexes: effects of receptor down-regulation and phosphorylation on coupling

Zhenhai GAO*, Anna S. ROBEVA* and Joel LINDEN*†¹

*Department of Molecular Physiology and Biological Physics, Health Sciences Center Box 449, University of Virginia, Charlottesville, VA 22908, U.S.A., and †Department of Internal Medicine, Health Sciences Center Box MR4 6012, University of Virginia, Charlottesville, VA 22908, U.S.A.

We examined the effects of exposing A₁ adenosine receptors (A₁ARs) to an agonist on the stability and phosphorylation state of receptor–guanine nucleotide-binding regulatory protein (R–G-protein) complexes. Non-denatured recombinant human A₁ARs extended on the N-terminus with hexahistidine (His₆) and the FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) epitope (H/F) were purified to near homogeneity from stably transfected Chinese-hamster ovary (CHO)-K1 cells. Purified receptors have pharmacological properties similar to receptors in membranes. G-proteins were co-purified with 15 ± 2% of H/F-A₁AR unless receptor–G-protein (R–G) complexes were uncoupled by pre-treating cell membranes with GTP. By silver staining, purified A₁AR–G-protein complexes contain receptors, G-protein α and β subunits and an unidentified 97 kDa protein. Pretreating intact cells with N⁶-cyclopentyladenosine (CPA) for 24 h decreased both the total number of receptors measured in membranes and

the number of purified A₁ARs by about 50%. In contrast, pretreating cells with CPA decreased the number of R–G complexes measured in membranes (54 ± 6%) significantly less than it decreased the number of purified R–G complexes (78 ± 3%) as detected by ¹²⁵I-N⁶-(4-aminobenzyl)adenosine binding or by Western blotting G α 2. The effect of CPA to decrease the fraction of receptors purified as R–G complexes was not associated with any change in low-level A₁AR phosphorylation (found on serine), or low-level phosphorylation of G-protein α or β subunits or the 97 kDa protein. These experiments reveal a novel aspect of agonist-induced down-regulation, namely a diminished stability of receptor–G-protein complexes that is manifested as uncoupling during receptor purification.

Key words: desensitization, FLAG, hexahistidine, purinergic, signalling.

INTRODUCTION

Heterotrimeric G-proteins of the G_{i/o} family are tightly coupled to heptahelical receptors in the rhodopsin family. Hence, when agonist affinity chromatography has been used to purify A₁ adenosine receptors (A₁ARs), G α 1, G α 2 and G β γ were co-purified from bovine brain [1,2]. Similarly, G α 1, G α 3 and G β γ were co-purified with agonist-occupied somatostatin receptors [3]. Using lectin affinity chromatography or co-immunoprecipitation techniques, G_{i/o} proteins were co-purified with receptors for formylpeptide chemoattractant [4], μ - and δ -opioid [5], heart and brain muscarinic [6], C5a [7] and AT2 angiotensin [8].

Ligand affinity chromatography or immunoprecipitation generally results in a low yield of purified receptors. Higher-efficiency receptor purification has been achieved recently using receptors genetically engineered to facilitate purification. Recombinant human adenosine receptors were double-tagged with hexahistidine (His₆) and the FLAG epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) (H/F) and purified to near homogeneity by sequential anti-FLAG-antibody and Ni-nitrilotriacetate (NTA)-affinity-chromatography steps [9]. Of the four G-protein-coupled adenosine receptors that have been cloned (A₁, A_{2A}, A_{2B} and A₃ [10]), the A₁ and the A₃ receptors are coupled to the G_{i/o} subfamily of G-proteins. In the present study we show that non-denatured A₁ARs can be purified to near homogeneity and that, by altering the coupling of A₁ARs to G-proteins before their solubilization and purification, H/F-A₁ARs can be purified with or without associated G-proteins.

Long-term exposure of G-protein-coupled receptors (GCRs) to agonists results in uncoupling of receptors from G-proteins. With continued agonist exposure the number of receptors is down-regulated [11,12]. Desensitization of GCRs is sometimes, but not always, preceded by receptor phosphorylation [13]. The A₁AR is controversial in this regard because it desensitizes slowly and there are conflicting reports about whether it is phosphorylated after exposure to an agonist (see the Discussion section). In the present study we confirmed that A₁ARs desensitize slowly ($t_{1/2} > 5$ h) and we show that receptor–G-protein (R–G) complexes derived from desensitized cells are prone to become uncoupled during purification. Highly purified H/F-A₁ARs that are unequivocally identified as receptors based on a characteristic shift in their electrophoretic mobility following deglycosylation are only weakly phosphorylated on serine residues. Although we detect low-level phosphorylation of the A₁AR, G-protein α and β subunits and a co-purified 97 kDa protein, we conclude that phosphorylation of these proteins is not responsible for the destabilization of R–G complexes that occurs following long-term exposure of A₁ARs to an agonist.

EXPERIMENTAL

Materials

N⁶-Cyclopentyladenosine (CPA), 5'-N-ethylcarboxamidoadenosine (NECA), 9-chloro-2-(2-furyl)-5,6-dihydro[1,2,4]triazolo[1,5-c]quinazoline-5-imine (CGS21680), xanthine amine con-

Abbreviations used: A₁AR, A₁ adenosine receptor; ADA, adenosine deaminase; FLAG (epitope), Asp-Tyr-Lys-Asp-Asp-Asp-Lys; H/F, His₆-FLAG; ABA, N⁶-(4-aminobenzyl)adenosine; azido-BW-A844, 8-cyclopentyl-3-azidophenethyl-1-propylxanthine; CPA, N⁶-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; CGS15943, 9-chloro-2-(2-furyl)-5,6-dihydro[1,2,4]triazolo[1,5-c]quinazoline-5-imine; GCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; CHO, Chinese-hamster ovary; R–G, receptor–G-protein (complex); GTP[S], guanosine 5'-[γ -thio]triphosphate; DMEM, Dulbecco's modified Eagle's medium; NTA, nitrilotriacetate.

