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RESEARCH PAPER

Deletion of the distal COOH-terminus of the A_{2B} adenosine receptor switches internalization to an arrestin- and clathrin-independent pathway and inhibits recycling

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Background and purpose: We have investigated the effect of deletions of a postsynaptic density, disc large and zo-1 protein (PDZ) motif at the end of the COOH-terminus of the rat A_{2B} adenosine receptor on intracellular trafficking following long-term exposure to the agonist 5′-(N-ethylcarboxamido)-adenosine.
Experimental approach: The trafficking of the wild type A_{2B} adenosine receptor and deletion mutants expressed in Chinese hamster ovary cells was studied using an enzyme-linked immunosorbent assay in combination with immunofluorescence microscopy.
Key results: The wild type A_{2B} adenosine receptor and deletion mutants were all extensively internalized following prolonged treatment with NECA. The intracellular compartment through which the Gln^{325}-stop receptor mutant, which lacks the Type II PDZ motif found in the wild type receptor initially trafficked was not the same as the wild type receptor. Expression of dominant negative mutants of arrestin-2, dynamin or Eps-15 inhibited internalization of wild type and Leu^{330}-stop receptors, whereas only dominant negative mutant dynamin inhibited agonist-induced internalization of Gln^{325}-stop, Ser^{326}-stop and Phe^{328}-stop receptors. Following internalization, the wild type A_{2B} adenosine receptor recycled rapidly to the cell surface, whereas the Gln^{325}-stop receptor did not recycle.
Conclusions and implications: Deletion of the COOH-terminus of the A_{2B} adenosine receptor beyond Leu^{330} switches internalization from an arrestin- and clathrin-dependent pathway to one that is dynamin dependent but arrestin and clathrin independent. The presence of a Type II PDZ motif appears to be essential for arrestin- and clathrin-dependent internalization, as well as recycling of the A_{2B} adenosine receptor following prolonged agonist addition.
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Keywords: A_{2B} adenosine receptor; internalization; COOH-terminus deletion; arrestin

Abbreviations: CHO, Chinese hamster ovary; DMEM, Dulbecco's Modified Eagle Medium; DNM, dominant negative mutant; ELISA, enzyme-linked immunosorbent assay; EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; NECA, 5′-(N-ethylcarboxamido)-adenosine; NHERF1, Na^+/H^+ exchange regulatory factor-1; PBS, phosphate-buffered saline; PDZ, postsynaptic density, disc large and zo-1 protein; ; RIPA buffer, radioimmunoprecipitation assay buffer; Ro 201724, 4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TE, Tris EDTA buffer; XAC, xanthine amine congener

Introduction

Desensitization of G protein-coupled receptor (GPCR) responses usually occurs following the prolonged or repeated exposure of the receptor to agonist (Krupnick and Benovic, 1998; Kelly *et al.*, 2008). The mechanisms underlying agonist-induced desensitization of GPCRs often involve phosphorylation by a family of G protein-coupled receptor kinases (GRKs), followed by binding of arrestins, which trigger

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desensitization by uncoupling the receptor from G-protein (Pitcher *et al.*, 1998; Premont and Gainetdinov, 2007). Following desensitization, many GPCRs appear to internalize by an arrestin-dependent process via clathrin-coated pits, although other internalization pathways for GPCRs also exist (Ferguson, 2001; Moore *et al.*, 2007). In most cases, GPCR internalization leads to the eventual dephosphorylation of the receptor and its re-insertion into the cell membrane in a re-sensitized state. More prolonged agonist activation generally leads to the redirection of internalized receptor to a lysosomal compartment with subsequent down-regulation, although some GPCRs appear to be targeted for down-regulation after relatively short agonist treatment times (Marchese *et al.*, 2008). However, many of the molecular signals that determine the pathways of GPCR desensitization, internalization or down-regulation remain to be clarified.

Four adenosine GPCRs have now been cloned, namely the A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (Olah and Stiles, 1995; Jacobson and Gao, 2006; Klaasse *et al.*, 2008; nomenclature follows Alexander *et al.*, 2008). Both the A_{2A} and A_{2B} adenosine receptors are G_s-coupled and stimulate cAMP formation, and the A_{2B} adenosine receptor subtype is thought to regulate such diverse processes as vascular tone and mast cell activation (Feoktistov and Biaggioni, 1997), and represents a potential therapeutic target (Jacobson and Gao, 2006). A_{2A} and A_{2B} adenosine receptors are subject to agonist-induced desensitization and internalization in various cell types (Klaasse *et al.*, 2008). We have previously shown (Matharu *et al.*, 2001), using a series of receptor deletion and point mutants, that amino acid residues close to the COOH-terminus of the A_{2B} adenosine receptor are critical for the rapid (<1 h) agonist-induced desensitization and arrestin-dependent internalization of the receptor.

The last four amino acids of the A_{2B} adenosine receptor (Ser-Leu-Ser-Leu in rat, Gly-Val-Gly-Leu in human) correspond to a Type II postsynaptic density, disc large and zo-1 protein (PDZ) motif (x-Ø-x-Ø, where x is any amino acid and Ø corresponds to a bulky hydrophobic reside). PDZ motifs, found at the very end of the COOH-terminus of a number of GPCRs, bind to PDZ domain-containing proteins, which have established roles in the localization and assembly of signalling complexes, and, more recently, have been suggested to control GPCR traffic (Weinman *et al.*, 2006; Marchese *et al.*, 2008; Houslay, 2009). In the present study, we investigated the role of the COOH-terminus of the A_{2B} adenosine receptor in regulation following more prolonged (up to 24 h) pretreatment with agonist. Our results indicate that removal of the PDZ motif on the COOH-terminus of the A_{2B} adenosine receptor causes internalization of the receptor to switch from an arrestin- and clathrin-dependent mechanism to one which is arrestin and clathrin independent, and also inhibits receptor recycling upon removal of agonist.

Methods

Cell culture, constructs and transfections

The cloning and stable expression in Chinese hamster ovary (CHO) cells of the N-terminus HA-tagged rat A_{2B} adenosine receptor and deletion mutants (Figure 1), as well as A_{2B} adenosine receptor-green fluorescent protein (GFP), have been previously described in detail (Matharu *et al.*, 2001). Stably transfected CHO cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) : F12 (50:50) medium, 10% fetal calf serum (FCS), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin supplemented with 600 μg mL^{-1} geneticin. For transient transfections with pcDNA3, wild type A_{2B} adenosine receptor, Phe^{328}-stop mutant, rat-4human chimera, with the dominant negative mutant constructs pcDNA3-arrestin-2-(319-418), pcDNA3-dynamin-K44A or enhanced green fluorescent protein-C2-Eps15 (EΔ95-295) (EGFP-C2-Eps15), with wild type construct pRK RhoA or dominant negative mutant construct pRK RhoA(T19N), or with myc-tagged Na^+-H^+ exchanger regulatory factor 1 (NHERF1), CHO cells were grown in 60 mm dishes to 60–80% confluency and transfected with 3–6 μg of DNA in 2 mL medium using Fugene-6 following the manufacturer's instructions. Cells were incubated with a DNA/Fugene mixture for 24 h, the media was replaced and the cells analysed 24–48 h after transfection.

Whole cell cAMP accumulation

CHO cells were seeded into 24-well multi-tray culture plates and grown at 37°C under 5% CO_2 and humidified conditions. In pretreatment experiments, medium was replaced by 0.5 mL of prewarmed medium containing 5′-(N-ethylcarboxamido)-adenosine (NECA; 10 μM) or water (control). At various times after this, the medium was removed and wells washed three times with 1 mL ice-cold phosphate buffered saline (PBS). Following this, 0.5 mL of prewarmed fresh medium (without FCS) containing 250 μM of the phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one (Ro 201724, 250 μM), was added to each well, followed immediately with either NECA (10 μM) or water (control). This was incubated for 20 min at 37°C, and the reaction was terminated by the addition of 20 μL of ice-cold trichloroacetic acid (100%). In non-pretreatment experiments, 0.5 mL of prewarmed fresh medium (without FCS) containing 250 μM Ro 201 724 was added to each well, followed 15 min later with either NECA (10 μM) or water (control). This was incubated for 20 min at 37°C, and the reaction was terminated by the addition of 20 μL of ice-cold trichloroacetic acid (100%).

Fifty microlitres of the supernatant was transferred to a fresh tube containing 50 μL of NaOH (1 M) and 200 μL of Tris (50 mM, pH 7.4) and EDTA (4 mM) (TE buffer). A further 100 μL of this mixture was transferred to a fresh tube containing 50 μL of TE buffer, 100 μL of [^{3}H]-cAMP in TE buffer (about 20 000 c.p.m.) and 100 μL of cAMP binding protein (to give a final concentration of ~750 μg of protein mL^{-1}, prepared from bovine adrenal cortex). Tubes containing 50 μL of cAMP (0.125–40 pmol) were used to construct a standard curve. After 90 min incubation at 4°C, 200 μL of TE buffer containing charcoal (50 mg mL^{-1} final concentration) and bovine serum albumin (BSA) (2 mg mL^{-1} final concentration) was added to each tube. After 15 min, bound and non-bound [^{3}H]-cAMP were separated by centrifugation at 2900× *g* for 15 min at 4°C. The resulting supernatant was transferred into vials for liquid scintillation spectroscopy. Standard curve data

