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Review

Adenosine receptors interacting proteins (ARIPs): Behind the biology of adenosine signaling

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ABSTRACT

Adenosine is a well known neuromodulator in the central nervous system. As a consequence, adenosine can be beneficial in certain disorders and adenosine receptors will be potential targets for therapy in a variety of diseases. Adenosine receptors are G protein-coupled receptors, and are also expressed in a large variety of cells and tissues. Using these receptors as a paradigm of G protein-coupled receptors, the present review focus on how protein–protein interactions might contribute to neurotransmitter/neuromodulator regulation, based on the fact that accessory proteins impinge on the receptor/G protein interaction and therefore modulate receptor functioning. Besides affecting receptor signaling, these accessory components also play a key role in receptor trafficking, internalization and desensitization, as it will be reviewed here. In conclusion, the finding of an increasing number of adenosine receptors interacting proteins, and specially the molecular and functional integration of these accessory proteins into receptorsomes, will open new perspectives in the understanding of particular disorders where these receptors have been proved to be involved.

Contents

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1. Introduction

Since Drury and Szent-Györgyi found 80 years ago that adenosine produced profound hypotension and bradycardia, as well as it affected kidney function in mammals [1], the physiological roles and potential therapeutic use of adenosine have been largely revised [2,3]. Adenosine is mainly produced by degradation of ATP and in lessen extend by *S*-adenosyl-L-homocysteine (SAH) metabolism. Also, adenosine can be formed extracellularly by the breakdown of ATP. Once it has been generated, it can either be eliminated out of the cells by means of ubiquitous nitrobenzylthioinosine-sensitive equilibrative nucleoside transporters (ENTs) or intracellularly phosphorylated to form AMP. In addition, adenosine can react with L-homocysteine to form SAH. And finally, both intra- and extracellular adenosine can be deaminated to form inosine by the action of intra- and ecto-adenosine deaminase, respectively.

Regarding adenosine functions, it has been largely considered a retaliatory metabolite [4] that increases oxygen supply and decreases oxygen consumption, thus modulating a large array of physiological processes including respiratory regulation, hormone action, neural function, platelet aggregation, lymphocyte differentiation and vascular tone. For instance, adenosine is able to induce the dilation of coronary arteries, but it can also lead to the contraction of other blood vessels like the ones in the kidney, resulting in a decreased renal filtration. Also, adenosine exerts a negative chronotropic and dromotropic effect on the heart, as well as it mediates the inhibition of neurotransmitters release and lipolysis. Overall, it has been assumed that this purine nucleoside is a mediator of metabolic distress, thus having considerable impact on homeostatic cellular functioning.

In 1972, it was shown that adenosine was released from brain slices upon electrical stimulation [5]. Interestingly, the released adenosine produced an increase in the intracellular levels of cAMP, an effect that was antagonized by methylxanthines such as caffeine and theophylline [6]. This fact was also observed in the heart [7]. Collectively, these observations constituted the first evidence suggesting that adenosine exerted its effects via specific plasma membrane receptors. Afterwards, it was demonstrated that the so described antilipolytic effect of adenosine on fat cells occurred with a concomitant reduction in cAMP [8]. The dual effects of adenosine on cAMP formation were further substantiated when it was demonstrated that adenosine could either inhibit or stimulate adenylyl cyclase. Overall, these observations ended with the first subclassification of adenosine receptors into R_i and R_a [9], or alternatively, A_1 and A_2 adenosine receptors [10].

In the Central Nervous System (CNS), adenosine has been shown to play a regulatory role, acting as a presynaptic, postsynaptic and/or non-synaptic neuromodulator [11]. Extracellular adenosine in the brain is related to the intracellular concentration of adenosine and nucleotides, such as ATP, AMP and cAMP [12]. In some brain areas, like the hippocampus, most of the extracellular adenosine seems to depend mostly on intracellular adenosine, the concentration of which is related to the rate of breakdown and synthesis of ATP [12]. Thus, adenosine is released as a neuromodulator [13] by the effector cells in response to an increased metabolic demand [14]. However, in the striatum, it has been suggested that the main source of extracellular adenosine is intracellular cAMP [15], which is metabolized to AMP by means of phosphodiesterases and then to adenosine by the ectoenzyme 5′nucleotidase. Since cAMP can only be generated by the action of the enzyme adenylyl cyclase, striatal extracellular adenosine would mostly reflect an increased activation of receptors positively linked to adenylyl cyclase.

