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Allosteric modulators affect the internalization of human adenosine A_1 receptors

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Abstract

To study the effect of allosteric modulators on the internalization of human adenosine A_1 receptors, the receptor was equipped with a C-terminal yellow fluorescent protein tag. The introduction of this tag did not affect the radioligand binding properties of the receptor. CHO cells stably expressing this receptor were subjected during 16 h to varying concentrations of the agonist N^6-cyclopentyladenosine (CPA) in the absence or presence of 10 μM of the allosteric enhancer PD 81,723 ((2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone) or the allosteric inhibitor SCH-202676 (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2*H*)-ylidene)methanamine). CPA itself was able to internalize 25% and 40% of the receptors at a concentration of 400 nM or 4 μM, respectively. Addition of either PD 81,723 or SCH-202676 alone had no effect on internalization. However, with PD 81,723 a slight amount of internalization was obtained already at 40 nM of CPA and at 400 nM CPA 59% of the receptors internalized. SCH-202676 on the other hand effectively prevented CPA-induced internalization of the receptor.

Keywords: Internalization; Allosteric modulation; PD 81,723; SCH-202676; Human adenosine A_1 receptor; YFP

1. Introduction

Adenosine receptors are members of the superfamily of G protein-coupled receptors (GPCRs) and are pharmacologically classified into four distinct types, namely the adenosine A_1, A_{2A}, A_{2B} and A_3 receptor. Adenosine A_1 and A_3 receptors are coupled to a G_i protein, thereby inhibiting the production of cAMP via adenylate cyclase. In contrast, adenosine A_{2A} and A_{2B} receptors are coupled to a G_s protein, thereby stimulating the production of cAMP (for review on adenosine receptors, see Fredholm et al. (2001).

Like other members of the GPCR family, adenosine receptors are subject to allosteric modulation (for reviews on allosteric modulation, see Soudijn et al. (2001) and Christopoulos and Kenakin (2002)). PD 81,723 ((2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone) is an allosteric enhancer selectively exerting its action on the adenosine A_1 receptor. It potentiates the agonist binding, thereby enhancing the functional effects of adenosine or its analogs (Musser et al., 1999). It has also been reported that PD 81,723 potentiates constitutive activity of the adenosine A_1 receptor (Kollias-Baker et al., 1997). SCH-202676 (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2*H*)-ylidene)methanamine) on the other hand, is a general allosteric inhibitor which inhibits both agonist and antagonist binding to a number of GPCRs such as human μ-, δ-, and κ-opioid, α- and β-adrenergic, muscarinic M_1 and M_2, and dopaminergic D_1 and D_2 receptors (Fawzi et al., 2001), including adenosine receptors (Van den Nieuwendijk et al., 2004; Gao et al., 2004).

Adenosine receptors, like other GPCRs, undergo internalization upon agonist stimulation. Internalization or sequestration is described as the loss of cell surface receptor number, determined by the combined effects of endocytosis and

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recycling (Koenig and Edwardson, 1997). In the present study, we focus on the internalization of the human adenosine A_1 receptor. The process of internalization has been shown to be initiated by the functional desensitization of the adenosine A_1 receptor. After a short period of agonist exposure (5–15 min), the adenosine A_1 receptors uncouple from the G_i proteins due to phosphorylation, catalyzed by specific receptor kinases (Ciruela et al., 1997; Ruiz et al., 1996; Saura et al., 1998; Nie et al., 1997). Especially serine and threonine residues close to the C-terminus of the receptor are susceptible to phosphorylation (Ciruela et al., 1997; Gao et al., 1999). Upon phosphorylation of the adenosine A_1 receptor, β-arrestins are attracted, which couple to the phosphorylated receptor. β-arrestins do not only desensitize the receptor but also function as clathrin adaptors thereby inducing the process of sequestration and internalization. Unlike the uncoupling, $t_{1/2}$ for the internalization process of the adenosine A_1 receptor is quite slow, 10±1 h (Saura et al., 1998). In contrast, $t_{1/2}$ for internalization of the other G_i-coupled adenosine receptor (A_3 receptor) is short, only 10 min (Ferguson et al., 2000).

