Non-ribose ligands for the human adenosine A1 receptor

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CHAPTER 2

Desensitization and Internalization of Adenosine Receptors

Until now, more than 800 distinct GPCRs are identified in the human genome. The four subtypes of the adenosine receptor (A_1-, A_{2A}-, A_{2B}- and A_3 receptor) belong to this large family of GPCRs that represent the most widely targeted pharmacological protein class. Since adenosine receptors are widespread throughout the body and involved in a variety of physiological processes and diseases, there is great interest in understanding how the different subtypes are regulated, as a basis for designing therapeutic drugs that either avoid or make use of this regulation. The major GPCR regulatory pathway involves phosphorylation of activated receptors by G protein-coupled receptor kinases (GRKs), a process that is followed by binding of arrestin proteins. This prevents receptors from activating downstream heterotrimeric G protein pathways, but at the same time allows activation of arrestin-dependent signaling pathways.

Upon agonist treatment, adenosine receptor subtypes are differently regulated. For instance, the A_1Rs are not (readily) phosphorylated and internalize slowly, showing a typical half-life of several hours, whereas the A_{2A}R and A_{2B}R undergo much faster downregulation, usually shorter than 1 hour. The A_3R is subject to even faster downregulation, often a matter of minutes. The fast desensitization of the A_3R after agonist exposure may be therapeutically equivalent to antagonist occupancy of the receptor. This review describes the process of desensitization and internalization of the different adenosine subtypes in cell systems, tissues and *in vivo* studies. In addition, molecular mechanisms involved in adenosine receptor desensitization are discussed.

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Introduction

Adenosine is an important neuromodulator involved in a variety of brain activities and it also serves many different functions in the periphery. This nucleoside, when extracellular, exerts its action via specific G protein-coupled receptors (GPCRs) of the P1 class, divided in four subtypes: A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R[1]. GPCRs consist of a single polypeptide, containing 7 α-helices which are oriented perpendicular to the membrane. The N-terminus is located at the extracellular side of the cell and often contains one or more glycosylation sites. The C-terminus is located intracellularly and contains phosphorylation and palmitoylation sites, which are involved in regulation of receptor desensitization and internalization[2]. All adenosine receptors, with the exception of the $A_{2A}R$, contain a palmitoylation site near the C-terminus. The $A_{2A}R$ is the only subtype with an extraordinary long C-terminus, 122 amino acids versus 36 amino acids in e.g. the A_1R[3]. All the adenosine receptors are glycosylated on the second extracellular loop, although glycosylation does not appear to influence ligand binding. The third intracellular loop and/or the C-terminus are involved in coupling the adenosine receptors to G-proteins. Phosphorylation of in particular intracellular loop 3 is involved in desensitization and internalization of adenosine receptors[4,5,6].

Adenosine and analogues

Adenosine, consisting of a purine ring connected to a ribose group, is the endogenous ligand for the adenosine receptors (Figure 2.1). Under normal conditions, adenosine is continuously formed extracellularly by dephosphorylation of ATP, ADP and/or AMP to adenosine by NTPDases (ecto-nucleoside triphosphate diphosphohydrolases). However, the A_3R can also be activated by inosine, a breakdown product from adenosine[5]. Most adenosine receptor agonists are analogues of adenosine, modified by N^6, C2 and C8 substitutions at the adenine base, and C5' modifications of the ribose moiety[5,6]. Antagonists lack the ribose group and usually possess a mono-, bi- or tricyclic core-structure, e.g. caffeïne, which contains a xanthine as basic structure (Figure 2.1). For extended reviews on high affinity agonists and antagonists for adenosine receptors and their structure-activity relationships, see Palmer and Stiles[1], Fredholm et al.[5], Jacobson and Gao[7], Beukers et al.[8], Müller[9] and Klotz[10].

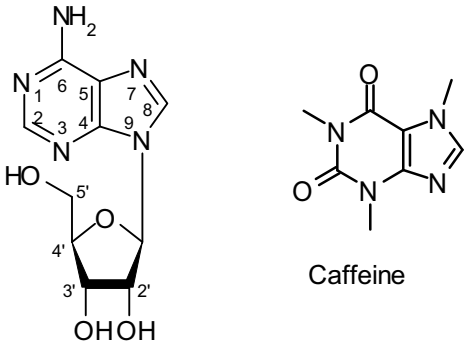

Figure 2.1. Chemical structures of the endogenous ligand adenosine and the antagonist caffeine.

Occurrence and Physiological functions of adenosine receptors

The adenosine receptors are widespread throughout the body, and exert many different functions both in the CNS and in the periphery.

The A_1R is particularly prevalent in the central nervous system, with high levels in the cerebral cortex, hippocampus, cerebellum, thalamus, brain stem and spinal cord. Numerous peripheral tissues also express the A_1R, including vas deferens, testis, white adipose tissue, stomach, spleen, pituitary, adrenal gland, heart, aorta, liver, eye and bladder. Low levels are found in the lung, kidney and small intestine[1,5,6]. The A_1R is involved in cardiovascular effects (e.g. reducing heart rate), inhibition of lipolysis and stimulation of glucose uptake in white adipocytes and the modulation of neurotransmitter release in the CNS[1]. The A_1R also plays a role in anxiety, hyperalgesia, bronchoconstriction and the glomerular filtration rate and renin release in the kidney[5,6,11].

In the CNS, the $A_{2A}R$ is highly expressed in the striatum and olfactory tubercle[1]. In the periphery, it is highly expressed in the spleen, thymus, leukocytes and blood platelets, and intermediate levels are found in the heart, lung and blood vessels[5,6]. The $A_{2A}R$ is involved in the onset of vasodilation, inhibition of platelet aggregation, exploratory activity, aggressiveness and hypoalgesia[1]. In addition, $A_{2A}R$ play a role in Parkinson's disease, Huntington's disease, Alzheimer's disease, ischemia, attenuation of inflammation, and neuroprotection, particularly in peripheral tissues[5,6]. A_{2A} receptor antagonists slow the neurodegeneration which occurs in Parkinson's- and Huntington's disease, and also prevent toxicity induced by beta-amyloid in the development of Alzheimer's disease[12-14].

The $A_{2B}R$ is widely expressed in the brain, but generally at very low levels. In the periphery, high levels of $A_{2B}R$ were detected in the cecum, large intestine and urinary bladder. Lower levels were observed in the lung, spinal cord, vas deferens, pituitary, adipose tissue, adrenal gland, kidney, liver and ovaries[5,6]. Since there is a lack of specific agonists for the $A_{2B}R$, little is known about the functional significance of this receptor. However, the $A_{2B}R$ plays a role in mediating vasodilation in a.o. the aorta, the renal artery and the coronary artery of different species. It is also involved in allergic and inflammatory disorders[5,6].

The A_3R is expressed in the CNS, but at relatively low levels, and only the hypothalamus and the thalamus have been reported to contain A_3R[15]. The highest levels of adenosine A_3R have been found in lung and liver, and somewhat lower levels were found in the aorta[1]. In addition, the A_3R was found in eosinophils, mast cells, testis, kidney, placenta, heart, spleen, uterus, bladder, jejunum, aorta, proximal colon and eye, although with pronounced differences in expression level between species[5,6,16]. The A_3R has been implicated in mediating allergic responses, airway inflammation and apoptotic events, however, the latter is dependent on the cell type

involved and/or the type of activation[5,16]. Furthermore, the A_3R is involved in the control of the cell cycle and inhibition of tumour growth both *in vitro* and *in vivo*[6]. In fact, adenosine A_3 receptors have been demonstrated to be more highly expressed in tumours than in healthy cells, suggesting a role for A_3R as a tumour marker[17].

Signal transduction of adenosine receptors

G protein-coupling and second messengers

Heterotrimeric G proteins are guanine-nucleotide regulatory protein complexes composed of α and $\beta\gamma$ subunits. They are responsible for transmitting signals from G protein-coupled receptors to effectors, e.g. adenylyl cyclase. Until now, 16 α, 5 β and 14 γ isoforms have been reported[18]. G proteins are divided in several subclasses with a specific activity profile: G_s proteins stimulate adenylyl cyclase, G_i proteins inhibit adenylyl cyclase and stimulate GIRK channels, G_0 proteins stimulate K^+ ion channels, $G_{q/11}$ proteins activate phospholipase C, G_{12} proteins activate Rho guanine-nucleotide exchange factors (GEFs) and the olfactory G protein, G_{olf}, stimulates adenylyl cyclase. Upon receptor activation, both the α subunit and the $\beta\gamma$ subunit can signal, but to different effectors[19-21]. For a recent review on G proteins, see Milligan and Kostenis[18].