¹ To whom correspondence should be addressed (e-mail jlinden@virginia.edu).

gener (8-{4-[(2-aminoethyl)aminocarbonylmethoxy]phenyl}-1,3-dipropylxanthine (XAC) and 8-sulphophenyltheophylline (8-SPT) were purchased from Research Biochemicals International (Natick, MA, U.S.A.); 8-cyclopentyl-1,3-dipropylxanthine (CPX) and N⁶-(4-aminobenzyl)adenosine (ABA) were gifts from Dr. Susan Daluge of Glaxo Wellcome (Research Triangle Park, NC, U.S.A.). [³H]CPX was from New England Nuclear (Boston, MA, U.S.A.); tissue-culture reagents were from Gibco-BRL (Grand Island, NY, U.S.A.); adenosine deaminase and N-glycosidase F from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.); FLAG peptide and anti-FLAG m2 affinity gel from Kodak IBI (New Haven, CT, U.S.A.); Ni-NTA-agarose from Qiagen (Chatsworth, CA, U.S.A.); enhanced chemiluminescence reagent and hyper-film MP from Amersham (Arlington Heights, IL, U.S.A.); centricon-30 concentrators from Amicon (Beverly, MA, U.S.A.); digitonin from Gallard-Schlesinger Industries (Carle Place, NY, U.S.A.). The synthesis and purification of [¹²⁵I]-ABA [14] and the preparation of stably transfected cells with His₆/FLAG-tagged human A₁ARs (H/F-A₁ARs) [9] have been described previously.

Cell culture and membrane preparation

Chinese-hamster ovary (CHO)-K1 cells stably transfected with H/F-A₁ARs were grown in Ham's F12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.5 mg/ml G418. In some instances cells were exposed to 20 µM CPA for various times. Cell monolayers were washed with PBS (3 × 10 ml after incubation with CPA) and harvested in buffer A (10 mM Hepes/20 mM EDTA, pH 7.4), supplemented with protease inhibitors (20 µg/ml benzamide, 100 µM PMSF and 2 µg/ml each of aprotinin, pepstatin and leupeptin). The cells were homogenized, centrifuged at 30000 g and washed twice with buffer HE (10 mM Hepes/1 mM EDTA, pH 7.4) containing protease inhibitors. The pellet was resuspended in HE and used immediately or frozen in aliquots at -80 °C.

Solubilization and purification of G-protein-coupled or uncoupled H/F-A₁AR

CHO-K1 cell membranes were resuspended in buffer B (25 mM Hepes/150 mM NaCl, pH 7.4) supplemented with 1 µM adenosine. For purification of receptors nearly free of G-proteins, the resuspended membranes were incubated with 10 mM GTP for 1 h at 21 °C. In preliminary experiments this concentration of GTP was found to be sufficient to produce maximal uncoupling. Digitonin was added at a detergent-to-membrane protein ratio of 2:1 and incubated on ice with rocking for 1 h. The solubilized receptors were collected after centrifugation at 100000 g for 1 h, and the detergent concentration was decreased to 0.1% by adding buffer B. The receptors were loaded on to a 1 ml anti-(FLAG M2) affinity column, washed with 3 × 12 ml of buffer C (buffer B, containing 0.1% digitonin) and eluted in 5 × 1 ml fractions with buffer C containing 400 µg of FLAG peptide. In most instances receptors from pooled fractions 2, 3 and 4 were applied to a 1 ml Ni-NTA affinity column, washed with three column vol. of buffer C, containing 1 mM imidazole, and eluted with buffer C supplemented with 250 mM imidazole, pH 7.4.

Photoaffinity labelling of H/F-A₁AR

Membranes of CHO-K1 cells expressing H/F-A₁AR were incubated in dim light with 1 nM [¹²⁵I]-azido-BW-A844 (a xanthine photoaffinity label) at room temperature for 1.5 h in the presence

of 10 µM guanosine 5'-[γ-thio]triphosphate (GTP[S]). The membranes were then irradiated with UV light for 15 min and centrifuged at 10000 g for 30 min. The resultant pellet was resuspended in 2 × buffer C. After solubilization, the photoaffinity-labelled H/F-A₁ARs were purified by anti-FLAG affinity chromatography as described above.

Protein phosphorylation studies

Transfected CHO-K1 or HEK-293 cells expressing the H/F-A₁AR from confluent 100 mm plates were washed with 2 × 10 ml of phosphate-free Dulbecco's modified Eagle's medium (DMEM) and incubated in the same medium at 37 °C, under 5% CO₂, for 45 min. The medium was replaced with 5 ml of phosphate-free DMEM containing 1 unit/ml adenosine deaminase (ADA) and ≈ 0.8 mCi/ml [³²P]P_i, and the cells were incubated at 37 °C, under 5% CO₂, for 2 h. Cells were then treated for 12 h with CPX (1 µM), CPA (10 µM) or vehicle (0.1% DMSO), washed with ice-cold PBS containing protease and phosphatase inhibitors (0.18 mg/ml vanadate, 2.1 mg/ml NaF, 21.2 mg/ml glycerol α,β-phosphate, and 1 nM okadaic acid). Following the addition of solubilization buffer (25 mM Hepes/150 mM NaCl/2% digitonin/protease and phosphatase inhibitor cocktail) and centrifugation at 100000 g for 1 h, R-G complexes were purified by anti-FLAG affinity chromatography as described above and phosphoproteins analysed by SDS/PAGE and autoradiography. Some samples were deglycosylated (see below).