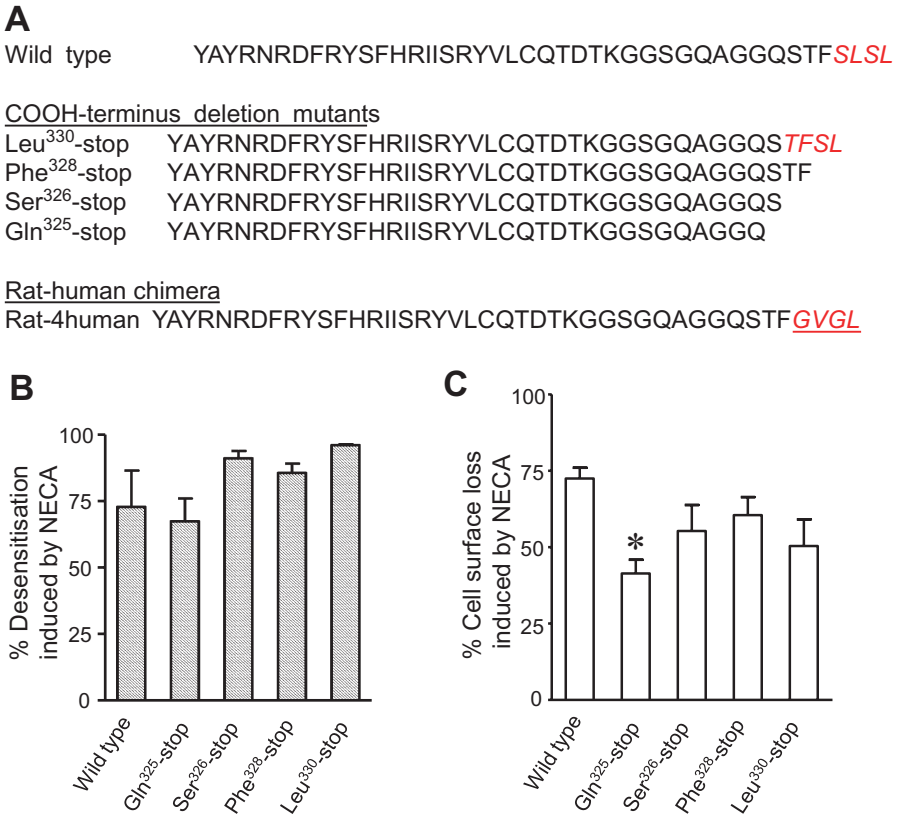

Figure 1 (A) Sequence comparison of the COOH-terminus domains of the rat A_{2B} adenosine receptor constructs studied. Ser^{329}, previously identified as crucial for rapid arrestin-dependent A_{2B} adenosine receptor desensitization and internalization (Matharu *et al.*, 2001), is marked in bold. The four amino acids from the human A_{2B}-AR adenosine receptor sequence that were added to the rat Phe^{328}-stop construct to form the rat-4human chimera are underlined. Residues that correspond to a Type II PDZ motif are in red italics. (B) Agonist-induced desensitization of wild type and mutant A_{2B} adenosine receptors in response to prolonged treatment with 5′-(N-ethylcarboxamido)-adenosine (NECA) (10 μM, 24 h). Following agonist pretreatment, cells were washed, and cyclic AMP accumulation measured in response to 10 μM NECA for 20 min. In each case, the level of 10 μM NECA-stimulated cAMP accumulation in non-pretreated cells was taken as 0% desensitization. Data are means ± SEM from three independent experiments. (C) Cell surface loss of wild type and mutant A_{2B} adenosine receptors in response to prolonged treatment with NECA (10 μM, 24 h). In each case, the cell surface loss was calculated by comparing levels in the presence of NECA to levels in the absence of NECA treatment (taken as 0% cell surface loss). Data are means ± SEM from three independent experiments. Note: In this series of experiments, the agonist-induced loss in cells expressing Gln^{325}-stop was less extensive than in cells expressing wild type A_{2B} adenosine receptor. *$P < 0.05$, one-way ANOVA with Dunnett's post-test.

were fitted to a one-site competition model: Y = Bottom + [(Top − Bottom)/1 + $10^{X-LogEC50}$] (GraphPad Prism, GraphPad Software, La Jolla, CA, USA). The protein content of the cell monolayers was determined, and cAMP accumulation expressed as pmol cAMP mg^{-1} protein, or as a % of the respective control NECA-stimulated cAMP accumulation.

Agonist-induced cell surface loss and recycling of A_{2B} adenosine receptor constructs

Loss of A_{2B} adenosine receptor constructs from the cell surface was assessed by enzyme-linked immunosorbent assay (ELISA), as described previously (Mundell *et al.*, 2000). Briefly, cells plated at a density of approximately 6×10^5 cells per 60 mm dish were split 24 h post-transfection into 24-well tissue culture dishes coated with 0.1 mg mL^{-1} poly-L-lysine. Twenty-four hours later, cells were incubated with DMEM containing NECA (0.1 μM–1 μM) for 0–24 h at 37°C. In experiments assessing receptor recycling, NECA-containing media was removed and replaced with DMEM containing the adenosine receptor antagonist xanthine amine congener (XAC; 10 μM). Reactions were stopped by removing the media and fixing the cells with 3.7% formaldehyde in Tris-buffered saline (TBS) (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM CaCl2) for 5 min at room temperature. Fixation over this period produces minimal cell permeabilization (Mundell *et al.*, 2002). Cells were washed three times with TBS, incubated for 45 min with TBS containing 1% BSA (TBS/BSA), then incubated with a primary antibody (anti-HA monoclonal HA-11 antibody, 1:1000 dilution in TBS/BSA) for 1 h at room temperature. Cells were washed three times with TBS, reblocked with TBS/BSA for 15 min at room temperature and incubated with secondary antibody (goat anti-mouse secondary antibody conjugated with alkaline phosphatase; 1:1000 dilution in TBS/BSA) for 1 h at room temperature. Cells were washed three times with TBS, and a colourimetric alkaline phosphatase substrate added. When adequate colour change was achieved, 100 μL of sample was added to 100 μL of 0.4 M NaOH to terminate the reaction, and the samples were read at 405 nm using a microplate reader.

Immunofluorescence microscopy and single cell imaging
To assess the cellular distribution of wild type and mutant A_{2B} adenosine receptor, CHO cells stably transfected with these constructs were grown in 6-well plates on coverslips. Cells were then incubated with primary antibody (anti-HA monoclonal HA-11; 1:200 dilution) for 1 h in DMEM supplemented with 1% BSA. For experiments analysing transferrin distribution, cells were also incubated for 1 h, at 4°C with 200 μg mL^{-1} rhodamine-conjugated transferrin. For experiments analysing lysosomal distribution, cells were also incubated for 1 h, at 37°C with 100 nM Lysotracker Red. Cells were washed twice with PBS, and then incubated at 37°C ± NECA (10 μM; 0–24 h) in DMEM with 0.5% BSA. There was no obvious difference in transferrin or Lysotracker Red localization in cells that were pre-incubated with these markers prior to addition of agonist or when added 30 min before cell fixation. The cells were then fixed with 3.7% formaldehyde in PBS for 15 min at room temperature, washed with PBS and permeabilized with 0.05% Triton X-100 in PBS for 10 min at room temperature. Non-specific binding was blocked with 'blotto' (0.05% Triton X-100 in PBS containing 5% non-fat dry milk) for 30 min at 37°C. Where appropriate, goat anti-mouse rhodamine- or fluorescein-conjugated secondary antibody was then added at a dilution of 1:150 in 'blotto' for 1 h at 37°C. In experiments assessing Rab7 or Rab11 localization, cells were incubated with rabbit polyclonal anti-Rab7 or anti-Rab11 antibodies at a 1:200 dilution in 'blotto' for 1 h at 37°C, followed by incubation with goat anti-rabbit rhodamine-conjugated secondary antibody at a dilution of 1:200 in 'blotto' for 1 h at 37°C. The cells were then washed six times with 0.05% Triton X-100/ PBS, and the last wash left for 37°C for 30 min. Finally, the cells were fixed again with 3.7% formaldehyde as described. Coverslips were mounted using Slow-Fade mounting medium and examined by microscopy on an upright Leica TCS-NT (Leica Microsystems, Milton Keynes, Bucks, UK) confocal laser scanning microscope attached to a Leica DM IRBE epifluorescence microscope with phase-contrast and a Plan-Apo 40 × 1.40 NA oil immersion objective. In experiments assessing the effect of dominant negative mutant Eps-15 expression on wild type A_{2B} and Gln^{325}-stop receptor redistribution, CHO cells stably expressing wild type or Gln^{325}-stop receptor were transiently transfected as described above with 5 μg EGFP-C2-Eps15 (EΔ95-295). Cells were then split onto coverslips in 6-well plates, and 24 h later receptor redistribution assessed as described above. All images were collected on Leica TCS-NT software for two- and three-dimensional image analysis and processed on Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA, USA).

Arrestin translocation
Arrestin-2-GFP re-distribution was assessed as described previously (Mundell *et al*., 2001). Briefly, CHO cells stably expressing receptor contructs grown on poly-L-lysine coverslips were transiently transfected with 0.5 μg of pEGFP-N1-arrestin-2 and incubated for 16 to 24 h before use. Cells were then washed three times with PBS before imaging, the coverslips being mounted in a heated (37°C) imaging chamber through which media and drugs could be added. Cells were examined by microscopy on an inverted Leica TCS-NT confocal laser scanning microscope attached to a Leica DM IRBE epifluorescence microscope with phase contrast and a Plan-Apo 60 × 1.40 numerical aperture oil immersion objective. Similar acquisition settings (laser intensity and channel gains) were used throughout. All images were collected on Leica TCS-NT software for two- and three-dimensional image analysis and processed using Photoshop 6.0.

Immunoprecipitation and Western blotting
Following agonist treatment, cells were washed three times in ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 10 mM EDTA, 150 mM NaCl, 0.5% deoxycholate, 0.1% sodium dodecyl sulphate, 1% Triton-X-100, pH 7.5) containing protease inhibitors (Roche). Antibody–protein complexes were precipitated by incubation in the presence of anti-HA antibody (HA-11; 3 μg/sample) and protein G/A–agarose for 24 h at 4°C. Beads were washed three times with buffer before addition of 2× Laemmli sample solvent and boiling for 5 min. Following transfer, myc-tagged NHERF1 was detected using an anti-myc antibody and enhanced chemiluminescence. The extent of immunoprecipitation was quantified by densitometric analysis of resulting autoradiographs.

Data analysis
Data are presented as mean ± SEM. The time courses of agonist-induced desensitization and internalization were analysed by fitting data from each experiment to a single-phase exponential decay: $Y = Span.e^{-K.X} + plateau$ (GraphPaD Prism) to provide individual values of $t_{0.5}$ and plateau, the mean ± SEM of which are presented in Table 1. For Figure 2, single exponentials were fitted to the mean data points for purposes of presentation. Statistical analysis was performed either by one-way analysis of variance with either Dunnett's or Bonferonni's post-test, or by Student's *t*-test. Differences were considered significant when $P < 0.05$.

Materials
The pcDNA3 vector was obtained from Invitrogen (Paisley, Scotland). The construct EGFP-C2-Eps15 (EΔ95-295) was a kind gift from Dr A Benmerah (Institut Cochin, Université Paris Descartes, Paris, France). Fugene-6 was obtained from Roche Molecular Biochemicals (Lewes, East Sussex, UK). DMEM : F12 (50:50) was from Life Technologies (Paisley, Scotland), fetal calf serum from Harlan Sera Labs (Loughborough, Leicestershire, UK) and geneticin (G418 sulphate) from Calbiochem (San Diego, CA, USA). The anti-HA monoclonal HA-11 antibody was from BAbCO (Richmond, CA, USA). Goat anti-mouse rhodamine-conjugated or fluorescein-conjugated secondary antibody, goat anti-rabbit rhodamine-conjugated secondary antibody, Lysotracker Red, rhodamine-conjugated transferrin and Slow-Fade Mounting Medium were all from Molecular Probes (Eugene, OR, USA). Rabbit-polyclonal Rab7 and Rab11 antibodies were purchased from Autogen Bioclear (Calne, Wiltshire, UK). The goat anti-

mouse secondary antibody conjugated with alkaline phosphatase was from Sigma-Aldrich (Poole, Dorset, UK). Colourimetric alkaline phosphatase substrate was from Biorad (Hemel Hempstead, Hertfordshire, UK). [^{3}H]-cAMP was obtained from Amersham International (Little Chalfont, Buckinghamshire, UK). All other biochemical reagents including NECA, XAC and Ro 201724 were obtained from Sigma-Aldrich.

