Selective inactivation of guanine-nucleotide-binding regulatory protein (G-protein) α and $\beta\gamma$ subunits by urea

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G-protein-coupled receptors activate signal-transducing G-proteins, which consist of an α subunit and a $\beta\gamma$ dimer. Membrane extraction with 5–7 M urea has been used to uncouple receptors from endogenous G-proteins to permit reconstitution with purified G-proteins. We show that α_i subunits are inactivated with 5 M urea whereas the $\beta\gamma$ dimer requires at least 7 M urea for its inactivation. There is no significant loss of receptors. Surprisingly, Western-blot analysis indicates that the urea-denatured α_i subunit remains mostly membrane-bound and that β is only partially removed. After 7 M urea treatment, both α_{i1} and $\beta\gamma$ subunits are

required to restore high-affinity agonist binding and receptor-catalysed guanosine 5'-[γ -thio]triphosphate binding. We demonstrate the generality of this approach for four G_i -coupled receptors (α_{2A} -adrenergic, adenosine A1, 5-hydroxytryptamine_{1A} and μ -opioid) expressed in insect cells and two mammalian cell lines. Thus a selectivity of urea for G-protein α versus $\beta\gamma$ subunits is established in both concentration and mechanism.

Key words: adenosine, α_2 adrenergic, 5-hydroxytryptamine, opioid, reconstitution.

INTRODUCTION

G-protein-coupled receptors are integral membrane proteins that convey signals from the outside of the cell to the interior [1]. Liganded receptor activates the G-protein, which consists of an α subunit and a $\beta\gamma$ dimer. The α subunit consists of two domains, termed the GTPase and helical domains [2]. The β subunit has an N-terminal α -helical segment followed by seven β -sheets forming a β -propeller structure. The γ subunit forms a tight coiled-coil interaction with the N-terminus of β and has contacts along blades five, six and seven of the β -propeller [3]. G-proteins require post-translational lipid modifications to associate with the membrane: the α subunit is acylated whereas γ is prenylated [4]. G-protein subunits modulate the activities of many intracellular effectors, which range from enzymes to ion channels [1]. In addition to receptor, G-protein and effector, there are other regulatory molecules, such as regulators of G-protein signalling (RGS) [5], G-protein receptor kinases and arrestins [6], which contribute to signalling at the inner surface of the plasma membrane.

Various techniques have been utilized to study the specificity of the interaction between receptors and G-proteins. These include agonist-stimulated covalent label incorporation on the α subunit [7,8], immunoneutralization [9,10], antisense techniques [11] and *in vitro* reconstitution of purified components [12,13]. Of these, only the reconstitution approach allows the precise manipulation of each signalling component at defined concentrations. A widely used approach is the reconstitution of signalling components with receptors expressed in cell membranes, obviating the need for receptor purification. However, receptors in membranes are able to couple to endogenous membrane

proteins. Such crosstalk is not desirable in experiments aimed at studying interaction of receptor with purified components, such as G-proteins of defined composition. Several methods have been used to inactivate G-proteins, including alkaline pH [14], pertussis toxin [15], CHAPS [16] and the chaotropic agent urea [17]. Northup and colleagues have adapted this latter technique to *Spodoptera frugiperda* Sf9 insect cell membranes [18] and rat fibroblast cell membranes [19,20] expressing G-protein coupled receptors. They have shown that urea treatment preserved antagonist binding [18,19] but abolished receptor coupling to endogenous G-proteins [18–20]. Upon reconstitution with purified G-proteins, there was restoration of high-affinity agonist binding and receptor-stimulated guanosine 5'-[γ -thio]triphosphate (GTP[S]) binding. The utility of this technique in receptor-coupling studies is clear, but the effects of different urea concentrations on different G-protein components and the optimal urea concentration for receptor uncoupling remains undefined. It is also unclear whether the loss of G-protein activity is due to denaturation *in situ* or if the subunits are physically removed from membranes.

In this report, we demonstrate that urea extraction mainly denatures G-proteins *in situ*, and that 5 M urea is sufficient to inactivate α subunits, while 7 M is required to functionally inactivate $\beta\gamma$. For multiple G-protein-coupled receptors and cell lines, 7 M urea treatment uncouples receptor from endogenous G-proteins, thereby enhancing the signal-to-noise ratio for high-affinity agonist binding and receptor-catalysed GTP[S] binding upon reconstitution with purified G-proteins. This technique should be applicable in studies that require a low background level to measure biochemical signals generated by receptor and reconstituted signalling components.