Phosphoamino acid analysis

³²P-labelled proteins were resolved by SDS/PAGE and transferred to Immobilon-P (PVDF membranes). Regions of the PVDF that contain ³²P-labelled deglycosylated receptors were identified by autoradiography, cut out and placed in screw-cap microcentrifuge tubes. After a quick rinse in methanol, 200–300 µl of 5.7 M HCl was added and the tubes were capped and incubated for 1 h at 110 °C. The hydrolysed samples were dried in a centrifugal vacuum concentrator and resuspended in 5–10 µl of pH 1.9 buffer [7.8% (v/v) acetic acid/2.5% (v/v) formic acid] containing 0.5–1 µg of unlabelled phosphoamino acid standards. The tubes were centrifuged briefly at room temperature to remove particulate debris and spotted on TLC plates dampened in pH 2.5 electrophoresis buffer [5.9% (v/v) acetic acid/0.8% (v/v) formic acid/0.3% (v/v) pyridine/0.3 mM EDTA]. The samples were electrophoresed for 45–60 min at a constant 750 V, air-dried under a fan for 20 min, sprayed with 0.25% ninhydrin in acetone, and dried at 65 °C for 15 min. The plates were exposed to X-ray films using intensifying screens at -70 °C for 2 days.

SDS/PAGE, Western-blot analysis and silver staining

Aliquots from the anti-FLAG affinity or Ni-NTA affinity chromatography were loaded on to 1.5 mm-thick SDS/12%-polyacrylamide gels and electrophoresed at 20 mA constant current. Some aliquots (30 µl) were deglycosylated by incubation overnight at 37 °C with N-glycosidase F (0.5 unit). The proteins were transferred to Westran PVDF membranes for 3 h at 35 mA constant current and incubated for 1 h with anti-FLAG antibody or specific anti-G_{iα2} monoclonal antibody generated against the purified recombinant G_{iα2} subunit. The Western blot was developed using the enhanced chemiluminescence (ECL[®], Amersham) method with sheep anti-mouse IgG antibody and developed by 30–60 s of exposure to Hyperfilm ECL auto-

radiography film. For quantification of G_iα₂, developed films were scanned and integrated using Image Quant™ software. Preliminary experiments using purified G_iα₂ as standards have established that the signals generated under our working conditions were within the linear range. In some cases, SDS/polyacrylamide gels were stained for protein [15].

Radioligand binding assays

Binding assays were performed using A₁-selective radioligands, the agonist ¹²⁵I-ABA and the antagonist, [³H]CPX, in the presence of 1.5 unit/ml ADA and 0.006% digitonin (for purified receptor only) with or without 4.9 mM MgCl₂ for agonist and antagonist binding respectively. Non-specific binding was measured in the presence of 1 μM CPX.

Down-regulation of H/F-A₁AR

CHO-K1 cells expressing H/F-A₁AR were pretreated with either vehicle (DMSO) or 20 μM CPA in the presence of 0.5 unit/ml ADA for various times, as indicated. Radioligand binding, membrane preparation, R–G complex solubilization and purification were performed as described above. Differences in the time courses of agonist and antagonist down-regulation of receptor number were evaluated as described by Motulsky and Ransnas [16]. This procedure determines if two data sets are significantly better fitted to two curves or to a single curve.

RESULTS

Affinity purification of G-protein-coupled and uncoupled H/F-A₁ARs

The purification of H/F-A₁ARs by sequential anti-FLAG antibody and Ni-NTA affinity chromatography results in a good yield of very highly purified receptors, as described previously [9]. In the present study, we sought to identify conditions optimal for the purification of receptors that are coupled to G-proteins (R–G) and receptors uncoupled from G-proteins (R). Uncoupling was achieved by pretreating membranes with GTP before receptor solubilization and purification. According to the 'ternary complex' model, GTP or GTP analogues uncouple receptors from G-proteins [17]. The addition of 10 mM GTP to membranes almost completely abolished subsequent co-purification of G-proteins with H/F-A₁ARs from affinity columns. Figure 1(A) shows silver-stained SDS/polyacrylamide gels of R and R–G purified by sequential anti-FLAG and Ni²⁺-NTA affinity columns. Upon treatment with N-glycosidase F, the diffuse H/F-A₁AR shifts from an apparent molecular mass of 38–42 kDa to 33–34 kDa, and the same shift in mass is detected in receptors photoaffinity-labelled with the xanthine ¹²⁵I-azido-BW-A844 (Figure 1C) or by Western blotting receptors using anti-FLAG antibodies (Figure 1D). Some purified receptors form aggregates which appear as 94–98 kDa (non-deglycosylated) and 66 kDa (deglycosylated) proteins respectively by silver staining, photoaffinity labelling and Western blotting. The receptors are highly purified, but there are some additional proteins that can be weakly stained with silver that are co-purified. The most abundant of these is an unidentified 97 kDa protein that was co-purified with R or with R–G (Figure 1A). Deglycosylation of purified R–G complexes also reveals 42 and 35 kDa proteins that are far less abundant in the R lane. These correspond in size to G-protein α and β subunits respectively (Figure 1A, lane 4). G_iα₂ immunoreactivity co-migrates with the 42 kDa protein found in purified R–G complexes, but not with uncoupled receptors (Figure 1B). By subjecting untagged (lacking His₆ and FLAG) A₁ARs to the purification protocol we established that there is no non-specific binding of receptors, G-proteins or the 97 kDa

