Naunyn-Schmiedeberg's Arch Pharmacol (1999) 359: 28–32

ORIGINAL ARTICLE

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Signaling via A_{2A} adenosine receptor in four PC12 cell clones

Received: 29 June 1998 / Accepted: 10 November 1998

Abstract PC12 cells are genetically labile and so-called wild-type cells comprise multiple subclones. We have examined the A_{2A} adenosine receptor signal transduction pathways in four such clones (denoted clones 1, 19, 21 and 27) of PC12 cells. Adenosine A_{2A}, A_{2B} and A_1 receptor mRNAs were detected in all four clones by RT-PCR, whereas no A_3 receptor mRNA was found. A_{2A} receptors were quantitated by radioligand binding using the antagonist radioligand [^{3}H]SCH 58261 ([^{3}H]-5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4 triazolo [1,5-c] pyrimidine). The B_{max} was highest in clone 1 followed by clones 21, 19 and 27. Whereas the amount of G_i protein appeared similar in all four clones, the amount of G_s protein was higher in clones 21 and 27 than in the other two clones. Maximal responses to the non-selective adenosine analogue NECA (5'-*N*-ethylcarboxamidoadenosine) were similar to those observed with the selective adenosine A_{2A} receptor agonist CGS 21680 (2-[*p*-(2-carbonylethyl) phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine), and were approximately equal in clones 1 and 21, but lower in clone 19 and very low in clone 27. For both compounds EC_{50} was significantly higher in clone 27 than in clone 1. In both clones the response to NECA could be competitively antagonized by a selective adenosine A_{2A} antagonist, SCH 58261.
The present results show that different clones of PC12 cells differ widely in the cAMP increase induced by adenosine analogues and that this is due to differences in the amount of adenosine A_{2A} receptor, G protein and effector. A large difference in receptor number resulted in differences in potency of an agonist.

Key words G proteins · Cyclic AMP · Signal transduction · RT-PCR

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Introduction

Adenosine is a ubiquitous endogenous modulator of several biological processes. It acts on four receptors, A_1, A_{2A}, A_{2B} and A_3, cloned from several mammalian species (see Fredholm et al. 1994). The first two of these receptors have a sufficiently high affinity for the endogenous ligand to be activated already under basal physiological conditions (Fredholm 1995). Adenosine A_{2A} receptors are found in several types of cells, such as endothelial cells, polymorphonuclear leukocytes and a subgroup of medium-sized spiny neurons in the caudate-putamen (Ongini and Fredholm 1996). PC12 cells, derived from a spontaneous rat pheochromocytoma (Greene and Tischler 1976), also possess A_{2A} and A_{2B} receptors (Guroff et al. 1981; Noronha-Blob et al. 1986; Hide et al. 1992; van der Ploeg et al. 1996; Kull et al. 1998).

PC12 cells are widely used to study, e.g., neuronal differentiation, effects of growth factors and the regulation of neurotransmitter release (Greene et al. 1991). It is well known that PC12 cells undergo spontaneous mutations, and heterogeneity in many functional aspects has been reported (Shafer and Atchison 1991). Indeed, the cell populations generally available may be composed of multiple subclones with widely different properties (Clementi et al. 1992).

We have examined signal transduction via A_{2A} adenosine receptors in four of the several clones isolated by Meldolesi and coworkers (Zacchetti et al. 1991; Clementi et al. 1992) from wild-type PC12 cells. We found a marked difference that could be explained by differences in adenosine receptors and/or subsequent steps in the signaling cascade.

Materials and methods

Chemicals. Cell culture media, horse and fetal calf serum, and cell culture flasks were from Nordcell (Bromma, Sweden). [^{3}H]Adenosine 3',5'-cyclic monophosphate ([^{3}H]cAMP) was from Amersham. Antibodies against G proteins were from Santa Cruz Biotechnology

(Santa Cruz, Calif., USA). Forskolin was from Sigma (St. Louis, Mo., USA). 5'-*N*-Ethylcarboxamidoadenosine (NECA) and 2-[*p*-(2-carbonylethyl) phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine (CGS 21680) were from Research Biochemicals International (Wayland, Mass., USA), 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4 triazolo[1,5-c]pyrimidine (SCH 58261) and [^{3}H]SCH 58261 (specific activity 28.4 Ci/mmol) were kindly provided by Dr. Ennio Ongini, Schering Plough (Milan, Italy). A purified mouse monoclonal antibody, obtained by immunizing with the purified full-length, non-denatured human adenosine A_{2A} receptor, was a kind gift from Dr. Joel Linden (University of Virginia, Charlottesville, Va., USA). Horseradish peroxidase conjugated antibodies were from Pierce (Rockford, Ill., USA).