The A_1R is usually coupled to a pertussis toxin-sensitive $G_{i\alpha}$ protein, which mediates inhibition of adenylyl cyclase and regulates calcium and potassium channels[1,3,5,6,11]. Both the third intracellular loop and the C-terminal tail of the A_1R are involved in $G_{i\alpha}$ coupling[5]. In addition, it has been reported that under certain conditions the A_1R couples to G_s to stimulate adenylyl cyclase, or to $G_{q/11}$ to stimulate inositol phosphate production. Apparently, the specific activity state of the receptor or the nature of the agonist determine which G protein class is activated by the A_1R[22,23]. The $A_{2A}R$ in the periphery is coupled to cholera toxin-sensitive $G_{s\alpha}$ proteins, which increase adenylyl cyclase activity upon receptor activation. The $A_{2A}R$ in the striatum is presumably coupled to G_{olf}[5,6]. The third intracellular loop, but not the C-terminus of the $A_{2A}R$, is involved in $G_{s\alpha}$ coupling[5]. The $A_{2B}R$ is coupled to $G_{s\alpha}$ proteins leading to stimulation of adenylyl cyclase upon receptor activation[6]. There is quite some evidence that $A_{2B}R$ can activate phospholipase C as well, via $G_{q/11}$ proteins[5]. The A_3R is coupled to pertussis toxin-sensitive $G_{i\alpha}$ proteins, which mediate inhibition of adenylyl cyclase. In addition, A_3R can stimulate phospholipase C via $G_{q/11}$ proteins[5,6]. For an extensive overview of adenosine receptor G protein-coupling, see Fredholm et al.[24].

Desensitization and internalization – general principles and players

Mechanisms to dampen GPCR signaling exist at every level in the cell. In this paragraph attention will be paid to the underlying principles of desensitization and internalization and the protein partners involved in these processes.

Receptor localisation

Depending on the localisation signal, GPCRs in the plasma membrane can be targeted to lipid rafts[1] or caveolae[2]. Different regions of GPCRs can influence not only the targeting to either lipid rafts or caveolae but may also enable an interaction of the receptor with constituents of these rafts and caveoli. For instance the extracellular part of the receptor might interact with GM1 gangliosides (glycosphingolipids) present in lipid rafts/caveoli. In addition, the C-terminal fatty acid acylation or palmitoylation may also affect targeting of GPCRs to either lipid rafts or caveoli. Finally, transmembrane regions may interact with cholesterol in the lipid rafts resulting in a change in conformation of the α-helices. Since the conformation of α-helices depends on the activation state of GPCRs, it may well be that agonist binding to the receptor may affect its localisation in lipid rafts by means of molecular transitions leading to receptor activation[25,26].

Desensitization

Desensitization reduces receptor activity and plays a role in signal duration, intensity and quality. Desensitization is initiated by phosphorylation of serine and/or threonine residues in the third intracellular loop and C-terminus of the receptor. Two types of desensitization occur, heterologous and homologous desensitization, and both are the result of receptor phosphorylation. Heterologous desensitization is induced by phosphorylation of the receptor by protein kinase A or C – sometimes even without agonist occupancy. On the other hand, homologous desensitization is specific for agonist-occupied receptors, and consists in most cases of two steps. Firstly, the receptor is phosphorylated by one of the G protein-coupled receptor kinases (GRKs 1-7); then it binds to β-arrestin, of which two subtypes exist, that exhibit high affinity for agonist-occupied, phosphorylated receptors. β-Arrestin serves to sterically inhibit

[1] **Lipid rafts** are planar domains of 25-100 nm in cell membranes enriched in specific lipids and proteins. They are in particular characterised by a high cholesterol and glycosphingolipid content in the outer leaflet of the lipid bilayer that gives them a gel-like liquid-ordered organisation in comparison with the surrounding phospholipid-rich disordered membrane[26].

[2] **Caveolae** are flask-shaped invaginations located at or near the plasma membrane with a 50-100 nm diameter. They are considered to be a non-planar subfamily of lipid rafts. The shape and structural organisation of caveolae are due to the presence of caveolin-1, -2 and -3, that self-assemble in high-mass oligomers to form a cytoplasmic coat on the membrane invaginations[25,26].

G protein coupling, thereby terminating the G protein activation, and may also target the receptor to clathrin-coated pits[3] for internalization (Figure 2.2)[19,25,27,28].

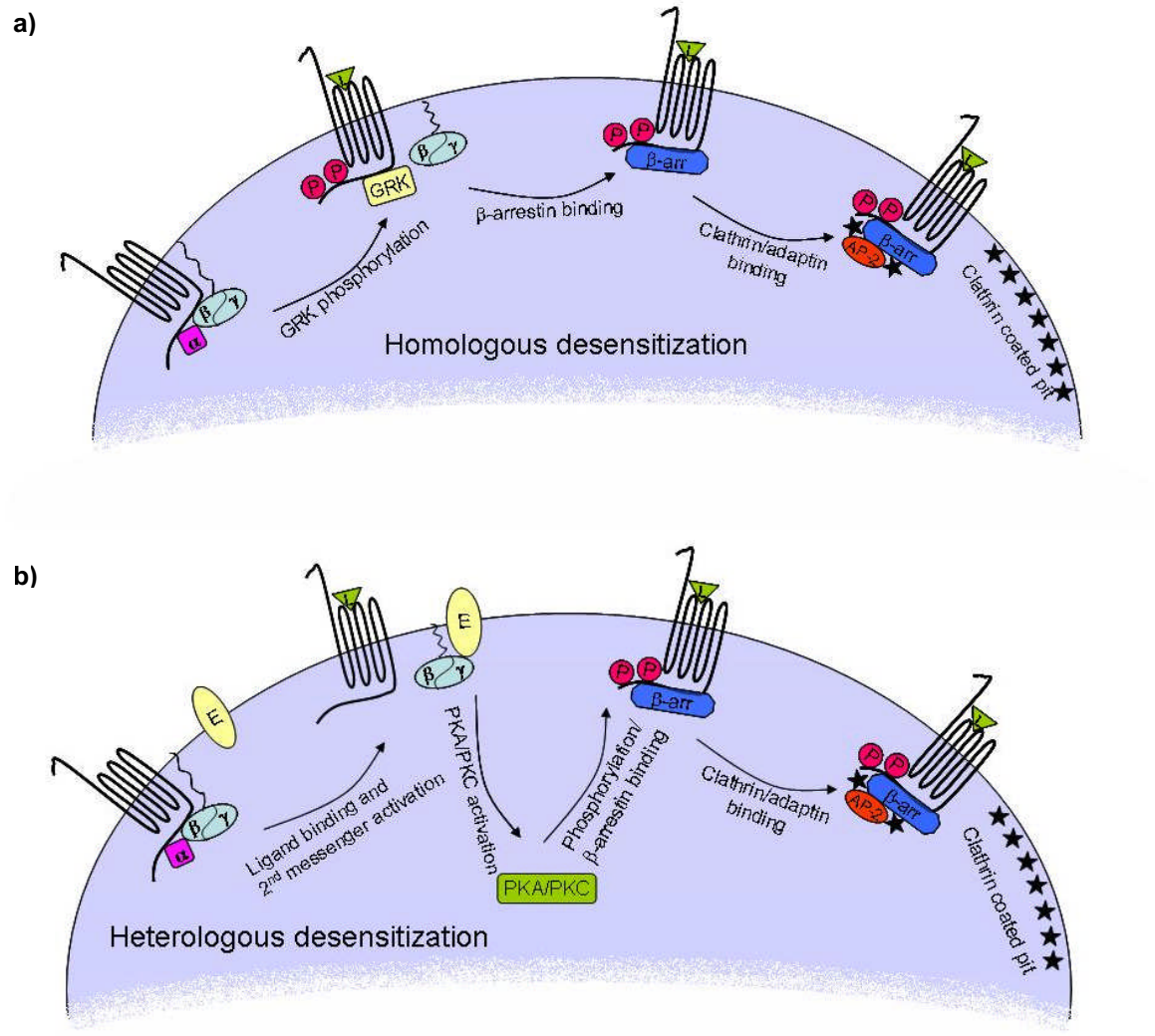

Figure 2.2. a) Homologous and b) heterologous desensitization and subsequent internalization of GPCRs. α = $G_{i\alpha}$ subunit, $\beta\gamma$ = $G_{i\beta\gamma}$ subunit, L = ligand, GRK = G protein-coupled receptor kinase, P = phosphorylated amino acids, β-arr = β-arrestin, AP-2 adaptin, E = effector, second messenger, PKA = protein kinase A, PKC = protein kinase C.

