Gene dose effect reveals no G_s-coupled A_{2A} adenosine receptor reserve in murine T-lymphocytes: studies of cells from A_{2A}-receptor-gene-deficient mice

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Agonist binding to extracellular A_{2A} adenosine receptors (A_{2A}Rs) inhibits the activation of virtually all tested functions of T-cells and can induce apoptosis in thymocytes. The evaluation of levels of expression of these immunosuppressive receptors is expected to clarify whether the absence of spare A_{2A}Rs (no 'receptor reserve') might be one of the mechanisms of attenuation of the effects of extracellular adenosine on T-cells. A_{2A} transcript is found in T-cells and functional receptors can be demonstrated, but the density of receptor on T-cells is too low to be detected by radioligand binding. Studies of direct radioligand binding to murine brain with the selective A_{2A}R agonist [^{3}H]CGS21680 (2-{4-[(2-carboxyethyl)-phenyl]ethylamino}-5′-*N*-ethylcarboxamidoadenosine) established that striata levels of A_{2A}R are virtually absent from A_{2A} knock-out mice. Mice that are heterozygous ($A_{2A}R^{+/-}$) for the A_{2A}R express significantly decreased levels of A_{2A}R. To test for the presence of spare receptors in T-cells we took advantage of this gene dose effect and examined whether the decrease in the number of receptors in thymocytes from $A_{2A}R^{+/-}$ mice was proportionately reflected in a decrease in the functional cAMP response of T-cells to adenosine. cAMP accumulation and apoptosis induced by adenosine and by A_{2A}R agonist are of a lower magnitude in T-cells from $A_{2A}R^{+/-}$ heterozygous mice than in T-cells from $A_{2A}R^{+/+}$ littermate control mice. These results indicate that there is no A_{2A}R reserve in murine T-cells. Strongly decreased adenosine-triggered cAMP increases were detected in thymocytes from $A_{2A}R^{-/-}$ mice, suggesting that A_{2B} adenosine receptors cannot fully compensate for the loss of A_{2A}Rs in murine T-cells. We conclude that the number of A_{2A}Rs is the limiting factor in determining the maximal cAMP response of T-lymphocytes to extracellular adenosine, thereby minimizing the immunosuppressive effects of extracellular adenosine.

Key words: apoptosis, cAMP, gene targeting, purinergic receptors, thymocytes.

INTRODUCTION

G-protein-coupled receptors for extracellular adenosine have been classified into A_1, A_{2A}, A_{2b} and A_3 subtypes [1]. The study of these receptors has contributed to our understanding of the mechanisms governing the expression and function of G-protein-coupled receptors in general. Interest in receptor-mediated effects of extracellular adenosine has been fuelled by evidence of their functions in cardioprotection, neuroregulation and the immune response [2–8].

It has been shown in biochemical studies that murine T-cells predominantly express the A_{2A} adenosine receptor (A_{2A}R) subtype [7,9,10]. T-cell A_{2A}Rs were found to be immunosuppressive and were shown to inhibit effector functions and expansion of peripheral T-cells and to inhibit T-cell differentiation in the thymus [7,9,10]. Aberrant signalling through adenosine receptors might contribute to immune defects that are observed in patients with adenosine deaminase activity severe combined immunodeficiency ('SCID') [8].

The evaluation of levels of expression of these immunosuppressive receptors is expected to clarify whether the absence of spare A_{2a}Rs ('receptor reserve') might be one of the mechanisms of attenuation of effects of extracellular adenosine on T-cells. To examine the expression and functional roles of adenosine receptors, biochemical and immunochemical experiments have been used.

Pharmacological and biochemical experiments provided initial working models of adenosine receptor function [2–7], whereas the use of monoclonal antibodies (mAbs) against adenosine receptors in flow cytometry and histochemical experiments established tissue-specific and T-cell-type-specific patterns of expression [11,12]. Levels of expression of A_{2A}R are much higher in T-cells than in B-cells [12] but only relative levels of A_{2A}R expression could be determined by this method.

Gene-targeting techniques have recently led to the development of A_{2A}R-gene-deficient mice [13,14], but these animals have been used mostly to resolve neurological and behavioural questions. Here we use gene targeting to investigate the role of the density of A_{2A}Rs on their function in T-cells.

The strong immunosuppressive properties of extracellular adenosine [7–10] led us to consider inhibitory A_{2A}Rs as important regulators of T-cell function. The low level of expression of

Abbreviations used: A_{2A}R, A_{2A} adenosine receptor; CGS21680, A_{2A}R agonist 2-{4-[(2-carboxyethyl)-phenyl]ethylamino}-5′-*N*-ethylcarboxamidoadenosine; mAb, monoclonal antibody; NECA, 5′-*N*-ethylcarboxamide adenosine; ZM241385, A_{2A}R antagonist 4-(2-{7-amino-2-(2-furyl)[1,2,4]triazolo[2,3a][1,3,5]triazinyl-amino}ethyl)-phenol.

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A_{2A}Rs in T-cells, found in preliminary studies, precluded the use of radioligand binding to quantify receptor density.

Although no results are available on the levels of A_{2A}R in T-lymphocytes, pharmacological experiments have revealed that there is a high density of A_{2A}Rs in other tissues [11,15–19].

The availability of wild-type ($A_{2A}R^{+/+}$) and homozygous ($A_{2A}R^{-/-}$) and heterozygous ($A_{2A}R^{+/-}$) A_{2A}R-deficient mice allowed us to perform genetic experiments and to determine whether the inactivation of one allele of the A_{2A}R gene results in a proportional decrease in the number of functional ligand-binding molecules of A_{2A}R. Theoretically, alterations in the A_{2A}R gene copy number could result in underexpression (haploid insufficiency), little change owing to enhanced translation of this gene product, or the up-regulation of compensating adenosine receptors such as A_{2B} [20].

In the beginning of the current study of A_{2A}R gene-deficient mice, we noticed a gene dose effect on the density of receptors expressed in tissues in which receptors could be quantified with only half of the normal wild-type receptor number expressed in heterozygous ($A_{2A}R^{+/-}$) mice. This provided us with an opportunity to consider $A_{2A}R^{+/-}$ heterozygous mice as manipulated to decrease the expression of A_{2A}R. The use of a combination of A_{2A}R wild-type ($A_{2A}R^{+/+}$) and heterozygous ($A_{2A}R^{+/-}$) cells allowed us to investigate the previously elusive issue of adenosine receptor reserve in T-lymphocytes by examining whether a decrease in the number of receptors is proportionately reflected in a decrease in the functional response to extracellular adenosine.