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; CHO, Chinese hamster ovary; PIC, *p*-iodoclonidine; YOH, yohimbine; CCPA, 2-chloro-*N*⁶-cyclopentyladenosine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; MPPF, 4-(2'-methoxy)-phenyl-1-[2'-(*N*-2'-pyridinyl)-*p*-fluorobenzamido]ethyl-piperazine; Gpp(NH)p, guanyl-5'-yl imidodiphosphate; RGS, regulators of G-protein signalling; 5-HT, 5-hydroxytryptamine; 5-HT_{1A}-R, 5-hydroxytryptamine_{1A} receptor; α_{2A} -AR, α_{2A} adrenergic receptor; DAMGO, Tyr-D-Ala-Gly-(Me)Phe-Gly-ol.

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EXPERIMENTAL

Materials

[¹²⁵I]*p*-Iodoclonidine ([¹²⁵I]PIC, 2200 Ci/mmol), [³H]yohimbine ([³H]YOH, 80 Ci/mmol), [³H]2-chloro-*N*⁶-cyclopentyladenosine ([³H]CCPA, 55 Ci/mmol), [³H]8-cyclopentyl-1,3-dipropylxanthine ([³H]CPX, 112 Ci/mmol), [³H]8-hydroxy-2-(di-*n*-propylamino)tetralin ([³H]8-OH-DPAT, 125 Ci/mmol), [³H]4-(2'-methoxy)-phenyl-1-[2'-(*N*-2''-pyridinyl)-*p*-fluorobenzamido]-ethyl-piperazine ([³H]MPPF, 78 Ci/mmol), [³H]Tyr-D-Ala-Gly-(Me)Phe-Gly-ol ([³H]DAMGO, 55 Ci/mmol), [³H]naloxone (55 Ci/mmol) and [³⁵S]GTP[S] (1250 Ci/mmol) were from DuPont-New England Nuclear (Wilmington, DE, U.S.A.). UK 14,304 [5-bromo-6-*N*-(2-4,5-dihydroimidazolyl)quinoxaline] was from Pfizer (Sandwich, Kent, U.K.), CPX was from RBI (Natick, MA, U.S.A.), urea was from Fisher Scientific (Fair Lawn, NJ, U.S.A.), whereas YOH, 5-hydroxytryptamine (5-HT), DAMGO and guanyl-5'-yl imidodiphosphate [Gpp(NH)p] were from Sigma (St. Louis, MO, U.S.A.). All other reagents were of reagent grade or better.

Construction of stable cell lines and recombinant baculoviruses

The α_2 Tag H/N vector including silent *Hind*III/*Nhe*I restriction sites was previously described by Wade et al. [21] and contains the porcine α_{2A} adrenergic receptor (α_{2A} -AR) with an N-terminal haemagglutinin tag derived from the pCMV4-TAG α_{2A} -AR construct, a gift from Dr Lee Limbird (Vanderbilt University, Nashville, TN, U.S.A.). C₆ glioma cells stably transfected with the rat μ -opioid receptor were a gift from Dr John Traynor (University of Michigan, Ann Arbor, MI, U.S.A.) [22].

For preparation of recombinant α_{2A} -AR baculovirus, a 1579 bp *Kpn*I restriction fragment from the α_2 Tag H/N vector was subcloned into the *Kpn*I site in the pFastBac baculovirus transfer vector (Gibco-BRL, Gaithersburg, MD, U.S.A.) and the orientation checked by restriction mapping. Virus stocks were prepared according to manufacturer's protocol. Recombinant baculovirus encoding the bovine adenosine A1 receptor was a gift from Dr James Garrison (University of Virginia, Charlottesville, VA, U.S.A.) and prepared as described in [23], while that encoding the human 5-hydroxytryptamine_{1A} receptor (5-HT_{1A}-R) was provided by Dr David Manning (University of Pennsylvania, Philadelphia, PA, U.S.A.) [24]. Viruses were amplified by infecting ≈ 100 ml of Sf9 cells at 2×10^6 cells/ml with a multiplicity of infection of 0.1.

Cell culture and receptor expression

Chinese hamster ovary (CHO) cells expressing the α_{2A} -AR were maintained in Ham's F-12 medium (Gibco-BRL) with 10% fetal bovine serum. C₆ glioma cells were grown in Dulbecco's modified Eagle's high-glucose Hepes (Irvine Scientific) with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine. Both mammalian cell lines were maintained at 37 °C with 5% CO₂.