2. Adenosine receptors

Adenosine mediates its actions by means of activation of specific G protein-coupled receptors (GPCRs), for which four subtypes (A_1R, A_{2A}R, A_{2B}R and A_3R) have been identified so far. These receptors have a distinctive pharmacological profile, tissue distribution and effector coupling [16], and its functioning has been extensively studied in the CNS (Table 1). The GPCRs comprise the largest family of membrane-bound receptors, containing more than 1000 different members which represent over 1% of the genome in vertebrates [17]. From a phylogenetic point of view, these receptors can be classified into five main families (GRAFS classification system), namely glutamate, rhodopsin, adhesion, frizzled/taste 2 and secretin [18]. Adenosine receptors (ARs) belong to the rhodopsin family, also called family I or A [19]. This receptor family has more than 700 different members that share some phylogenetic and intrinsic characteristics. For instance, within their sequence, all adenosine receptors contain the widely conserved NPxxY(x)5,6F and the DRY motifs [20,21]. Thus, adenosine-mediated conformational change in the ARs core domain affects the conformation of the intracellular loops, which in turn determines the binding and activation of specific G proteins (Table 1). Stimulation of G proteins is then responsible for activation of different intracellular signaling pathways associated to adenosine function (Table 1).

Interestingly, A_1Rs and A_{2A}Rs are primarily responsible for the central effects of adenosine [22]. The most abundant and homogeneously distributed adenosine receptor in the brain is the A_1R, which is functionally coupled to members of the pertussis toxin-sensitive family of G proteins (G_{i1}, G_{i2}, G_{i3} and G_o) and whose activation regulates several membrane and intracellular proteins such as adenylyl cyclase, Ca^{2+} channels, K^+ channels and phospholipase C (Table 1) [23]. On the other hand, A_{2A}R is expressed at high levels in only a few regions of the brain, namely primarily striatum, olfactory tubercle and nucleus accumbens [15]. A_{2A}Rs are mostly coupled to G_s/G_{olf} proteins [24], thus activating adenylyl cyclase which in turn converts ATP into AMPc (Table 1). Depending on the cell type studied, the A_{2A}R may also signal via a pathway that might be independent of heterotrimeric G proteins; such is the case of the activation of the mitogen-activated protein kinase (MAPK) signaling cascade [25]. Next, the A_{2B}R is positively coupled to adenylyl cyclase and PLC through a G_s and G_q protein, respectively [2] (Table 1). A_{2B}R is thought to be fairly ubiquitous in the brain, and the association of this receptor to specific physiological or behavioral responses remains quite

Table 1
Adenosine receptors in the brain.

Receptor	Adenosine affinity	G protein	Transduction mechanisms[a]	Physiological actions in brain
A_1R	~ 70 nM	$G_{i1,2,3}$	Inhibits AC (↓cAMP) Activates PLC (↑IP_3/DAG)	Inhibits synaptic transmission; hyperpolarizes neurons
			Activates PLA2 (↑AA) Activates PLD (↑PEtOH) Activates GIRKs Inhibits Ca^{2+} channels	
		G_o		
A_{2A}R	~ 150 nM	G_s[b]	Activates AC (↑cAMP)	Facilitates transmitter release; regulation of sensorimotor integration in basal ganglia
		G_{olf}	Activates AC (↑cAMP)	
		$G_{15,16}$[c]	↑IP_3 Inhibits Ca^{2+} channels	
A_{2B}R	~ 5000 nM	G_s[b]	Activates AC (↑cAMP) Activates Ca^{2+} channels	Increases in cAMP in brain slices
		$G_{q/11}$[c]	Activates PLC (↑IP_3/DAG)	
A_3R	~ 6500 nM	$G_{i2,3}$	Inhibits AC (↓cAMP)	Uncouples A_1R and mGlu receptors
		$G_{q/11}$	Activates PLC (↑IP_3/DAG)	

[a] AC, adenylyl cyclase; PLC, phospholipase C; IP3, inositol triphosphate; DAG, diacylglycerol; PLA2, phospholipase A2; PEtOH, phosphatidylethanol; GIRKs, G protein-dependent inwardly rectifying K+ channels; AA, arachidonic acid.
[b] Main mechanism of coupling.
[c] Receptor transfected cell system.

difficult because of the paucity of $A_{2B}R$ agonists or antagonists [26]. Lastly, the A_3R has also been found to be coupled to classical second-messenger pathways such as inhibition of adenylyl cyclase, stimulation of PLC and calcium mobilization (for review see [2]) (Table 1). Finally, it is important to mention at this point that different classes of proteins, other than G proteins, are being recognized to interact with GPCRs. The binding of GPCRs to some of these proteins will determine interactions with different intramembrane and intracellular elements, which in turn will result, not only in changes in receptor localization and function, but also in G protein-independent signaling [27]. These proteins include serine–threonine or tyrosine protein kinases, β-arrestins and scaffolding proteins within others.