Results

Agonist-induced desensitization and cell surface loss of A_{2B} adenosine receptor constructs

CHO cells stably expressing wild type or mutant A_{2B} adenosine receptors (Figure 1A) have been characterized previously (Matharu *et al*., 2001). Sequential deletion of the COOH-terminus of this receptor has produced constructs that possess, as does the full-length receptor, a type II PDZ motif (Leu^{330}stop), a type I PDZ motif (Phe^{328}stop), or a receptor that lacks any PDZ-binding motif (Ser^{326}stop and Gln^{325}stop). Pretreatment of cells stably expressing wild type, Gln^{325}-stop, Ser^{326}-stop, Phe^{328}-stop or Leu^{330}-stop A_{2B} adenosine receptor constructs with the adenosine-receptor agonist NECA (10 μM) for 24 h led to a marked loss (>40% in each case) of cell surface receptor as measured by ELISA (Figure 1C). This pretreatment (NECA, 10 μM for 24 h) also led to extensive desensitization (>70% in each case) of subsequent NECA-stimulated (10 μM; 20 min) cAMP accumulation for all constructs (Figure 1B). The time course of desensitization and cell surface loss in response to different concentrations of NECA in cells expressing wild type or Gln^{325}-stop A_{2B} adenosine receptor constructs was characterized in more detail (Figure 2; Table 1). Desensitization of both constructs was agonist concentration dependent, with the Gln^{325}-stop receptor response desensitizing more slowly, but after 24 h pretreatment with 1 or 10 μM NECA, the level of desensitization for each construct was similar at 70–80% (Figure 2A, C and E; Table 1). Cell surface loss of both wild type and Gln^{325}-stop A_{2B} adenosine receptors was also time- and agonist concentration dependent, with cell surface loss of the Gln^{325}-stop mutant being somewhat slower than that of the wild type A_{2B} adenosine receptor (Figure 2B, D and F; Table 1). In most cases, for each concentration of NECA, the rate of desensitization was not faster than the rate of cell surface loss for either the wild type A_{2B} adenosine receptor or the Gln^{325}-stop receptor mutant (Table 1).

Subcellular localization of internalized receptor constructs

Confocal imaging was then undertaken in order to investigate the intracellular trafficking of A_{2B} adenosine receptor constructs following prolonged incubation with agonist. In a first series of experiments, co-localization of A_{2B} adenosine receptor constructs and transferrin receptors was studied before and after prolonged incubation of cells with NECA (10 μM). Cells were incubated with rhodamine-labelled transferrin and anti-HA antibody for up to 1 h at 4°C. After this time, cells were incubated in the presence or absence of NECA for 1, 6 or 24 h, and the co-localization of receptor construct and rhodamine-labelled transferrin, which labels early endosomes and the endocytic recycling compartment (Cao *et al*., 1998) examined in fixed cells. Without agonist addition, wild type receptor (green) was localized to the cell membrane, while rhodamine-labelled transferrin bound to the constitutively internalizing transferrin receptor (red) was detected intracellularly (Figure 3, top left panel). Addition of NECA (10 μM) to the cells for 1 or 6 h caused extensive internalization of wild

Table 1 Time- and concentration-dependent desensitization and internalization of the wild type A_{2B} adenosine receptor and the Gln^{325}-stop receptor mutant

A) NECA concentration	*$t_{0.5}$ (h)*			
	Desensitization		*Internalization*	
	Wild type	*Gln^{325}-stop*	*Wild type*	*Gln^{325}-stop*
0.1 μM	4.1 ± 1.0	–	2.3 ± 0.6	6.0 ± 1.8[a]
1 μM	1.5 ± 0.5	5.3 ± 1.7	1.6 ± 0.7	3.6 ± 1.2
10 μM	0.5 ± 0.1	2.4 ± 0.5	0.9 ± 0.2	1.4 ± 0.3
B) NECA concentration	*Plateau (% of control)*			
	Desensitization		*Internalization*	
	Wild type	*Gln^{325}-stop*	*Wild type*	*Gln^{325}-stop*
0.1 μM	13.7 ± 6.6	–	60.4 ± 3.2	71.4 ± 3.7
1 μM	15.9 ± 7.8	17.1 ± 5.9	42.9 ± 4.9	58.7 ± 5.3
10 μM	16.9 ± 7.0	28.2 ± 7.0	34.7 ± 7.1	43.2 ± 10.5

Data from individual experiments were fitted to one phase exponential decay curves to obtain values of (A) $t_{0.5}$ and (B) plateau (i.e. maximum reduction). The $t_{0.5}$ and plateau values are presented in the table as mean ± SEM from three individual experiments for desensitization and four to five individual experiments for internalization.

[a]The difference in $t_{0.5}$ for internalization between wild type and Gln^{325}-stop cells was significant at 0.1 μM NECA (one-way ANOVA with Bonferroni post-test). The differences between wild type and Gln^{325}-stop cells for desensitization or internalization did not reach statistical significance at other NECA concentrations. It was not possible to fit data to one phase exponential decay curves for Gln^{325}-stop cell desensitization at 0.1 μM NECA.

NECA, 5′-(N-ethylcarboxamido)-adenosine.

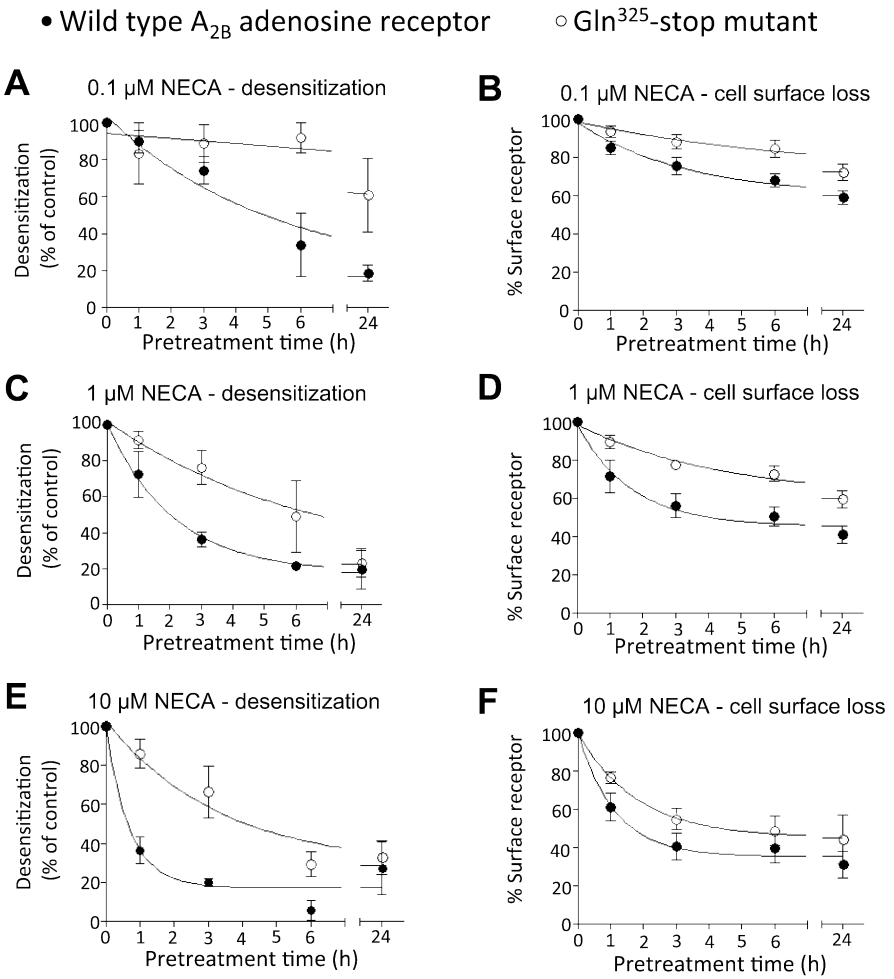

Figure 2 Time course and agonist concentration dependence of desensitization (A, C and E) or cell surface loss (B, D and F) of wild type A_{2B} adenosine receptor and the Gln^{325}-stop deletion mutant. Chinese hamster ovary cells stably expressing wild type A_{2B} adenosine receptor or Gln^{325}-stop receptor were incubated with 0.1 μM (A, B), 1 μM (C, D) or 10 μM (E, F) NECA for up to 24 h. To assess desensitization, cells were washed, and 10 μM 5′-(N-ethylcarboxamido)-adenosine (NECA)-stimulated cAMP accumulation measured. In each case, 10 μM NECA-stimulated cAMP accumulation in non-pretreated cells was taken as 100%. Cell surface receptor loss was measured by enzyme-linked immunosorbent assay, with cell surface receptor expression in non-pretreated cells being taken as 100%. Values are means ± SEM from three independent experiments for desensitization and four to five independent experiments for cell surface loss. In this figure, for each condition, mean data were fitted to the equation for a one phase exponential decay curve.

type receptor to transferrin receptor-positive compartments, seen as a yellow co-localization of receptor and rhodamine-labelled transferrin (Figure 3, wild type A_{2B} 1 h and 6 h). After 24 h with agonist, wild type receptor could still be detected intracellularly, but the degree of co-localization with transferrin was less than at earlier time points (Figure 3, wild type A_{2B} 24 h; see also Supporting Information Figure S1). In the absence of agonist, the Gln^{325}-stop receptor mutant was also localized to the cell membrane (Figure 3, bottom left). Upon agonist addition, the mutant receptor internalized extensively, however, at 1 h or 6 h after agonist addition, it was not co-localized with rhodamine-labelled transferrin (note absence of yellow overlap in Figure 3, Gln^{325}-stop 1 h and 6 h, as compared to yellow overlap in wild type A_{2B} 1 h and 6 h). However, as with wild type (Figure 3 wild type A_{2B} 24 h), after 24 h treatment with NECA, the Gln^{325}-stop receptor mutant appeared to congregate in central compartments that were not rhodamine-labelled transferrin positive (Figure 3, Gln^{325}-stop 24 h). Trafficking of the Ser^{326}-stop receptor mutant was similar to that of the Gln^{325}-stop receptor mutant (Supporting Information Figure S1).