In the present study, we have examined whether allosteric modulators are able to influence the internalization of the human adenosine A_1 receptor. To visualize this process, we engineered a yellow fluorescent protein (YFP) C-terminal of the human adenosine A_1 receptor. Our findings indicate that the presence of the allosteric enhancer PD 81,723 lowers the threshold of agonist concentration at which receptor internalization occurs. In contrast, the presence of the allosteric inhibitor SCH-202676 almost completely prevented internalization of the human adenosine A_1 receptor.

2. Materials and methods

2.1. Materials

N^6-cyclopentyladenosine (CPA) was obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX-specific activity 124 Ci/mmol) was purchased from NEN (Du Pont Nemours, 's Hertogenbosch, The Netherlands). G418 (neomycin) was obtained from Stratagene (Cedar Creek, U.S.A.). (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluoromethyl)phenyl]methanone (PD 81,723) and (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2*H*)-ylidene) methanamine (SCH-202676) were synthesized in our own laboratory as described by Van der Klein et al. (1999) and Van den Nieuwendijk et al. (2004), respectively. All other chemicals were of analytical grade and obtained from standard commercial sources.

2.2. Construction of human adenosine A_1YFP receptor

The human adenosine A_1 receptor cDNA (1.3 kb) was isolated from pcDNA3 (Invitrogen BV, Breda, The Netherlands) and inserted into the pBluescript II SK(−) vector (Stratagene, Cedar Creek, USA) using *Hind*III/*Xba*I sites. A reverse primer was designed to mutate the stop codon and introduce an extra base and an *Apa*I site at the 3′ end: 3′ gggtcttctctccggactactgc*cccggg*cgc 5′ (*Apa*I restriction site in *italics*). The human adenosine A_1 receptor cDNA was amplified by a polymerase chain reaction (PCR) using a universal forward primer (un-4) for pBlueScript II SK(−) and the designed reverse primer. The PCR product was purified on gel and subcloned into the peYFP-N1 vector digested with *Hind*III/*Apa*I (kindly provided by C. Backendorf, Leiden University), resulting in a human adenosine A_1YFP receptor construct.

2.3. Cell culture

CHO cells were cultured in a humidified atmosphere at 37 °C and 5% CO_2 in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 medium, containing 10% newborn calf serum, 50 IU/ml penicillin, 50 μg/ml streptomycin and 0.8 μg/ml G418 for selection. CHO cells were stably transfected with the human adenosine A_1YFP receptor construct, using 1 mg/ml *N*-(2,3-dioleoyloxy-1-propyl)trimethylammonium methyl sulfate (DOTAP) as described by Beukers et al. (2004). In short, 5×10^4 cells per well (24-well plate) were seeded the day before transfection. For each well, 2.3 μl DOTAP (1 mg/ml) plus 0.7 μg human adenosine A_1YFP receptor cDNA was added to 50 μl DMEM without serum. Liposomes were formed during 20 min at room temperature. Cells were washed twice with DMEM without serum. Next, 50 μl DMEM without serum was added followed by the transfection mix. Cells were left for 2 h at 37 °C, 5% CO_2. Subsequently, the cells were washed with phosphate buffered saline (PBS) and treated with 5% dimethyl sulfoxide (DMSO) in PBS for 3 min. The DMSO mixture was removed and 500 μl of a 1:1 mixture of DMEM/F12 medium, containing 10% newborn calf serum, 2 mM glutamax, 50 IU/ml penicillin and 50 mg/ml streptomycin was added per well. G418 (0.8 mg/ml) was added to the medium to select for cells that had taken up the plasmid. The medium was replaced every other day. After 10 days, individual colonies were selected and transferred to separate wells in a 24-well plate. Radioligand binding experiments were performed to select a clone with a sufficiently high expression level of the human adenosine A_1YFP receptor. This clone was used for internalization experiments. Cells were subcultured twice a week (1:40).

2.4. Preparation of cell membranes

CHO cells stably expressing the wt human adenosine A_1 receptor were obtained from A. Townsend-Nicholson (Townsend-Nicholson and Shine, 1992). Confluent CHO cells expressing either the wt human adenosine A_1 receptor or the human adenosine A_1YFP receptor were scraped in PBS and centrifuged for 10 min at 250 g. The cell pellets were resuspended in ice-cold Tris–HCl buffer, 50 mM, pH 7.4, and homogenized on ice for 5 s at position 8 using an Ystral homogenizer. The homogenates were centrifuged for 45 min at 17,000 *g* at 4 °C. The resulting pellets were resuspended in Tris–HCl buffer, 50 mM, pH 7.4, and 2 IU/ml adenosine deaminase was added. Aliquots were stored at −80 °C. The

protein concentration of the membranes was measured using the BCA method (Smith et al., 1985).