All other reagents were of the best purity commercially available.

Cell culture. The clones of PC12 cells used in this study were a generous gift from Dr. Emilio Clementi. Cells were grown in DMEM supplemented with 5% fetal calf serum, 10% horse serum, 2 mM L-glutamine, penicillin (100 µg/ml) and streptomycin (100 µg/ml), at 37°C in 5% CO_2 and 95% air. Cells were detached and subcultured three times weekly at a density of 500,000 cells per ml.

Measurement of cyclic AMP accumulation. The cells were washed twice in HEPES-buffered DMEM before incubation with drugs. After 10-min incubation, cAMP was extracted with perchloric acid (final concentration 0.4 M). The protein-free supernatant, obtained by centrifugation, was neutralized with 4 M KOH/1 M Tris. Cyclic AMP was assayed as described by Nordstedt and Fredholm (1990), using rapid filtration over Whatman GF/B filters by means of a semi-automatic cell harvester (Skatron A/S, Norway).

A_{2A} receptor binding. PC12 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in 50 mM Tris-HCl buffer, pH 7.4, sonicated (4 times 10 s) and centrifuged at 1000 *g* for 10 min at 4°C. The resulting supernatant was recentrifuged at 30,000 *g* for 60 min at 4°C and the pellet obtained was resuspended in Tris-HCl. Aliquots were rapidly frozen and stored at –20°C.

Thawed membranes were resuspended in the same buffer to a final protein concentration of 0.6 mg/ml. Adenosine deaminase (2 U/ml) was added for 30 min at 37°C before radioligand binding assay to remove endogenous adenosine. Binding of [^{3}H]SCH 58261 to A_{2A} receptors was performed as previously described (Zocchi et al. 1996). Assays were carried out in GF/B filter Millipore plates in triplicates in a final volume of 0.3 ml. Non-specific binding was determined in the presence of 50 µM NECA. After 1-h incubation at 25°C, samples were filtered through a Pall Manifold and rinsed with 0.6 ml 50 mM Tris/HCl, pH 7.4. Twenty microliters of Optiphase Supermix scintillation fluid (Wallac, Turku, Finland) was added and samples were counted in a 1450 Trilux Microbeta (Wallac, Turku, Finland).

Protein analysis by Western blotting. Cell membranes were prepared in the same way as those for binding assays. Proteins were resolved on a denaturing 12% polyacrylamide gel, blotted onto a membrane (Immobilon; Millipore, Bedford, Mass., USA) and non-specific binding was blocked by an overnight incubation in Tris-buffered saline (Tris-HCl, pH 7.6, 150 mM NaCl) containing Tween-20 (0.5%) and 5–10% dry milk. Membranes were incubated with primary antibodies for 1 h, rinsed several times in Tris-buffered saline, incubated with a secondary antibody (horseradish peroxidase conjugated donkey anti-rabbit or donkey anti-mouse) and developed with an enhanced chemiluminescence system (Amersham, UK). The bands were quantitated by means of a digital image analysis system (MCID; Imaging Research, Canada) by summing the density readings along a strip of the gel.

PCR analysis of A_1, A_{2A}, A_{2B} and A_3 receptor mRNA. Cellular mRNA was isolated with a PolyAttract System 1000 (Promega, Madison, Wis., USA). RNA was resuspended in diethyl pyrocarbonate-treated water. RNA concentration was measured spectrophotometrically (Beckman DU-64; Beckman Instruments, Fullerton, Calif., USA) and RNA was diluted to a final concentration of 0.4 µg/µl. cDNA was synthesized by reverse transcription of total RNA. The resulting single-stranded cDNA was submitted to 35 cycles of PCR under standard conditions. The PCR product was separated in a 1.5% agarose gel (BRL, Life Technologies, Gaithersburg, Md., USA) and stained with ethidium bromide.