The expected result of such an experiment would be that a functional response to a high dose of agonist much greater than 50 % of normal would occur in $A_{2A}R^{+/-}$ cells if there were spare A_{2A}Rs in T-cells. Indeed, in all examples of A_{2A}R reserve described so far in tissues with a high density of receptors, the occupation of even a very small proportion of receptors is sufficient to elicit a maximal response, indicating that studies of T-cells with 'depleted' A_{2A}Rs in $A_{2A}R^{+/-}$ mice might provide a definitive indication of the existence or absence of adenosine receptor reserves in lymphoid cells.

The results reported here suggest that there is no significant reserve of spare A_{2A}Rs in murine T-cells. Transcriptional and translational regulation of A_{2A}Rs on T-cells might be important determinants of how T-lymphocytes respond to adenosine.

EXPERIMENTAL

Animals

DBA/2 and C57BL/6 mice were purchased from Charles River Laboratory (Wilmington, MA, U.S.A.). To generate mice lacking the A_{2A}R, a gene-targeting vector was constructed with 10 kb of the A_{2A}R gene disrupted by a positive selection marker, PGK-Neo [14]. The replacement of a crucial stretch of nucleotides at the junction of exon 1 and its second intron with the Neo cassette was designed to ensure that the resulting mutant gene did not encode a functional A_{2A}R. The A_{2A}R genotypes of mice generated with this vector were determined by Southern blot analysis, yielding the expected 7.5 and 5.0 kb labelled restriction fragments for wild-type and mutant alleles respectively.

Littermates of wild-type ($A_{2A}R^{+/+}$), heterozygous ($A_{2A}R^{+/-}$) and homozygous deficient ($A_{2A}R^{-/-}$) mice were used in experiments for better reproducibility of results.

Antibodies

Anti-CD3 mAbs (2C11) were purchased from PharMingen (San Diego, CA, U.S.A.).

Reagents

Adenosine was purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). 2-{4-[(2-Carboxyethyl)-phenyl]ethylamino}-5′-*N*-ethylcarboxamidoadenosine (CGS21680) and 5′-*N*-ethylcarboxamide adenosine (NECA) and the A_{2A}R antagonist 4-(2-{7-amino-2-(2-furyl)[1,2,4]triazolo[2,3a][1,3,5]triazinyl-amino}ethyl)-phenol (ZM241385) were purchased from Research Biochemicals International (RBI, Natick, MA, U.S.A.).

Cells and medium

Thymocytes were isolated from adult organs *ex vivo* and incubated in RPMI-1640 medium (Biofluids, Rockville, MD, U.S.A.) supplemented with 5 % (v/v) dialysed fetal calf serum (heat-inactivated) and 100 i.u./ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1 mM Hepes, non-essential amino acids and 50 μM 2-mercaptoethanol, essentially as described previously [8].

Receptor autoradiography

Receptor autoradiography of A_{2A}Rs with the A_{2A}R agonist [^{3}H]CGS21680 was described previously [21–23]. In brief, mice were killed by decapitation, and whole brains were quickly removed and immediately frozen on solid CO_2. Brains were sectioned through the striatum on a cryostat (10 μm); sections were preincubated in Tris buffer containing 2.0 units/ml adenosine deaminase for 30 min and then incubated at room temperature for 60 min with the same buffer containing 5.0 nM ^{3}H-CGS21680 in the presence of adenosine deaminase. Slides were then washed twice with ice-cold Tris/HCl buffer and once with cold water, each for 1 min.

Non-specific binding of A_{2A}Rs was determined by co-incubating ^{3}H-labelled ligands with 25 μM 2-chloroadenosine. Air-dried slides were then exposed to ^{3}H-Hyperfilm for 1–3 weeks.

Preparation of lymphocytes for cAMP measurement assays

Single-cell suspensions of murine thymocytes, splenocytes and lymph node cells were isolated by standard procedures. Cells were washed and incubated with ACK lysing buffer (BioWhittaker, Walkersville, MD, U.S.A.) at 10^8 cells/ml for 1 min at 37 °C to remove red blood cells. The cells were then washed and resuspended in RPMI-1640, supplemented as described above, at 2×10^6 cells/ml for cAMP assays.

Measurement of adenosine-triggered accumulation of cAMP

Thymocytes (2×10^6 cells/ml) were incubated in the presence or absence of different concentrations of adenosine or adenosine analogues at 37 °C in an incubator under air/CO_2 (93:7). After 30 min the incubations were stopped by the addition of 10 % (v/v) 1 M HCl and transferred to −70 °C. After thawing and centrifugation at 10000 ***g*** to remove cellular debris, supernatants were assayed for cAMP using a BIOTRAK cAMP EIA kit from Amersham Pharmacia (Buckinghamshire, England). K_D and B_{max} were determined with GraphPad Prism 2.0 scientific graphing software (GraphPad Software, San Diego, CA, U.S.A.). Error bars represent S.D.

Southern blot analysis of A_{2a}R-deficient mice

Genetic identification of mice was done by Southern blot analysis. A Gentra DNA Isolation Kit (Minneapolis, MN, U.S.A.) was used in isolating genomic DNA.

After digestion with *Bam*H1 restriction endonuclease, DNA

was transferred to a nitrocellulose membrane. The $A_{2A}R$ DNA probe was labelled by using a protocol based on the Amersham Pharmacia Biotech Oligolabelling Kit. The ^{32}P-labelled $A_{2A}R$ probe was purified on an Amersham Pharmacia Biotech Nick Column and hybridized overnight with the immobilized DNA before exposure for autoradiography.

Wild-type $A_{2A}R$ DNA resolved into a single DNA band (7.5 kb), whereas heterozygous $A_{2A}R$ DNA resolved into two DNA bands (7.5 and 5.0 kb) and mutant (or knock-out) $A_{2A}R$ DNA resolved into a single DNA band (5.0 kb).

Northern blot analysis of $A_{2A}R$ mRNA

Total RNA was isolated from excised organs and resolved on 1% (w/v) agarose gels at 100 V with a horizontal electrophoresis unit (Life Technologies). RNA was then transferred from the gel to Opitran nitrocellulose paper (Schleicher & Schuell, Keene, NH, U.S.A.) in the same manner as the DNA. $A_{2A}R$ DNA probe was labelled and used during hybridization overnight to the immobilized RNA and followed by exposure for an autoradiograph.

Control Northern blots for glyceraldehyde-3-phosphate dehydrogenase 'housekeeping' mRNA were performed, both to test for predicted changes in the size of transcripts in $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice and to demonstrate the differences in the level of expression of the $A_{2A}R$ gene between brain and lymphoid tissues.