Figure 1 Silver-stained SDS/PAGE and Western analysis of purified H/F-A₁ARs without (R) or with (R–G) associated G-proteins

H/F-A₁AR derived from 70 mg of CHO-K1 membranes pretreated with or without 10 mM GTP were solubilized and purified through sequential affinity-chromatography columns (anti-FLAG and Ni-NTA) as described in the Experimental section. (A) Aliquots containing 5.3% of the purified receptors free of G-proteins (R) or R–G complexes were resolved by SDS/PAGE, incubated with or without N-glycosidase F (N-Gly F), and stained with silver. (B) Western blots of G_iα₂ in 1% of the R and R–G purified fractions. (C) Autoradiograph of purified photoaffinity-labelled H/F-A₁ARs. (D) Western analysis of purified H/F-A₁AR using anti-FLAG m2 antibody.

protein to the affinity-chromatography columns (results not shown). From these experiments we conclude that H/F-A₁ARs can be purified by sequential anti-FLAG and Ni-NTA affinity columns either as pure receptors or as a mixture of uncoupled receptors and R–G complexes from membranes pretreated with or without GTP respectively. Furthermore, there is no non-specific purification of proteins that are not associated with H/F-A₁ARs.

Table 1 summarizes the recovery and specific activity of H/F-A₁AR–G-protein complexes during affinity chromatography. We found that purified R–G complexes have a specific activity (antagonist [³H]CPX-binding sites) of 5 nmol/mg, which is 20% of the theoretical specific activity for a homogeneous 38 kDa H/F-A₁AR. This specific activity is less than theoretical, owing to the presence of G-proteins, the 97 kDa protein, other minor contaminants, and receptors that are denatured during solubilization and purification. The coupling between receptors and G-proteins [calculated as the ratio of high-affinity agonist- [¹²⁵I-ABA] to [³H]CPX-binding sites] is similar throughout the purification process, suggesting that the R–G complexes are stable following detergent solubilization.

We have previously demonstrated that extension of the N-termini of ARs with His₆ and the FLAG epitope has no effect on the binding affinity of ligands, the coupling of receptors to G-

Table 1 Purification of H/F-A₁AR-G-protein complexes

	³ H]CPX						¹²⁵ I-ABA					
	Protein (mg)	K _D (nM)	Total receptor (pmol)	Specific activity (pmol/mg)	Yield (%)	Purification (fold)	K _D (nM)	Total R-G complexes (pmol)	Specific activity (pmol/mg)	Yield (%)	Purification (fold)	% Coupled
Membrane	90.3	2.80	2070	23.0	100	1	1.0	266.4	2.95	100	1	12.8
Solubilization	35.6	14.0	911	25.6	44	1.11	0.9	79.4	2.23	29.8	0.76	8.7
FLAG elution	0.12	14.8	530	4417	25.6	191	0.9	55.3	512	20.7	174	11.6
Ni-NTA elution	0.09	14.8	438	4815	21	209	0.9	51.8	569.8	19.4	193	11.8

Table 2 Summary of K_i values of compounds to compete for radioligand binding to membranes and affinity purified recombinant H/F-A₁AR-G-protein complexes

¹²⁵I-ABA was used as the radioligand. Values are means ± S.E.M. Agonists are indicated by *italics*. K_i values for agonists reflect high-affinity binding sites. *h* is the Hill coefficient.

Competitor	Purified R-G			Membrane*		
	K _i (nM)	<i>h</i>	<i>n</i>	K _i (nM)	<i>h</i>	<i>n</i>
CPX (K _D)	14.8 ± 1.8	1.13 ± 0.08	5	2.8 ± 0.6	1.17 ± 0.14	3
XAC	122 ± 15	1.12 ± 0.06	3	50.6 ± 7.0	0.96 ± 0.08	5
8-SPT	6460 ± 40	1.07 ± 0.02	3	6210 ± 2580	0.84 ± 0.06	3
<i>CPA</i>	1.00 ± 0.08	1.01 ± 0.06	4	0.31 ± 0.08	0.79 ± 0.02	3
<i>NECA</i>	1.06 ± 0.13	0.91 ± 0.14	4	1.27 ± 0.36	0.97 ± 0.06	5
<i>CGS21680</i>	154 ± 13	0.95 ± 0.05	6	416 ± 130	0.96 ± 0.07	5

* Values are derived from [9].

proteins or the receptor-mediated changes in cyclic AMP accumulation [9]. Here we show that, with the exception of very hydrophobic ligands (CPX and CGS15943), which show decreased affinity for detergent-solubilized receptors, purified H/F-A₁ARs have pharmacological properties similar to those in membranes (Table 2). The selective effect of detergent solubilization on hydrophobic compounds is most likely explained by differential partitioning of lipophilic ligands into membranes versus detergent solution. These data suggest that the purification of recombinant H/F-A₁ARs by affinity chromatography results in functional receptors suitable for further studies of receptor structure and function.

Effect of prolonged agonist-pretreatment on receptor-G-protein coupling

A₁AR desensitization has been extensively studied in transfected cells [18], isolated tissues [11,19] and whole animals [20]. In each case, chronic exposure of A₁AR to agonists is manifested as a slow decrease in receptor-G-protein coupling and a gradual down-regulation of total receptor number. We examined agonist-induced down-regulation in CHO-K1 cells stably transfected with H/F-A₁ARs. Intact cells were exposed to 20 μM CPA for various times, as indicated in Figure 2. The total number of receptors on membranes was determined by measuring [³H]CPX binding (Figure 2A), and the number of GCRs was determined by measuring high-affinity [¹²⁵I]-ABA binding (Figure 2B). Figure 2(C) shows the kinetics of down-regulation of both total receptor number and the number of GCRs during agonist exposure. The *t*_{1/2} for the down-regulation of agonist binding (5.6 h) is significantly lower than the *t*_{1/2} for the loss of antagonist binding

(10.9 h). After 24 h of exposure to CPA there is a similar fractional decrease in the number of agonist- and antagonist-binding sites detected on membranes, so the coupling ratio is minimally changed from control (Figure 2C).