Oligonucleotide primers for the rat A_{2A} receptor ([5'-CGAATTC-AACCTGCAGAACGTCACC-3', sense] and [5'-TCGAATTCGCG-GTC(G/A)ATGGCGAT(A/G)-3', antisense]), for the rat A_{2B} receptor ([5'-CAGAC(G/C)CCCACCAACTACTT-3', sense] and [5'-GCCA-CCA(G/T)GAAGAT(C/T)TT(A/G)ATG-3', antisense]), for the rat A_1 receptor ([5'-TCGAATTCCAGGCTGCCTACATTGGCAT-3', sense] and [5'-TCGAATTCCAGAAAGGTGACCCGGAACT-3', antisense]) and for the rat A_3 receptor ([5'-AGGTTTGAGTCCAAA-GAATCCG-3', sense] and [5'-AATACCACGACGAGTGCCTTGT-3', antisense]) were chosen using Oligo primer Analysis Software (4.0), and were expected to code for products of 301 (A_1), 216 (A_{2A}), 513 (A_{2B}) and 922 (A_3) bp. For each reaction we ran two negative controls, one sample with no RNA and one sample with no reverse transcriptase enzyme in the RT reactions.

Statistical analysis. Binding parameters were estimated using GraphPadPrism 2 software (Graph Pad, San Diego, Calif., USA). This program package was also used for other statistical analyses.

Results

The previous finding that PC12 cells express adenosine A_{2A} and A_{2B} receptors was confirmed using RT-PCR (Fig. 1). In addition we tested primers for A_1 and A_3 receptors and found that these cells have a message for adenosine A_1 but not for A_3 receptors. Although the amount of A_1 receptor seemed to differ between the cell clones, this has not been studied further because we have shown previously that A_1 receptors do not significantly modify the cAMP responses even in a subclone (clone 1) that appeared to have a relatively large amount of A_1 receptor mRNA (Arslan et al. 1997).

Since the cAMP increase elicited by adenosine analogues in PC12 cells is mostly mediated by activation of A_{2A} receptors (see Hide et al. 1992; van der Ploeg et al. 1996), A_{2A} receptor expression was examined first. Experiments with the A_{2A}-specific antagonist [^{3}H]SCH 58261 showed that receptor binding was significantly higher in clones 1 and 21 than in clones 19 and 27. The K_d-values were approximately 1.5–3 nM in clones 1, 19 and 21. The estimated B_{max}-values were 2.3 (2.13–2.52) pmol/mg protein for clone 1, 0.3 (0.27–0.33) pmol/mg for clone 19 and 0.7 (0.61–0.82) pmol/mg for clone 21. In clone 27 the binding was too low (less than 0.2 pmol/mg) to allow for an accurate estimate of the K_d- and B_{max}-values.

Receptor protein expression was also quantitated by Western blotting (Fig. 2) which confirmed that the receptor is much more abundant in clone 1, followed by clones 21 and 19. The receptor was hardly detectable in clone 27.

Western blotting was also employed in order to assess the content of G protein α-subunits (Fig. 2). $G_{\alpha s}$ was most abundant in clone 27 followed by clone 21, less abundant in clone 1, and markedly so in clone 19. $G_{\alpha o}$ and $G_{\alpha i}$ content in the cells was similar, with the exception of clone 27, which contained very little $G_{\alpha o}$. Forskolin was used to test for differences in adenylyl cyclase activity. The stimulatory effect was largest in clones 21 and 1, whereas cAMP pro-