Next, the co-localization of A_{2B} adenosine receptor constructs and low-pH lysosomal compartments was studied after prolonged incubation of cells with NECA. Cells were incubated with Lysotracker Red to label lysosomes (Tarasova *et al.*, 1997) and anti-HA antibody for 1 h at 37°C. After this time, NECA was added to cells for 6 or 24 h, and the co-localization of receptor construct and Lysotracker Red examined in fixed cells. After 6 h of agonist addition, wild type receptor partially co-localized with Lysotracker Red (Figure 4A, top left panel), but this co-localization was extensive after 24 h of agonist addition (yellow colour in Figure 4A, top right panel). In a similar way, treatment of cells expressing Gln^{325}-stop and Ser^{326}-stop receptor mutants with NECA for 6 h led to partial co-localization of receptor and Lysotracker Red (Figure 4A, middle left and lower left panels), while treatment with NECA

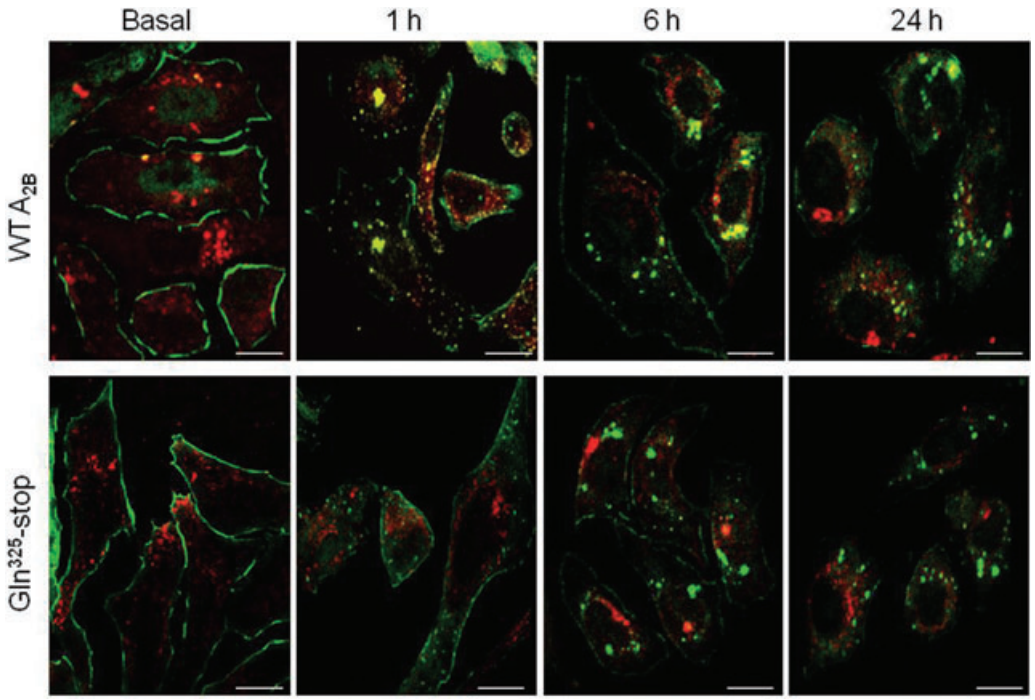

Figure 3 Cellular redistribution of wild type A_{2B} adenosine receptor (top panels), or Gln^{325}-stop mutant (lower panels) following prolonged agonist addition: co-localization of receptor with rhodamine-labelled transferrin. Chinese hamster ovary cells stably expressing the above constructs were incubated with an anti-HA antibody and rhodamine-labelled transferrin at 4°C for 1 h. Subsequently, receptor construct (green) and rhodamine-labelled transferrin (red) were visualized under basal conditions without agonist addition (left panels) or following addition of 10 μM 5′-(N-ethylcarboxamido)-adenosine for 1, 6 or 24 h at 37°C. Receptor and rhodamine-labelled transferrin localization was determined by immunofluorescence in fixed cells as described under Methods. A significant degree of wild type receptor/rhodamine-labelled transferrin co-localization (yellow) is clearly visible following agonist addition for 1 and 6 h. Data shown are representative of three independent experiments with >10 fields of cells captured per experiment. White scale bars represents 10 μm.

for 24 h led to extensive co-localization of the deletion mutants with Lysotracker Red (Figure 4A, middle right and lower right panels). It is possible that the anti-HA antibody dissociates from the receptor during the prolonged (24 h) agonist incubation period. To assess this possibility, CHO cells stably transfected with wild type A_{2B}-GFP receptor (Matharu *et al.*, 2001) were transiently transfected with HA-tagged wild type A_{2B} adenosine receptor. The cells were then incubated with anti-HA antibody for 1 h, then NECA for 24 h. Under these conditions, the A_{2B}-GFP receptor and anti-HA antibody were co-localized intra-cellularly, as evidenced by the yellow colour observable within the cells (Figure 4B). This strongly suggests that the anti-HA antibody remains attached to the receptor after 24 h of incubation.

Mechanism of loss of A_{2B} adenosine receptor constructs from the cell surface

Cells were transiently transfected with dominant negative mutant constructs of arrestin-2 [arrestin-2-(319-418)], dynamin (dynamin K44A) or Eps-15 (EΔ95-295). The cells were then incubated with 10 μM NECA for 24 h, and the loss of receptor from the cell surface assessed (Figure 5A). Whereas the agonist-induced cell surface loss of the wild type and Leu^{330}-stop receptors was strongly inhibited by all three dominant negative mutants, only dominant negative mutant dynamin inhibited cell surface loss of the Gln^{325}-stop, Ser^{326}-stop and Phe^{328}-stop mutants. The findings with dominant negative mutant Eps-15 were confirmed by confocal microscopy of NECA-treated (10 μM; 24 h) cells co-expressing dominant negative mutant Eps-15 and either wild type receptor or the Gln^{325}-stop construct (Figure 6). The transiently transfected dominant negative mutant Eps-15 construct was distributed in a similar fashion in cells stably expressing wild type (upper middle panel) or Gln^{325}-stop receptor (lower middle panel). Whereas only cell surface localization of wild type receptor can be seen in a cell transiently transfected with dominant negative mutant Eps-15 and incubated with 10 μM NECA for 24 h (Figure 6, upper left panel), widespread intracellular Gln^{325}-stop localization is observed in a similarly dominant negative mutant Eps-15 transfected and NECA-treated cell (lower left panel). Thus the dominant negative mutant Eps-15 strongly inhibits agonist-induced cell surface loss of the wild type A_{2B} adenosine receptor, but not the Gln^{325}-stop receptor mutant.

A dynamin-dependent, but clathrin-independent, pathway for the internalization of interleukin 2 receptors that is blocked by a dominant negative mutant of the small G protein RhoA has been described (Lamaze *et al.*, 2001). We therefore assessed whether transient overexpression of the dominant negative mutant form of RhoA (RhoA T19N) could block agonist-induced cell surface loss of the Gln^{325}-stop receptor. However, neither overexpression of wild type RhoA nor RhoA T19N was able to affect NECA-induced cell surface loss of wild type A_{2B} adenosine receptor or the Gln^{325}-stop receptor mutant (Figure 5B).

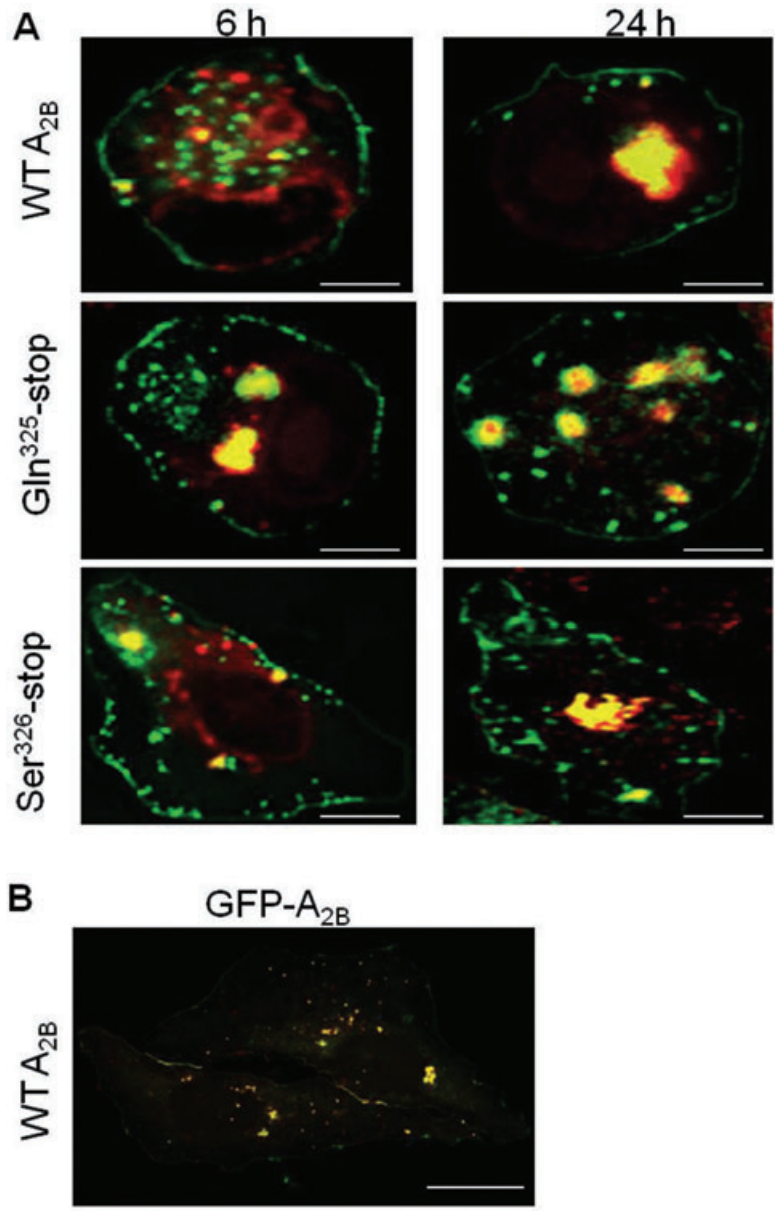

Figure 4 Co-localization of A_{2B} adenosine receptor constructs with lysosomal marker. (A) Cellular redistribution of wild type A_{2B} adenosine receptor (top panels), Gln^{325}-stop (middle panels) and Ser^{326}-stop receptors (lower panels) following prolonged agonist addition: co-localization of receptor with Lysotracker Red. CHO cells stably expressing the above constructs were incubated with an anti-HA antibody and Lysotracker Red at 37°C. Subsequently, receptor construct (green) and Lysotracker Red (red) were visualized following 10 μM 5′-(N-ethylcarboxamido)-adenosine (NECA) for 6 h or 24 h. Receptor and Lysotracker Red localization was determined by immunofluorescence in fixed cells as described under Methods. A high degree of receptor/Lysotracker Red co-localization (yellow) is clearly visible for all three receptor constructs following agonist addition for 6 or 24 h. Data shown are representative of three independent experiments with >10 fields of cells captured per experiment. White scale bars represent 10 μm. (B) Co-localization of wild type A_{2B}-green fluorescent protein (GFP) receptor and anti-HA antibody in Chinese hamster ovary cells stably transfected with wild type A_{2B}-GFP receptor and transiently transfected with HA-tagged wild type A_{2B} adenosine receptor. The cells were incubated with anti-HA antibody at 4°C for 1 h, then NECA for 24 h at 37°C. Under these conditions, the A_{2B}-GFP receptor and anti-HA antibody co-localized in intracellular compartments, as demonstrated by the yellow colour observable within the cells. Data shown are representative of three independent experiments with >10 fields of cells captured per experiment. White scale bar represents 10 μm.