3. Adenosine receptors interacting proteins (ARIPs)

In the present work, we underscore the ARs ability to interact with proteins different from receptors and how these accessory proteins affect receptor function, in the view that these protein–protein interactions might control receptor trafficking (e.g. cell surface expression, internalization and recycling) and function (e.g. G protein-coupling and desensitization) [27]. In addition, the stoichiometry, the affinity of these partners and the presence of cellular modulators (e.g. Ca^{2+}) can determine the dynamics of these protein–protein interactions. Finally, it is proposed that the intracellular portions of adenosine receptors, namely the C-terminal tail and intracellular loops, might become signal integrators where interacting proteins would be associated or dissociated depending on cellular inputs, thus making up the adenosine receptorsome.

3.1. Adenosine A_1 receptor interacting proteins (A_1RIPs)

Within the numerous neurophysiological actions of adenosine, the inhibition of glutamate neurotransmission has been largely studied in several brain regions [22]. This adenosine-mediated glutamate release inhibition might be a consequence of the activation of presynaptic A_1Rs, which on its turn lead to a direct inhibitory effect of G protein βγ-subunits on voltage-dependent Ca^{2+} channels [28,29]. Thus, presynaptic A_1Rs are the prototype of GPCRs the stimulation of which decreases the probability of neurotransmitter release (Table 1). On the other hand, the A_1R turns out to be the predictable example of GPCR where the identification of interacting proteins other than the typical GPCR-associated proteins (e.g. G proteins, β-arrestin, GRKs, etc.) became successful. For instance, four A_1RIPs have been described so far, namely adenosine deaminase (ADA), heat shock cognate protein 73 (HSC-73), Caveolin-1 and Protein 4.1.

3.1.1. Adenosine deaminase (ADA)

ADA is the enzyme that metabolizes the physiological agonist of adenosine receptors, thus reducing the effective concentration of adenosine in the cellular milieu and precluding adenosine-mediated receptor stimulation. In addition, it has been demonstrated that ADA behaves like an ecto-enzyme that is anchored to the cell surface through different plasma membrane proteins [30]. Interestingly, apart from being the enzyme that can deaminate extracellular adenosine, ADA can also have a non-enzymatically role through direct protein–protein interactions [31]. Firstly, by means of confocal laser microscopy, co-immunoprecipitation and affinity chromatography experiments performed in DDT1MF-2 cells, an ADA/A_1R interaction was demonstrated [32], and it was shown to occur within the extracellular loops of the receptor (Fig. 1). Subsequently, the existence of this interaction in native cell membranes was also confirmed, where it seemed that ADA was necessary for the coupling of A_1R to heterotrimeric G proteins [32,33]. Moreover, the ADA/A_1R complex was found in the cell-surface of cortical cultured neurons [34], and afterwards it was suggested that ADA would be targeted to the plasma membrane by A_1R [35].