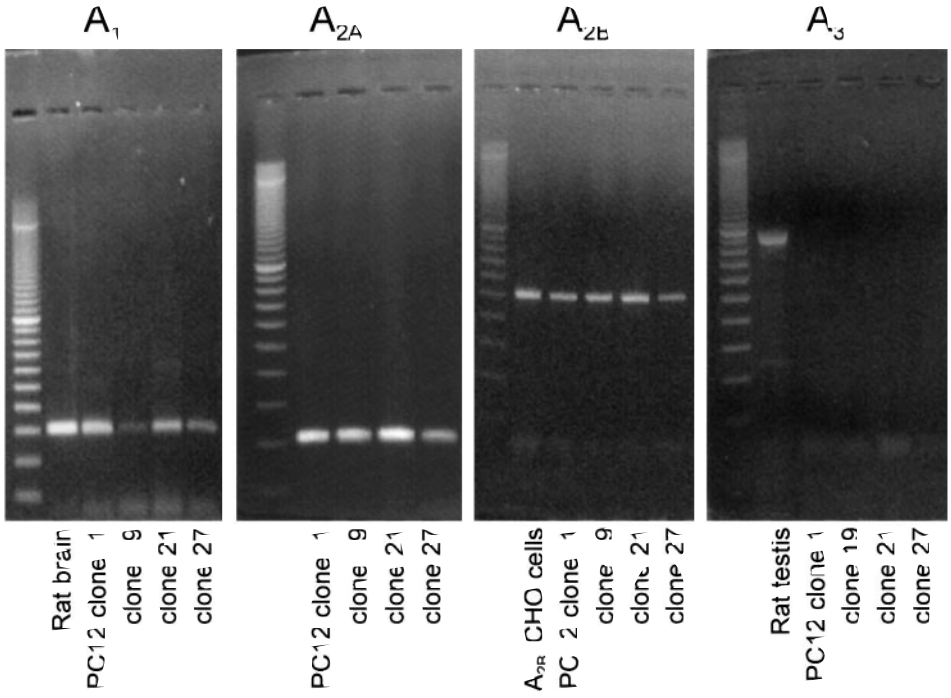

Fig. 1 Presence of adenosine A_1, A_{2A} and A_{2B}, but not A_3, receptor mRNA in PC12 cells. PCR analysis of cDNA obtained by reverse transcription of cellular mRNA showing the expression of A_1 (301 bp), A_{2A} (216 bp), A_{2B} (513 bp) and A_3 (922 bp) receptors in the four clones of PC12 cells. cDNA from rat brain, CHO cells transfected with the human A_{2B} receptor and rat testis were employed as positive controls for A_1, A_{2B} and A_3 receptors, respectively. A 100-bp ladder is shown to the *left* in each panel

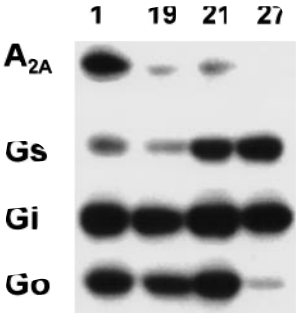

Fig. 2 Insets from Western blots showing the abundance of A_{2A} receptor protein, and of $G_{\alpha s}$, $G_{\alpha o}$ and $G_{\alpha i}$ proteins in the four PC12 cell clones. This figure is representative of three different experiments that led to similar results

duction was much lower in clones 19 and 27 (Fig. 3). All these data are summarized in Table 1.

A_{2A} receptors were next tested from a functional point of view, measuring cAMP responses induced by CGS 21680. This compound, which shows a very high degree of selectivity for rat A_{2A} over rat A_{2B} receptors (see Fredholm et al. 1994), was much more efficacious in clones 1 and 21 than in clones 19 and 27 (Fig. 3). The response was particularly small in clone 27 and even the difference between clones 27 and 19 was statistically significant ($P<0.01$). The EC_{50}-values did, however, differ somewhat. They were similar in clones 1 and 21: 9.1 (5.6–14.9) nM and 7.9 (2.7–23.1) nM (mean and 95% confidence intervals). In

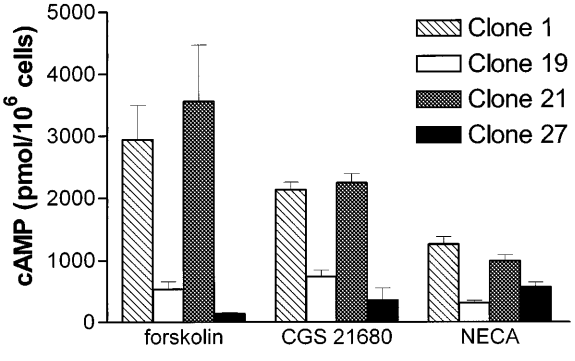

Fig. 3 Effect of CGS 21680 (1 μM), forskolin (30 μM) and NECA (1 μM) on accumulation of cAMP in PC12 cells. The histogram shows the effects of CGS 21680, NECA and forskolin on cAMP accumulation after 10-min incubation in the four clones of PC12 cells. Results are means ± SEM of two to three separate experiments each performed in duplicate. The efficacy of NECA and CGS 21680 in clones 19 and 27 showed a statistically significant difference ($P<0.001$ and $P<0.01$, respectively)