We next sought to determine if down-regulation has any effect on the fraction of receptors that are purified as R-G complexes. Following down-regulation for 24 h by exposing intact cells to 20 μM CPA, receptors and R-G complexes were purified by anti-FLAG affinity chromatography. The number of total receptors and R-G complexes was determined from antagonist [³H]CPX (Figure 3A) and high-affinity agonist [¹²⁵I]-ABA binding (Figure 3B) respectively. Following down-regulation, there was a 47 ± 6 % decrease in the total number of receptors, and a similar 54 ± 4 % decrease in the number R-G complexes measured in membranes. There was a similar decrease in the number of purified receptors, 54 ± 6 %, but a substantially larger decrease in the number of purified R-G complexes, 78 ± 3 % (Figure 3C). These experiments reveal a novel aspect of agonist-induced down-regulation, namely a diminished stability of R-G complexes that is manifested as R-G uncoupling during receptor purification.

Down-regulation decreases the amount of G_{iα2} that is co-purified with H/F-A₁AR

We next sought to confirm that down-regulation decreases the amount of G-proteins that can be co-purified with H/F-A₁AR. Following 24 h of down-regulation of intact cells with 20 μM CPA, the same number of purified receptors from control and desensitized cells (based on [³H]CPX-binding sites and confirmed by Western analysis using anti-FLAG M2 antibody; results not shown) were subjected to SDS/PAGE followed by Western transfer. Co-purified G_{iα2} was quantified by Western analysis using a previously characterized monoclonal antibody [21] (results not shown). When normalized to receptor number, there is a 50 % decrease in G_{iα2} following down-regulation. Taking into account the 54 % decrease in the total number of receptors purified from agonist-treated cells, the overall decrease of G_{iα2} co-purified with desensitized receptors was calculated as 74 %, which is comparable with the 78 % decrease in [¹²⁵I]-ABA binding sites noted above. These data are consistent with the expectation that decreased high-affinity agonist binding following down-regulation reflects a decrease in the amount of G-proteins co-purified with receptors.

Phosphorylation of H/F-A₁AR

For many GCRs, receptor phosphorylation results in uncoupling from G-proteins and functional desensitization. We set out to determine whether H/F-A₁ARs can be phosphorylated and

Figure 2 Down-regulation of radioligand binding to membranes from CHO-K1 expressing H/F-A₁AR following treatment with CPA

Intact cells derived from a single confluent 150 mm plate were pretreated with 20 μ M CPA in the presence of 0.5 unit/ml ADA for the times indicated. After extensive washes, membranes were prepared and used for binding isotherms with [³H]CPX (A) or [¹²⁵I]-ABA (B). The binding isotherms (A and B) are representative of two independent experiments. The time-dependence of the loss in radioligand binding was pooled from two independent experiments each done in triplicate and fitted to mono-exponential equations (C). The $t_{1/2}$ for the loss of binding was 5.6 h for [¹²⁵I]-ABA and 10.9 h for [³H]CPX. The time courses differed significantly ($P < 0.05$) (see the Experimental section).

whether phosphorylation is regulated by pretreatment of intact cells with an agonist or an antagonist. Transfected cells were metabolically labelled with [³²P]phosphate and receptors were purified over anti-FLAG affinity columns. As shown in Figures 4(A) and 4(B), deglycosylation revealed a distinct, weakly phosphorylated protein with a molecular mass of $\approx$ 34 kDa, suggesting that this phosphoglycoprotein might be the H/F-A₁AR. Without deglycosylation, receptor phosphorylation was not easily detected because of the diffuseness of glycosylated receptors and the existence of other weakly phosphorylated proteins, possibly including G-protein α and β subunits. Several

Figure 3 Effect of CPA-induced down-regulation on radioligand binding to purified H/F-A₁AR–G-protein complexes

Receptors were purified from 20 150-mm-diameter plates of CHO-K1 cells expressing H/F-A₁AR and pretreated with either vehicle or 20 μ M CPA for 24 h. (A) and (B) show typical equilibrium binding isotherms for the antagonist, [³H]CPX, and the agonist, [¹²⁵I]-ABA, respectively. S.E.M. bars are smaller than the symbols. Each fraction contains 4 μ l from 2 ml of purified R–G complexes. (C) Comparison of CPA-induced down-regulation (24 h) of [³H]CPX and [¹²⁵I]-ABA binding to H/F-A₁AR measured in membranes or purified R–G complexes. Data are normalized to specific binding sites in membranes or purified from control cells in parallel experiments. Specific-binding data are means $\pm$ S.E.M. ($n = 3$).

high-molecular-mass phosphoproteins also were detected by autoradiography, including a 97 kDa phosphoprotein. Prolonged antagonist or agonist (12 h) pretreatment did not significantly alter the amount of [³²P]phosphate incorporated into the receptors, G-proteins or the 97 kDa protein. Phosphoamino acid analysis indicated that H/F-A₁ARs are phosphorylated predominantly on serine residue(s), with a very low level of phosphothreonine; no phosphotyrosine was detected (Figure 4C). These data suggest that H/F-A₁ARs, G-protein α - and β -subunits, as well as the 97 kDa protein, are constitutively and weakly phosphorylated in transfected cells and that the amount of phosphorylation is not changed by exposure to CPA.