Internalization

Receptor desensitization, initiated by phosphorylation of the receptor by different protein kinases (A or C) or GRKs, can be subsequently followed by receptor internalization. Upon phosphorylation, β-arrestin 1 or 2 is attracted to the receptor[31]. β-Arrestins not only interact with the phosphorylated receptor, but also bind to the heavy chain of clathrin, to the β2-adaptin subunit of the clathrin adaptor protein AP-2,

[3] **Clathrin-coated pit** is a specialized region of the cell surface that mediates the internalization of extracellular macromolecules and GPCRs. Coated pits derive their name from the presence of a distinctive polygonal lattice that decorates the inner surface of the membrane. This lattice is composed of multiple triskelion-shaped subunits that contain three clathrin heavy chains (180 kD) and three clathrin light chains (30-40 kD) plus a family of associated poteins with molecular sizes of 50 kD and 110 kD [29,30].

and to phosphoinositides. These interactions direct the phosphorylated receptor to punctate clathrin-coated pits in the cell membrane, which are internalized by action of the GTPase dynamin. Upon internalization, receptors can either be rapidly recycled to the plasma membrane, targeted to larger endosomes and slowly recycled, or degraded in lysosomes. The final destination of the internalized receptors largely depends on the β-arrestin subtype (1 or 2) that is recruited by the receptor upon phosphorylation and the duration of β-arrestin binding. In this way, internalization may regulate receptor resensitization and contributes to a positive regulation of receptor signalling[19,25,31].

Internalization pathways

From internalization studies with several receptors, it appears that the internalization pathway is specific for receptor type, cell type, metabolic state of the cell, cell-specific factors etc. Receptor trafficking can be regulated in different ways (Figure 2.3): a) the receptor resides mainly in lipid rafts/caveolae and enters the cell via this pathway by default; b) the receptor is in lipid rafts, but leaves these upon agonist binding to be internalized via clathrin-coated pits; c) the receptor moves into lipid rafts upon agonist binding and is internalized via this pathway; d) the receptor moves into lipid rafts after agonist binding to activate certain signalling events, but is eventually moved out of the lipid rafts to be internalized via clathrin-coated pits. Internalization can even be achieved via uncoated vesicles or by a combination of two or more of the afore mentioned pathways. For example, β1-AR is internalized via both lipid rafts and clathrin-coated pits. PKA phosphorylation directs β1-AR to a clathrin-coated pit, whereas GRK phosphorylation directs the receptor to lipid raft-mediated internalization[19,25,26].

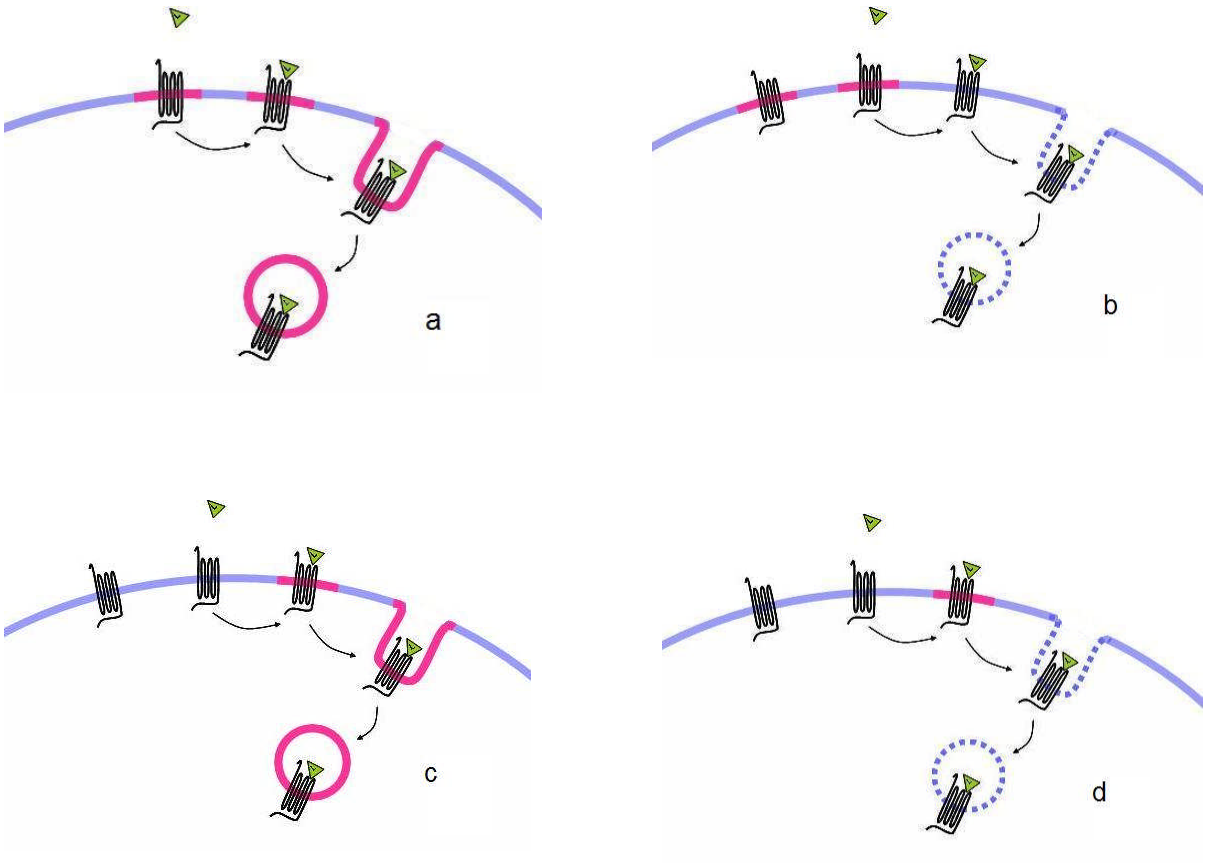

Figure 2.3. Different internalization pathways, adapted from Chini and Parenti, 2004[25]. Internalization via a) lipid rafts/caveolae b) upon agonist binding, the receptor moves to clathrin-coated pits to be internalized c) the receptor moves into lipid rafts upon agonist binding and is internalized d) the receptor moves into lipid rafts upon agonist binding to activate certain signalling events, but is eventually moved out to be internalized via clathrin-coated pits. Ligand (L, green triangle), clathrin-coated pits (dotted blue lines) and lipid rafts (solid pink lines) are indicated.

Function of lipid rafts/caveolae

The existence of lipid rafts/caveolae serves different functions. First of all, lipid rafts act as 'stations' in which GPCRs accomplish specific signalling tasks by meeting a selected set of signalling molecules, e.g. G proteins and adenylyl cyclases. Another possible function of lipid rafts is the protection of receptors from rapid constitutive or agonist-induced internalization, thus allowing their coupling to specific signalling pathways. In addition, caveolin may regulate the constitutive activity of receptors[32]. Finally, the endocytic pathways that GPCRs choose may depend on cell-specific factors. Switching the internalization pathway from lipid rafts/caveolae to clathrin-coated pits may alter the final receptor destination[19,25,26]. Research on adenosine receptors probably provided the first account of receptor internalization via caveolae and lipid rafts, as alternative to the well described β-arrestin pathway[33].

G protein-coupled receptor kinases (GRKs)

Upon agonist binding to GPCRs, not only G proteins are recruited to the activated receptor conformation, but also two other protein families, namely the G protein-coupled receptor kinases (GRKs) and the β-arrestins[31]. The GRK family consists of seven different genes. The GRKs have been divided into three protein subfamilies, based on sequence similarity. GRK1 and -7 belong together. The second subfamily consists of GRK2 and -3 since their membrane recruitment depends on interaction with Gβγ subunits and phosphatidylinositol 4,5-bisphosphate. GRK2 and -3 are primarily responsible for agonist-dependent receptor phosphorylation, β-arrestin recruitment and functional uncoupling of the receptor. GRK4, -5 and -6 form the third subfamily and are constitutively associated with the membrane. GRK1 and -7 are expressed mainly in the retinal rods and cones, whereas GRK4 expression is limited to the cerebellum, testis and kidney. In contrast, GRK2, -3, -5 and -6 are widely expressed in mammalian tissues[31].