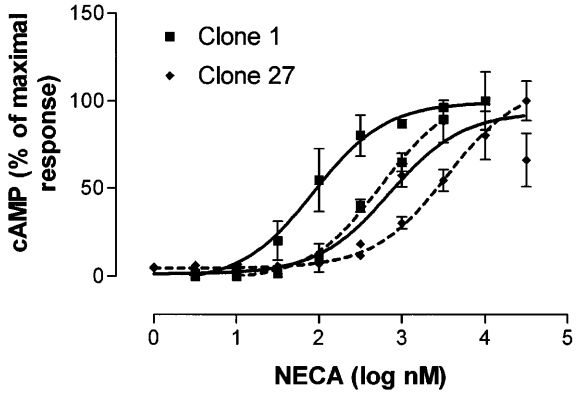

Fig. 4 cAMP response in clone 1 (■) and clone 27 (◆) induced by increasing doses of NECA in the absence (*full line*) or presence (*dotted line*) of 20 nM SCH 58261. Data are from two to three separate experiments each performed in duplicate

Table 1 Differences between subclones of PC12 cells with respect to parameters related to adenosine receptor expression and induced increases in cyclic AMP. For comparison all results are expressed as relative magnitude, the values obtained for clone 1 being set to unity. For details on the individual data see text

Parameter	Clone 1	Clone 19	Clone 21	Clone 27
Adenosine receptor-induced increases in cAMP				
CGS 21680 (A_{2A} receptor)	1	0.3	1	0.2
NECA (A_{2A} and A_{2B} receptors)	1	0.2	0.8	0.5
A_{2A} receptor expression				
[^{3}H]SCH 58261 binding	1	0.13	0.3	<0.06
Western blotting	1	0.2	0.4	<0.05
Amount of G proteins				
$G_{\alpha s}$	1	0.4	3.5	5.6
$G_{\alpha o}$	1	1	1.5	0.1
$G_{\alpha i}$	1	0.7	1.2	1
Adenylyl cyclase activity				
Forskolin-stimulated cAMP	1	0.2	1.2	0.05

clone 27 the value was considerably higher, however: 204 (113–369) nM. Thus the significant, more than 18-fold difference in the number of A_{2A} receptors translates into a significant, approximately 21-fold difference in the EC_{50}-value for a selective agonist.

NECA is not a selective A_{2A} receptor agonist: it also stimulates A_{2B} receptors, albeit much less potently. NECA was most efficacious in clones 1 and 21, whilst clones 19 and 27 displayed much smaller responses (Fig. 3). The EC_{50}-values for NECA were lower in clones 1 and 21 [34 (21–54) nM and 26 (16–44.5) nM, respectively] than in clones 19 and 27 [142 (89–230) nM and 240 (131–440) nM, respectively].

It was, however, important to test if the cAMP response evoked by a non-selective adenosine analogue was partly due to activation of A_{2B} receptors, since clone 1 contains mRNA for this receptor as well. As seen in Fig. 4, the dose-response curve was shifted to the right by SCH 58261, showing that NECA could be competitively antagonized. This competitive antagonism was observed for both clones 1 and 27, despite the large difference in the position of the concentration-response curve. Additional concentrations of the antagonist were used to generate a Schild regression plot. This was linear and had a slope of close to unity, and the estimated pA_2-value was 8.48 in clone 1 cells and 8.30 in clone 27 cells.

Discussion

Cell lines are useful in pharmacological studies because they offer an opportunity to study signal transduction and regulation of receptors under more controlled conditions than in a tissue, which contains multiple cell types. However, immortalized cells tend to undergo spontaneous mutations, thereby generating heterogeneity. PC12 cells are well known to exhibit such heterogeneity (see Introduction) and were examined here. Indeed, we found a surprisingly large variability in the ability of four subclones of a laboratory stock of so-called wild-type PC12 cells to respond to adenosine analogues via adenosine A_{2A} receptors. The reason for this variability appeared not to be the same in all the subclones, as summarized in Table 1.