Measurement of CGS21680-induced apoptosis of thymocytes

A single-cell suspension of murine thymocytes was isolated by standard procedures and cultured in 96 well plates [$(0.5–1) \times 10^6$ cells per well] as described [7]. After incubation for 16–18 h, or as indicated, cells were harvested and analysed by flow cytometry.

Flow cytometric quantification of live, apoptotic and dead cells was performed with a modified flow cytometry procedure as described [7]. The effects of adenosine on thymocytes were studied after incubating thymocytes *ex vivo* in short-term culture. The analysis of surviving dead and apoptotic cells was based on the gating of cells by their size (side and forward scatter), plasma membrane integrity (staining with propidium iodide) and the redistribution of plasma membrane phosphatidylserine (annexin V binding). The Annexin V binding assay was performed as described [7]. In brief, $(0.6–1) \times 10^6$ cells from a 96-well plate were resuspended in 100 μl of buffer containing 10 mM Hepes, pH 7.3, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$, then incubated with 0.3 μg/ml FITC-conjugated annexin V and 5 μg/ml propidium iodide for 15 min. After incubation, samples were diluted 1:3 with buffer containing 1.8 mM $CaCl_2$ and analysed by FACScan. Annexin V–FITC was purchased from Trevingen (Gaithersburg, MD, U.S.A.) and BioWhitaker (Walkersville, MD, U.S.A.).

Statistical analysis of triplicate sample measurements was performed with the StatView statistic program (Abacus-Concepts, Berkeley, CA, U.S.A.). S.D. values of triplicate measurements within the same experiment were usually lower than 1%.

The acquisition and analysis of flow cytometry data were done on a FACScan with FACScan research software and CellQuest programs (Becton–Dickinson, San Jose, CA, U.S.A.).

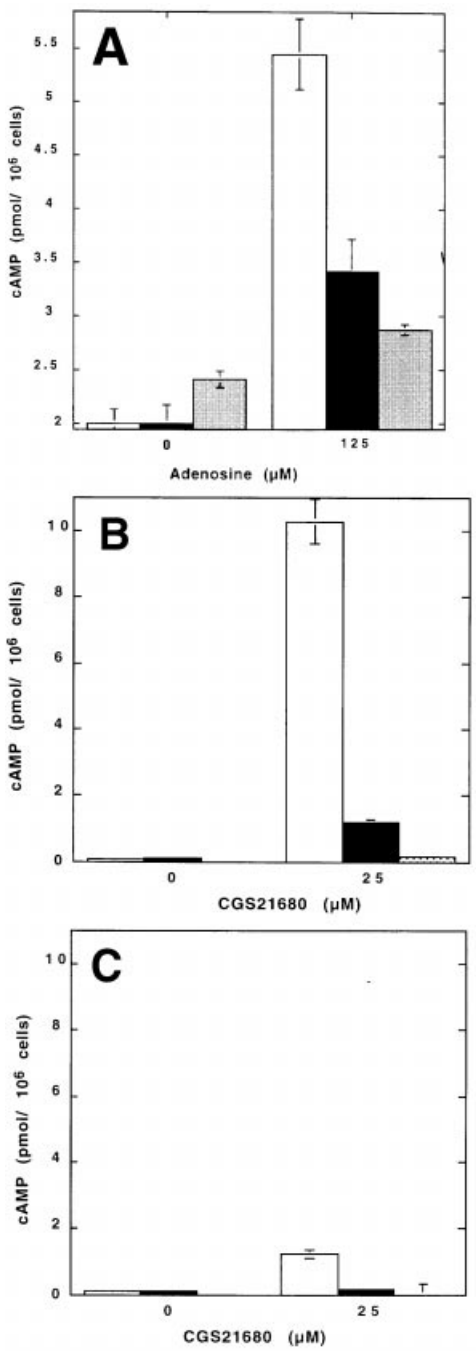

Figure 1 Pharmacological and genetic evidence for predominant expression of $A_{2A}R$ in T-cells

(**A**) Lymphocytes isolated *ex vivo* were incubated with or without extracellular adenosine in the presence or absence of the $A_{2A}R$ antagonist ZM241385 at the following concentrations: open columns, 0 μM; filled columns, filled columns, 0.1 μM; grey or stippled columns, 1.0 μM. (**B**, **C**) Thymocytes isolated *ex vivo* were incubated with or without the $A_{2A}R$ agonist CGS21680 in the presence or absence of $A_{2A}R$ antagonist ZM241385 [concentrations as in (**A**)] followed by measurements of the accumulation of cAMP in thymocytes from wild-type (**B**) or $A_{2A}R$ gene-deficient (**C**) mice. Experiments were performed in triplicate.

RESULTS

Murine T-cells respond to the addition of adenosine by accumulating cAMP [6–10]; studies with selective agonists and antagonists indicate the G_s-coupled $A_{2A}R$ mediate this response in murine T-cells. Figure 1(A) shows extracellular, adenosine-stimulated, cAMP accumulation in T-cells and strong inhibition of the effect of adenosine by ZM241385, and these results justify the focus on studies of $A_{2A}R$ signalling in lymphocytes with $A_{2A}R$ gene-deficient mice.

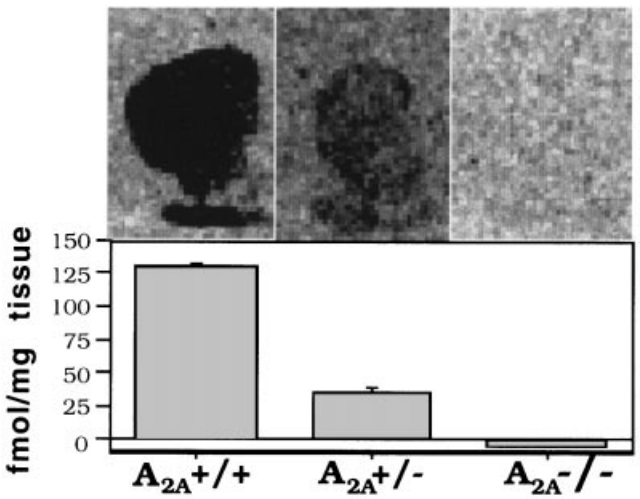

Figure 2 Receptor autoradiography of $A_{2A}R$ in wild-type, heterozygous, and homozygous A_{2A} knock-out mice

Heterozygous and homozygous A_{2A} knock-out amd wild-type mice were derived from a near congenic line (N6) as described elsewhere [14]. Control sections of brains from drug-naive adult mice show receptor binding densities for A_{2A}Rs, as determined by receptor autoradiography with ^{3}H-CGS21680 as a specific ligand. Receptor binding densities were quantified by an image analysis system and are expressed as fmol/mg of tissue after subtraction of non-specific binding and calibration with a ^{3}H-receptor-binding standard as described previously [22,27].