The proposed physiological role of such protein–protein interaction is to make receptors more sensitive to adenosine. Thus, this heterotypic interaction would be necessary for A_1Rs to achieve high-affinity binding of agonists and subsequently for allowing efficient coupling to the signal transduction machinery. In fact, it was recently demonstrated that human ADA markedly enhanced the agonist and antagonist affinity and abolished the negative cooperativity on agonist binding to human striatal A_1Rs [36]. In addition, apart from reducing the adenosine concentration and preventing A_1R desensitization, ADA binds to A_1R and behaves as an allosteric effector that markedly enhances agonist affinity and increases receptor functionality (Fig. 1). Interestingly, when the enzymatic activity of ADA was knocked down by incubation of the enzyme with low Hg^{2+} concentrations, an ADA/A_1R interaction was still confirmed, thus suggesting that this interaction would be enzymatic activity-independent and proposing the contention that the docking of ADA to A_1R might promote receptor conformational changes that would allow a more efficient receptor-mediated signaling [32,36,37]. On the other hand, in cell-based assays performed in different cell lines, the aggregation of both A_1R and ADA was reported after agonist challenge, which preceded the translocation of both proteins into intracellular compartments. These results suggested that the receptor–enzyme complex was sufficiently stable to allow the simultaneous internalization of both proteins [37]. In addition, preincubation with an A_1R agonist resulted in co-aggregation of the dopamine D_1 receptor (D_1R) and A_1R together with ADA in cells expressing both receptors, suggesting that after A_1R agonist challenge a higher-order oligomeric structure leaded by A_1R was formed [35]. Indeed, more research will be needed to unravel the molecular mechanism by which ADA mediates the formation of a D_1R/A_1R oligomer as this might have dramatic consequences in adenosine–dopamine functional cross-talk. Precisely, this information will become relevant to understand the molecular basis of some antagonistic adenosine–dopamine interactions in the central nervous system that might be relevant in the treatment of some CNS pathological conditions.

3.1.2. Heat shock cognate protein 73 (HSC-73)

A different A_1R interacting protein, a chaperone protein, which has been shown to play a key role in ligand binding and receptor signaling, is the heat shock cognate protein 73 (HSC-73). Heat shock proteins (HSPs), or stress proteins, are highly conserved and present in all cells of all organisms. HSPs, also known as chaperones, play crucial roles in the folding/unfolding of proteins, assembly of multiprotein complexes, transport/sorting of proteins into correct subcellular compartments, cell-cycle control and signaling, and protection of cells against stress/apoptosis (for review see [38]). The 70-kDa members of the HSPs family, including HSC-73, have been found to play a role in the movement of proteins into the nucleus, mitochondria, endoplasmic reticulum and lysosome (for review see [38]). HSC-73, specifically, has been observed to facilitate the transport of proteins into the lysosome after the withdrawal of serum from cells in culture. HSC-73 has additional cellular functions apart from its role as a molecular chaperone, namely it associates with cell cycle regulatory proteins, retinoblastoma, p53 and Bcl-2 and also it has an ATPase activity for the uncoating of clathrin-coated vesicles during endocytosis and vesicle recycling (for review see [38]).

The interaction between HSC-73 and A_1R, which takes place within the third intracellular loop of the receptor, reduces agonists binding and also prevents receptor-G protein activation (Fig. 1), this last action being completely prevented by ADA, the extracellular receptor partner. In fact, although ADA interacts extracellularly and HSC-73 interacts in the cytoplasmic side of the receptor, it seems that both proteins compete for binding to A_1R, thus modulating the functionality of the receptor [39].

On the other hand, it has been described that the HSC-73/A_1R interaction might also be implicated in A_1R trafficking and receptor downregulation. A distinctive co-distribution of HSC-73 and A_1R, but