RT-PCR revealed transcripts for A_1, A_{2A} and A_{2B} adenosine receptors in all four clones, whereas no A_3 receptor transcript was detected. We have confirmed and extended previous findings that the functional response to an agonist capable of stimulating all these receptors (NECA) is almost exclusively accounted for by A_{2A} receptor activation. Therefore this receptor and its signaling via G_s and adenylyl cyclase have been the focus of our investigations.

The number of A_{2A} receptors appeared to differ between the four subclones. Data obtained with the A_{2A} receptor-selective antagonist [^{3}H]SCH 58216 and the A_{2A} receptor antibody were in good agreement and indicated highest A_{2A} receptor content in clone 1, followed by clone 21. Much lower levels were determined in clones 19 and 27 (Table 1). The potency of CGS 21680 in the functional assay, in the three subclones with the highest number of A_{2A} receptors, agreed well with its reported affinity for A_{2A} receptors in a binding assay using membranes from a PC12 cell clone (Belardinelli et al. 1996). In cells of clone 27, however, the potency was markedly reduced. The maximal cAMP response was also variable (Table 1), being lowest in clone 27, indicating that the low amount of A_{2A} adenosine receptors expressed in clone 27 leads to a reduction of both maximal response and EC_{50}.

The magnitude of the cAMP response depends not only on the number of receptors but also on the amount of available G protein and the activity of the effector enzyme. We have shown elsewhere (see Kull et al. 1998) that phosphodiesterase activity does not affect the magnitude of the response in assays with the duration used in the present studies. We examined the additional parameters (G_s content and adenylyl cyclase activity) that account for the magnitude of the responses. Clone 27 showed a low adenylyl cyclase activity and a relatively high expression of G_s, which, however, did not compensate for the relative lack of the two other important components. Clone 27 appears therefore to express less A_{2A} receptor and cyclase effector and also shows lower functional responses than all the other clones. The rather small response of clone 19 as compared to clone 1 appears to be due to a relatively low receptor number, G_s content and adenylyl cyclase activity. By contrast, the fact that clone 21 showed an overall response similar to that of

clone 1 despite the lower number of A_{2A} receptors results from a higher amount of both G_s and adenylyl cyclase.

From the data summarized in Table 1 it may therefore be concluded that the maximal response is determined to a major extent by the amount of adenylyl cyclase effector, as assessed by forskolin stimulation. On the other hand, the number of adenosine A_{2A} receptors can determine the position of the dose-response curve. From a comparison of clones 1 and 21 it would appear that small differences in receptor number may be compensated for by an increased amount of the relevant G protein. If, however, the number of receptors is drastically reduced, as in clone 27, there is a large shift in the dose-response curve that is apparently not compensated for by a major (over fivefold) increase in G_s protein.

NECA was tested to find out whether A_{2B} receptors could contribute to the response to adenosine analogues in these cells. In clone 19 and 27 cells the EC_{50}-value for NECA was higher than in clones 1 and 21. This could indicate that A_{2B} receptors might contribute to the cAMP response in the two former clones. Indeed, in clone 27 cells NECA was more efficacious than CGS 21680; this is also compatible with a substantial contribution by A_{2B} receptors. However, when the NECA dose-response curve was antagonized by the A_{2A}-specific antagonist SCH 58261, the shift to the right was virtually identical in clones 1 and 27, showing that contribution of A_{2B} receptors to the functional responses is at most marginal.

Our results suggest that at least some of the reported variability in responses to adenosine in PC12 cells could be due to the fact that "wild-type" PC12 cells used in different laboratories are populations of cells, comprising variable proportions of clones that express receptors and other relevant proteins at high and low abundance. It is obvious that the interpretation of concentration-response data is very much complicated if the overall response is composed of several different types of responses. We have also shown that clone 1 presents a prototypical cAMP response to A_{2A} adenosine receptor stimulation, which indicates that this clone can be an appropriate tool for studying this receptor.

Acknowledgements We thank Dr. Emilio Clementi for providing us with the PC12 cell clones and Dr. Ennio Ongini for providing cold and radiolabeled SCH 58261. We are grateful to Janet Holmén for proofreading this manuscript. The monoclonal antibody against the adenosine A_{2A} receptor was a kind gift from Dr. Joel Linden. These studies were supported by the Swedish Medical Research Council (project no. 2553 and 12707), the Swedish Foundation for Strategic Research and by Wallenberg stiftelsen.

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