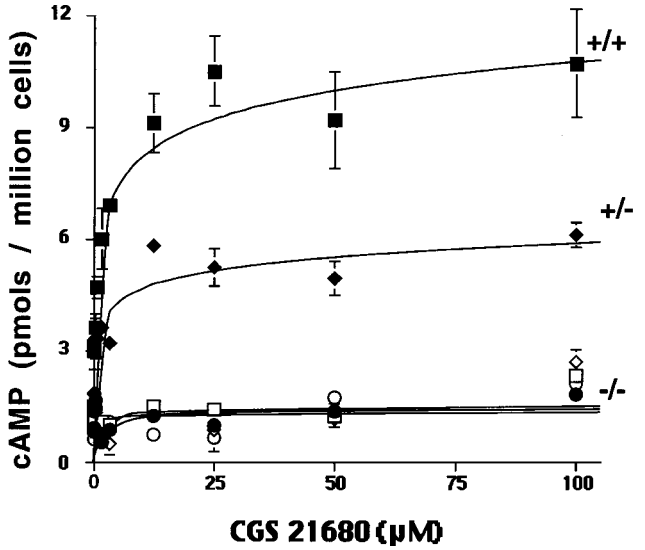

Figure 4 cAMP responses to $A_{2A}R$ agonist CGS21670 in thymocytes from age-matched littermates mice with $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ genotypes

Thymocytes isolated *ex vivo* were incubated for 30 min with 0.2, 0.4, 0.8, 1.6, 3.25, 6.25, 12.5, 25, 50 and 100 μM CGS21680 at 37 °C, followed by cAMP assay as described in the Experimental section. Symbols: ■, ◆, ●, incubation with CGS21680 alone; □, ◇, ○, incubation with both CGS21680 and ZM241385; ■, □, $A_{2A}R^{+/+}$; ◆, ◇ $A_{2A}R^{+/-}$; ●, ○, $A_{2A}R^{-/-}$. Each point was replicated in triplicate.

The $A_{2A}R$ gene was disrupted by a DNA-targeting construct as described by Chen et al. [14]. The $A_{2A}R$ genotypes of mice generated with this vector were determined by Southern blot analysis. The screening of mice to identify wild-type ($A_{2A}R^{+/+}$), heterozygous ($A_{2A}R^{+/-}$) or homozygous deficient ($A_{2A}R^{-/-}$) mice was based on discriminating between DNA fragments by using a Southern blot analysis with expected patterns of 7.5 kb DNA bands from $A_{2A}R^{+/+}$ mice, both 7.5 and 5.5 kb bands in $A_{2A}R^{+/-}$ mice, and one 5.5 kb band in DNA from $A_{2A}R^{-/-}$ mice. The size of mRNA transcripts from $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice was higher than in $A_{2A}R^{+/+}$ wild-type mice because of a replacement of nucleotides at the junction of exon 1 with the Neo cassette, resulting in more nucleotides in the resulting transcript. The Northern blot analysis of RNA from different tissues of $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice revealed that $A_{2A}R$ mRNA expression in $A_{2A}R^{+/+}$ mice was highest in the brain and lymphoid organs (spleen and thymus), with much less $A_{2A}R$ mRNA detectable in other organs (results not shown).

Inactivation of the $A_{2A}R$ gene resulted in a marked decrease in cAMP response in thymocytes of $A_{2A}R^{-/-}$ mice (Figure 1C) compared with the strong response of thymocytes from wild-type ($A_{2A}R^{+/+}$) mice (Figure 1B). These experiments confirm that there are no functional $A_{2A}Rs$ in $A_{2A}R^{-/-}$ mice.

The absence of ligand binding to $A_{2A}Rs$ in $A_{2A}R$ knock-out mice was demonstrated by receptor autoradiography with the $A_{2A}R$ agonist [^{3}H]CGS21680 (Figure 2). Wild-type ($A_{2A}R^{+/+}$) mice show specific labelling of $A_{2A}Rs$ in the striatum, nucleus accumbens and olfactory bulb, whereas homozygous ($A_{2A}R^{-/-}$) knock-out mice show virtually no [^{3}H]CGS21680 binding in these regions. Significant but decreased binding was detected in brains of heterozygous ($A_{2A}R^{+/-}$) mice.

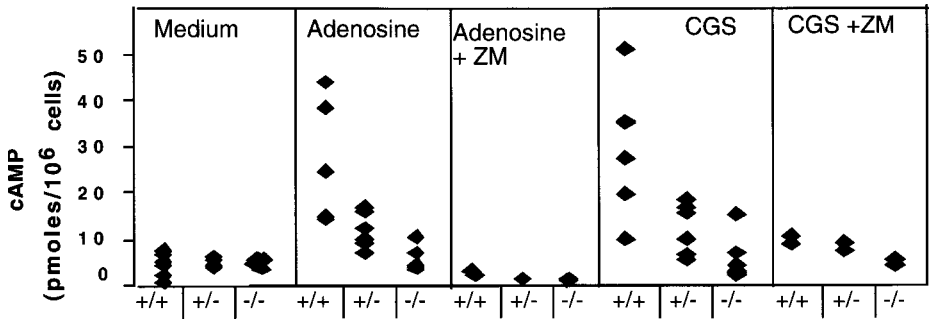

Figure 3 Comparison of cAMP accumulation induced by adenosine and CGS21680 in thymocytes of $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice

Multiple triplets of $A_{2A}R^{+/+}$ (+/+) $A_{2A}R^{+/-}$ (+/−) and $A_{2A}R^{-/-}$ (−/−) mice were analysed for their cAMP response to adenosine (50 μM), or CGS21680 (CGS, 50 μM) in the presence or absence of the $A_{2A}R$ antagonist ZM2413815 (ZM). Each point represents the response from an individual mouse.