Figure 4 Phosphorylation of H/F-A₁AR

One confluent 100-mm-diameter plate of transfected CHO-K1 cells expressing 25–35 pmol/mg of protein of recombinant human H/F-A₁AR was preincubated with 4 mCi of [³²P]phosphate in phosphate-free DMEM medium for 2 h; 1 μ M CPX (A) or 10 μ M CPA (B) was added for an additional 12 h. Receptors were purified as described in the Experimental section and were subject to SDS/PAGE without (–) or with (+) prior treatment with N-glycosidase F (N-Gly F). (C) Phosphoamino acid analysis of purified deglycosylated ³²P-labelled H/F-A₁AR. Abbreviations: pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

DISCUSSION

In the present study we have examined factors that control receptor desensitization and the coupling of receptors to G-proteins using purified recombinant human A₁ARs that have been modified to facilitate their purification. We show that either uncoupled receptors or receptors that are partially coupled to G-proteins can be purified under conditions which preserve their ability to bind radioligands.

Our data suggest that recombinant human A₁ARs can only undergo long-term (slow) desensitization following exposure to an agonist (Figure 2). Interestingly, during this process, there is a significantly faster decrease in the number of high affinity A₁AR binding sites for the agonist, [¹²⁵I]-ABA, than for the antagonist, [³H]CPX, (Figure 2). Agonist binding may diminish more quickly than antagonist binding because the loss of agonist binding results from both decreased coupling of receptors to G-proteins and from down-regulation of receptor number, whereas antagonist binding primarily reflects receptor number and is only slightly affected by receptor coupling to G-proteins. A comparison of the number of receptors and R–G complexes purified from control and desensitized cells indicates that long-term exposure of intact cells to an agonist results in a weakened

interaction between receptors and G-proteins and facilitates uncoupling during the solubilization and purification process. The destabilizing effect of prolonged agonist exposure on R–G complexes is manifested as a decrease in the fraction of purified receptors that can bind [¹²⁵I]-ABA (presumed R–G complexes), and decreased amounts of G_i2 co-purified with receptors. It is noteworthy that this destabilization is not accompanied by a change in the phosphorylation state of receptors, the phosphorylation state of co-purified G-protein α - and β - subunits or the 97 kDa protein. These findings imply that there is an unknown factor or factors that regulate(s) the affinity of the interaction between the A₁ARs and G-proteins.

Searching for factors that modulate R–G coupling is an area of intense current interest, and several proteins that regulate coupling have been identified. These include arrestin [22], phosducin [23], recoverin [24] and regulators of G-protein signalling ('RGSs') [25]. In the case of the A₁AR, a putative 'coupling cofactor' partially purified from cortical brain membranes ($\approx$ 150 kDa) was reported to stabilize A₁AR–G-protein complexes and to trap the A₁AR in a high-affinity agonist-binding state [26]. Removal of this protein from membranes was reported to increase the sensitivity of high-affinity agonist binding to inhibition by GTP[S], resulting in weakened R–G coupling [27]. On silver-stained gels of purified A₁AR–G-protein complexes we did not detect co-purified proteins corresponding to the molecular masses of these various factors. This would suggest that the regulation of the interaction between receptors and G-proteins is the result of a modification of the receptor itself, a G-protein or the 97 kDa protein that was co-purified with receptors.

Phosphorylation of receptors secondary to agonist exposure by either GCR kinases (GRKs) or second-messenger-activated protein kinases, such as protein kinases A and kinase C, has been reported to influence the stability of various R–G complexes, and have been implicated in both short-term uncoupling and desensitization processes. Receptor phosphorylation by a GRK promotes the binding of one of four arrestin family members to agonist-occupied receptor molecules that are engaged in G-protein activation [28]. However, endogenous levels of GRKs are sufficient to phosphorylate some G-protein-coupled receptors more efficiently than others. For example, human endothelin A and B receptors overexpressed in HEK-293 cells undergo rapid agonist-induced phosphorylation and desensitization, and these effects are only somewhat enhanced by overexpression of GRKs [28,29]. In contrast, desensitization of the chemokine receptor CCR-5 does not occur at all in HEK-293 cells in the absence of GRK overexpression [30]. α -Adrenergic receptor subtypes display different rates of phosphorylation and desensitization. Unlike the α 2C10 adrenergic receptor, the α 2C4 receptor is not phosphorylated by any of the GRKs and does not undergo rapid agonist-induced desensitization when overexpressed in HEK-293 cells [13]. Mutant m2 muscarinic receptors that can not be phosphorylated in an agonist-dependent manner also do not display rapid desensitization [31]. Hence, there are substantial differences in the susceptibility of various GCRs to phosphorylation by GRKs and correlated differences in rapid agonist-induced desensitization.

A₁ARs do not undergo rapid agonist-induced desensitization. In transfected CHO-K1 cells, Palmer et al. [32] could not detect rapid (10 min) agonist-stimulated A₁AR phosphorylation. In parallel experiments, recombinant A₃ARs displayed substantial agonist-stimulated phosphorylation and desensitization. These findings agree with the results of the present study and suggest that the A₁AR belongs to a subset of GCRs that are not substrates or are poor substrates for GRKs and do not undergo

rapid desensitization. Teleologically, A₁ARs may be resistant to acute agonist-dependent phosphorylation because adenosine receptors protect excitable tissues from ischaemia, and this protective response may have evolved to be resistant to rapid and large-scale desensitization characteristic of certain other GCRs, including the A₃AR [32].