The effect of hypertonic sucrose, reported to inhibit clathrin-coated pit formation (Heuser and Anderson, 1989) on agonist-induced cell surface loss of selected A_{2B} adenosine receptor constructs was also assessed (Figure 5C). Long-term treatment with hypertonic sucrose is toxic to cells, so for these experiments, treatment with agonist for 2 h instead of 24 h was used. Interestingly, hypertonic sucrose (0.45 M) was able to strongly inhibit the agonist-induced cell surface loss of not only the wild type A_{2B} adenosine receptor, which is clathrin-dependent, but also the Gln^{325}-stop and Ser^{326}-stop mutants, which undergo clathrin-independent internalization (Figure 5C).

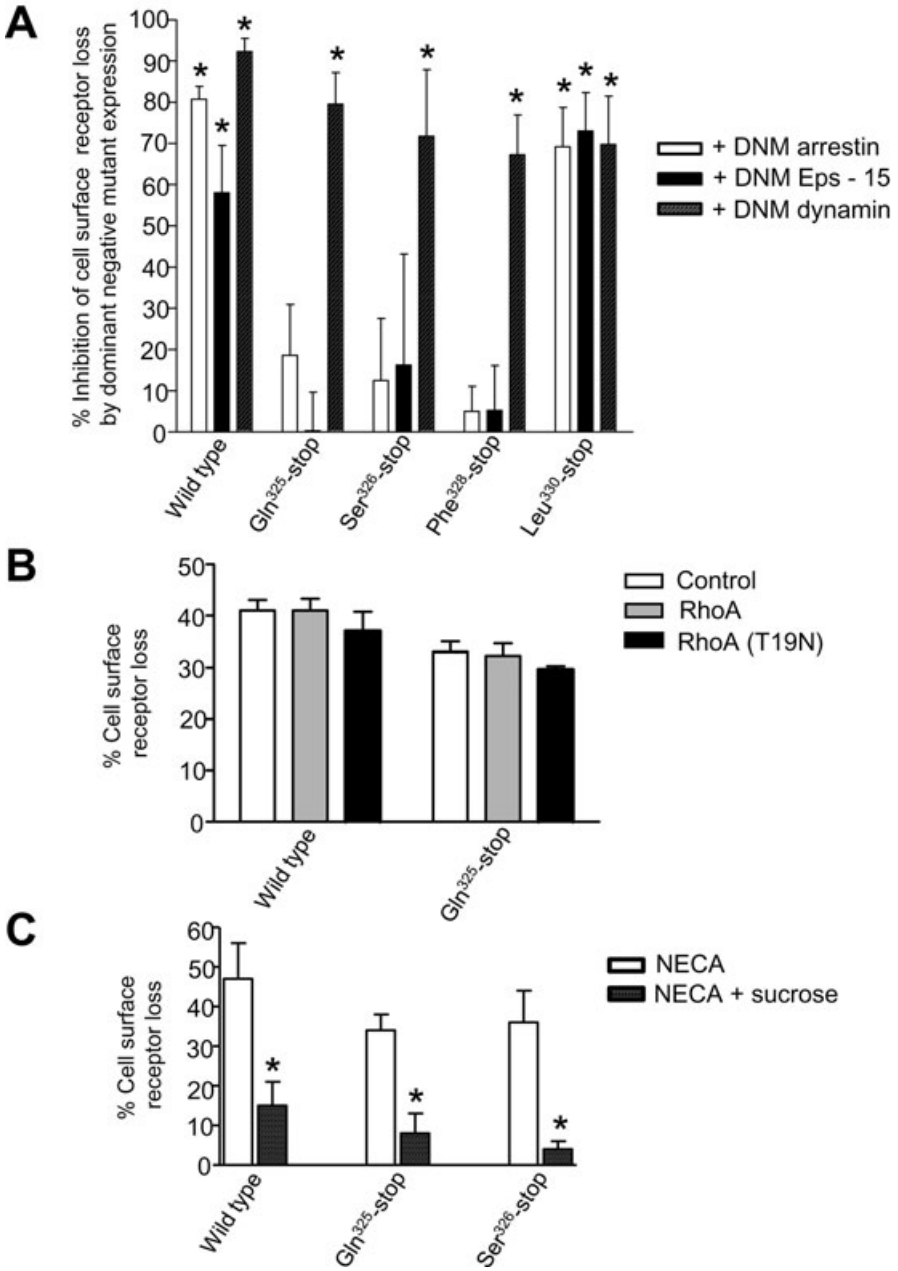

Figure 5 (A) Ability of transient transfection with dominant negative mutants (DNM) of arrestin-2, Eps-15 and dynamin to inhibit 5′-(N-ethylcarboxamido)-adenosine (NECA)-induced cell surface loss of wild type A_{2B} adenosine receptor, Gln^{325}-stop, Ser^{326}-stop, Phe^{328}-stop and Leu^{330}-stop receptor constructs stably expressed in Chinese hamster ovary cells. At 48 h following transient transfection with dominant negative mutant constructs, cells were challenged with NECA (10 μM; 24 h) and surface receptor loss assessed by enzyme-linked immunosorbent assay (ELISA). Note that all three dominant negative mutant constructs strongly inhibit agonist-induced cell surface loss of wild type A_{2B} adenosine receptor and the Leu^{330}-stop receptor construct, whereas only dominant negative mutant dynamin is able to inhibit the agonist-induced internalization of the Gln^{325}-stop, Ser^{326}-stop, Phe^{328}-stop receptor constructs. The data represent mean ± SEM of at least four independent experiments in each case. *$P < 0.05$ different from vector-transfected control cells, one-way ANOVA with Bonferroni post-test. (B) Lack of effect of wild type RhoA or dominant negative mutant RhoA T19N on the NECA-induced cell surface loss of wild type A_{2B} adenosine receptor or Gln^{325}-stop mutant. Cells were transiently transfected with wild type RhoA or RhoA T19N, and 48 h later used in the ELISA assay to determine NECA-induced (10 μM; 2 h) cell surface receptor loss. Neither RhoA construct affected cell surface loss of the receptor constructs. Values are means ± SEM for three independent experiments. (C) Hypertonic sucrose blocks the agonist-induced cell surface loss of the wild type A_{2B} adenosine receptor, as well as the Gln^{325}-stop and Ser^{326}-stop receptors. Cells were pretreated in the presence or absence of 0.45 M sucrose for 15 min before addition of 10 μM NECA for a further 2 h in the continued presence or absence of sucrose. Cell surface receptor loss was measured by ELISA. Controls (0% cell surface loss) were constituted by cells not treated with NECA. Values are means ± SEM for four independent experiments. *$P < 0.05$ different from corresponding values for NECA alone, Student's *t*-test.

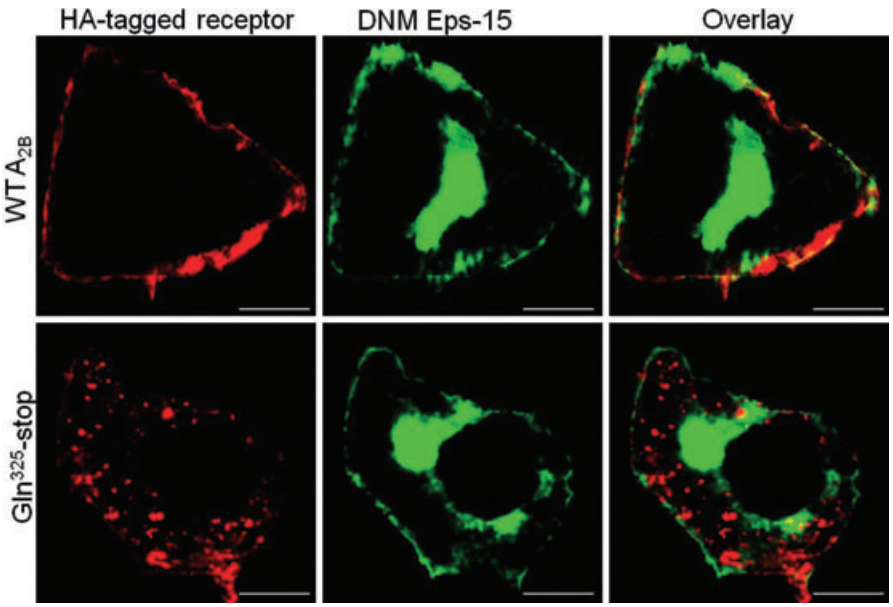

Figure 6 Cellular redistribution of wild type A_{2B} adenosine receptor (top panels) or Gln^{325}-stop (lower panels) following 24 h agonist addition in the presence of dominant negative mutant (DNM) Eps-15-GFP. Chinese hamster ovary cells stably expressing the above receptor constructs were transiently transfected with dominant negative mutant Eps-15-green fluorescent protein (GFP); 48 h later, cells were challenged with 5′-(N-ethylcarboxamido)-adenosine (10 μM; 24 h). Following this, receptor construct (left panels, red) and dominant negative mutant Eps-15-GFP (middle panels, green) localization was determined by immunofluorescence in fixed cells as described under Methods. The overlay of receptor construct and dominant negative mutant Eps-15-GFP is shown in the right-hand panels. Note that dominant negative mutant Eps-15-GFP completely blocks agonist-induced wild type A_{2B} adenosine receptor internalization, but there is still extensive Gln^{325}-stop internalization. Data shown are representative of three independent experiments with >10 fields of cells captured per experiment. White scale bars represent 10 μm.

A_{2B} adenosine receptor construct recycling and NHERF1 interaction

The A_{2B} adenosine receptor was previously reported to interact with a member of the Na^+-H^+ exchanger regulatory factor family, NHERF2 (Sitaraman *et al.*, 2002). Since NHERF proteins contain PDZ protein interaction domains and can regulate the trafficking of some GPCRs (Weinman *et al.*, 2006), we examined whether overexpression of NHERF1 affected the agonist-induced cell surface loss of the A_{2B} receptor constructs. Transient overexpression of NHERF1 markedly inhibited the NECA-induced cell surface loss of wild type A_{2B} adenosine receptor, but had no effect on that of the Gln^{325}-stop receptor (Figure 7A). In addition, in cells overexpressing NHERF1, when the receptor constructs were immunoprecipitated from CHO cells with an anti-HA antibody, NHERF1 could be detected in the immunoprecipitate with wild type A_{2B} receptor, but not with Gln^{325}-stop receptor (Figure 7B). Interestingly, the interaction of NHERF1 with the wild type A_{2B} adenosine receptor was also reduced upon acute agonist addition. Together, these results indicate that the PDZ motif at the COOH-terminus of the A_{2B} adenosine receptor is necessary for the physical and functional interaction of NHERF1 with the receptor.