2.5. Receptor binding assays

Radioligand displacement experiments were performed with minor modifications as described previously for the wt human adenosine A_1 receptor in CHO cells (Kourounakis et al., 2001). In brief, membranes (10 μg protein) were incubated in 400 μl Tris–HCl buffer, 50 mM, pH 7.4, in the presence or absence of 10 μM PD 81,723 or 10 μM SCH-202676 and in the presence of 1.6 nM [^{3}H]DPCPX and increasing concentrations of CPA. Non-specific binding was determined in the presence of 1×10^{-4} M CPA. For saturation experiments, concentrations of [^{3}H]DPCPX ranged from 0.1 to 10 nM. Samples were incubated at 25 °C for 1 h in a shaking water bath and the incubation was terminated by adding 1 ml of ice-cold Tris–HCl, 50 mM, pH 7.4 followed by rapid filtration over Whatman GF/B glass-fiber filters. Filters were washed three times with 2 ml of ice cold Tris–HCl buffer and placed in scintillation vials. For saturation experiments, filters were washed six times with 2 ml of ice cold Tris–HCl buffer. Scintillation fluid (Emulsifier Safe, Packard BioScience), 3.5 ml, was added and, after 2 h extraction, radioactivity was counted in an LKB Wallac 1219 rackbeta scintillation counter. Experiments were performed in triplicate.

2.6. Internalization experiments

CHO cells stably expressing the human adenosine A_1YFP receptor were plated on coverslips in 24-well plates at a density of 3×10^4 cells/well in a volume of 0.5 ml. The cells were allowed to attach for 24 h, after which the medium was aspirated and the cells were exposed for 16 h to 0 nM, 40 nM, 400 nM, and 4 μM CPA in the presence or absence of 10 μM PD 81,723 or 10 μM SCH-202676. Cells were washed once with 0.5 ml PBS and fixed with 0.5 ml 4.0% formaldehyde (pH 7.0–7.2) for 10 min at room temperature. Coverslips containing the cells were washed another 3 times with 0.5 ml PBS and subsequently mounted on object glasses using Aqua Polymount® (Polysciences). Coverslips were allowed to dry on air in darkness and stored at 4 °C for short-term storage or −20 °C for long-term storage. Images of the incubated cells were obtained using confocal microscopy (Nikon Eclipse TE 2000-U), excitation at 520 nm, emission at 532 nm, 60× oil enlargement.

2.7. Quantification of internalization

The computer program Image Pro Plus (MediaCybernetics, Germany) was used to quantify the amount of fluorescence in representative transsections of cells. The amount of fluorescence in the cell membrane was determined and expressed as percentage of the total fluorescence and was corrected for the differences in surface area of the transsection. Transsections of at least three different cells were analyzed.

2.8. Quantification of internalization through radioligand binding

In addition, the amount of internalization was quantified with the help of radioligand binding experiments. Therefore, confluent 10 cm plates of CHO cells stably expressing the human adenosine A_1YFP receptor were incubated with or without 4 μM CPA for 16 h. Both were washed once with 5 ml PBS and subsequently scraped in 5 ml PBS. The preparation of the membranes was performed as described in Section 2.4. The only modification was that the homogenates were centrifuged for 2×20 min at 17,000 *g* at 4 °C. Radioligand binding studies were performed on membranes of the control cells as well as the exposed cells as described in Section 2.5.

2.9. Data analysis

Data of radioligand binding and saturation experiments were analyzed using the non-linear regression curve fitting program Prism v. 3.0 (GraphPad, San Diego, CA, USA). Apparent inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng and Prusoff equation $K_i = IC_{50}/(1+[L^*]/K_d)$ where $[L^*]$ is the concentration of the radioligand and K_D its dissociation constant (Cheng and Prusoff, 1973).