Sf9 insect cells were from the American Type Culture Collection (ATCC CRL-1711). They were grown in suspension with Sf-900 II SFM serum-free media (Gibco-BRL) and maintained at 28 °C with agitation at 136 rev./min in an orbital shaker (Forma Scientific, Marietta, OH, U.S.A.). For protein expression, cells (2×10^6 /ml) were infected with the appropriate baculovirus at a multiplicity of infection ratio of 1 and harvested 48 h later.

Membrane preparation

Confluent monolayers of CHO and C₆ glioma cells from 25 tissue-culture dishes (150 mm) were washed with PBS and lysed (at 4 °C for 15 min) in 1 mM Tris/HCl, pH 7.4, with protease inhibitors (10 μ g/ml leupeptin/2 μ g/ml aprotinin/20 μ g/ml benzamidine/0.1 mM PMSF). Cells were scraped from dishes and homogenized by 10 strokes of a glass-Teflon homogenizer then sedimented at 1000 *g* for 10 min. The post-nuclear supernatant was centrifuged at 40000 *g* for 30 min, and the pellet was resuspended in buffer A (50 mM Tris/HCl/5 mM MgCl₂/1 mM EDTA, pH 7.6) and re-homogenized. Protein was determined by the Bradford method [25]. Aliquots were snap-frozen in liquid nitrogen and stored at -80 °C for up to 12 months before use.

Sf9 cell pellets from 500–800 ml of cell suspension were harvested by two washes in PBS and resuspension in lysis buffer (10 mM Tris/HCl/1 mM EDTA/100 mM NaCl/1% glycerol/protease inhibitors, pH 8) at 4 °C. Cells were burst by N₂ cavitation [Parr bomb, 600 p.s.i. (4140 kPa), 20 min] followed by sedimentation of nuclei (750 *g*, 10 min). Membranes were obtained by pelleting the post-nuclear supernatant (28000 *g*, 30 min) and redispersing in buffer A and protease inhibitors. These were homogenized by 10 strokes of a glass-Teflon homogenizer and stored as described for mammalian cell membranes.

Urea treatment

Urea stock solutions were made freshly by dissolution of urea crystals in buffer A at room temperature and then kept on ice before use. Membranes were pelleted (10000 *g* for 10 min) and resuspended to ≈ 0.75 mg/ml in urea solution of the desired molarity. After a 30 min incubation on ice, membranes were centrifuged at 140000 *g* for 30 min. The pellet was redispersed in (urea-free) resuspension buffer then incubated and centrifuged as before. The final pellet (≈ 1 mg/ml) was resuspended, homogenized and stored as described above.

G-protein purification

Myristoylated α_{11} was purified from *Escherichia coli* BL21/DE3 following the method of Mumby and Linder [26]. Specific activity was 20 nmol/mg, as monitored by [³⁵S]GTP[S] binding following the method of Sternweis and Robishaw [27]. Bovine brain $\beta\gamma$ was isolated from cortex synaptosomal membranes (a gift from Dr Tetsufumi Ueda, University of Michigan) essentially by the method of Sternweis and Robishaw [27], with modifications as described in [21].

Reconstitution of receptor with G-proteins

Membranes expressing receptors were pelleted in an Eppendorf centrifuge 5415 (12000 *g*, 10 min at 4 °C) and resuspended in reconstitution buffer (buffer A supplemented with 0.1% sodium cholate) in a 1.5 ml Eppendorf tube at ≈ 0.03 –0.13 mg/ml protein. After addition of the indicated amounts of G-proteins, the reaction mix was incubated on ice for 1 h before being diluted 5-fold for ligand binding or 2-fold for [³⁵S]GTP[S] binding.