Since NHERF proteins can modulate the intracellular trafficking of some GPCRs by interacting with receptor COOH-terminus PDZ motifs (Weinman *et al.*, 2006), we investigated whether removal of the PDZ motif could affect A_{2B} adenosine receptor recycling. Following pretreatment of cells with NECA for 1 h, cell surface loss of both wild type A_{2B} adenosine receptor and Gln^{325}-stop receptor could be detected (Figure 8A). As we have previously reported (Mundell *et al.*, 2000), over the 90 min period following removal of agonist, the amount of cell surface wild type receptor returned to over 90% of that observed before agonist administration at time 0. In contrast, there was no recovery of the level of cell surface Gln^{325}-stop receptor (Figure 8A). To further investigate the intracellular location of the internalized receptors, the co-localization of the small G proteins Rab11 and Rab7 (markers for recycling endosome and endosome to lysosome pathways, respectively; Seachrist and Ferguson, 2003) with the wild type A_{2B} adenosine receptor and Gln^{325}-stop receptor was determined using anti-Rab11 and anti-Rab7 antibodies. Following agonist addition (NECA; 10 μM for 2 h), the co-localization of Rab11 with wild type A_{2B} adenosine receptor was evident (yellow colour indicated by white arrows in Figure 8B, top left panel), but no co-localization of Rab11 with Gln^{325}-stop could be detected (Figure 8B, bottom left panel). On the other hand, both wild type A_{2B} adenosine receptor and Gln^{325}-stop receptor co-localized with Rab7 (yellow colour in Figure 8B, right-hand panels). Together, these results indicate that following agonist-induced internalization, the wild type A_{2B}-adenosine receptor can recycle back to the cell surface, but with prolonged agonist addition, it is eventually targeted to lysosomes, whereas the Gln^{325}-stop receptor internalizes and does not recycle but is targeted to lysosomes. It is also possible that the presence and absence of recycling for wild type and Gln^{325}-stop receptor respectively means that we have underestimated the difference in internalization rates between the two constructs, as recycling of wild type receptor may lead to a lower estimation of internalization than would be if it did not recycle.

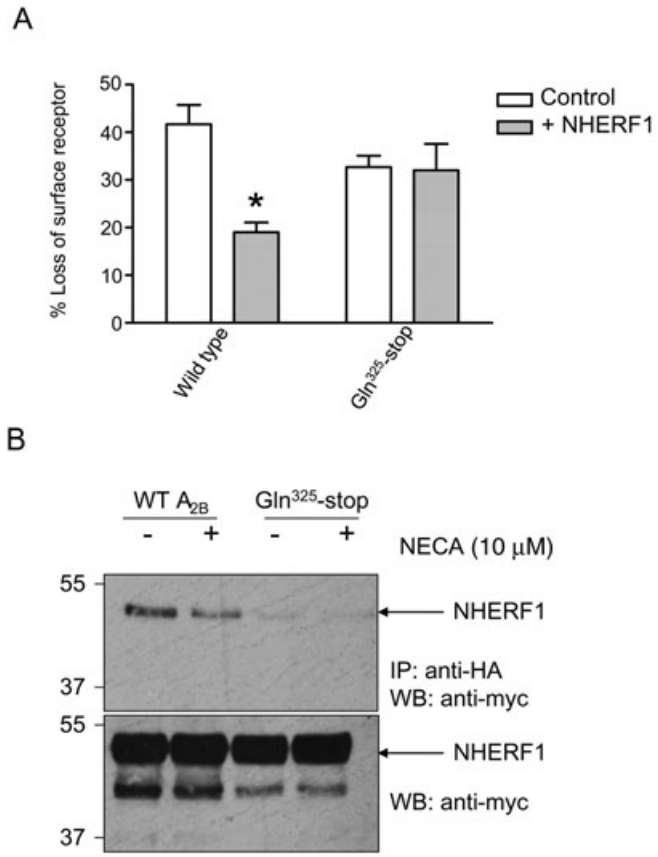

Figure 7 Functional interaction of Na^+/H^+ exchange regulatory factor-1 (NHERF1) with the A_{2B} adenosine receptor. (A) Transient overexpression of NHERF1 inhibits the 5′-(N-ethylcarboxamido)-adenosine (NECA)-induced (10 μM; 2 h) internalization of the wild type A_{2B} adenosine receptor, but not that of the Gln^{325}-stop receptor mutant. Values are means ± SEM for three independent experiments. *$P < 0.05$ different from respective NECA in vector only-transfected cells, Student's *t*-test. (B) NHERF1 coimmunoprecipitates with wild type A_{2B} but not Gln^{325}-stop receptor. Chinese hamster ovary cells transiently overexpressing myc-tagged NHERF1 and stably expressing either wild type A_{2B} adenosine receptor or Gln^{325}-stop receptor mutant were incubated in the presence or absence of NECA (10 μM; 30 min). HA-tagged receptors were immunoprecipitated, and samples were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis. Expression of NHERF1 in cell lysates (10% input), detected with an anti-myc antibody, is shown in the lower panel. Agonist exposure reduced the association of NHERF1 with the wild type receptor. The experiment was repeated one further time with the same result.

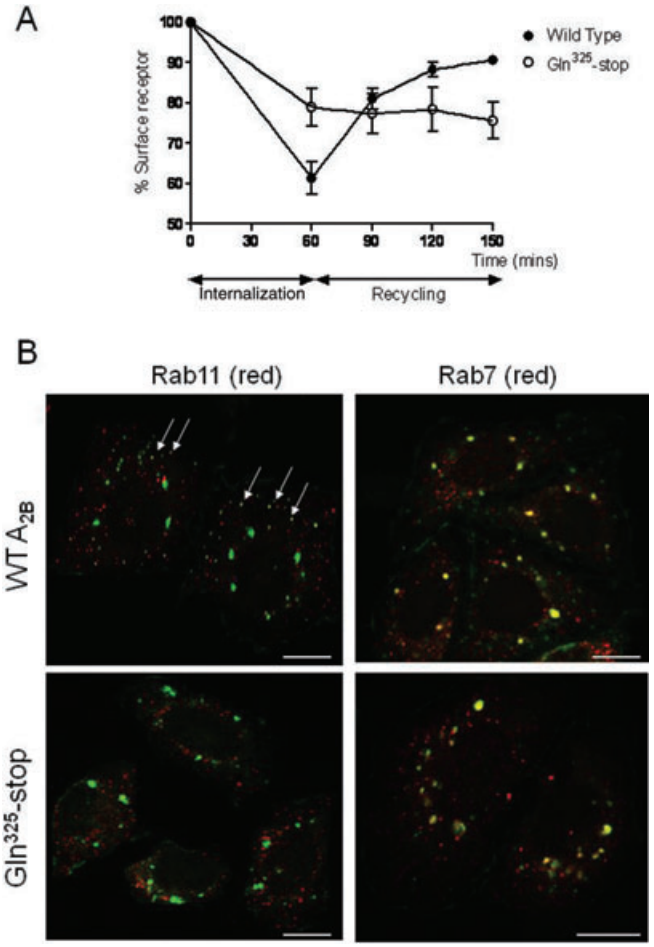

Figure 8 A_{2B} adenosine receptor recycling. (A) Cell surface loss of the wild type and Gln^{325}-stop receptor was induced by pretreatment with 5′-(N-ethylcarboxamido)-adenosine (NECA) (10 μM; 1 h), and recovery was evaluated for 90 min following agonist removal and addition of the A_2 adenosine receptor antagonist, xanthine amine congener (10 μM). Changes in cell surface expression were measured by enzyme-linked immunosorbent assay. Data represent mean ± SEM of three independent experiments. (B) Cellular redistribution of wild type A_{2B} adenosine receptor (top panels) or Gln^{325}-stop (lower panels) following prolonged agonist addition: co-localization of receptor with Rab11 (marker for recycling endosome; left-hand panels) or Rab7 (marker for endosome to lysosome pathway; right-hand panels). Chinese hamster ovary cells stably expressing the above constructs were incubated with an anti-HA antibody 4°C for 1 h. Subsequently, receptor construct (green) and rhodamine-labelled Rab proteins (red) were visualized following addition of NECA (10 μM; 6 h). Receptor and antibody-labelled Rab protein localization was determined by immunofluorescence in fixed cells as described under Methods. A significant degree of wild type receptor/rhodamine-labelled Rab11 co-localization (yellow) is visible (top left panel, white arrows), but none was evident in cells expressing Gln^{325}-stop receptor (bottom left panel). On the other hand, rhodamine-labelled Rab7 co-localized with both wild type and Gln^{325}-stop receptor (right hand panel). Data shown are representative of three independent experiments with >10 fields of cells captured per experiment. Scale bars represent 10 μm.

A_{2B} adenosine receptor chimera function

An intriguing final question is whether Ser^{329} in the PDZ motif of the A_{2B} adenosine receptor represents a phospho-acceptor site for phosphorylation by a GRK or other kinase, which then facilitates arrestin association with the receptor. However, we have been unable to detect agonist-induced phosphorylation of the wild type A_{2B} adenosine receptor, even when GRK2 has been transiently overexpressed in the cells (data not shown). In the absence of evidence for receptor phosphorylation, we examined receptor trafficking using a chimeric receptor containing a Type II PDZ motif, which does not contain a phospho-acceptor site. Accordingly, we replaced the final four residues (SLSL) of the rat A_{2B} adenosine receptor with those of the human A_{2B} adenosine receptor (GVGL). This construct, a rat-human A_{2B} adenosine receptor chimera (rat-4human), was transiently expressed in CHO cells and underwent rapid agonist-induced desensitization; following treatment for 1 h with 10 μM NECA; subsequent 10 μM NECA-stimulated cAMP

accumulation was 61.5 ± 16.4% of non-treated cells for wild type A_{2B} adenosine receptor and 72.0 ± 6.2% of non-treated cells for rat-4human receptor (means ± SEM, $n = 3$). In addition, rat-4human also internalized in response to agonist more efficiently than the Phe^{328}-stop mutant; following treatment for 1 h with 10 μM NECA, cell surface receptor expression had decreased to 38.6 ± 6.6% of non-treated control for wild type A_{2B} adenosine receptor, 61.6 ± 4.2% of non-treated control for rat-4human cells and 84.0 ± 4.4% of non-treated control for Phe^{328}-stop receptor cells (means ± SEM, $n = 3$). Most interestingly, like the wild type A_{2B} adenosine receptor, the chimera supported NECA-induced arrestin-2-GFP translocation, whereas the Phe^{328}-stop mutant did not (Figure 9).