3. Results

3.1. Binding profile of wt human adenosine A_1 receptors and human adenosine A_1YFP receptors stably expressed in CHO cells

First, we assessed the affinity and binding capacity of the radioligand [^{3}H]DPCPX for the human adenosine A_1YFP receptor. In Table 1, the K_d and B_{max} values of [^{3}H]DPCPX for the human adenosine A_1YFP receptor in the presence or absence of the allosteric modulators are shown. The K_d value of [^{3}H]DPCPX for the human adenosine A_1YFP receptor was hardly affected by the addition of allosteric modulators. The B_{max} value was slightly but not significantly lowered in the presence of PD 81,723. Subsequently, radioligand binding studies were performed on membranes of human adenosine A_1YFP-CHO cells to characterize the binding properties of the newly constructed human adenosine A_1YFP receptor. In Fig. 1, displacement curves for the human adenosine A_1YFP receptor

Table 1
Saturation parameters of [^{3}H]DPCPX on CHO-hA_1YFP cell membranes

Compound	K_d (nM)	B_{max} (fmol/mg)
Control	1.45±0.05	862±50
+PD 81,723 (10 μM)	1.51±0.15	669±55
+SCH-202676 (10 μM)	1.21±0.09	805±127

B_{max} and K_d values of [^{3}H]DPCPX in the absence or presence of PD 81,723 (10 μM) or SCH-202676 (10 μM) on CHO-hA_1YFP cell membranes were determined. Values are the means (±S.E.M.) of three independent experiments, performed in duplicate. None of the determined values is significantly different compared to the control values, $P>0.05$.

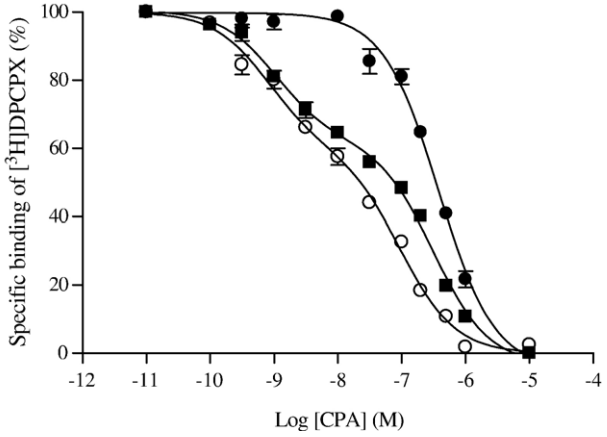

Fig. 1. Displacement of [^{3}H]DPCPX with CPA (■) only or in the presence of 10 μM PD 81,723 (○) or 10 μM SCH-202676 (●). Membranes (10 μg) were incubated for 60 min at 25 °C as described in Section 2.5. The graph is the mean of three displacement curves of the specific binding of [^{3}H]DPCPX to CHO-hA$_1$YFP membranes, each performed in duplicate.

are shown. Displacement experiments were performed using the reference agonist CPA alone and CPA in the presence of 10 μM PD 81,723 or 10 μM SCH-202676. [^{3}H]DPCPX was used as radioligand. The binding properties of the human adenosine A$_1$YFP receptors were compared with the binding profile of the wt human adenosine A$_1$ receptors. Like the wt human adenosine A$_1$ receptor, the human adenosine A$_1$YFP shows a biphasic behaviour towards CPA binding and is also susceptible to allosteric modulation. PD 81,723, the allosteric enhancer, shifts the CPA-curve to the left (gain of affinity) whereas in the presence of the allosteric inhibitor SCH-202676 the CPA-curve is shifted to the right (loss of affinity). In addition, the high-affinity state of the human adenosine A$_1$YFP receptor was shifted to the low-affinity state in the presence of SCH-202676. K_i values of CPA in the presence or absence of the two allosteric modulators for the human adenosine A$_1$YFP receptor are shown in Table 2. The shifts in the K_i values of CPA were 0.24 and 4.0 for the human adenosine A$_1$YFP receptor in the presence of PD 81,723 or SCH-202676, respectively.

3.2. Internalization of the human adenosine A_1YFP receptors

The internalization of human adenosine A$_1$YFP receptors induced by CPA and the influence of allosteric modulators on this process were investigated. First, we determined the time-course needed to observe proper internalization. We incubated CHO cells stably expressing the human adenosine A$_1$YFP receptor at increasing CPA concentrations for the following time points, 0, 2, 4, 6, 8, 16, and 24 h, and observed them with the help of fluorescence microscopy. From these experiments it appeared that clear internalization was only observed after at least 16 h of incubation (results not shown), which is in agreement with the fact that the $t_{1/2}$ for the internalization process of the adenosine A$_1$ receptor is 10±1 h (Saura et al., 1998). Additionally, we observed that the presence of the allosteric modulator PD 81,723 (10 μM) did not accelerate the occurrence of internalization but only increased the amount of internalized receptors (data not shown).