Radioligand binding

Radioligand in buffer A (0.03% adenosine deaminase was added for adenosine A1 receptor binding experiments) was dispensed

into 96-well plates. Assays with single radioligand concentrations used final concentrations of 1 nM [125 I]PIC, 2 nM [3 H]CCPA, 2 nM [3 H]8-OH-DPAT or 5 nM [3 H]DAMGO. Non-specific binding was less than 10 % of total binding, as determined in the presence of the respective unlabelled ligands 10 μ M YOH, 2 μ M CPX, 2 μ M 5-HT and 5 μ M DAMGO. To initiate binding, membranes (typically 20 μ l, with 2–15 μ g of protein) with or without reconstituted G-proteins were diluted into wells containing 80 μ l of the radioligand. After incubation at room temperature for 1 h (YOH, PIC, DAMGO, naloxone and MPPF), 1.5 h (8-OH-DPAT), 2 h (CPX) or 3 h (CCPA), samples were filtered over Brandel GF/C filters using a Brandel cell harvester. After two washes with 2 ml of wash buffer (50 mM Tris/HCl/10 mM MgCl₂, pH 7.6), filters were air-dried and scintillation counting was performed with 4 ml of Scintiverse cocktail (Fisher Scientific) in a Beckman LS 5801 Liquid Scintillation System. Receptor density of the α_{2A} -AR was determined by binding of 10 nM [3 H]YOH based on a K_d of 3 nM.

[35 S]GTP[S] binding

Membranes were reconstituted with G-proteins as described above, except that 5 ml 75 $\times$ 12 mm tubes (Sarstedt, Newton, NC, U.S.A.) were used. Also, the reconstitution buffer was supplemented with 100 mM NaCl/1 μ M GDP (GTP[S] reconstitution buffer). This reconstitution mix was diluted one-to-one on ice with GTP[S] reconstitution buffer minus cholate but with 0.4 nM [35 S]GTP[S]. To initiate GTP[S] binding, tubes were placed in a 25 $^{\circ}$ C water bath and, after 5 min, UK 14,304 was added. At the indicated time points, 100 μ l aliquots (5 μ g of membrane protein with 0.2 nM α_{2A} -AR) were diluted in 3 ml of ice-cold wash buffer (20 mM Tris/HCl/25 mM MgCl₂/100 mM NaCl, pH 7.7) then rapidly filtered over Whatman GF/C filters using a Hoefer filtration apparatus. After two 3 ml washes, filters were air-dried and scintillation counting performed as above. For $\beta\gamma$ concentration response curves, each tube contained 5 μ g of Sf9 membrane protein (0.6 nM α_{2A} -AR) and the contents of each tube was filtered 40 min after addition of UK 14,304.

Immunoblotting

CHO membrane proteins were separated on SDS/polyacrylamide (10%) gels prepared according to Laemmli [28] and transferred to Immobilon-P[®] nitrocellulose membranes from Millipore (Bedford, MA, U.S.A.). α Subunit was detected using anti- α_i antibody (AS/7, New England Nuclear), whereas the β subunit was probed with anti- β -common antibody (SW/1, New England Nuclear). Both primary antibodies were detected with anti-rabbit peroxidase conjugate (A-0545, Sigma). All antibodies were used at a dilution of 1:1000. Secondary antibody was visualized using ECL detection reagents (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) developed on X-OMAT[®] imaging film (Kodak, Rochester, NY, U.S.A.) for 3–6 s. Quantification of band densities of immunoblots was carried out using a GS300 scanning densitometer (Hoefer Scientific Instruments) with the GS365W version 3b software.

Data analysis

Curve fitting used the non-linear least-squares method in the Prism computer program (GraphPad Software, San Diego, CA, U.S.A.). Data points are shown as means $\pm$ S.E.M. (or $\pm$ S.D. for $n = 2$).

RESULTS

Based on previous reports of 5 M urea to strip endogenous α and $\beta\gamma$ off retinal rod disk membranes [29], we extracted Sf9 membranes expressing α_{2A} -AR at $\approx$ 10 pmol/mg of protein with 5 M urea. Protein recovery after urea extraction is typically $\approx$ 30 % while mock extraction with buffer yields 70 % recovery, indicating removal of $\approx$ 60 % of membrane proteins by urea. Binding of the radiolabelled antagonist [3 H]YOH and the partial agonist [125 I]PIC was performed before (Figure 1A) and after (Figure 1B) urea treatment. As expected, urea treatment left antagonist binding intact (K_d 6 ± 2 versus 7 ± 4 nM and B_{max} 12 ± 3 versus 26 ± 11 pmol/mg, $n = 2$). However, binding of the partial agonist [125 I]PIC was decreased markedly after 5 M urea extraction. The B_{max} for [125 I]PIC decreased from 19 ± 8.2 % to 2.1 ± 0.2 % ($n = 2$) of the YOH-binding sites. The K_d of [125 I]PIC increased from 0.44 ± 0.25 to 1.0 ± 0.08 nM after urea treatment