β-arrestins

β-arrestins belong to the arrestin family of which four members have been identified. Arrestin-1 and arrestin-4 are expressed in the visual system in retinal photoreceptor cells; arrestin-2 (also called β-arrestin1) and arrestin-3 (also called β-arrestin2) are more widely expressed and are involved in the regulation of nonvisual GPCRs. The β-arrestins were originally discovered as molecules that bind to and desensitize the activated and phosphorylated form of G protein-coupled receptors as described before[19,25,27,28,31]. The β-arrestin protein consists of an N and a C domain, which are almost entirely composed of antiparallel β sheets, connected by a linker of 12 amino acids. A 'polar core' is embedded between those two domains, and its disruption by the phosphorylated C-terminus of the activated receptor leads to conformational changes in the β-arrestin. The C tail of the β-arrestin is then released, exposing both the clathrin and the AP2-binding domains. Recently, it appeared that β-arrestins not only are involved in the desensitization of G protein-coupled receptors, but also act as signal transducer on their own. As a consequence, they emerge as multifunctional adaptor/scaffold proteins which mediate cellular processes such as chemotaxis, apoptosis and metastasis besides receptor signalling and trafficking. For an extensive and recent review on the interactions of β-arrestins with other cellular proteins, see Lefkowitz et al.[34].

GRKs and β-arrestins orchestrate GPCR activities at three different levels: 1) silencing: the functional uncoupling of the receptor from its G protein by a mechanism known as 'homologous desensitization', 2) trafficking, which involves receptor internalization, resensitization and/or degradation, 3) cross-signalling: activation or

inhibition of intracellular signalling pathways, independent of heterotrimeric G proteins[31].

Dampening adenosine receptor signals

In this section we will discuss the evidence for and mechanisms of desensitization and internalization of the four adenosine receptor subtypes. For each receptor we will first summarize results from *in vitro*, *ex vivo* and *in vivo* studies. This will be followed by a more biochemical approach in which we will focus on the molecular mechanisms of adenosine receptor desensitization and internalization by paying attention to the role of accessory proteins, the influence of receptor mutations, etc. We will refer to earlier seminal publications, but mostly focus on more recent evidence for adenosine receptor desensitization and internalization.

A_1 receptor

Cellular and physiological studies

The early evidence for adenosine A_1 receptor desensitization was largely obtained from primary cells, cell lines, tissues or tissue slices, and intact animals that were exposed to varying concentrations of adenosine receptor agonists (either A_1-selective or not), often examined over several time periods.

Stiles and co-workers were among the first to study A_1 receptor desensitization in detail (1991). Ramkumar et al. pretreated DDT1 MF-2 cells, a smooth muscle cell line expressing both A_1 and A_{2A} receptors, with (*R*)-*N*^{6}-(2-Phenylisopropyl)adenosine (*R*-PIA) for up to 24 h, after which the adenylyl cyclase activity was reduced by approx. 50%. This was associated with a significant decrease in cell membrane-bound A_1 receptors, and a concomitant increase of receptor number in intracellular compartments. The authors also showed an increase in receptor phosphorylation, nicely paralleling the time course of adenylyl cyclase modulation[35]. In a later study, Nie et al. reported similar findings in the same cell line, although desensitization occurred at a somewhat faster pace (4 h)[36]. Interestingly, Palmer et al. were unable to demonstrate desensitization in CHO cells expressing the human A_1R[37]. Klaasse et al., however, were able to show internalization of the human A_1R tagged with a C-terminal yellow fluorescent protein (hA_1YFP-R), stably expressed in CHO cells. Exposure of these cells for 16 h to 400 nM or 4 μM CPA resulted in 25% and 40% receptor-internalization, respectively. Addition of 10 μM of the allosteric enhancer PD81,723 did not accelerate the internalization process, but lowered the threshold-

concentration at which internalization occurred. Under those conditions a small degree of internalization was observed already at a concentration of 40 nM CPA, and at 400 nM CPA, 59% of the receptors internalized[38].

Exposure of cerebellar granule cells, endogenously expressing A_1R, to 100 nM *R*-PIA for 2 to 48 h led to a blunting of the inhibition of adenylyl cyclase. Along with this observation, a decrease of [^{3}H]cyclohexyladenosine ([^{3}H]CHA) binding to intact cerebellar granule cells, and an increase of [^{3}H]CHA binding in microsomes was detected. Simultaneously, a decrease in the steady-state level of $G_{i\alpha}$ in plasma membrane and microsomes was observed. These findings point not only to homologous desensitization, but also to subsequent internalization of the A_1R in the microsomal fraction of cerebellar granule cells upon long-term agonist exposure. However, no change in mRNA level was observed, suggesting that post-transcriptional regulation underlies receptor desensitization[39].

In hippocampal neurons, A_1R are present on both presynaptic and postsynaptic terminals, as well as on the cell body and dendrites where they exert different actions. To address the question whether desensitization was influenced by subcellular localization, neurons were exposed to the agonist 2-chloroadenosine (CADO, 20 μM) from 2 up to 96 h. It was found that upon agonist exposure in cultured hippocampal neurons, the presynaptic A_1Rs desensitize less quickly (>12 h) than the postsynaptic A_1Rs (2 h). In accordance, the recovery of desensitized presynaptic A_1Rs also requires more time (48 h vs. 8 h for postsynaptic A_1R). Desensitization of the postsynaptic A_1Rs apparently occurs at the level of the receptor, because the other elements of the signal transduction machinery appeared to be fully functional upon receptor desensitization. All in all, these results suggest that the extent and the kinetics of agonist-induced desensitization of A_1Rs depend on the subcellular localization of the receptors[40].

Using a more intact preparation, Coelho et al. found that the density of A_1R in rat hippocampal slices was decreased with 30% upon 60 min of imposed hypoxia. This desensitization could be mimicked by adding the A_1R agonist CADO (10 μM) for 60 min, and was prevented by adding the A_1R antagonist DPCPX. These results suggest that hypoxia leads to an increase in extracellular adenosine levels, and a subsequent, quite rapid (<90 min) desensitization, possibly followed by subsequent internalization of the A_1R in nerve terminals[41].

Rat *in vivo* studies also demonstrated A_1R desensitization. In a study by Parsons and Stiles rats were chronically (6 d) infused with *R*-PIA. Examination of adipocyte membranes of both treated and control rats revealed a 40% lower inhibition of adenylyl cyclase in the treated animals, coinciding with a reduced number of A_1Rs (68% agonist-occupied receptors remaining) as determined by radioligand binding[42]. Upon further study it was noticed that the effects on adenylyl cyclase were not A_1R

specific but were controlled at the level of adipocyte G proteins. G_i levels appeared downregulated, whereas the amount of G_s in the preparation was increased, although not at the level of their mRNA, suggesting a heterologous form of desensitization[43].

Chronic exposure of rats to *R*-PIA (6 d) also led to A_1R desensitization in the brain, and subsequent reduced inhibition of adenylyl cyclase. This loss of response was accompanied by a significant decrease in both total numbers of A_1R and $G_{i\alpha}$ proteins in synaptic plasma membranes in the brain, paralleling the finding by Stiles and co-workers in adipocytes[44]. As a consequence, a significant increase of A_1R was observed in microsomes and coated vesicles, which suggested a role for coated vesicles in the internalization of A_1R. Similarly, chronic agonist exposure of rats to NECA (6 d) resulted in a significant decrease of A_1R in the high-affinity state in the rat brain, however without changes in adenylyl cyclase activity or inhibition of the $G_{i\alpha}$ proteins[45].

Molecular mechanisms

As discussed in the general introduction to this review, receptor posttranslational modifications, receptor phosphorylation, recruitment of arrestins, and the formation of clathrin-coated pits are elements of the molecular machinery of desensitization and internalization. In addition, other potential protein partners in the two processes have been studied for the various adenosine receptor subtypes, which will also be discussed.

Effect of receptor posttranslational modifications on desensitization and/or internalization

Gao et al. studied the effects of preventing palmitoylation of the A_1R. It appeared that the $Cys^{309}Ala$ mutation, thus removing the palmitoylation site of the human A_1R, had no effect on internalization[46]. These findings were later confirmed by Ferguson et al.[47].