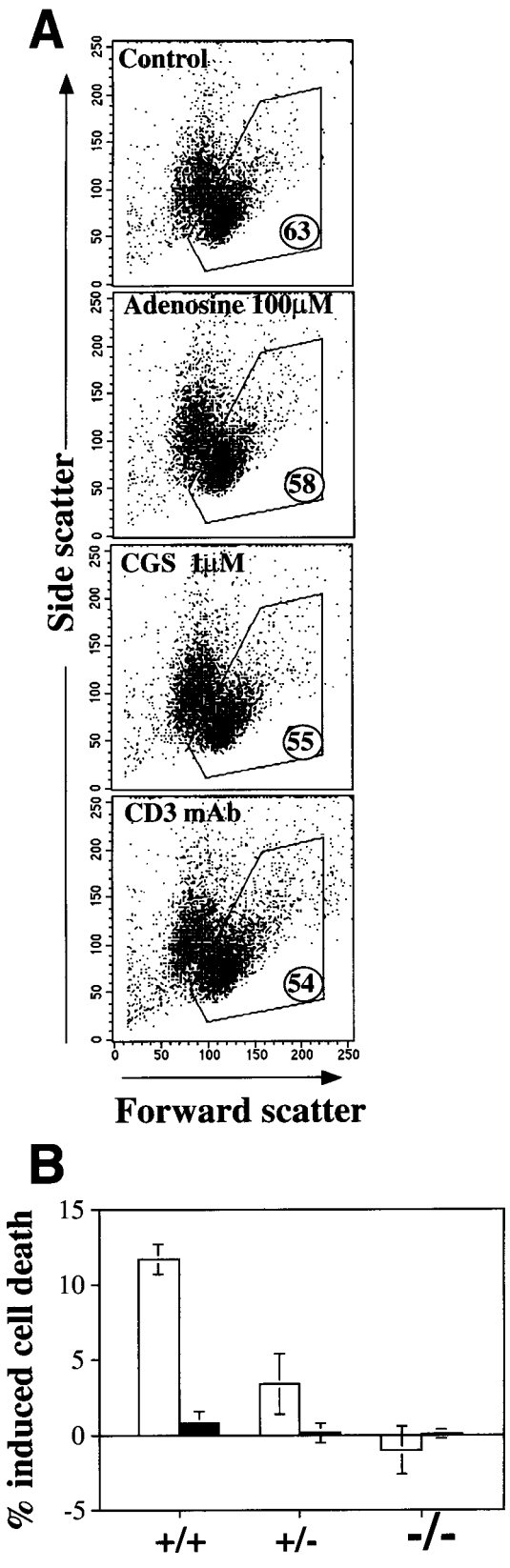

Figure 5 Gene-dose effect in A_{2A}R-induced apoptosis and cell death in thymocytes

(**A**) A_{2A}R agonist CGS21680 (CGS, 1 μM) and adenosine (100 μM) and anti-CD3 mAb (5 μg/ml) induce the death of thymocytes during 16 h of incubation *in vitro*. Thymocytes isolated *ex vivo* were incubated with adenosine, CGS21680 or anti-CD3 mAb as described in the Experimental section; after 16 h, live and dead cells were identified with the use of side scatter versus forward scatter or annexin (results not shown) flow-cytometry assays. Circled numbers inside gates identify the proportions of live thymocytes in the control (medium only) and with adenosine, CGS21680 and anti-CD3 mAb. Decreased numbers of live cells provide an estimate of death-inducing signalling through A_{2A}Rs or T-cell receptors. (**B**) Decrease in CGS21680-triggered and A_{2A}R-mediated apoptosis in thymocytes from heterozygous (+/−) mice. Thymocytes isolated *ex vivo* from $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice were incubated for 24 h with CGS21680 as described for (**A**) and the percentage of CGS-induced cell death was estimated by annexin V apoptosis assay as described in the Experimental section. Results are means ± S.D. for parallel assays. Open columns, CGS21680; filled columns, CGS21680 plus ZM241385.

It was found in our preliminary experiments with ^{125}I-labelled A_{2A}R ligand ZM241385 that the low level of A_{2A}R expression in thymocytes was not sufficient to detect A_{2A}R specific binding (results not shown) but was sufficient for reproducible assays of cAMP accumulation induced by extracellular adenosine.

The availability of specific agonists and antagonists of the A_1, A_{2A} and A_3 adenosine receptors was instrumental in measurements of A_{2A}R-mediated cAMP accumulation. The 2-substituted adenosine derivative CGS21680 is widely accepted as a specific A_{2A}R agonist, whereas specific antagonists for A_{2A}R receptors include 8-chlorostyrylcaffeine and ZM241385 [2]. The A_{2A}R agonist CGS21680 and A_{2A}R antagonist ZM241385 were used in the experiments described below.

In detailed time course studies (results not shown) the peak of cAMP accumulation was most often observed after 15–30 min. Therefore 30 min incubation assays were chosen in studies of the mechanisms of A_{2A}R-mediated signalling in short-term assays *in vitro*.

In our studies to evaluate the role of A_{2A}R in extracellular-adenosine-mediated signalling, thymocytes from $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ or $A_{2A}R^{-/-}$ mice were incubated with adenosine or the adenosine agonist CGS21680 in the presence or absence of the selective A_{2A}R antagonist ZM241385. Results of analysis of thymocytes from several individual sets of $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice are summarized in Figure 3. These results reveal that, whereas both adenosine and CGS21680 are able to trigger cAMP accumulation in wild-type mice, they are not effective in triggering cAMP accumulation in thymocytes from homozygous $A_{2A}R^{-/-}$ mice. The addition of ZM241385 strongly inhibited the cAMP accumulation induced both by adenosine and by CGS21680, confirming the earlier conclusion that the A_{2A}R is the predominantly expressed adenosine receptor in murine T-cells [10] and providing additional genetic evidence of the selectivity and specificity of the A_{2A}R agonists and antagonists used here in studies of A_{2A}R *in vitro*.

Comparison of results of cAMP measurements in adenosine-exposed cells from $A_{2A}R^{+/+}$ and $A_{2A}R^{+/-}$ mice established that cells from $A_{2A}R^{+/-}$ mice have decreased cAMP levels in comparison with cells from wild-type mice, thereby suggesting a gene dose effect on the functioning of A_{2A}R in thymocytes. Although thymocytes from virtually all tested wild-type mice responded to exposure to extracellular adenosine or CGS21680 by cAMP accumulation, the extent of cAMP increases varied between individual animals, as illustrated in Figure 3. These results clearly demonstrate the necessity of using age-matched animals or littermates.

Therefore, to confirm and quantitatively explore the possibility of a gene dose effect in the expression and function of A_{2A}R and to diminish the influence of differences in age, sex and activity of animals we performed analyses with thymocytes only from littermates with the $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ or $A_{2A}R^{-/-}$ genotype.

The experiment described in Figure 4 demonstrates the differences between $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ gene-targeted

thymocytes in their responses to the $A_{2A}R$ agonist CGS21680. The dose–response curve at low concentrations of CGS21680 was linear and reached a plateau at approx. 12.5 μM for thymocytes isolated from $A_{2A}R^{+/+}$ mice. By plotting the data with non-linear regression analysis (Cricket Graph, Islandia, NY, U.S.A.), we were able to determine that thymocytes isolated from $A_{2A}R^{+/-}$ female mice responded with a much smaller CGS21680-induced cAMP accumulation than those from their $A_{2A}R^{+/+}$ female littermates (Figure 4, and data not shown). CGS21680 concentrations needed to trigger half-maximal cAMP responses were identical between the two curves (1.3 μM), indicating that the difference in responses was probably due to changes in receptor number and an absence of spare receptors. Similar results were obtained in experiments in which thymocytes were incubated with adenosine and the adenosine analogue NECA.