Subsequently, human adenosine A$_1$YFP-CHO cells were plated on coverslips and incubated for 16 h with CPA in a concentration range of 0 nM, 40 nM, 400 nM and 4 μM, in the presence or absence of 10 μM PD 81,723 or 10 μM SCH-202676. Several confocal images were made from each incubation and representative transsections are shown in Fig. 2. Control human adenosine A$_1$YFP-CHO cells show a clear, equally distributed membrane staining. The lowest CPA concentration (40 nM, approximate K_i value) did not induce internalization of the human adenosine A$_1$YFP receptor. At 400 nM CPA (approx. 10×K_i), internalization of the human adenosine A$_1$YFP receptor was apparent as green fluorescent dots in the cytoplasm. This effect was more distinct at the highest concentration CPA (4 μM, approx. 100×K_i). In the presence of the allosteric enhancer PD 81,723 (10 μM) at the lowest CPA concentration (40 nM), membrane staining was not as equally distributed as in the control cells. The internalization of the human adenosine A$_1$YFP receptor at CPA concentrations of 400 nM and 4 μM in the presence of PD 81,723 was more substantial than internalization with CPA alone. Incubation of human adenosine A$_1$YFP-CHO cells in the presence of the allosteric inhibitor SCH-202676 (10 μM) almost completely prevented the internalization of the human adenosine A$_1$YFP receptors. No internalization was observed, except for the highest concentration of CPA (4 μM) where SCH-202676 was not able to completely prevent the internalization of the human adenosine A$_1$YFP receptors. The allosteric modulators alone did not induce internalization.

3.3. Quantitative analysis of internalization

The amount of receptor internalization was quantified with the help of the computer program Image Pro Plus. In Fig. 3, the remaining fluorescence in the cell membrane is represented as a bar graph. At least three transsections of independent cells were quantified. Control human adenosine A$_1$YFP-CHO cells as well as cells exposed to either one of the allosteric modulators showed between 84% and 88% fluorescent membrane staining.

Table 2
K_i values of CPA in the presence or absence of 10 μM PD 81,723 or 10 μM SCH-202676 for hA$_1$YFP receptors expressed in CHO cells

Compound	CHO-A$_1$YFP				
	K_i low (nM)	K_i high (nM)	*Fraction high (%)*	K_i (nM)	*Shift*
CPA	154±9.5	0.61±0.20	*37±2.0*	42.5±7.2	
CPA+PD 81,723	47±7.8	0.40±0.12	*40±5.4*	10.4±3.3	*0.24*
CPA+SCH-202676				172±28	*4.0*

The affinity of CPA for the hA$_1$YFP-receptors in the presence or absence of different modulators was determined by its displacement of [^{3}H]DPCPX binding. Membranes (10 μg) were incubated for 60 min at 25 °C with CPA (in the presence or absence of allosteric modulators) as described in Section 2.5. K_i values±S.E.M. were calculated from three independent experiments. Shifts were calculated by dividing the K_i value in the presence of allosteric modulator by the K_i value of CPA alone. To calculate the K_i values, the respective K_d values (Table 1) were used.