Figure 1 Inactivation of α subunit by 5 M urea

Saturation binding of the antagonist [3 H]YOH ($\blacktriangle$, 2.5–35 nM) and the partial agonist [125 I]PIC ($\triangle$, 0.25–3.5 nM) to α_{2A} -AR in Sf9 membranes before (A) and after 5 M urea treatment (B and C). (C) Saturation [125 I]PIC binding (0.1–3 nM) to 5 M urea-treated Sf9 membranes expressing the α_{2A} -AR before ($\triangle$) and after reconstitution with 3 nM myristoylated α_i ($\blacktriangle$) or both myristoylated α_i plus 15 nM $\beta\gamma$ ($\square$). Non-specific binding determined in the presence of 10 μ M YOH was less than 10 % of total binding. Specific binding is presented as a Scatchard transformation. Data are from one experiment that is representative of two. Lines were generated by linear regression of Scatchard transformation of binding data (GraphPad Prism software).

Figure 2 Inactivation of $\beta\gamma$ requires a higher concentration of urea than does inactivation of α

Sf9 membranes expressing the α_{2A} -AR were extracted with the indicated urea concentrations. (A) Binding of 10 nM [³H]YOH was measured without reconstituted G-proteins. Binding of 1 nM [¹²⁵I]PIC was measured after reconstitution with (B) bovine brain $\beta\gamma$ (4–9 nM; 1:50 receptor/ $\beta\gamma$) or (C) myristoylated α_{11} (1–3 nM; 1:15 receptor/ α). Data show mean specific binding $\pm$ S.D. (A, $n = 2$) or $\pm$ S.E.M. (B and C, $n = 3$) of triplicate determinations.

($n = 2$). This loss of high-affinity agonist binding appears to reflect uncoupling of receptor from the endogenous Sf9 G-proteins. Surprisingly, myristoylated α_{11} subunits alone increased the [¹²⁵I]PIC $B_{\max}$ from 6 ± 2 to $21 \pm 2.5\%$ of total [³H]YOH $B_{\max}$, and there was a further increase to $36 \pm 2\%$ after co-reconstitution with α_{11} and $\beta\gamma$ ($n = 3$, Figure 1C). The affinity of [¹²⁵I]PIC increased upon addition of G-proteins (K_d of 1.1 ± 0.39 to 0.46 ± 0.11 nM, $n = 3$). Since α subunits alone were able to restore high-affinity agonist binding, it appeared that 5 M urea did not fully inactivate the endogenous Sf9 $\beta\gamma$.

To determine what concentration of urea could functionally inactivate α and $\beta\gamma$ subunits, we treated Sf9 membranes expressing α_{2A} -AR with urea concentrations from 0 to 7.5 M. Higher urea concentrations were not tested due to precipitation at 0 °C. Binding of the antagonist [³H]YOH was not significantly reduced by urea treatment (Figure 2A). As mentioned above, binding of the partial agonist [¹²⁵I]PIC was markedly reduced by 5 M urea. Figure 2(B) shows that reconstitution with $\beta\gamma$ does not restore agonist binding, indicating that the endogenous α subunit was inactivated by 5 M urea. While reconstitution with myristoylated α_{11} subunits restored high-affinity agonist binding to membranes treated with 5 or 6 M urea it did not for membranes treated with 7 M urea and above (Figure 2C). This indicates that

Figure 3 Both α and $\beta\gamma$ subunits are required to reconstitute high-affinity agonist binding after 7 M urea

Sf9 membranes expressing the α_{2A} -AR were extracted with 0 M (A) or 7 M urea (B). Where indicated, membranes were reconstituted with 2 nM myristoylated α_{11} and/or 10 nM bovine brain $\beta\gamma$ and binding of 1 nM [¹²⁵I]PIC determined. The sample with both α and $\beta\gamma$ was also tested with 10 μ M Gpp(NH)p. (C) Urea-extracted membranes were reconstituted with 2 nM myristoylated α_{11} and the indicated amounts of $\beta\gamma$ subunits. Specific binding of 1 nM [¹²⁵I]PIC was determined. Data are the means $\pm$ S.E.M. of triplicate determinations of a representative experiment repeated three times with similar results.

inactivation of $\beta\gamma$ subunits occurs at a higher concentration of urea than inactivation of α subunits.