In the present study we have examined the effect of prolonged agonist exposure on A₁AR phosphorylation, R–G coupling and desensitization. The role of receptor phosphorylation in the long-term desensitization has been examined in only a limited number of studies. Phosphorylation of β_2 -adrenergic receptors by protein kinase A regulates the rate and extent of agonist-induced receptor down-regulation. McGraw et al. [33] recently demonstrated persistent phosphorylation of β_2 -adrenergic receptors by GRK during 24 h of exposure to an agonist. Removal of the GRK phosphorylation sites attenuates, but does not abolish, long-term desensitization. In the case of M3 muscarinic receptors, a threonine residue in the C-terminal tail appears to regulate the rate of long-term receptor down-regulation [34]. The results of the present study indicate that long-term down-regulation of the A₁AR and destabilization of R–G complexes can occur in the absence of a change in the receptor phosphorylation state. This supports the notion that phosphorylation may in some instances modulate, but is not required for, receptor down-regulation.

There is conflicting prior literature regarding agonist-induced phosphorylation and desensitization of A₁ARs. Partially purified receptors were reported to undergo rapid (5 min) [35] or slow (18 h) agonist-stimulated phosphorylation [36]. However, in this type of experiment it may be difficult to distinguish minor contaminating ³²P-labelled phosphoproteins from phosphorylated receptors in instances in which receptors are impure. This point is illustrated in Figure 4. Phosphorylated receptors cannot be resolved from contaminating non-receptor phosphoproteins unless they are deglycosylated. Using deglycosylation to confirm the identity of the putative receptor phosphoprotein has not been used in previous studies, such as in the study by Ciruela et al. [35], who reported that native A₁ARs partially purified by immunoprecipitation from DDT₁MF₂ cells display a > 7-fold increase in [³²P]phosphotyrosine into a 36 kDa putative receptor within minutes. These results are surprising in view of studies which show that desensitization of A₁ARs on DDT₁MF₂ cells takes several hours [36]. Moreover, in the present study we found no incorporation of ³²P into receptor tyrosine; rather the low-level phosphorylation of A₁ARs that was observed was predominantly on serine residues. Using transfected CHO-K1 cells, Palmer et al. were not able to detect any rapid agonist-induced phosphorylation of A₁ARs [32]. Given the great discrepancies in the literature regarding A₁AR phosphorylation, we believe that deglycosylation is a useful and important method to unequivocally identify poorly phosphorylated receptors.

In addition to receptor phosphorylation, another potential mechanism of regulating the stability of R–G complexes is via phosphorylation of G-proteins. G α_2 , but not G α_3 from hepatocytes, was found to be phosphorylated on serine residues by protein kinase C both *in vivo* and *in vitro* [37–39]. Elevated phosphorylation of G α_2 was correlated with decreased ability to inhibit adenylate cyclase [40,41]. Tyrosine phosphorylation of G α_q [42], G α_s [43] and functional consequences have also been reported. G β also has been reported to be regulated by phosphorylation [44,45]. One possibility that is consistent with our data is that phosphorylation of G-proteins causes them to become uncoupled from receptors, in which case the phosphorylated G-proteins would not be purified as R–G complexes.

Another possible mode of regulating R–G coupling that we did not explore in the present study is that the phosphorylation

state of the γ subunit of heterotrimeric G-proteins influences coupling to receptors. Our gels were run under conditions that were not designed to retain the small γ subunits. The γ_{12} subunit is phosphorylated by protein kinase C in response to activation of various GCRs [46]. Recently, the phosphorylation of γ_{12} by protein kinase C was reported to increase the stability of A₁AR–G-protein complexes [47]. To be consistent with the results of the present study, long-term exposure to an agonist would decrease the phosphorylation of γ_{12} to destabilize R–G complexes. It is also possible that, during long-term exposure to an agonist, there is a change in the complement of G-protein $\beta\gamma$ subunits associated with receptors. Finally, additional unknown modifications of receptors or G-proteins other than phosphorylation may play a role in influencing the stability of R–G complexes.

This work was supported by grant no. R0137942 from the National Institutes of Health. We thank Dr. James Garrison for helpful discussions.