GRKs and arrestins

A_1R phosphorylation by GRKs has been subject to debate. Palmer et al. and Ferguson et al. failed to demonstrate GRK-2 phosphorylation of A_1R; in the latter study it was found that nonphosphorylated A_1R redistributes arrestin-3 from the cytoplasm into punctate clusters at the plasma membrane[37,47]. Nie et al., however, reported that within 1 hr of exposure of DDT_1MF-2 cells to *R*-PIA, rapid translocation of GRKs was observed from the cytosol to the cytoplasm[36]. In a further biochemical approach with both purified receptor and GRK-2, a phosphorylated receptor was

obtained that showed enhanced affinity for arrestins over G proteins. The same receptor kinase, upon overexpression in FRTL-5 cells, influenced A_1R signaling, however, not via $G_{i\alpha}$-mediated adenylyl cyclase but through $G_{i\beta\gamma}$-mediated MAP kinase activation[48]. It may be concluded that under physiological conditions A_1R phosphorylation does not (readily) take place, which would be a rationale for the long time periods required for receptor internalization.

Other protein partners

The ecto-enzyme adenosine deaminase (ADA) regulates the extracellular adenosine concentration by converting excess adenosine into inosine. However, ADA also plays a role in the desensitization and internalization of A_1R in smooth muscle DDT_1MF-2 cells[49,50] and LLC-PK_1 epithelial cells[51], acting as a receptor activity modifying protein (RAMP). Upon agonist exposure (100 nM *R*-PIA, 2h), ADA and A_1R formed complexes on the cell surface, clustered and internalized together to intracellular compartments. Such clustering of adenosine A_1 receptors prior to internalization was also reported by Ciruela et al. and Saura et al.[49,52].The intracellular vesicles contained the lipid raft marker protein caveolin. Filipin, an agent that disrupts rafts or caveolae, inhibited A_1R internalization. In contrast, acidic treatment disrupting clathrin-coated vesicles, did not inhibit agonist-induced internalization of A_1R. These results indicate that ADA and A_1R form a stable complex in the cell membrane of LLC-PK_1 cells, internalizing upon agonist exposure via lipid rafts, in a clathrin-independent pathway. Furthermore, a direct interaction of the C-terminus of A_1R with caveolin was demonstrated[50]. These and other data suggest that the mode of receptor compartmentalisation in response to agonist stimulation may be governed by both receptor subtype and cell type[51].

Another accessory protein, the heat shock cognate protein 73 (hsc73), a member of the hsp70 family, was identified as a cytosolic component able to interact with the third intracellular loop of the A_1R. The interaction between hsc73 (30 nM) and A_1R led to a marked reduction in affinity of ligands for the A_1R and also prevented activation of the G proteins, even more so than the addition of GTP analogues or GTP itself (100 μM). These effects were completely prevented by the addition of 25 nM ADA. A high percentage of A_1R was coupled to hsc73 in cell lysate, according to immunoprecipitation experiments. A remarkable feature upon internalization of the receptor in DDT_1MF-2 cells was found; A_1Rs internalized via two different vesicle types, one in which A_1R and hsc73 are colocalized and another in which hsc73 was absent. These observations open the possibility that signalling via GPCRs is regulated at least to some extent by heat shock proteins[53].

Receptor-receptor interactions

Dunwiddie et al. found that activation of A_3R with a selective A_3R agonist resulted in subsequent heterologous desensitization of the A_1R, as observed in electrophysiological experiments in the CA1 region of rat hippocampus. Similar results were obtained after A_3R occupancy via a brief superfusion with a high concentration of adenosine[54].

Lopes et al. investigated how activated $A_{2A}R$ influenced A_1R function and whether this interaction was modified in aged rats. In hippocampal and cortical nerve terminals, the $A_{2A}R$ agonist CGS 21680 (30 nM) was able to lower the binding affinity of the A_1R selective agonist CPA, and this was taken as proof for A_1R desensitization. The effect was counteracted by the addition of the $A_{2A}R$ antagonist ZM 241385 (20 nM). This reduction in A_1R function could only be detected in young adult rats (6 weeks), but not in old rats (24 months). The addition of a PKC inhibitor, chelerythine (6 μM), also prevented the desensitization of A_1R[55]. Similarly, Ciruela et al. (2006) demonstrated that A_1R-$A_{2A}R$ heteromers were able to modulate the glutamatergic neurotransmission in the rat striatum. The main biochemical characteristic of this heteromer is the ability of the agonist-occupied $A_{2A}R$ to reduce the agonist affinity of the A_1R[56].

By binding to cannabinoid CB_1 receptors in Purkinje fibers in the cerebellum, Δ^9-tetrahydrocannabinol inhibits adenylyl cyclase and consequently motor coordination. Long-term Δ^9-tetrahydrocannabinol treatment resulted in CB_1R downregulation, desensitization of the $G_{i\alpha}$ protein and desensitization of adenylyl cyclase. G protein activation by A_1R, however, was unaffected. Surprisingly, heterologous attenuation of A_1R-mediated inhibition of adenylyl cyclase was observed. These results indicate that long-term Δ^9-tetrahydrocannabinol administration produces a disruption of inhibitory receptor control of cerebellar adenylyl cyclase and suggest a potential mechanism of cross-tolerance to the motor effects of cannabinoid and A_1 agonists[57].

D_1R and A_1R have been shown to form functionally interacting heteromeric complexes in engineered cell lines and in cortical neurons in culture. Pretreatment with an A_1R agonist caused complex formation of both receptors. Combined pretreatment with selective agonists for both receptors (but not one agonist alone) substantially reduced cAMP accumulation induced via the D_1R, indicative for this receptor's desensitization[58].

A_{2A} receptor

Cellular and physiological studies

DDT_1 MF-2 cells, expressing both A_1R and $A_{2A}R$, were exposed to an A_{2A}-selective agonist, which resulted in a rapid loss ($t_{1/2}$=45 min) of agonist-stimulated cAMP production in these cells. This receptor desensitization, however, did not involve a reduction in cell membrane receptor number or a change in ligand affinity[35].

Prolonged exposure of PC12 cells, expressing both $A_{2A}R$ and $A_{2B}R$, to AR agonists led to a fast (30 min) and significant inhibition of $A_{2A}R$ stimulation by the $A_{2A}R$-selective agonist CGS21680. This effect appeared to occur at the level of adenylyl cyclase, since no change was observed in receptor number or in CGS21680's affinity for the receptor. This conclusion was corroborated by the finding of reduced activation of adenylyl cyclase by forskolin. Longer agonist exposure (12-20h) led to a reduction of $G_{s\alpha}$ levels, whereas no changes occurred in the short-term protocol[59].

NG108-15 neuroblastoma x glioma hybrid cells express both $A_{2A}R$ and $A_{2B}R$[60,61]. Treatment of the cells with the non-selective agonist NECA followed by its washout, led to a rapid ($t_{1/2}$=20 min) and pronounced reduction in cAMP production by the $A_{2A}R$-selective agonist CGS21680 when compared to vehicle-treated cells.

Palmer et al. (1994) generated CHO cells solely expressing the recombinant (canine) $A_{2A}R$ and examined the desensitization process of this receptor. Cells exposed to NECA showed a rapid desensitization of $A_{2A}R$-stimulated adenylyl cyclase activity with no obvious difference between pretreatment of 30 min or 24 h. This was associated with a slightly reduced affinity of the receptor for the $A_{2A}R$-selective radiolabeled agonist [^{3}H]CGS21680. Cell surface receptor numbers only diminished significantly (up to 40%) upon longer-term pretreatment ($t_{1/2}$ = 8 h)[62].

Using a tissue preparation, Conti et al. investigated whether prolonged exposure of $A_{2A}R$ to the non-selective agonist NECA, or to the selective $A_{2A}R$ agonists CGS21680 and 2HE-NECA, influenced $A_{2A}R$ desensitization. The authors used the porcine coronary artery as a sensitive vascular model, expressing among others $A_{2A}Rs$. The arteries were first precontracted by adding 3 μM PGFα. NECA, CGS21680 and 2HE-NECA showed high affinities for the $A_{2A}R$ (EC_{50}'s of 72, 40 and 20 nM respectively) inducing vasorelaxation. Next, coronary arteries were pretreated with 10 μM NECA for 30 min or 2 h. After a two hour washout period, the functional response was assessed. It appeared that preincubation with NECA did not hamper the vasorelaxing effects of CGS21680 and 2HE-NECA. However, NECA response curves were shifted to the right after NECA pretreatment[63]. These results seem inconclusive, since NECA pretreatment might 'hit' other adenosine receptor subtypes.