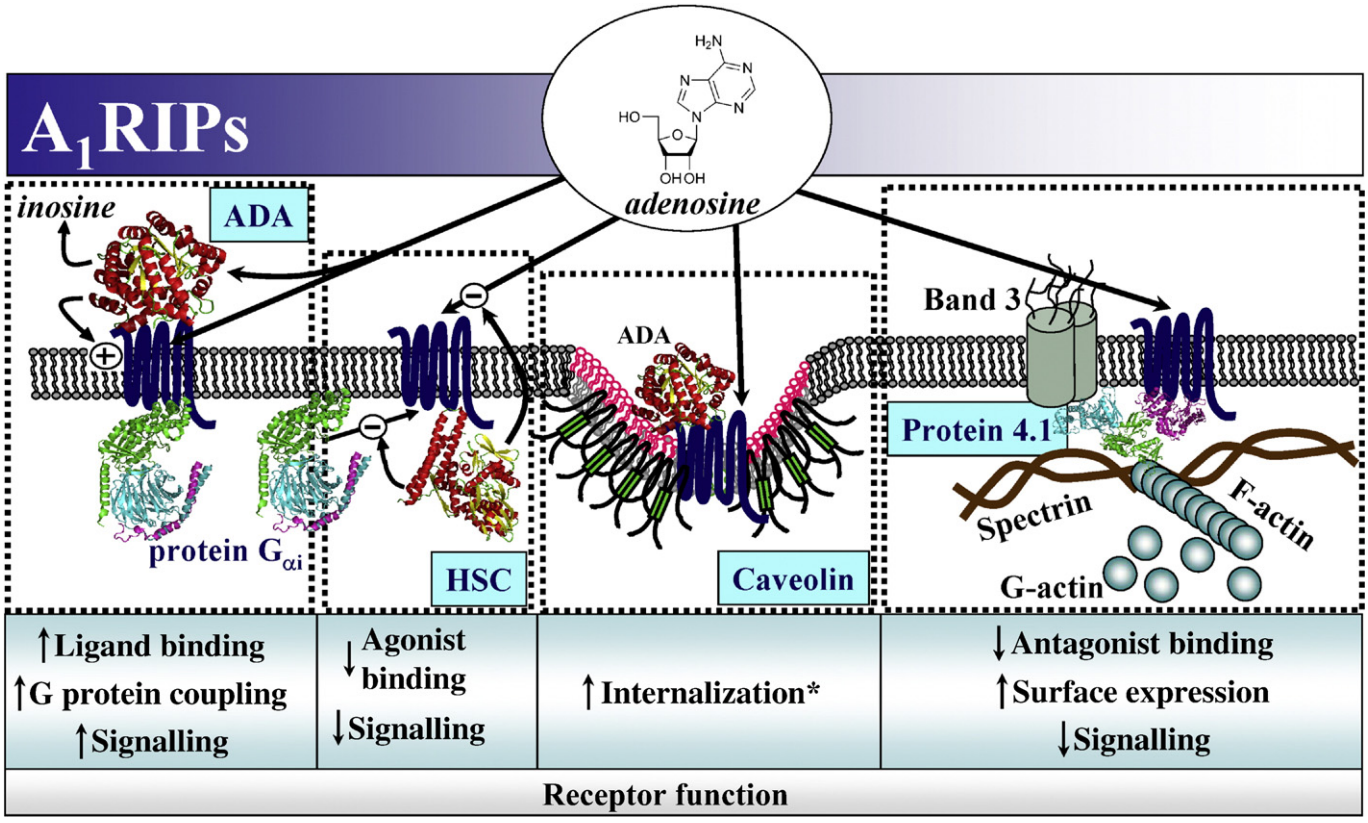

Fig. 1. Schematic representation of A_1R interaction with ADA, HSC, Caveolin-1 and Protein 4.1 proteins. Proposed interacting model where ADA interacts with the extracellular domain and HSC-73 with the third intracellular loop of the receptor. Caveolin proteins homo- and hetero-oligomerize and form caveolae. The differential segregation of lipids in caveolae, glycosphigolipids (pink) in the outer face of the plasma membrane and cholesterol in the inner face of the plasma membrane is also depicted in the figure. Protein 4.1G participates in receptor plasma membrane anchoring and signaling. Cartoon diagrams are from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (www.pdb.org) with the following references: G protein heterotrimer (PDB ID: 1gg2); ADA (PDB ID: 1vfl); HSC (PDB ID: 3fzf); Protein 4.1 (PDB ID: 1gg3). The schematic diagrams are prepared by using PyMOL (The PyMOL Molecular Graphics System. DeLano Scientific, San Carlos, CA, U.S.A.). *Necessary for internalization.

not with other members of the G protein-coupled receptor superfamily, was detected in specific regions of rat cerebellum and in the soma of cortical neurons but not in dendrites or synapses [39]. Related to this, and by means of confocal microscopy, it was observed that chronically agonist-treated cells led to internalization of the A_1R into intracellular vesicles, some of them also containing HSC-73 [37]. In conclusion, it seems reliable that HSC-73, without being the major responsible is a necessary accessory protein to ensure the correct function of A_1Rs, since it is implied in all the receptor's cycle, from agonist binding and signal transduction to receptor down-regulation.