REFERENCES

- Munshi, R. and Linden, J. (1989) *J. Biol. Chem.* **264**, 14853–14859
- Munshi, R., Pang, I. H., Sternweis, P. C. and Linden, J. (1991) *J. Biol. Chem.* **266**, 22285–22289
- Luthin, D. R., Eppler, C. M. and Linden, J. (1993) *J. Biol. Chem.* **268**, 5990–5996
- Polakis, P. G., Uhing, R. J. and Snyderman, R. (1988) *J. Biol. Chem.* **263**, 4969–4976
- Georgoussi, Z., Milligan, G. and Zioudrou, C. (1995) *Biochem. J.* **306**, 71–75
- Matesic, D. F., Manning, D. R., Wolfe, B. B. and Luthin, G. R. (1989) *J. Biol. Chem.* **264**, 21638–21645
- Wennogle, L. P., Conder, L., Winter, C., Braunwalder, A., Vlattas, S., Kramer, R., Cioffi, C. and Hu, S. I. (1994) *J. Cell Biochem.* **55**, 380–388
- Zhang, J. and Pratt, R. E. (1996) *J. Biol. Chem.* **271**, 15026–15033
- Robeva, A. S., Woodard, R., Luthin, D. R., Taylor, H. E. and Linden, J. (1996) *Biochem. Pharmacol.* **51**, 545–555
- Linden, J., Jacobson, M. A., Hutchins, C. and Williams, M. (1994) in *Handbook of Receptors and Channels: G-Protein Coupled Receptors* (Peroutka, S. J., ed.), pp. 29–44, CRC Press, Boca Raton, FL
- Shryock, J., Patel, A., Belardinelli, L. and Linden, J. (1989) *Am. J. Physiol.* **256**, H321–H327
- Freedman, N. J. and Lefkowitz, R. J. (1996) *Recent Prog. Horm. Res.* **51**, 319–351
- Jewell-Motz, E. A. and Liggett, S. B. (1996) *J. Biol. Chem.* **271**, 18082–18087
- Linden, J., Patel, A. and Sadek, S. (1985) *Circ. Res.* **56**, 279–284
- Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310
- Motulsky, H. J. and Ransnas, L. A. (1987) *FASEB J.* **1**, 365–374
- Limbird, L. E., Gill, D. M. and Lefkowitz, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 775–779
- Bhattacharya, S. and Linden, J. (1996) *Mol. Pharmacol.* **50**, 104–111
- Parsons, W. J. and Stiles, G. L. (1987) *J. Biol. Chem.* **262**, 841–847
- Dennis, D. M., Shryock, J. C. and Belardinelli, L. (1995) *J. Pharmacol. Exp. Ther.* **272**, 1024–1035
- Luthin, D. R., Eppler, C. M. and Linden, J. (1993) *J. Biol. Chem.* **268**, 5990–5996
- Ferguson, S. S., Barak, L. S., Zhang, J. and Caron, M. G. (1996) *Can. J. Physiol. Pharmacol.* **74**, 1095–1110
- Wehmeyer, A. and Schulz, R. (1998) *Life Sci.* **62**, L127–L134
- Faurobert, E., Chen, C. K., Hurley, J. B. and Teng, D. H. (1996) *J. Biol. Chem.* **271**, 10256–10262
- Dohlman, H. G. and Thorner, J. (1997) *J. Biol. Chem.* **272**, 3871–3874
- Nanoff, C., Waldhoer, M., Roka, F. and Freissmuth, M. (1997) *Neuropharmacology* **36**, 1211–1219
- Nanoff, C., Mitterauer, T., Roka, F., Hohenegger, M. and Freissmuth, M. (1995) *Mol. Pharmacol.* **48**, 806–817
- Freedman, N. J., Ament, A. S., Oppermann, M., Stoffel, R. H., Exum, S. T. and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 17734–17743
- Freedman, N. J., Liggett, S. B., Drachman, D. E., Pei, G., Caron, M. G. and Lefkowitz, R. J. (1995) *J. Biol. Chem.* **270**, 17953–17961
- Aramori, I., Zhang, J., Ferguson, S. S., Bieniasz, P. D., Cullen, B. R. and Caron, M. G. (1997) *EMBO J.* **16**, 4606–4616
- Pals-Rylaarsdam, R., Xu, Y., Witt-Enderby, P., Benovic, J. L. and Hosey, M. M. (1995) *J. Biol. Chem.* **270**, 29004–29011
- Palmer, T. M., Benovic, J. L. and Stiles, G. L. (1996) *J. Biol. Chem.* **271**, 15272–15278

-
- 33 McGraw, D. W., Donnelly, E. T., Eason, M. G., Green, S. A. and Liggett, S. B. (1998) *Cell. Signal.* **10**, 197–204
- 34 Yang, J., Logsdon, C. D., Johansen, T. E. and Williams, J. A. (1993) *Mol. Pharmacol.* **44**, 1158–1164
- 35 Ciruela, F., Saura, C., Canela, E. I., Mallol, J., Lluís, C. and Franco, R. (1997) *Mol. Pharmacol.* **52**, 788–797
- 36 Ramkumar, V., Olah, M. E., Jacobson, K. A. and Stiles, G. L. (1991) *Mol. Pharmacol.* **40**, 639–647
- 37 Morris, N. J., Bushfield, M., Lavan, B. E. and Houslay, M. D. (1994) *Biochem. J.* **301**, 693–702
- 38 Bushfield, M., Lavan, B. E. and Houslay, M. D. (1991) *Biochem. J.* **274**, 317–321
- 39 Bushfield, M., Murphy, G. J., Lavan, B. E., Parker, P. J., Hruby, V. J., Milligan, G. and Houslay, M. D. (1990) *Biochem. J.* **268**, 449–457
- 40 Strassheim, D. and Malbon, C. C. (1994) *J. Biol. Chem.* **269**, 14307–14313
- 41 Bushfield, M., Griffiths, S. L., Murphy, G. J., Pyne, N. J., Knowler, J. T., Milligan, G., Parker, P. J., Mollner, S. and Houslay, M. D. (1990) *Biochem. J.* **271**, 365–372
- 42 Liu, W. W., Mattingly, R. R. and Garrison, J. C. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8258–8263
- 43 Hausdorff, W. P., Pitcher, J. A., Luttrell, D. K., Linder, M. E., Kurose, H., Parsons, S. J., Caron, M. G. and Lefkowitz, R. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5720–5724
- 44 Feng, Y., Song, L. Y., Kincaid, E., Mahanty, S. K. and Elion, E. A. (1998) *Curr. Biol.* **8**, 267–278
- 45 Cole, G. M. and Reed, S. I. (1991) *Cell* **64**, 703–716
- 46 Asano, T., Morishita, R., Ueda, H., Asano, M. and Kato, K. (1998) *Eur. J. Biochem.* **251**, 314–319
- 47 Yasuda, H., Lindorfer, M. A., Myung, C.-S. and Garrison, J. C. (1998) *J. Biol. Chem.* **273**, 21958–21965
-

Received 17 August 1998/25 November 1998; accepted 18 December 1998