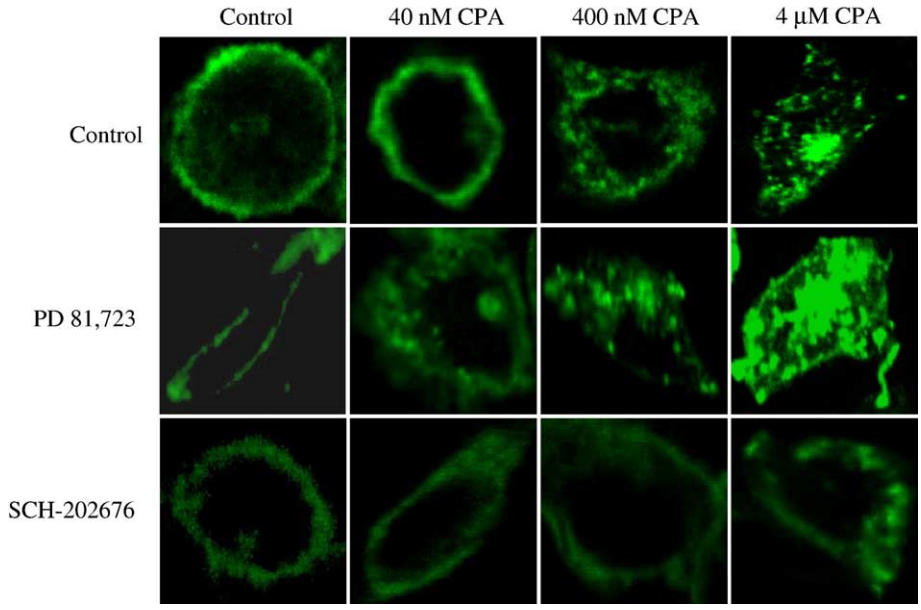

Fig. 2. Confocal fluorescent pictures of internalized hA_1YFP receptors in CHO cells. hA_1YFP CHO cells were incubated for 16 h with increasing concentrations of CPA in the presence or absence of the allosteric modulators PD 81,723 (10 μM) and SCH-202676 (10 μM). Pictures were taken from a representative transsection of the cell.

The 40 nM CPA incubation showed only 11% internalization in the presence of 10 μM PD 81,723; the membrane staining was reduced from 88% to 77%. However, this reduction was not significant. CPA alone (40 nM) and in the presence of SCH-202676 (10 μM) showed values comparable to control cells for membrane staining (respectively, 87% and 84%). Exposure to 400 nM CPA reduced the membrane staining from 88% to 63% (25% internalization), whereas the addition of 10 μM PD 81,723 reduced the membrane staining even further to 29% (59% receptor internalization). SCH-202676 counteracted the effect of 400 nM CPA such that no internalization was observed (90% fluorescence in the membrane). Exposure to 4 μM CPA reduced membrane staining from 88% to 48%, whereas the addition of 10 μM PD 81,723 reduced this percentage to 27% (61% internalized human adenosine A_1YFP receptors), yielding approximately the same amount of internalized receptors as obtained after incubation with 400 nM CPA plus PD 81,723. Incubation with 4 μM CPA in the presence of 10 μM SCH-202676 led to a marginal, non-significant reduction of membrane staining from 84% to 74%.

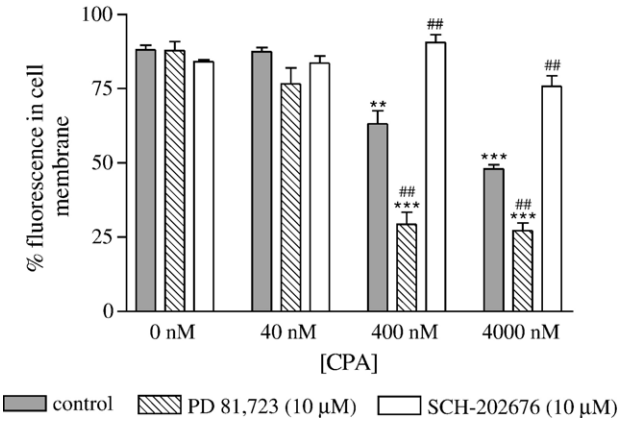

Fig. 3. Quantitative analysis of the internalization of human adenosine A_1YFP-receptors in CHO cells. This bar graph corresponds with the confocal pictures shown in Fig. 2. The bars represent the percentage fluorescence in the cell membrane. Transsections of at least three different cells were analyzed. Values are means ± S.E.M. $^{**}P \leq 0.005$, $^{***}P \leq 0.0005$, percentage fluorescence compared to the percentage fluorescence of corresponding incubations in the absence of CPA. $^{\#\#}P \leq 0.005$ compared to the percentage fluorescence of the respective internal control (equal CPA concentration).

Confirmation of these results was obtained by performing radioligand binding studies on plasma membranes of human adenosine A_1YFP-CHO cells, exposed to 4 μM CPA for 16 h. The percentage of internalization measured as decrease of radioligand binding compared to control (i.e. cells treated identically but without CPA present) was 32 ± 1%, corresponding very well to the 40% internalization measured by confocal microscopy.

4. Discussion

The introduction of a yellow fluorescent protein at the C-terminus of the human adenosine A_1 receptor and stable transfection of this human adenosine A_1YFP receptor into CHO cells provided us with a tool to study internalization of the human adenosine A_1 receptor. By making the human adenosine A_1 receptor visible, there is no need to add fluorescent antibodies or gold particles as has been described before to visualize the internalization of the adenosine A_1 receptors (Navarro et al., 1999; Escriche et al., 2003; Gines et al., 2001; Ciruela et al., 1997; Saura et al., 1998).