Functional response (cAMP) assays with CGS21680 and adenosine were also conducted on lymph node cells and splenocytes isolated from $A_{2A}R^{+/+}$, $A_{2A}R^{+/-}$ and $A_{2A}R^{-/-}$ mice. These assays demonstrated a similar gene dose effect (results not shown), but detailed dose–response experiments were not performed with these tissues.

To test whether the gene-dose effect of $A_{2A}R$ expression could be detected at the level of a complex cellular response, we tested whether adenosine-induced apoptotic processes were different in $A_{2A}R^{+/-}$ heterozygous mice. Figure 5(A) shows that 5 % fewer thymocytes from normal mice survived a 16 h incubation with adenosine (58 % compared with 63 %), whereas CGS21680 caused the death of 8 % of thymocytes, almost as much as anti-CD3 mAb (9 %). These observations of apoptosis induced by extracellular adenosine and by CGS21680 confirm our findings [7–10,12] and provided an appropriate experimental assay with which to test the possible gene dose effect in $A_{2A}R$-deficient mice. Flow cytometry permits discrimination between live, early and late apoptotic and dead cells by an analysis of forward and side scatter, propidium iodide permeability and DNA breaks as detected by TUNEL (terminal transferase deoxytidyl uridine end labelling) assay [7]. Death induced by adenosine or CGS21680 was detected as a decrease in the number of surviving thymocytes (Figure 5A); it was comparable with that induced by a known apoptotic agent, anti-CD3/TCR mAb.

The gene dose effect of a decrease in $A_{2A}R$ function is demonstrated in Figure 5(B) in a thymocyte apoptosis assay, which demonstrated that CGS21680-triggered and $A_{2A}R$-mediated signalling in thymocytes from $A_{2A}R^{+/-}$ mice was approximately half as efficient in triggering apoptosis as that in thymocytes from $A_{2A}R^{+/+}$ mice. The results in Figure 5(B) are representative of several experiments and are in agreement with observations that $A_{2A}R^{+/+}$ thymocytes responded to CGS21680 by cAMP accumulation, which was blocked by an $A_{2A}R$ antagonist. Thus gene dose effects of $A_{2A}R$ signalling were proportionally reflected in other $A_{2A}R$-mediated functions.

It is noteworthy that no adenosine-triggered cAMP increases and no adenosine-induced apoptosis (Figure 5B) were detected in thymocytes from $A_{2A}R^{-/-}$ mice, directly demonstrating the absence of compensation for $A_{2A}Rs$ by cAMP-increasing A_{2B} adenosine receptors.

DISCUSSION

The main findings and conclusions of this study are the following: (1) there is a gene dose effect in the expression of ligand-binding to $A_{2A}Rs$ in brain; (2) there is a gene dose effect in the signal transduction events triggered by $A_{2A}Rs$ on thymocytes and T-cells; (3) there is no adenosine $A_{2A}R$ reserve for the induction of cAMP in thymocytes and mature T-cells; (4) there is no, or there are very low levels of, cAMP-inducing A_{2B} receptor compensation for the depletion of $A_{2A}R$ in $A_{2A}R^{-/-}$ mice. These observations extend our understanding about the mechanism of regulation of expression of G_s-coupled membrane receptors.

The genetic model used here supplements classical biochemical and pharmacological methods for studying receptor reserve and G-protein-coupled receptor functioning by taking advantage of a gene dose effect on $A_{2A}R$ expression. A gene dose effect on protein expression had previously been discussed for other G-protein-coupled receptors with regard to cellular responses of gene-targeted animals [22] but it had to be established with $A_{2A}R$ in this model of both proximal and late stages of the transmembrane signalling pathway. Indeed, it would not be expected from first principles that a decrease in ploidy in $A_{2A}R^{+/-}$ mice would result in a 50 % decrease in expression of surface $A_{2A}R$ protein. It was possible that the phenomenon of haploid insufficiency [20] would be reflected in the expression of $A_{2A}R$ at levels less than 50 % of wild type. Similarly, a decrease in the expression of $A_{2A}R$ does not necessitate a decrease in the functional response to adenosine because (1) a receptor reserve would still allow a maximal functional response in the presence of fewer surface receptors, (2) there was a possibility of up-regulation of compensating and cAMP-triggering adenosine A_{2B} receptors, and (3) factors beyond the receptor number might limit the response, such as the amount of G protein or other effectors.

Thus, because the level of $A_{2A}R$ expression in $A_{2A}R^{+/-}$ mice is less than 50 % of wild-type levels, haploid insufficiency cannot be ruled out as a factor (Figures 2 and 5). These results also indicate that there are no compensatory changes in $A_{2A}R$ gene expression, thereby supporting the general view [24] that changes in the number of active genes alter the amount of gene product.

Decreasing the number of $A_{2A}Rs$ in $A_{2A}R^{+/-}$ mice has the effect of decreasing agonist-induced signalling in thymocytes and T-cells (Figure 4), suggesting that there is little or no receptor reserve in these cells. Studies of $A_{2A}R$ reserve in T-cells would not be not possible without the genetic model used here because the classical approach to demonstrate a surplus of receptors involves measuring receptor occupancy and agonist response as functions of agonist concentration, in which the surplus is quantified by dividing the K_a of the binding curve by the EC_{50} of the response curve [19]. In this method, quotients greater than one are interpreted as a measure of the magnitude of the surplus. Such an approach is not possible with $A_{2A}Rs$ in T-cells because their level is so low that we could not measure binding to T-cells even by using very sensitive ligand-binding assays with the ^{125}I-labelled $A_{2A}R$ antagonist ZM241385 (results not shown). Results are shown for CGS-21680-induced cAMP accumulation in Figure 4; however, very similar results were obtained with adenosine and the $A_{2a}R$ agonist NECA (results not shown).

In some instances the occupation of only a very small proportion of receptors causes a maximal response. An early example of this spare-receptor phenomenon was demonstrated when it was shown that the occupancy of only 1 % of histamine receptors in the guinea-pig ileum was required to elicit maximum contractile response. There is also a more than 99 % receptor reserve of β-adrenergic receptors in rat C6 glioma cells and atrium [18]. Thus the loss of $A_{2A}Rs$ to a degree observed in $A_{2A}R^{+/-}$ mice would not be expected to decrease the maximal response if there were an $A_{2A}R$ reserve in T-cells; the use of $A_{2A}R^{+/-}$ mice provided a convenient genetic model with which to test this.