A_{2A}Rs, next to A_1R, are present on presynaptic baroreceptor afferent terminals within the nucleus tractus solitarius (NTS) in the brain[64]. It was observed that these A_{2A}R modulate 5-HT release as a mechanism of baroreflex control mediated by the NTS. Low concentrations of the non-selective agonist NECA (0.3-3 nM), briefly exposed (5 min) to NTS brain slices, induced the release of 5-HT caused by the activation of the A_{2A}R present at presynaptic nerves. This effect could be blocked by the addition of the adenosine A_{2A}R antagonist 8-(3-chlorostyryl)caffeine (CSC; 100 nM). Longer exposure of NECA (20 min) to the NTS slices resulted in inhibition of the 5-HT release, probably caused by quick desensitization of the A_{2A}R (15 min) and subsequent involvement of the A_1R[65]. These findings were corroborated in a similar study with CGS21680, an A_{2A}R-selective agonist[66].

The effects on AR fate upon chronic agonist exposure were studied *in vivo* in rat brain[45]. After 6 days of NECA treatment, the adenylyl cyclase activity in synaptic plasma membranes was decreased, suggesting a desensitization of A_2Rs, although the authors did not specify or study which A_2R subtype was involved. G_s protein levels were decreased indicating G_s down-regulation as the mechanism of desensitization.

Interestingly, Rekik and Mustafa (2003) showed that chronic *antagonist* treatment (3 days) of porcine coronary arteries with ZM241,385 led to a decreased agonist responsiveness. Although A_{2A} receptor expression went up, it appeared that the levels of G_s had decreased, altogether leading to a functional desensitization of the relaxing response by e.g. CGS21680[67]. It should be kept in mind that ZM241,385 is also a potent antagonist for the adenosine A_{2B} receptor.

Molecular mechanisms

Effect of receptor C-terminus on desensitization and/or internalization

To investigate the importance of the (120 amino acid residues) long C-terminus of the A_{2A}R receptor in inducing desensitization and internalization, Palmer and Stiles introduced several mutations and deletions into the receptor tail. It appeared that deletion of the last 95 amino acids of the C-terminus, containing 10 possible phosphorylation sites, did not have any effect on radioligand binding, adenylyl cyclase activity or desensitization kinetics compared to the wild-type A_{2A}R. However, when two possible phosphorylation sites (Thr 298 and Ser 305) just upstream the 95 deleted amino acids were mutated to Ala, short term (30 min) agonist-induced desensitization was attenuated, while the long-term (24h) desensitization was not affected. Single mutations revealed that mutation of Thr 298 alone was sufficient to reduce receptor phosphorylation and agonist-induced short-term desensitization.

This study also shows that short-term and long-term desensitization have distinct structural requirements and do not occur via the same mechanism[4].

GRKs and arrestins

Which GRK isoforms are involved in the phosphorylation of the $A_{2A}R$ is not entirely clear yet. However, a putative role for GRK2 and/or GRK5 has been suggested[4]. The role of GRK2 in agonist-induced phosphorylation and subsequent desensitization of $A_{2A}R$ and $A_{2B}R$ was thoroughly investigated by Kelly and coworkers. Wild-type GRK2 was stably expressed in NG108-15 cells, which endogenously express $A_{2A}R$ and $A_{2B}R$. The acute stimulation of adenylyl cyclase by activation of $A_{2A}R$ was markedly reduced in NG108-15 cells overexpressing wild-type GRK2. This was probably caused by GRK2-dependent pre-desensitization of the $A_{2A}R$ by extracellular adenosine. This effect could be reversed by pretreating the cells 24h with 0.5 unit/mL ADA[68]. The same research group investigated the effect of a dominant-negative GRK2 mutant on the desensitization of the $A_{2A}R$[60]. Stable transfection of a GRK2Lys220Arg mutant in NG108-15 cells reduced desensitization of the $A_{2A}R$ by 50% following a 30 min treatment with the adenosine agonist NECA.

TNF-α treatment of human monocytoid THP-1 cells expressing the $A_{2A}R$ prevented desensitization of this receptor, occurring under control conditions upon pretreatment with CGS21680 or NECA[69]. It was discovered that the TNF-α treatment prevented GRK2 translocation to and decreased GRK2 association with the plasma membranes of these cells as a consequence of the activation of a sphingomyelinase-dependent pathway.

In another study, Mundell and Kelly investigated the effect of inhibitors of receptor internalization on desensitization and resensitization of ARs in NG108-15 cells[70]. Before agonist exposure, cells were pretreated with hypertonic sucrose or concanavalin A, both inhibitors of internalization. This pretreatment did not affect the agonist-induced desensitization of the $A_{2A}R$. However, the resensitization of the $A_{2A}R$ upon agonist removal was abolished in the presence of con A or sucrose.

Arrestins-2 and -3 have been implicated in the downstream desensitization process. CGS21680 stimulation of a tagged $A_{2A}R$ transiently transfected in HEK293 cells induced the translocation of GFP-tagged arrestins-2 and -3 towards the plasma membrane. A dominant-negative arrestin-2 mutant inhibited agonist-induced internalization[71].

Other protein partners

The long C terminus of the $A_{2A}R$ has been coined a ‘coincidence detector’ as it recognizes quite a number of other proteins such as α-actinin and ARNO, the Arf nucleotide site opener (for a review see Gsandtner and Freissmuth[72]). α-Actinin may

play a role in receptor internalization, not unlikely for a protein involved in cytoskeletal arrangements[71], however ARNO does not[73].

Receptor-receptor interactions

The $A_{2A}R$ colocalizes with D_2 dopamine receptors in the basal ganglia, and their interaction has been documented on several occasions[74]. For instance, the protein-protein interaction between $A_{2A}R$-D_2R was confirmed in HEK293T cells. The most likely mode of interaction is that helix 5 and/or helix 6 and the N-terminal portion of I3 in the D_2R approach helix 4 and the C-terminus of the $A_{2A}R$[75]. Within the scope of this review fits the observation that activation of the D_2 receptor actually sensitizes $A_{2A}R$-mediated increases in cAMP production, in CHO cells expressing both receptors[76] as well as in CAD and NS20Y neuroblastoma cells in which the D_2 receptor was expressed[77]. In SH-SY5Y neuroblastoma cells co-stimulation of $A_{2A}R$ and D_2R accelerated D_2R desensitization, probably by causing or enhancing D_2R internalization[80]. Recently it was found that in these SH-SY5Y neuroblastoma cells the $A_{2A}R$ also formed heteromers with the endogenously expressed CB_1R. The CB_1R is negatively coupled to adenylyl cyclase, and requires previous or simultaneous activation of $A_{2A}R$ to signal in these cells[79].

A_{2B} receptor

Cellular and physiological studies

The adenosine A_{2B} receptor ($A_{2B}R$) is endogenously expressed on most artificial cell lines, such as COS and HEK293 cells. The exception is formed by CHO cells that lack this adenosine receptor subtype.

The $A_{2B}R$, like the other three adenosine receptor subtypes, is subject to agonist-induced desensitization. This was measured on the level of cAMP production by Peters et al.[80]. Pretreatment of COS7 cells endogenously expressing A_{2B} receptors with the nonselective agonist NECA (1 μM) for 1 up to17 h, led to a significant reduction in cAMP production upon acute agonist challenge, already after 1 h of pretreatment. Maximal reduction of cAMP production was reached after 4 h of pretreatment. CHO cells stably expressing a 5' FLAG epitope-tagged A_{2B} receptor showed the same result, albeit that maximal reduction of cAMP response was already achieved after 1 h of pretreatment. Also mouse 3T3-L1 cells and human HEK293 cells, endogenously expressing the $A_{2B}R$, showed a decreased cAMP response after 2 h and 5.5 h of pretreatment with 1 μM NECA, respectively[80]. In NG108-15 cells, expressing both $A_{2A}R$ and $A_{2B}R$, the rate of desensitization for both receptor subtypes appeared similar, with a half-life of 15-20 min. Pretreatment with

NECA of these cells (0.1 mM, 30 min) reduced stimulation of adenylyl cyclase by $A_{2B}R$ by approx. 50%[60,61].

Rat pheochromocytoma PC12 cells also endogenously express both $A_{2A}R$ and $A_{2B}R$. Prolonged exposure (14 h) to 100 nM NECA or 1 μM CGS21680 significantly inhibited the cAMP response of the cells to subsequent stimulation with the A_{2A}-selective agonist CGS21680. Although a 100-fold higher NECA-concentration is needed to stimulate the $A_{2B}R$ compared to $A_{2A}R$, Chern et al. found that the A_{2B} receptor cAMP response was also desensitized upon desensitization of the A_{2A} receptor. This observation can be explained by the fact that the $G_{s\alpha}$ protein level and the adenylyl cyclase activity were diminished upon long-term desensitization of the $A_{2A}R$ by CGS21680[59].