To test directly the requirement for α and $\beta\gamma$ subunits for reconstitution of high-affinity α_{2A} -AR agonist binding after 7 M urea treatment, we added α_{11} and $\beta\gamma$ separately and together. Figure 3(B) shows the dramatic increase in agonist binding to 7 M urea-treated membranes with α and $\beta\gamma$ together (a 21 ± 3.7 -fold increase, $n = 3$). The binding was abolished by addition of Gpp(NH)p and neither α nor $\beta\gamma$ alone was sufficient to reconstitute high-affinity binding. This degree of reconstitution was not evident in 0 M urea-treated Sf9 membranes because of the high 'blank' binding ($9.3 \pm 1.8\%$ of [³H]YOH sites, $n = 6$) due to coupling to endogenous G-proteins (Figure 3A). The $\beta\gamma$ concentration dependence for reconstitution of 7 M urea-treated membranes is shown in Figure 3(C).

The functional uncoupling of α and $\beta\gamma$ subunits from receptor after 7 M urea treatment may have been due to denaturation, removal from the membrane, or both. To test this, we treated CHO cell membranes expressing the α_{2A} -AR with 0 or 7 M urea and performed Western blotting of G-protein subunits in the

Figure 4 Urea does not fully remove G-protein subunits from the membrane

CHO membranes expressing α_{2A} -AR were extracted with 7 M urea in buffer A or buffer A alone. Samples of membrane pellet (buffer-treated in lane 1, urea-treated in lane 2; reading left to right) and supernatant (buffer-treated in lane 3, urea-treated in lane 4) which represented equal amounts of starting material were loaded on to 10% polyacrylamide gels for SDS/PAGE. Top panels: Western blotting was performed as described in the Experimental section using (top panel) anti- α_i antibody (AS/7) or (bottom panel) anti- β -common antibody (SW/1). Densitometric scanning of immunostains is shown for α (B) and β (C). Data shown are from one extraction repeated with similar results.

pellet and supernatant. Immunostaining of α_i was of similar density in both the 0 and 7 M urea-treated pellets, but staining for β was moderately reduced after 7 M urea treatment (Figure 4). Neither α_i nor β reactivity was seen in the supernatants from control (0 M urea) extractions. The 7 M urea supernatant showed minimal immunostaining for α_i but a strong immunoreactivity for the β subunit. The total β subunit immunostaining from the sum of values for the pellet and supernatant was slightly greater than that for the starting membranes. Excess β staining in the 7 M urea supernatant may be due to increased epitope exposure in the presence of urea. Surprisingly, the β subunit is removed from the membrane to a greater extent than the α subunit.

We also tested this system using a functional response, receptor-catalysed [35 S]GTP[S] binding. Nucleotide binding was minimal after urea treatment and there was no stimulation upon α_{2A} -AR activation (Figure 5A). Reconstitution with either α or $\beta\gamma$ subunits alone gave no enhancement (results not shown), but on addition of heterotrimer, the α_{2A} -AR full agonist UK 14,304 stimulated GTP[S] binding on α subunits in a time-dependent manner (Figure 5B). The $\beta\gamma$ concentration dependence is shown in Figure 5(C). Half-saturation occurred at ≈ 10 nM $\beta\gamma$.

Finally, we explored the utility of 7 M urea treatment for reconstitution of three other receptors in Sf9 membranes and a

Figure 5 Urea (7 M)-extracted membranes reconstituted with G-proteins support agonist-regulated GTP[S] binding

Sf9 membranes expressing the α_{2A} -AR were extracted with 6.5 M urea and reconstituted with 3 nM α and 20 nM $\beta\gamma$. Membranes without (A) or with G-protein reconstitution (B) were incubated in the presence ($\blacktriangle$) or absence ($\triangle$) of 100 nM UK 14,304 for the indicated times and bound [35 S]GTP[S] determined, as described in the Experimental section. (C) Urea-extracted membranes were reconstituted with 2 nM α and the indicated amounts of $\beta\gamma$, and incubated with ($\blacktriangle$) or without ($\triangle$) 1 μ M UK 14,304. [35 S]GTP[S] binding was assayed at 40 min. All data are the means $\pm$ S.E.M. of triplicate determinations from one experiment representative of two.

mammalian cell line: adenosine A1 receptor, 5-HT $_{1A}$ -R and μ -opioid receptor. High-affinity specific agonist binding was assayed as described in the Experimental section, using [3 H]CCPA for adenosine A1 receptor, [3 H]8-OH-DPAT for 5-HT $_{1A}$ -R or [3 H]DAMGO for μ -opioid receptor. The α_{2A} -AR was expressed in CHO cells for comparison with the results in previous Figures where the receptor was expressed in Sf9 cells. As can be seen in Figure 6, for the four receptors in three cell systems, 7 M urea treatment resulted in very low agonist binding, which could be increased substantially upon reconstitution with heterotrimer. The increase in agonist binding ranged from 4- to 10-fold, with $\approx 50\%$ of the total antagonist sites bound by the agonist.