3.1.3. Caveolin-1

Another confirmed A_1R interacting protein is caveolin-1. This protein has been proposed to be involved in receptor anchoring, trafficking and down-regulation (Fig. 1), thus complementing the function of GRKs and β-arrestins. Lipid rafts are divided into two main structural types: caveolae, which are flask-like invaginations of the plasma membrane containing caveolin proteins, and planar lipid raft. Caveolae are present in most cell types but are especially abundant in adipocytes, endothelial cells, fibroblasts and smooth muscle cells [40]. In adipocytes and smooth muscle cells, they represent up to 20% of the total plasma membrane surface area. The main membrane proteins present in caveolae are caveolins, which are necessary for caveolae formation [41] and constitute, as a matter of fact, the molecular marker for these domains. Caveolin proteins (-1, -2 and -3) have cytoplasmic N and C termini palmitolylation sites, and a scaffolding domain that facilitates interaction and organization of signaling molecules [42]. Although these three mammalian subtypes of caveolin proteins are similar in structure and function, they differ in tissue distribution; while caveolin-1 and -2 are abundant in caveolae-rich non-muscle cells, caveolin-3 is found in skeletal muscle and in some smooth-muscle cells [43]. The formation of caveolae by caveolin involves oligomerization and association of these proteins with cholesterol-rich lipid-raft domains. For instance, caveolin-1 binds to one or two cholesterol molecules [44] and it is also palmitoylated in the C-terminal domain [45]. Interestingly, caveolae are disrupted when cholesterol is depleted in the cell, suggesting a key role of caveolin/cholesterol interaction on caveolae formation [46]. Nevertheless, caveolae are involved in cellular processes such as signal transduction and receptor-mediated transport, including endocytosis, transcytosis and potocytosis. As a consequence, it has been proposed that caveolae serve as organizing hubs for cellular signal transduction, according to which the signaling machinery found in caveolae would be upstream of constituents as GPCRs, receptor tyrosine kinases and steroid hormone receptors, and downstream of components as heterotrimeric and low-molecular-weight G proteins, effector enzymes and ion channels. Consequently, it is believed that caveolae in general and caveolin proteins in particular might be necessary to provide a coordinated and efficient signal transduction, thus their altered localization or expression of signaling molecules in lipid rafts (e.g. caveolae) might be at the rear of diseases associated with aberrant signaling (for review see [42]). It is important to mention here that while lipid rafts/caveolae domains might facilitate $G_{\alpha i}$-coupled receptor-mediated signaling (e.g. A_1R) for some $G_{\alpha s}$-coupled receptors, these domains might have an opposite effect, thus being highly effective in silencing $G_{\alpha s}$ functioning (e.g. cAMP production) and mediating $G_{\alpha s}$ internalization [47]. Therefore, one should take this into account when the functional effects of caveolae into A_1R and $A_{2A}R$ are

studied as these receptors are coupled to $G_{\alpha i}$ and $G_{\alpha s}$, respectively (Table 1). Interestingly, a direct interaction between caveolin-1 and the C-terminal domain of A_1R was demonstrated by means of pull down experiments and co-localization assays [37,48,49] (Fig. 1). Moreover, it was reported that agonist-induced internalization of the stable complex ADA/A_1R was mediated by clathrin-independent endocytosis. Thus, detailed electron microscopy in DDT1MF-2 cells revealed that ADA/A_1R complexes internalized through caveolae and that these complexes were differentially sorted in intracellular endosomes which were recycled back to the plasma membrane separately [49]. Overall, these results indicate that desensitization and internalization of A_1R are modulated by ADA and both proteins internalize after agonist challenge via non-coated vesicles, namely caveolae, due to the caveolin-1/A_1R interaction (Fig. 1).

3.1.4. Protein 4.1G

The last described adenosine A_1R interacting protein is a member of the 4.1 family of cytoskeletal-associated proteins, the protein 4.1G. The protein 4.1 superfamily is classified into two main groups according to their cellular expression pattern: the red blood cells which express an erythroid form, also called 4.1R, and the non-erythroid cells that present the 4.1G (general type), 4.1B (brain type) and 4.1N (neuron type) forms. Despite being encoded by different genes, proteins 4.1R, 4.1G, 4.1N and 4.1B all share similar structural features, such as the presence of three highly conserved domains: a N-terminal conserved FERM (Four.1 protein, Ezrin, Radixin, Moesin) domain that links this cytoskeletal scaffold to the plasma membrane via interactions with band 3 and glycophorin C, a spectrin–actin-binding domain (SABD) that potentiates the interactions of spectrin tetramers with F-actin and a C-terminal domain (CTD) that binds FKBP13 (13 kDa FK506-binding protein) [50]. These multifunctional proteins are critical components of the spectrin/actin cytoskeleton and provide attachments between cytoskeleton and cell membranes, thus playing an important structural and regulatory role in the stabilization and assembly of these membranes.