The domain in which the receptor resides is also important for desensitization events, according to Sitaraman et al. NECA (10 μM) added to either the apical or basolateral side of T84 intestinal cells resulted in desensitization of the $A_{2B}R$ on the corresponding side within 2-3 h. However, whereas applying NECA to the apical side of the membrane had no effect on the basolateral $A_{2B}R$, basolateral NECA induced a complete desensitization of the apical receptor. Since receptor trafficking may play a role in this cross-desensitization process, this effect may contribute to the desensitization and subsequent downregulation of the $A_{2B}R$[81].

Trincavelli et al. observed a very rapid desensitization ($t_{1/2}$=5 min) of $A_{2B}R$ in a human astrocytoma cell line. Both G protein coupling efficiency and cAMP production were diminished after pretreatment of the cells with NECA in the presence of an $A_{2A}R$-selective antagonist[82].

Mundell et al. reported that acute exposure of human airway smooth muscle (ASM) cells to adenosine receptor agonists resulted in a rapid accumulation of cAMP, most probably via $A_{2B}R$. Treatment with adenosine deaminase (ADA) suggested that ASM cells produce adenosine which feeds back on the cells' $A_{2B}Rs$ thereby regulating basal cAMP levels and inducing a small degree of $A_{2B}R$ desensitization. Chronic treatment with adenosine agonists had a dual effect; both $A_{2B}R$ desensitization and adenylyl cyclase sensitization (an increased responsiveness of adenylyl cyclase upon stimulation) were observed[83].

Haynes et al. (1999) studied $A_{2B}R$ desensitization in both pulmonary artery smooth muscle cells and a more integrated preparation, the isolated perfused lung. Pretreatment of the smooth muscle cells with NECA for 45 min abrogated the increase in cAMP response otherwise observed for both NECA and isoproterenol, suggesting heterologous desensitization. Indeed, experiments with cholera toxin showed that the desensitization took place at the level of the G_s protein-adenylyl cyclase complex. In the lung preparation NECA, when dosed acutely, caused a rapid

vasodilation, which was not observed when the tissue was treated again with the same compound 45 min after the first dose[84].
Taken together, these results suggest that desensitization of the A_{2B} receptor is a robust phenomenon, rather independent from the cell type or tissue in which it is expressed.

Molecular mechanisms

Effects of receptor mutations on desensitization and/or internalization
Matharu et al. studied the regions of the $A_{2B}R$ which are responsible for the desensitization and internalization of the $A_{2B}R$[85]. For this purpose they introduced point mutations or deletions in the C-terminus of the $A_{2B}R$. Deleting the final two amino acids (Leu^{330}-stop) did not affect the internalization properties of the $A_{2B}R$. The Phe^{328}-stop and Gln^{325}-stop mutants however, were resistant to agonist-induced desensitization and internalization. GFP-tagged arrestin-2 did not translocate from the cytosol to the plasma membrane upon agonist stimulation of these two truncated receptors. From a single point mutation in the C-terminus, Ser^{329}Gly, it became clear that this serine residue is responsible for the rapid agonist-induced desensitization and internalization. Surprisingly, a further deletion mutant Ser^{326}-stop was able to undergo rapid agonist-induced desensitization and internalization, however, arrestin-2 was not attracted to the plasma membrane upon agonist stimulation. It appeared that internalization of this truncated receptor occurred via an arrestin- and clathrin-independent pathway, probably via caveolin-mediated internalization since the internalization of Ser^{326}-stop was dynamin-dependent. The destination of this truncated receptor was also different; a compartment close to the plasma membrane, instead of early endosomes. Whether the receptor will undergo rapid agonist-induced desensitization and internalization, or influence the rate, extent and mechanism of internalization depends on specific sites in the C-terminus of the $A_{2B}R$ and can be influenced by their mutation or deletion.

GRKs and arrestins
The $A_{2B}R$, endogenously expressed in NG108-15 cells, is desensitized through phosphorylation by GRK2. This GRK2 effect was selective for $A_{2A}R$ and $A_{2B}R$, since the other endogenously expressed receptors (secretin R and IP-prostanoid R) were not desensitized in this way[68]. In addition, stable transfection of a dominant negative GRK2(Lys220Arg) mutant in the NG108-15 cells reduced the NECA-induced (30 min, 100 μM) desensitization of the $A_{2B}R$ by 50%[60]. Peters et al. studied the role of phosphorylation by PKA and PKC rather than GRK2 in agonist-induced

desensitization in COS-7 cells transiently expressing the 5'-FLAG A_{2B}R. They concluded that these second messenger dependent kinases are not involved in A_{2B}R phosphorylation[80]. A similar conclusion for A_{2B}R phosphorylation in NG108-15 cells was reached by Mundell and Kelly[61]. Trincavelli et al. found that treatment of astrocytoma cells with TNF-α increased the A_{2B}R functional response, without changing receptor protein or mRNA levels. TNF-α treatment appeared to markedly reduce agonist-dependent receptor phosphorylation and also attenuated agonist-mediated A_{2B}R desensitization. These effects could be inhibited by the addition of the A_{2B}R antagonist MRS 1706[82]. These findings resemble data obtained with the A_{2A}R (*vide supra*).

Mundell et al. found that within the arrestin family, both arrestin-2 and arrestin-3 are involved in the internalization process of the A_{2B}-receptor. Within 1 minute of agonist exposure, both GFP-tagged arrestin isoforms were translocated to the plasma membrane. Longer agonist exposure (>10 min) however, revealed that only arrestin-2 colocalizes with the receptor in the early endosomes. One explanation for this may be a higher affinity of the A_{2B}R for arrestin-2 compared to arrestin-3. Interestingly, arrestins are not only involved in the internalization of the A_{2B}-receptor, but also in its recycling. It appeared that the expression of stable arrestin anti-sense constructs, reducing the levels of endogenous arrestins, not only resulted in less internalization of the receptors, but also impaired the recycling process significantly. In contrast to the internalization process, arrestin-3 induced a significantly faster rate of A_{2B}R recycling compared to arrestin-2[86].

Penn et al. also studied the behaviour of GFP-tagged arrestins, now in human airway smooth muscle cells. Arrestin-2 and arrestin-3 were able to reduce the cAMP production upon stimulation of endogenous A_{2B}R by NECA and stimulation of β_2-AR by isoproterenol. In addition, a punctate clustering was observed in the membrane upon exposure to either NECA (100 μM) or isoproterenol (1 μM), indicating that arrestins play a role in receptor trafficking. On the contrary, signaling and trafficking of the prostaglandin E_2-R, another endogenously expressed receptor, was not affected by arrestin-2 or arrestin-3[27].

A_3 receptor

Adenosine A_3 receptors (A_3R) evoke considerable interest as novel drug targets to address cerebral/cardiac ischemia, cancer, inflammation, asthma and chronic obstructive pulmonary disease. So far, potent and selective antagonists for the hA_3R have been identified; the disadvantage however is that those antagonists show extremely low binding affinity for the rodent A_3R, typically 1000 times lower than in

human. Since rodent models are essential for the pharmacological evaluation of new therapeutic agents, this forms a serious drawback.
In addition, the adenosine A_3 receptor (A_3R) sequence holds a nuclear localization signal in its C-terminal tail[87]. This typical stretch of 4 amino acids (KKFK) in helix 8 may direct the receptor to the cell nucleus, not necessarily as a consequence of desensitization or internalization. This should be kept in mind when appreciating the studies described below.