Saturation experiments revealed that the addition of YFP to the C-terminus of the adenosine A_1 receptor did not affect the K_d value of [^{3}H]DPCPX for the human adenosine A_1YFP receptor

(1.45±0.05 vs. 1.6±0.1 nM for the wt human adenosine A_1 receptor, Kourounakis et al., 2001). The addition of the allosteric modulators PD 81,723 or SCH-202676 also hardly affected the K_d value (1.51±0.15 and 1.21±0.09 nM, respectively, see Table 1). However, in the presence of PD 81,723, the B_{max} value was slightly lower, 669±55 vs. 862±50 fmol/mg for control, although these values did not differ significantly. Similar effects of PD 81,723 on B_{max} and K_d values for the wt human adenosine A_1 receptor had been found by Bhattacharya and Linden (1996) and Kollias-Baker et al. (1997).

As is shown in Fig. 1 and Table 2, the addition of the C-terminal YFP did not markedly influence the binding properties of the adenosine A_1 receptor. The human adenosine A_1YFP receptor showed a two-state binding curve, similar as described for the wildtype human adenosine A_1 receptor (Dalpiaz et al., 1998). The fraction of high-affinity receptors was 37–40% for the human adenosine A_1YFP receptors, stably expressed in CHO cells. Slightly higher percentages of wildtype human adenosine A_1 receptors in the high-affinity state were found by Musser et al. (1999) and Dalpiaz et al. (1998), 52% and 74%, respectively. The addition of PD 81,723 did not increase the number of human adenosine A_1YFP receptors in the high-affinity state, in accordance with experiments by Musser et al. (1999).

The affinity of CPA for the human adenosine A_1YFP receptor is in the same order of magnitude as reported in the literature for the wt human adenosine A_1 receptor. The K_i value for the low-affinity state was 154±9.5 vs. 76±6 nM for the wt human adenosine A_1 receptor and the K_i value for the high-affinity state was 0.61±0.2 vs. 2.8±0.2 nM for the wt human adenosine A_1 receptor, respectively (Dalpiaz et al., 1998). We observed a shift in affinity caused by the addition of PD 81,723 of 0.30 for the low-affinity state and of 0.66 for the high-affinity state, respectively. This corresponds well with the values found by Musser et al. (1999), reporting shifts for the wt human adenosine A_1 receptor of 0.29 and 0.57, respectively. The addition of SCH-202676 caused a shift towards the low-affinity state, resulting in a one-site binding curve with a K_i value of 172±28 nM. This is in the same order of magnitude as the K_i value found by Heitman (personal communication) for binding of CPA in the presence of SCH-202676 to the wt human adenosine A_1 receptor, 225±8 nM. SCH-202676 inhibited the binding of the agonist CPA to the human adenosine A_1YFP receptor with a shift of 4. This is in accordance with the results found by van den Nieuwendijk et al. (2004), who observed that the binding of the agonist radioligand [^{3}H] 2-Cl-N^6-cyclopentyladenosine ([^{3}H]CCPA) to human adenosine A_1 receptors was reduced to only 10% in the presence of 10 μM SCH-202676. Gao et al. (2004) found that the dissociation of the antagonist [^{3}H] DPCPX was decreased almost 4 times by the presence of SCH-202676.

These binding experiments showed us that the human adenosine A_1YFP receptor retains its pharmacological profile and that the allosteric modulators PD 81,723 and SCH-202676 have the same effect on the wt and the YFP-tagged adenosine A_1 receptor.