Discussion

Using a series of A_{2B} adenosine receptor deletion and point mutants, we have previously shown that a serine residue (Ser^{329}), four residues from the COOH-terminus and within a type II PDZ motif (SLSL), of the A_{2B} adenosine receptor, is critical for rapid agonist-induced receptor desensitization and internalization (Matharu *et al.*, 2001). In that study, two deletion mutants lacking this PDZ motif were identified (Phe^{328}-stop and Gln^{325}-stop), which displayed relatively little desensitization or internalization following treatment with agonist for up to 1 h. In the present study, the effect of more prolonged agonist treatment on the trafficking of wild type and deletion mutant A_{2B} adenosine receptors was examined. Of these deletion mutants, Leu^{330}, like the wild-type receptor, possesses a type II PDZ motif. Following treatment of CHO cells stably expressing wild type, Gln^{325}-stop, Ser^{326}-stop, Phe^{328}-stop or Leu^{330}-stop A_{2B} adenosine receptors with NECA for 24 h, each displayed extensive desensitization and internalization. This indicates that Ser^{329} and/or the PDZ motif are important determinants of rapid agonist-induced desensitization and internalization, but not for regulation following more prolonged agonist treatment. This is similar to findings with the A_{2A} adenosine receptor (Palmer and Stiles, 1997), where mutation of a single threonine residue in the COOH-terminus of the receptor inhibited rapid agonist-induced desensitization, but not that following 24 h agonist addition. In the present study, the rate and extent of desensitization and internalization of the wild type and Gln^{325}-stop receptor in response to treatment with different agonist concentrations for up to 24 h was compared. This revealed that the Gln^{325}-stop receptor desensitizes and internalizes more slowly than wild type receptor. This is in line with findings from our previous study (Matharu *et al.*, 2001), where the acute (up to 1 h) agonist-induced desensitization and internalization of the wild type A_{2B} adenosine receptor was much more extensive than that of the Gln^{325}-stop receptor. This suggests that the mechanism of internalization of the Gln^{325}-stop receptor may be different from that of the wild type receptor, and since the Gln^{325}-stop receptor is, unlike the wild type receptor, not able to support arrestin translocation from cytosol to cell membrane (Matharu *et al.*, 2001), it seems likely that different mechanisms underlie the internalization of these two receptor constructs.

The results from ELISA experiments in the current study are presented as 'cell surface loss' rather than 'internalization'. Although it is likely that most of the cell surface receptor loss is due to internalization, we cannot always assume this, particularly following prolonged agonist incubation times of up to 24 h. Altered receptor recycling, down-regulation, synthesis of new receptors and the trafficking of new receptors to the cell membrane could also play a part in determining the overall cell surface receptor level. This is important when considering that PDZ motifs are considered to be important for intracellular trafficking of GPCRs (Cao *et al.*, 1999), particularly since we report here that removal of the PDZ motif inhibits receptor recycling. Notwithstanding, we have referred to cell surface loss as internalization in this discussion for the sake of simplicity.

Many GPCRs have been shown to internalize via a clathrin-dependent pathway (Moore *et al.*, 2007), utilized also by transferrin receptors (von Zastrow and Kobilka, 1992; Tsao *et al.*, 2001). In the present study, agonist addition for 1 or 6 h led to the co-localization of the wild type receptor with rhodamine-labelled transferrin, which labels early endosomes and the endocytic recycling compartment (Cao *et al.*, 1998). We have previously observed a similar co-localization for the A_{2B} adenosine receptor with transferrin in early and recycling endosomes in HEK293 cells (Mundell *et al.*, 2000). In contrast, for mutant receptor constructs lacking the PDZ motif, there was little or no co-localization of Gln^{325}-stop or Ser^{326}-stop with rhodamine-labelled transferrin at 1 or 6 h after agonist addition, suggesting that these two deletion mutants internalize via a clathrin-independent pathway. Following 24 h of agonist addition, none of the constructs (wild type, Ser^{326}-stop and Gln^{325}-stop) was co-localized with rhodamine-labelled transferrin, but found in lysosomes as assessed by co-localization with Lysotracker Red after 6 h, and particularly so after 24 h agonist treatment. Therefore, although the initial trafficking pathway utilized by the receptor constructs lacking a PDZ motif was different from that used by wild type receptors, they were eventually delivered to the same lysosomal compartment. This data would suggest that the presence of a PDZ motif in the A_{2B} adenosine receptor, although not required for down-regulation of receptor into lysosomes, is certainly critical for determining the initial endocytic route taken by this receptor.

The different internalization pathways utilized by the wild type A_{2B} adenosine receptor and Leu^{330}-stop receptors as opposed to the Gln^{325}-stop, Ser^{326}-stop and Phe^{328}-stop receptors was confirmed by the use of dominant negative mutant constructs of arrestin-2, dynamin and Eps-15. Dominant negative mutant arrestin-2 [arrestin-2-(319-418)] contains the clathrin binding domain of arrestin-2, but not the putative receptor interaction site; it constitutively interacts with clathrin and blocks the internalization of a number of GPCRs (Krupnick *et al.*, 1997; Mundell *et al.*, 2001). Dominant negative mutant dynamin (dynamin K44A) does not express the GTPase activity required for this protein to promote the formation of clathrin-coated vesicles (Damke *et al.*, 1994), and inhibits the internalization of many GPCRs (Mundell *et al.*, 2001; Mundell *et al.*, 2006). The Eps15 protein binds to the clathrin adaptor protein AP-2 and is required for normal formation of clathrin-coated pits (Benmerah *et al.*, 1998); the dominant negative mutant Eps-15 (EΔ95-295) blocks clathrin-coated pit formation and the internalization of transferrin

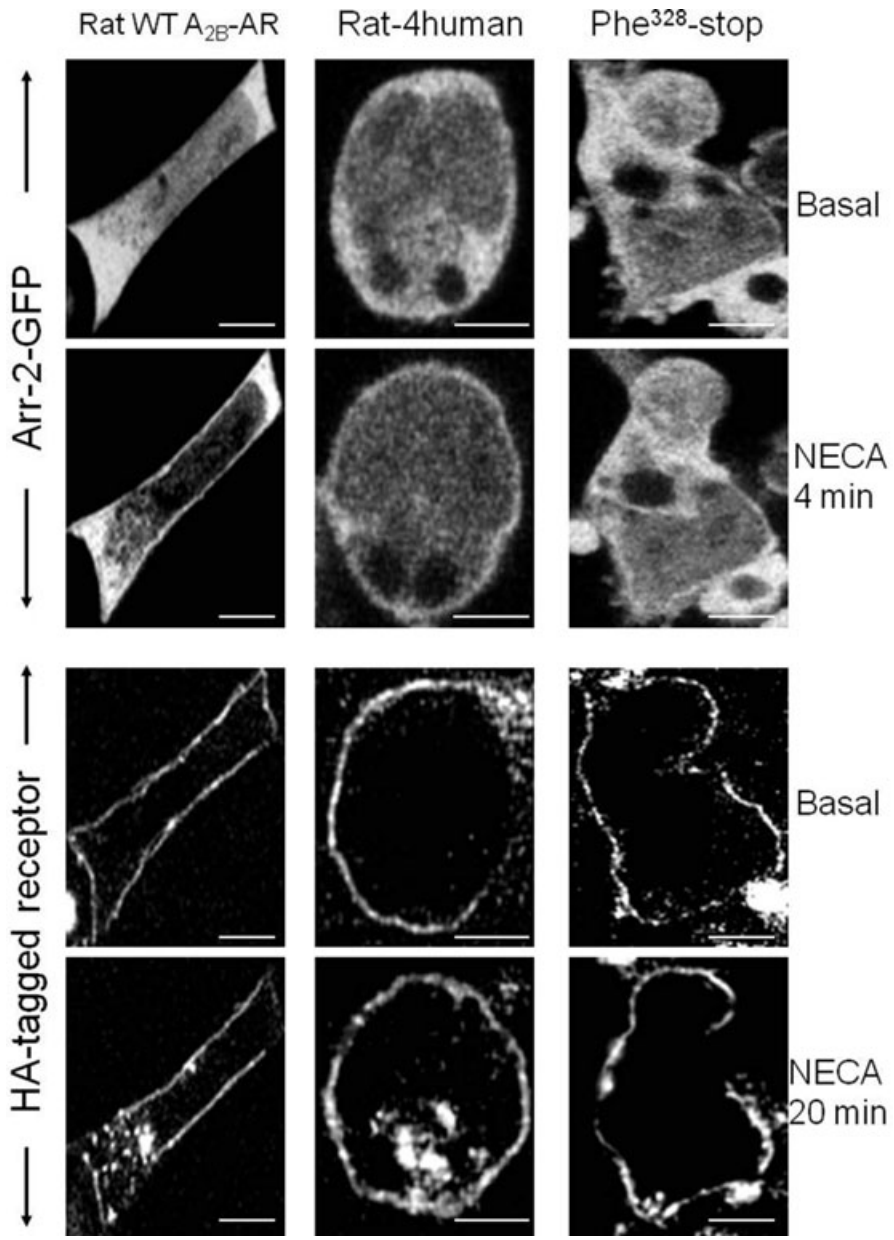

Figure 9 Live cell imaging of arrestin-2-green fluorescent protein (GFP) translocation (upper panels) and receptor trafficking (lower panels) following agonist activation of the rat-4human A_{2B} adenosine receptor chimera. CHO cells stably expressing wild type A_{2B} adenosine receptor, the rat-4human A_{2B} adenosine receptor chimera or the Phe^{328}-stop receptor construct were co-transfected with arrestin-2-GFP (Arr-2-GFP). The same cell is shown in each of the three columns in the figure, with the initial diffuse cytoplasmic distribution of arrestin-2-GFP being evident (top panels, basal). 5′-(N-ethylcarboxamido)-adenosine (NECA) (10 μM) was added for 4 min and the redistribution of arrestin-2-GFP monitored in real time. Agonist-induced arrestin-2-GFP translocation was only observed in cells expressing the wild type A_{2B} adenosine receptor and the rat-4human A_{2B} adenosine receptor chimera, but not in cells expressing the Phe^{328}-stop receptor construct. Receptor trafficking (lower panels) was monitored before agonist addition and then 20 min following NECA using a rhodamine-conjugated anti-HA antibody. The initial plasma membrane location of the receptor constructs is shown prior to NECA addition (basal), then NECA was added for 20 min, and the redistribution of receptor constructs visualized. Internalization of the wild type A_{2B} adenosine receptor and the rat-4human A_{2B} adenosine receptor chimera could clearly be seen, but not for the Phe^{328}-stop receptor construct. Note that the same cells are shown in the upper and lower panels for each construct. Data shown are representative of three independent experiments, with >10 fields of cells captured per experiment. White scale bars represent 10 μm.

receptors (Benmerah *et al*., 2000), as well as a number of GPCRs (Mundell *et al*., 2001; Mundell *et al*., 2006). We have previously shown that the agonist-induced rapid internalization of wild type A_{2B} adenosine receptor is blocked by dominant negative constructs of each of these proteins (Matharu *et al*., 2001). In the present study, all three dominant negative mutant constructs also blocked the long-term agonist-induced internalization of the wild type and Leu^{330}-stop receptor, but only dominant negative mutant dynamin blocked internalization of Gln^{325}-stop, Ser^{326}-stop and Phe^{328}-stop receptors. This supports the findings with rhodamine-labelled transferrin, and indicates that receptor constructs

lacking the Type II PDZ motif internalize via a dynamin-dependent but arrestin- and clathrin-independent pathway. Such an internalization pathway has been described for the interleukin 2 receptor, and is inhibited by a dominant negative mutant (T19N) of the small GTP binding protein RhoA (Lamaze *et al.*, 2001). However, the lack of effect of this mutant on Gln^{325}-stop internalization indicates that another clathrin-independent, dynamin-dependent pathway (Doherty and McMahon, 2009; Hansen and Nichols, 2009) is involved, the nature of which remains to be identified.