DISCUSSION

The denaturing effect of the chaotropic agent urea has been used in procedures ranging from protein unfolding (at 2–8 M) [30] to extraction of peripheral membrane proteins (5 M) [31]. This led

Figure 6 Urea (7 M) extraction in reconstitution studies using multiple cell systems and receptors

Membranes expressing the indicated receptor were extracted with 7 M urea. Receptor (0.3–2 nM) was reconstituted with a G-protein subunit/receptor ratio of $\approx 30:1$ α_{11} (10–45 nM) and/or $\approx 100:1$ bovine brain $\beta\gamma$ (30–150 nM) as described in the Experimental section. Following a 1 h incubation, membranes were diluted 5-fold and radiolabelled agonist binding performed as described in the text. (A) [3 H]CCPA (2 nM) binding to adenosine A1 receptor (A1-R) in Sf9 membranes. (B) [3 H]8-OH DPAT (2 nM) binding to 5-HT_{1A}-R in Sf9 membranes. (C) [125 I]PIC (1 nM) binding to α_{2A} -AR in CHO membranes. (D) [3 H]DAMGO (5 nM) binding to μ -opioid receptor (μ -R) in C₆ glioma membranes. Specific binding data are means $\pm$ S.E.M. of triplicate determinations (or $\pm$ S.D. of duplicate determinations for [125 I]PIC) from a single experiment representative of two or more independent experiments.

to its use in receptor–G-protein reconstitution studies where urea extraction uncoupled receptor from endogenous G-proteins without loss of receptor function while allowing recoupling to exogenously added G-proteins [18–20,32]. These experiments utilized from one to three extractions with 5, 6 or 7 M urea. The biochemical basis for urea-mediated uncoupling was unclear, as was the exact urea concentration required. By single extractions with a range of urea concentrations, we show in Sf9 cells that while the α subunit is functionally inactivated by 5 M urea, at least 7 M is required to attenuate $\beta\gamma$ activity. In contrast, receptor function is unaffected, as shown by the ability to couple to reconstituted G-proteins. Although urea extraction removed peripheral membrane proteins, urea-denatured G-proteins largely remain attached to the membrane. A key result of this study is the differential concentration dependence of urea-induced denaturation in the uncoupling of receptor from the two G-protein subunits.

Chaotropic agents denature proteins by disrupting hydrophobic interactions that normally stabilize native conformation. Urea reportedly binds proteins at amide groups and aromatic side chains [33] and binding to amide units is via hydrogen bonds [34]. This promotes hydrogen bonding of water molecules to hydrophilic groups [35] in the protein, leading to increased solvation and unfolding. Urea may not unfold proteins completely, as two studies have reported the presence of organized secondary structures in proteins even in 7–8 M urea [36,37]. There is evidence with hen egg-white lysozyme [38] and immunoglobulins [36] that unfolding is not a gradual phenomenon but follows a two-state behaviour with an abrupt transition zone at

midpoints of 5.6 and 6.5 M urea, respectively. Our data on functional inactivation of the α subunit with 5 M urea and the $\beta\gamma$ dimer at 7 M suggest that a similar two-state model applies in G-proteins.

Urea treatment does not attenuate receptor activity. This is the basis for its utility in studies involving receptors, or other transmembrane spanning proteins such as ion channels. It has been reported that stripping by 5 M urea of retinal rod outer-disk membranes removes peripheral proteins but not rhodopsin, as revealed by the ability to recover all photolysable rhodopsin activity after treatment [29]. Huber et al. [31] have shown by Western blotting that stripping by 5 M urea of *Calliphora* blowfly photoreceptor membranes removes peripheral proteins like InaD but not the Trp Ca²⁺ channel. This integral membrane protein is removed from the membrane only by detergent treatment, which disrupts the lipid bilayer [31]. Furthermore, urea treatment does not alter receptor specificity for ligand. Northup et al. have reported that after 6 M urea extraction of 5-HT_{2C} receptor and gastrin-releasing peptide receptor in membranes, receptors retain the predicted potency rank order for antagonist [18] and agonist [19].