Desensitization and presumed internalization of adenosine A_1 receptors after long term exposure to N^6-(R-phenylisopropyl)adenosine (R-PIA) was observed earlier in other tissues such as rat brain (Ruiz et al., 1996), DDT_1 MF-2 cells (Ciruela et al., 1997; Nie et al., 1997) and rat adipocytes (Longabaugh et al., 1989). Human adenosine A_1 receptors, stably expressed in HEK293 cells and pretreated with 10 μM CPA for 24 h, showed a downregulation of 48% (Gao et al., 1999). In these experiments, the desensitization was quantified using radioligand binding experiments on plasma membranes. Here, we describe the internalization of the human adenosine A_1YFP receptor in CHO cells induced by different concentrations of CPA and monitored by confocal microscopy and confirm the results with radioligand binding experiments. After 16 h of incubation with 400 nM CPA, which is approximately 10×K_i of CPA, 25% of the receptors were internalized. It is striking that CPA is not able to induce receptor internalization at a concentration of 40 nM, close to CPA's K_i value (42.5 nM) at which half of the receptor population is assumed to be occupied. This is in contrast to DDT_1MF-2 cells exposed to 50 nM R-PIA for 12–24 h, which showed clear internalization (Ciruela et al., 1997). The K_i value of R-PIA for the adenosine A_1 receptor in DDT_1MF-2 cells is 76 nM (Nie et al., 1997). This difference may be explained to a different regulation of receptor internalization for each cell type the adenosine A_1 receptors are expressed in. Apparently, over 50% of the human adenosine A_1YFP receptors have to be occupied by CPA before the receptors start to cluster, subsequently followed by internalization. Internalization became more apparent (40%) at a concentration of 4 μM CPA (approx. 100×K_i). Radioligand binding experiments performed on membranes of this incubation confirmed this percentage (32±1%). In the presence of the allosteric enhancer PD 81,723 (10 μM), however, some internalization (11%) was already observed at a concentration of 40 nM CPA. The internalization at 400 nM and 4 μM became more distinct in the presence of 10 μM PD 81,723 (59% and 61%, respectively). Allosteric enhancers, such as PD 81,723, have the property to bind to a site distinct from the orthosteric ligand binding site. By doing so, they change the 3D-conformation of the receptor and facilitate ligand binding. In other words, they enhance the affinity of agonists for the receptor. Our data show that this change in receptor conformation, induced by PD 81,723, also enhances clustering and internalization of the human adenosine A_1YFP receptor upon agonist binding, thereby increasing the amount of internalized receptors. This results in a higher percentage of internalized receptors in the presence of PD 81,723 at the same CPA concentration. The action of PD 81,723 is synergistic. PD 81,723 (10 μM) alone has no effect on receptor distribution. This is in accordance with Bhattacharya and Linden (1996) who did not observe a decrease in membrane binding sites after 24 h of pretreatment with 20 μM PD 81,723. Pretreatment of CHO cells stably expressing recombinant human adenosine A_1 receptors with either 10 μM CPA or 10 μM CPA plus 20 μM PD 81,723 resulted in loss of membrane binding sites of over 40%.

Recently, it has been shown that the enzyme adenosine deaminase (ADA) acted as a receptor activity modifying protein

(RAMP) on the adenosine A_1 receptor, and stimulated R-PIA-induced A_1 receptor internalization (Escriche et al., 2003). Thus, ADA also appears to regulate A_1 receptor function allosterically. However, the role of ADA was different compared to that of PD 81,723. ADA accelerated the internalization of adenosine A_1 receptors, $t_{1/2}$ decreased from 10 to 2.9 h, (Saura et al., 1998), whereas PD 81,723 lowers the treshold of agonist concentration at which internalization occurs. ADA did not increase the number of receptors which are internalized (Escriche et al., 2003), whereas PD 81,723 increases the percentage of internalized receptors from 40% to 61% at the highest concentration of CPA tested (4 μM).

Next to the allosteric enhancer PD 81,723 we also studied the allosteric inhibitor SCH-202676. In contrast to PD 81,723, the allosteric inhibitor SCH-202676 (10 μM) completely prevented the internalization of the human adenosine A_1YFP receptors at all CPA concentrations, except for 4 μM CPA. At the highest CPA concentration, some clustering of human adenosine A_1YFP receptors was observed (10%). Even at a concentration of 4 μM CPA, the percentage of occupied receptors is not high enough to induce substantial internalization due to the presence of 10 μM SCH-202676. To the best of our knowledge the effects of this modulator on receptor processing have not been addressed before.

In conclusion, in this study we present the effect of allosteric modulators on the internalization of the human adenosine A_1 receptor. PD 81,723, an allosteric enhancer, is able to lower the threshold of agonist concentration for inducing internalization. Already at a concentration of 40 nM CPA, some internalization is observed. On the contrary, the allosteric inhibitor SCH-202676 is a strong inhibitor of agonist binding, thereby preventing internalization of the human adenosine A_1YFP receptors.

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