Other GPCRs are reported to internalize by a dynamin-dependent but arrestin-independent pathway (Claing *et al.*, 2000; Paing *et al.*, 2002; van Koppen and Jakobs, 2004), while the M_2 muscarinic acetylcholine receptor can internalize via a dynamin-dependent but clathrin- and caveolae-independent pathway (Roseberry and Hosey, 2001). More recently, the D_3 dopamine receptor was shown to undergo PKC-mediated internalization in an arrestin- and caveolin-independent but dynamin-dependent fashion that involves the actin-binding protein filamin A (Cho *et al.*, 2007). Whatever the mechanisms of internalization of the A_{2B} adenosine receptor constructs used in the present study, it appears that they all eventually accumulate in a lysosomal compartment after a number of hours of agonist treatment. This indicates that the mutant A_{2B} adenosine receptors still contain the necessary sequence information to traffic to this intracellular compartment. The A_{2B} adenosine receptor does possess a motif (YVLC) in the COOH-terminus (Figure 1A) that somewhat resembles the YXXØ (where Ø is a bulky hydrophobic residue) sequence implicated in endosome/lysosome targeting of diverse proteins (Bonifacino and Traub, 2003) and recently shown to target the GAL_1 galanin receptor to lysosomes (Xia *et al.*, 2008). It is unclear if the cysteine, in the YVLC motif of the A_{2B} receptor, although hydrophobic, is sufficiently bulky to promote such targeting. Unfortunately, we have been unable to quantify down-regulation of A_{2B} adenosine receptor constructs in the present study, since the HA-tagged receptors are not detected by Western blot (data not shown), and a suitable radioligand for the A_{2B} adenosine receptor remains to be characterized. However, it seems likely that the wild type A_{2B} adenosine receptor and the deletion mutants all down-regulate in lysosomes.

In the presence of dominant negative mutant arrestin or dominant negative mutant Eps-15, the wild type receptor did not appear to undergo significant internalization via the non-clathrin route, even after 24 h agonist treatment. This indicates that the internalization mechanism of the A_{2B} adenosine receptor, at least in CHO cells, is strictly controlled by the COOH-terminus sequence. The residue Ser^{329} found within the PDZ motif is obligatory for agonist-induced arrestin- and clathrin-dependent internalization, and the presence of this residue (or at least the PDZ motif found in Leu^{330} stop) appears to suppress entry into the non-clathrin pathway. Only when the receptor loses this PDZ motif and is truncated beyond Ser^{329} does the receptor switch significantly to the clathrin-independent pathway.

The results of this study also indicate that care must be taken when analysing the effects of mutations on GPCR trafficking, since at least for the A_{2B} adenosine receptor, COOH-terminus truncation not only alters the kinetics of internalization, but also the mechanism and pathway involved in the trafficking, including the recycling. Furthermore, although hypertonic sucrose is still widely used to selectively inhibit clathrin-dependent GPCR internalization, we show here that hypertonic sucrose can inhibit both clathrin-dependent and clathrin-independent GPCR internalization, and conclusions drawn from its use should therefore be treated with caution.

The PDZ motif of the A_{2B} adenosine receptor has been shown to bind to the PDZ domain-containing protein E3KARP/NHERF2, which regulates the signalling of this receptor (Sitaraman *et al.*, 2002). It has been suggested that this interaction may not only anchor the A_{2B} adenosine receptor to the plasma membrane, but may also function to stabilize the receptor in a signalling complex at the plasma membrane (Sitaraman *et al.*, 2002). To investigate the effects of NHERF protein expression on A_{2B} adenosine receptor trafficking, NHERF1 was overexpressed in CHO cells and was found to inhibit wild type receptor internalization, but not that of the Gln^{325}-stop receptor. In this respect, the effects of NHERF1 on the A_{2B} adenosine receptor are similar to those on the PTH_1 parathyroid hormone receptor, which is also dependent upon a PDZ motif at the COOH terminus of this receptor (Sneddon *et al.*, 2003; Wang *et al.*, 2009). In addition, NHERF protein interaction with some GPCRs is reported to regulate their recycling and down-regulation, as found for β_2- adrenoceptors (Cao *et al.*, 1999) and κ-opioid receptors (Li *et al.*, 2002), respectively. It was therefore of interest to find that the Gln^{325}-stop receptor did not recycle, suggesting that interactions of scaffolding proteins such as NHERF1 with the PDZ motif at the COOH terminus of the wild type A_{2B} receptor are crucial for receptor recycling. Interestingly, NHERF1 expression enhances the recycling of both β_2-adrenoceptors and κ-opioid receptors following internalization (Cao *et al.*, 1999; Li *et al.* 2002.) The idea that a scaffolding protein can regulate A_{2B} adenosine receptor trafficking is further supported by the data presented here showing that transiently expressed NHERF1 is constitutively associated with the wild type receptor. Although our data suggests that the Gln^{325}-stop receptor does not associate with NHERF1, indicating that it is the PDZ motif of the A_{2B} adenosine receptor that interacts with this scaffolding protein, additional detailed studies with more robust controls will be required to demonstrate this conclusively. Further studies using other approaches, the use of GST-fusion proteins for example, will be required to establish a direct interaction between the wild type A_{2B} adenosine receptor and NHERF1.

Finally, the present results call into question our previous suggestion (Matharu *et al.*, 2001) that GRK-mediated phosphorylation of Ser^{329} in the COOH-terminus of the receptor could mediate the desensitization and internalization of the wild type A_{2B} adenosine receptor. The fact that we were unable to detect receptor phosphorylation is in itself not conclusive, since it is possible that the levels of phosphorylation required are below the detection limits of the assay we used. However, it is of interest that knockdown of GRK2/3 in airway smooth muscle cells was recently reported not to enhance coupling of endogenous A_{2B} adenosine receptors to cAMP formation (Kong *et al.*, 2008), questioning the role of GRKs in regulation of this receptor. On the other hand, replacement of the PDZ

motif (SLSL) of the rat A_{2B} adenosine receptor COOH-terminus with the corresponding PDZ motif (GVGL) of the human A_{2B} adenosine receptor maintained agonist-induced arrestin translocation. Since the sequence from the human receptor does not include a phosphoacceptor site, it is possible that arrestin association with the wild type A_{2B} adenosine receptor either does not require receptor phosphorylation, or that the receptor is phosphorylated at a residue other than Ser^{329}, and that the extreme COOH-terminus is then required to initiate or stabilize the association with arrestins. Further molecular studies will be required to resolve this issue, as well as possible functional interactions between arrestins and NHERF1 or other adapter/scaffolding proteins at the COOH terminus of the receptor.

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Conflict of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Cellular redistribution of wild type A_{2B} adenosine receptor (top panels), Gln^{325}-stop (middle panels) and Ser^{326}-stop receptors (lower panels) following prolonged agonist addition: co-localization of receptor with rhodamine-labelled transferrin. Chinese hamster ovary cells stably expressing the above constructs were incubated with an anti-HA antibody and rhodamine-labelled transferrin at 4°C for 1 h. Subsequently, receptor construct (green) and rhodamine-labelled transferrin (red) were visualized under basal conditions after 24 h incubation without agonist addition (left panels) or following addition of 10 μM 5′-(N-ethylcarboxamido)-adenosine for 1, 6 or 24 h. Receptor and rhodamine-labelled transferrin localization was determined by immunofluorescence in fixed cells as described in Methods. A high degree of wild type receptor/rhodamine-labelled transferrin co-localization (yellow) is visible following agonist addition for 1 and 6 h, but not for Gln^{325}-stop and Ser^{326}-stop receptor mutants. Data shown are representative of three independent experiments with >10 fields of cells captured per experiment. White scale bars represent 10 μm.

Figure S2 Single channel and overlay images of the cellular redistribution of wild type A_{2B} adenosine receptor (left-hand panels) or Gln^{325}-stop (right-hand panels) in relation to rhodamine-labelled transferrin following incubation with 5′-(N-ethylcarboxamido)-adenosine (NECA) (10 μM; 6 h). Chinese hamster ovary cells stably expressing the above constructs were incubated with an anti-HA antibody and rhodamine-labelled transferrin at 4°C for 1 h, then with NECA (10 μM) for 6 h at 37°C. Subsequently, receptor construct (green; top panels), rhodamine-labelled transferrin (red; middle panels) and the degree of co-localization (yellow; bottom panels) was visualized. White arrows in the left-hand panels highlight examples of wild type A_{2B} adenosine receptor clusters co-localized with rhodamine-labelled transferrin, seen as yellow in the bottom panel. White arrows in the right-hand panels highlight examples of Gln^{325}-stop receptor clusters that were not co-localized with rhodamine-labelled transferrin, note absence of yellow in the bottom panel. Receptor and rhodamine-labelled transferrin localization was determined by immunofluorescence in fixed cells as described in Methods. Data shown are representative of three independent experiments with >10 fields of cells captured per experiment. White scale bars represents 10 μm.

Figure S3 Single channel and overlay images of the cellular redistribution of the wild type A_{2B} adenosine receptor and Rab11 (marker for recycling endosome; left-hand panels) or Rab7 (marker for endosome to lysosome pathway; right-hand panels) following prolonged incubation with 5′-(N-ethylcarboxamido)-adenosine (NECA). Chinese hamster ovary cells stably expressing the above constructs were incubated with an anti-HA antibody at 4°C for 1 h. Subsequently, receptor construct (green, top panels), rhodamine-labelled Rab proteins (red; middle panels) and their co-localization (yellow; bottom panels) were visualized following addition of NECA (10 μM) for 2 h at 37°C. White arrowheads in the left-hand panels highlight examples of wild type A_{2B} adenosine receptor clusters co-localized with rhodamine-labelled Rab11, seen as yellow in the bottom panel. White arrowheads in the right-hand panels highlight examples of wild type A_{2B} adenosine receptor clusters co-localized with rhodamine-labelled Rab 7, seen as yellow in the bottom panel. Receptor and rhodamine-labelled Rab protein localization was determined by immunofluorescence in fixed cells as described in Methods. Data shown are representative of three independent experiments with >10 fields of cells captured per experiment. Scale bars represent 10 μm.

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