Whereas urea treatment does not abrogate receptor function, it inactivates G-proteins. Urea treatment of *Calliphora* membranes has been reported to remove almost all of the peripheral membrane proteins InaD, NorpA and InaC [31]. Thus one possible mechanism for urea-mediated inactivation of G-proteins is via removal of G-proteins from membranes. However, our immunoblotting data do not support this hypothesis. They provide the first evidence that G-protein α_i subunit, and to some

degree the β subunit, remain membrane-bound after urea extraction. Hence urea-mediated uncoupling is mainly due to *in situ* denaturation of G-proteins. Acylation of the α subunit and prenylation of the γ subunit is required for the membrane localization of the α subunit and $\beta\gamma$ dimer respectively [4]. The inability of urea to remove G-proteins from membranes may be due to the high affinity of these lipid anchors for membrane lipids. The presence of β subunits in the 7 M urea-treated membrane supernatant could indicate a greater susceptibility of prenylated proteins (i.e. γ) to urea extraction compared with acylated proteins (i.e. α). Alternatively, some β subunits may have dissociated from the lipid-anchored γ . Urea (8 M) does dissociate β from γ , in a reaction mixture that additionally contains 1.5 M thiocyanate and 2 M NaCl [39].

It is interesting that while both receptor and G-proteins are exposed to urea, G-proteins but not receptor become inactivated. This differential result may be related to the structure and localization of these proteins. Anfinson [40] has shown in ribonuclease A that, after urea denaturation, removal of urea allows refolding to an active conformation. The urea-free incubation in our urea-treatment protocol may have led to sufficient renaturation of the receptor to regain function while the structure of G-proteins may be such that once unfolded it is unable to renature sufficiently for activity. Alternatively, receptor may be spared because its lipid-embedded transmembrane portion is less exposed to urea than are the peripheral proteins. Urea is known to be able to diffuse through the lipid bilayer [41] and is proposed to perturb the polar or hydrophobic interactions in the bilayer [42] but it has not been reported to solubilize membranes.

We have demonstrated a selective urea concentration-dependence for G-protein subunit inactivation. Although 5–7 M urea has been used for receptor uncoupling, we now show that 5 M urea extraction is sufficient for inactivation of $G\alpha$, but not $G\beta\gamma$. The latter is only inactivated by 7 M urea with this treatment protocol. This is in agreement with the observation that while 5 M urea extraction of rod outer-disk membranes inactivates α , there is residual $\beta\gamma$ activity [43]. Also, in Sf9 membranes treated with 6 M urea, a 3-fold enhancement of 5-HT_{2c}-activated GTP[S] binding occurred upon reconstitution with α alone, but not for $\beta\gamma$ alone [18]. Thus we show that urea inactivation of peripheral membrane proteins is concentration-dependent and must be optimized for the specific protein in question. We have previously inactivated G-proteins in platelet membranes by treatment with pH 11.5, but have found that it was critically dependant on the exact pH [14]. CHAPS has been used to strip $\beta\gamma$ in S49 cyc⁻ membranes, but there was significant reduction in forskolin and β -adrenergic receptor-stimulated cyclase activity [16]. In contrast, urea-mediated denaturation is less harsh and more reproducible. We have shown the utility of this technique in multiple cell types and receptors. Variants of this technique that have been reported include three consecutive urea extractions [18] or agonist preincubation followed by two consecutive urea extractions [19]. These steps are probably unnecessary as we demonstrate that a single extraction with 7 M urea is sufficient to functionally inactivate G-proteins.

Urea treatment of membranes inactivates G-proteins without attenuating receptor function. The utility of this technique has been demonstrated in various studies, including receptor- α subtype specificity [19,20], inverse agonist efficacy [18] and mutant receptor-G-protein coupling [21]. Although urea extraction removes membrane proteins, we provide evidence that G-protein inactivation is not just due to physical removal but to denaturation *in situ*. We further demonstrate that G-protein α and $\beta\gamma$ subunits are inactivated at differing concentrations of urea. Thus with optimization, it would be possible to use selective

urea concentrations to inactivate specific peripheral membrane proteins. This would allow the extension of this technique to study other members of the signalling network, including effectors like phospholipase C and regulatory components such as RGS.

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