However, it should be noted that the use of a gene dose effect is the most appropriate method to establish the absence of a

receptor reserve because the obvious limitation of relying on a gene dose effect to detect and quantify receptor reserves is the situation with cells that have large receptor reserves. Indeed, if a large reserve of surplus receptors existed, maximal function responses would be diminished very little in cells from $A_{2A}R^{+/-}$ animals. The use of a gene dose effect in evaluation of the existence of spare A_{2A}Rs was successful here because there are no spare A_{2S}R in T-cells. Thus the use here of heterozygous $A_{2A}R^{+/-}$ mice was justified to establish the absence of an A_{2A}R reserve.

There are numerous reports describing systems with spare receptors [19,23,25–27]; a wide variability exists in the reserve of G-protein-coupled receptors. There also is substantial variability between tissues. Furchgott [17] noted that progressive alkylation of adrenergic receptors in vascular smooth muscle results in a shift of less than 3-fold for adrenaline (epinephrine) signalling before a decrease in the maximal response is observed, indicative of low reserve. No spare β-adrenoreceptors have been found in the human heart [28] but a substantial receptor reserve is found in human lung mast cells [26]. Our results support the view that the same receptors can have substantially different reserve levels in different tissues.

Whereas some of the reports describing a lack of receptor surpluses were based solely on the K_a/EC_{50} quotient [28–31], others were based on the use of heterozygous and knock-out animals [32,33]. In contrast with earlier reports, the read-outs in our experiments with cells from $A_{2A}R^{+/-}$ and $A_{2A}R^{+/+}$ mice included both functional cellular responses (apoptosis) and assays of the most proximal events of transmembrane signalling through G-protein-coupled receptors by measuring cAMP induction and accumulation. The results provided here provide the first demonstration that the diminished expression of G-protein-coupled adenosine A_{2A}R translates into a proportional decrease in cAMP accumulation in the cellular responses of thymocytes.

The number of expressed A_{2A}Rs in T-cells might be such that there are no spare A_{2A}Rs for such functions as cAMP accumulation and apoptosis induction but it remains to be determined whether there are some other A_{2A}R-related functions for which there is an excess of these receptors in T-cells. Indeed, the lack of an A_{2A}R reserve for one function can apparently coexist with a surplus of these same receptors for another function [19,34]. It remains to be determined whether there are T-cell functions that respond equally to signalling through A_{2A}Rs in $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice.

The CGS21680-induced apoptosis of thymocytes from $A_{2A}R^{+/-}$ mice was found to be diminished in comparison with those from wild-type mice (Figure 5). This was probably due to a decrease in A_{2A}R-triggered activation of PKA (cAMP-dependent protein kinase) rather than due to a PKA-independent mediator such as the cAMP-binding guanine nucleotide exchange factor ('cAMP-GEF') proteins described recently, because cAMP-GEF mRNA was not detected in thymus [35]. However, cAMP-GEF was detected in spleen and lymph node, so PKA-independent processes cannot be ruled out for the cAMP-mediated regulation of peripheral T-cells.

No, or a markedly inhibited, cAMP increase triggered by extracellular adenosine was detected in thymocytes from $A_{2A}R^{-/-}$ mice, suggesting that there is no redundancy of receptors for extracellular adenosine. In other words, the loss of functional A_{2A}Rs was not compensated for by an increase in the expression of cAMP-increasing A_{2B} or any other adenosine receptors. This is an interesting observation because a redundancy of receptors was shown for other immunologically relevant proteins and receptors [36]. The absence of redundancy in the expression of A_{2A}Rs suggests that their expression level regulates T-cell sensitivity to adenosine. It is noted in this regard that there are many more lymphokine-producing cells among cells expressing A_{2A}Rs than among cells not expressing A_{2A}Rs [12]. This was interpreted as a reflection of A_{2A}R-mediated signalling in the control of cytokine production in activated T-lymphocytes. Signalling through A_{2A}R in T-cells seems to be inhibitory (immunosuppressive) at higher concentrations of extracellular adenosine but low-level signalling by low concentrations of extracellular adenosine might be permissive for the propagation of lymphokine responses driven by antigen receptors.

Most studies with gene-targeted mice have been performed only with wild-type ($A_{2A}R^{+/+}$) and gene-deficient ($A_{2A}R^{-/-}$) mice without much emphasis on the biochemical behaviour of cells from mice heterozygous for the gene of interest. Our continuing studies of A_{2A}R gene-deficient mice promise to shed light on the functional roles of these receptors in regulating immune responses *in vivo*.

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REFERENCES

1 Olah, M. E. and Stiles, G. L. (1995) Adenosine receptor subtypes: characterization and therapeutic regulation. Annu. Rev. Pharmacol. Toxicol. **35**, 581–606
2 Jacobson, K. A., Von Lubitz, D. K., Daly, J. W. and Fredholm, B. B. (1996) Adenosine receptor ligands: differences with acute versus chronic treatment. Trends Pharmacol. Sci. **17**, 108–113
3 Jacobson, K. A., Hoffmann, C., Cattabeni, F. and Abbracchio, M. P. (1999) Adenosine-induced cell death: evidence for receptor-mediated signaling. Apoptosis **4**, 197–211
4 Van der Ploeg, I., Ahlberg, S., Parkinson, F. E., Olsson, R. A. and Fredholm, B. B. (1996) Functional characterization of adenosine A2 receptors in Jurkat cells and PC12 cells using adenosine receptor agonists. Naunyn Schmiedebergs Arch. Pharmacol. **353**, 250–260
5 Olsson, R. A. (1996) Adenosine receptors in the cardiovascular system. Drug Dev. Res. **39**, 301–307
6 Apasov, S., Koshiba, M., Redegeld, F. and Sitkovsky, M. (1995) Role of extracellular ATP and P1 and P2 classes of purinergic receptors in T-cell development and cytotoxic T lymphocyte effector functions. Immunol. Rev. **146**, 5–19
7 Apasov, S. G., Koshiba, M., Chused, T. M. and Sitkovsky, M. V. (1997) Effects of extracellular ATP and adenosine on different thymocyte subsets: possible role of ATP-gated channels and G protein-coupled purinergic receptor. J. Immunol. **158**, 5095–5105
8 Apasov, S. and Sitkovsky, M. (1999) The extracellular versus intracellular mechanisms of inhibition of TCR-triggered activation in thymocytes by adenosine under conditions of inhibited adenosine deaminase. Int. Immunol. **11**, 179–189
9 Huang, S., Koshiba, M., Apasov, S. and Sitkovsky, M. (1997) Role of A_{2a} extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. Blood **90**, 1600–1610
10 Koshiba, M., Kojima, H., Huang, S., Apasov, S. and Sitkovsky, M. V. (1997) Memory of extracellular adenosine A_{2A} purinergic receptor-mediated signaling in murine T cells. J. Biol. Chem. **272**, 25881–25889
11 Rosin, D. L., Robeva, A., Woodward, R. L., Guyenet, P. G. and Linden, J. (1998) Immunohistochemical localization of adenosine A_{2A} receptors in the rat central nervous system. J. Comp. Neurol. **401**, 163–186
12 Koshiba, M., Rosin, D. L., Hayashi, N., Linden, J. and Sitkovsky, M. V. (1999) Patterns of A_{2A} extracellular adenosine receptor expression in different functional subsets of human peripheral T cells. Flow cytometry studies with anti-A_{2A} receptor monoclonal antibodies. Mol. Pharmacol. **55**, 614–624
13 Ledent, C., Vaugeois, J. M., Schiffmann, S. N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J. J., Costentin, J., Heath, J. K., Vassart, G. and Parmentier, P. (1997) Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A_{2a} receptor. Nature (London) **388**, 674–678
14 Chen, J. F., Huang, Z., Ma, J., Zhu, J., Moratalla, R., Standaer, D., Moskowitz, M. A., Fink, J. S. and Schwarzchild, M. (1999) A_{2A} adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. J. Neurosci. **19**, 9192–9200
15 Kaelin-Lang, A., Lauterburg, T. and Burgunder, J. M. (1999) Expression of adenosine A2a receptors gene in the olfactory bulb and spinal cord of rat and mouse. Neurosci. Lett. **261**, 189–191
16 Peterfreund, R. A., MacCollin, M., Gusella, J. and Fink, J. S. (1996) Characterisation and expression of the human A2a adenosine receptor gene. J. Neurochem. **66**, 362–368