Cellular and physiological studies
Ramkumar et al. studied the characteristics of rat A_3R, endogenously expressed on RBL-2H3 mast cells. In one of their experiments the authors pretreated the cells with NECA, and found a partial desensitization of the initial Ca^{2+} response to NECA[88]. Trincavelli et al. studied the relationship between agonist-induced desensitization, internalization and resensitization of hA_3R in transfected CHO cells. Agonist-induced endocytosis of hA_3R was investigated by immunogold electron microscopy of plasma membranes and intracellular vesicles and shown to occur with a half-life of 17 min. Subsequent removal of the agonist led to recycling of 90% of the receptor population to the cell surface, with a half-life of 35 min. Short-term exposure to agonist caused rapid desensitization, as assessed in cAMP assays. Removal of the agonist led to resensitization of the cAMP signal to 90% of the original signal within 120 min. Internalization did not affect signal transduction, as was demonstrated after blockade of internalization and recycling. Internalization occurred via clathrin-coated pits. These results show that hA_3R undergoes agonist-induced endocytosis, which is not responsible for desensitization. Moreover, in the case of hA_3R, receptor sequestration rather than desensitization seems to be the first step in a cycle of internalization, dephosphorylation and recycling to the plasma membrane[89]. A similar cell line was used by Palmer et al. In stably transfected CHO cells prolonged treatment with NECA (10 μM, 20 h) induced uncoupling of recombinant hA_3R from G_i proteins and a functional desensitization. Upon this A_3R desensitization, an approximately 2-fold increase in adenylyl cyclase activity was found in the presence or absence of forskolin. This sensitization of adenylyl cyclase activity was not caused by an altered level of inhibitory or stimulatory G protein expression. The occurrence of the sensitization was also not due to new protein synthesis, but probably to an increased coupling efficiency between G_s and adenylyl cyclase. Compared to control cells, long-term exposure of stably transfected CHO cells to NECA caused an increase in phosphorylation of the cAMP-responsive element binding protein upon addition of suboptimal concentrations of forskolin. The sensitization of adenylyl cyclase activity upon long-term treatment might provide a molecular basis for the observation that for several adenosine receptor-mediated events, acute agonist

exposure produces opposite effects to those after chronic agonist treatment[90]. Ferguson et al. studied the fate of the rat A_3R expressed in CHO cells. The receptors internalized rapidly after treatment with NECA or *R*-PIA over a time frame ($t_{½}$=10 min) that followed receptor phosphorylation ($t_{½}$=1 min)[91].

The desensitization, internalization and down-regulation of hA_3R was also investigated in human astrocytoma cells, natively expressing hA_3R. Short-term (15 min) exposure of the cells to 100 nM Cl-IBMECA, an A_3R-selective agonist, caused rapid receptor desensitization, subsequently followed by receptor internalization within 30 min. With help of immunogold electron microscopy, the localization of the A_3R was revealed. After 10 min of exposure, the A_3R was found in smooth-surfaced pits and in uncoated vesicles in the cytoplasm. After 30 min of exposure, the A_3R was found in vesicular endosomes. Upon removal of the agonist, resensitization of the A_3R occurred within 120 min through receptor recycling to the cell surface. Long-term agonist exposure (1-24 h) resulted in a marked down-regulation of A_3R to 22 $\pm$ 3% of control after 24 h. In conclusion, multiple, temporally distinct and sequential processes are involved in the regulation of hA_3R upon short- and long-term exposure to agonists[92].

A_3Rs were found to be highly expressed on murine B16-F10 melanoma cells[93]. The authors examined the association between A_3R trafficking and receptor functionality and tumour growth inhibition upon activation with the A_3R-selective agonist IB-MECA. Exposure to 10 nM IB-MECA (5 min) led to rapid internalization of A_3R to the cytosol, upon which receptors were directed to the endosomes for recycling or to lysosomes for degradation. The addition of 100 nM MRS 1523 (5 min), an A_3R antagonist, was able to counteract the internalization process as well as the modulation of the Wnt pathway leading to proliferation, thereby emphasizing the involvement of A_3R in this process. When the melanoma cells were injected into nude mice, tumours rapidly developed. Tumour growth was significantly inhibited after administration of IB-MECA to the animals, paralleled by a decrease in A_3R expression in tumour lesions. In hypoxic human A172 and U87MG glioblastoma cell lines it was found that adenosine up-regulates the expression of hypoxia-inducible factor-1α (HIF-1α) and VEGF via the A_3 receptor. HIF-1α is a key regulator in the development of tumours. The addition of the A_3-antagonist MRE 3008F20 inhibited the adenosine-induced HIF-1α and VEGF accumulation in these hypoxic cells, thereby showing a putative role in inhibiting tumour growth [94].

To circumvent the species differences mentioned in the introduction to the A_3R paragraph, Yamano et al. generated A_3R humanized ($A_3AR^{h/h}$) mice in which the A_3R was replaced by its human counterpart. The expression level of hA_3R in the humanized ($A_3AR^{h/h}$) mice was equal to the expression levels of A_3R in WT mice. A_3R agonists were able to elevate the intracellular Ca^{2+} concentration in bone

marrow-derived mast cells from the humanized ($A_3AR^{h/h}$) mice. However, the rate of hA_3R internalization was markedly reduced compared with that of mA_3R in these mast cells[95].

Molecular Mechanisms

Receptor phosphorylation, GRKs and arrestins

Palmer and Stiles, and Ferguson et al. investigated which amino acid residues in the C-terminus are responsible and crucial for the rapid desensitization of the A_3R[96,91]. A triple mutant (Thr^{307}, Thr^{318} and Thr^{319} to Ala) exhibited dramatically reduced phosphorylation, desensitization and internalization of the rat A_3AR. Individual mutation of each Thr residue showed that Thr^{318} and Thr^{319} were the most important sites for phosphorylation. In addition, phosphorylation of Thr^{318} was necessary to observe phosphorylation of Thr^{319}, but not vice versa. Moreover, changing Thr^{318} for a negatively charged residue (Glu) was not sufficient to retain phosphorylation at Thr^{319}. Mutating two predicted palmitoylation-sites, $Cys^{302,305}$ to Ala resulted in agonist-independent basal phosphorylation of the rat A_3AR. Such findings strongly suggest that palmitoylation of these Cys residues is an important factor in controlling accessibility of the C-terminus of the A_3R in the process of recruiting GRKs. In fact, the palmitoylation sites are highly conserved between different species, e.g. rat, mouse, human, dog, sheep. Taking these results together, it appears that GRK-mediated phosphorylation of the A_3R C-terminus follows a sequential mechanism, with the receptor palmitoyl moieties in an important regulatory role, and phosphorylation of Thr^{318} being particularly crucial as an essential first step. In an earlier study Palmer et al. replaced the carboxyl terminus of the A_1R by that of the A_3R[37]. This chimaeric construct was able to undergo agonist-stimulated phosphorylation and functional desensitization, similar to A_3R. It was also found that purified GRK2, GRK3 and GRK5 were all able to enhance agonist-dependent phosphorylation of A_3R as well as the A_1-A_3 chimaera.

Trincavelli et al. studied the involvement of extracellularly regulated kinases (ERK1 and 2), members of the mitogen-activated protein kinase (MAPK) family, in A_3R phosphorylation. It was found that within 5 minutes of exposure to 10 μM NECA, ERK 1 and 2 were already phosphorylated in CHO cells stably expressing hA_3R. An inhibitor of MAPK activation (PD98059) also caused inhibition of A_3R phosphorylation, desensitization and internalization, probably by preventing the membrane translocation of GRK2. These results indicate that the MAPK cascade is involved in A_3R regulation by a feedback mechanism which controls GRK2 activity and probably involves direct receptor phosphorylation[97].

The rat basophilic leukaemia cell 2H3 cell line (RBL-2H3 cells) endogenously expresses equal levels of arrestin-2 and arrestin-3. Both arrestin isoforms also have a high and comparable affinity for clathrin, thereby promoting agonist-induced internalization. RBL-2H3 cells also endogenously express a high level of A_3R, which, however, did neither recruit arrestin-3 nor arrestin-2 upon stimulation with NECA. Also no changes in A_3R distribution were observed. One explanation is that A_3R follow an endocytic mechanism upon agonist stimulation that does not involve arrestin-mediated clathrin-coated pit internalization. Another possible explanation is that arrestin recruitment was below the limit of detection in the RBL-2H3 cells[98]. Ferguson et al. observed that upon phosphorylation of A_3R by GRK arrestin-3 is redistributed into punctate vesicles both at the plasma membrane and within the cytoplasm. Nevertheless, these authors also noticed that there was no colocalization between A_3R and arrestin-3[47].

Conclusions

The adenosine receptor subtypes are differentially regulated when exposed to agonist treatment. A_1Rs are not (readily) phosphorylated and slowly internalize with a typical half-life of several hours. This feature may cause less than average tachyphylaxis upon chronic agonist administration, for instance in type II diabetes. A_{2A}R and A_{2B}R show much faster downregulation with similar kinetics, usually < 1h for short-term desensitization. Agonists for the A_{2A} receptor, currently profiled in wound healing, may thus suffer from declining efficacy upon chronic administration, in contrast to those for the A_1 receptor. The A_3R undergoes even faster downregulation, often a matter of minutes. The latter receptor also holds a nuclear localization signal in its carboxy terminal tail, possibly obscuring true agonist-induced downregulation. The fast desensitization of the A_3R after agonist exposure may be therapeutically equivalent to antagonist occupancy of the receptor.

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