17 Furchgott, R. F. (1955) The pharmacology of vascular smooth muscle. Pharm. Rev. **7**, 183–235
18 Terasaki, W. L., Linden, J. and Brooker, G. (1979) Quantitative relationship between β-adrenergic receptor number and physiologic responses as studied with a long-lasting β-adrenergic antagonist. Proc. Natl. Acad. Sci. U.S.A. **76**, 6401–6405
19 Shryock, J. C., Snowdy, S., Baraldi, P. G., Cacciari, B., Spalluto, G., Monopoli, A., Ongini, E., Baker, S. P. and Belardinelli, L. (1998) A_{2A}-adenosine receptor reserve for coronary vasodilation. Circulation **98**, 711–718
20 Tang, B., Bottinger, E. P., Jakowlev, S. B., Bagnali, K. M., Mariano, J., Anver, M., Letterio, J. J. and Wakefield, L. M. (1998) Transforming growth factor-β1 is a new form of tumor suppressor with true haploid insufficiency. Nat. Med. (N.Y.) **4**, 802–807
21 Chen, J. F., Aloyo, V. J. and Weiss, B. (1993) Continuous treatment with the D2 dopamine receptor agonist quinpirole decreases D2 dopamine receptors, D2 dopamine receptor messenger RNA and proenkephalin messenger RNA, and increases μ opioid receptors in mouse striatum. Neuroscience **54**, 669–680
22 Rohrer, D. K. and Kobilka, B. K. (1998) G protein-coupled receptors: functional and mechanistic insights through altered gene expression. Physiol. Rev. **78**, 35–52
23 Gunst, S. J., Stropp, J. Q. and Flavahan, N. A. (1987) Analysis of receptor reserves in canine tracheal smooth muscle. J. Appl. Physiol. **62**, 1755–1758
24 Graves, J. A., Disteche, C. H. and Toder, R. (1998) Gene dosage in the evolution and function of mammalian sex chromosomes. Cytogenet. Cell Genet. **80**, 94–103
25 Bognar, I. T. and Enero, M. A. (1988) Influence of a receptor reserve on the inhibition by calcium channel blockers of alpha adrenoceptor-mediated responses in rat isolated vascular tissues. J. Pharmacol. Exp. Ther. **245**, 673–681
26 Drury, D. E., Chong, L. K., Ghahramani, P. and Peachell, P. T. (1998) Influence of receptor reserve on beta-adrenoceptor-mediated responses in human lung mast cells. Br. J. Pharmacol. **124**, 711–718
27 Cox, R. F., Meller, E. and Waszczak, B. L. (1993) Electrophysiological evidence for a large receptor reserve for inhibition of dorsal raphe neuronal firing by 5-HT1A agonists. Synapse **14**, 297–304
28 Schwinger, R. H., Bohm, M. and Erdmann, E. (1990) Evidence against spare or uncoupled beta-adrenoceptors in the human heart. Am. Heart J. **119**, 899–904
29 Agneter, E., Singer, E. A., Sauermann, W. and Feuerstein, T. J. (1997) The slope parameter of concentration–response curves used as a touchstone for the existence of spare receptors. Naunyn-Schmiedeberg's Arch. Pharmacol. **356**, 283–292
30 Meller, E. and Bohmaker, K. (1994) Differential receptor reserve for 5-HT1A receptor-mediated regulation of plasma neuroendocrine hormones. J. Pharmacol. Exp. Ther. **271**, 1246–1252
31 Camps, M., Guma, A., Vinals, F., Testar, X., Palacin, M. and Zorzano, A. (1992) Evidence for the lack of spare high-affinity insulin receptors in skeletal muscle. Biochem. J. **285**, 993–999
32 Hashimoto, N., Suzuki, F., Tamai, I., Nikaido, H., Kuwajima, M., Hayakawa, J-I. and Tsuji, A. (1998) Gene-dose effect on carnitine transport activity in embryonic fibroblasts of JVS mice as a model of human carnitine transporter deficiency. Biochem. Pharmacol. **55**, 1729–1732
33 Baik, J. H., Picetti, R., Saiardi, A., Thiriet, G., Dierich, A., Depaulis, A., Lemeur, M. and Borrelli, E. (1995) Parkinsonian-like locomotor impairment in mice lacking dopamine D2 receptors. Nature (London) **377**, 424–428
34 Dennis, D., Jacobson, K. and Belardinelli, L. (1992) Evidence of spare A1-adenosine receptors in guinea pig atrioventricular node. Am. J. Physiol. **262**, H661–H671
35 Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E. and Graybiel, M. (1998) A family of cAMP-binding proteins that directly activate Rap1. Science **282**, 2275–2279
36 Paul, W. E. and Seder, R. A. (1994) Lymphocyte responses and cytokines. Cell **76**, 241–251

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