Protein 4.1G binds to the third intracellular loop of A_1R (Fig. 1), and is involved in the trafficking and down-regulation of the receptor [51]. As the spectrin–actin-binding domain of protein 4.1 G can form a complex with actin and spectrin, it is believed that protein 4.1 G would anchor associated molecules, such as intramembranous proteins (e.g. A_1R), to the cytoskeleton. Lu and collaborators showed that protein 4.1G, by interacting with A_1R (Fig. 1), reduced receptor-mediated inhibition of cAMP accumulation and intracellular calcium release, thus interfering with the receptor signal transduction [51]. In addition, protein 4.1G also altered the cell-surface expression of the receptor. Interestingly, by means of immunostaining techniques, it was demonstrated that in the CNS protein 4.1 G co-localized with markers for microglia, such as CD45, OX-42 and ionized calcium-binding adapter molecule 1 (Iba1), but not with markers for neuronal or other glial cells [52]. In the same work, a co-localization between protein 4.1 G and A_1R in mouse cerebrum was also shown [52], leading to the belief that a further understanding of the protein 4.1 G distribution in the CNS might help to clarify some of the functions of A_1Rs.

3.2. Adenosine A_{2A} receptor interacting proteins (A_{2A}RIPs)

Quite the opposite to A_1Rs, the adenosine A_2 receptors subtype has been extensively shown to have an excitatory action [53]. The stimulation of $A_{2A}Rs$ results in a calcium-dependent release of glutamate and acetylcholine, by means of a mechanism that may involve P-type channels [54] (Table 1). However, due to the fact that A_1Rs are more abundant and show higher affinity for adenosine (Table 1), the A_1Rs effects (e.g. reduced excitotoxicity by reducing glutamate release [54]) prevail under most of circumstances. Nevertheless, some evidences indicate that these two receptors, which are coupled to apparently opposite signaling pathways, when co-expressed in the same cells such as in glutamatergic neurons of the hippocampus [55] and striatum [56] might establish a molecular and/or functional cross-talk [57,58]. Indeed, it has been shown that $A_{2A}R$ and A_1R oligomerize when co-expressed in the same cell (e.g. striatal neurons) [56], thus suggesting that these two receptors might form part of a shared molecular transduction complex or signalosome. Therefore, the $A_{2A}R/A_1R$ heterodimer will allow the $A_{2A}R$ to overcome the A_1R functional dominance by means of an intramembrane receptor–receptor communication phenomenon where the $A_{2A}R$ activation will promote a transinhibition of A_1R function [56]. Within this line of inquiry, it is laudable to hypothesize that A_1R and $A_{2A}R$ interacting proteins might potentially impinge in this intramembrane receptor–receptor communication. Thus, these accessory proteins, by driving receptor cell surface expression and plasma membrane anchoring, might participate directly in the regulation of this transconformational-transinhibitory switch, a phenomenon that will only occur in specific subcellular domains were these two receptors concur. Indeed, A_1R and $A_{2A}R$ interact with F-actin binding proteins (e.g. Protein 4.1 and α-actinin, respectively) that anchor receptors to the cell surface by using the F-actin cytoskeleton. Conversely, it is also feasible that the stimulation of a protomer within the $A_{2A}R/A_1R$ heteromer can in theory modulate not only the interaction with its own accessory proteins but also to impinge in the counterpart protomer's ability to interact with other proteins. Overall, the *in vivo* existence of an $A_{2A}R/A_1R$ heteromer would allow adenosine to exert a fine-tuning modulation of glutamatergic neurotransmission, providing a switch mechanism by which low and high concentrations of adenosine would inhibit and stimulate, respectively, glutamate release [59]. Thus, the role of $A_{2A}R/A_1R$ interacting proteins in this switch mechanism will constitute by sure an issue to be study in the future.

Whereas $A_{2B}R$ is widely spread within the brain, the $A_{2A}R$ is primarily located in the dopamine-rich areas of the basal ganglia, as well as in the *nucleus accumbens* and olfactory bulb [60]. In these brain areas, the $A_{2A}Rs$ are involved in processes that include neuronal plasticity and development. Interestingly, $A_{2A}R$ antagonists have been shown to possess strong neuroprotective effects as well as to prevent apoptosis. Consequently, in recent years this subtype of adenosine receptor has been the centre of a growing number of studies because of its potential therapeutic use [2,3].

The intracellular carboxyl-terminal tail of the $A_{2A}R$ is unusually long when compared to the C-terminal tail of the other adenosine receptors: 122 amino acids in contrast to the 34 residues of the A_1R, the 39 of the $A_{2B}R$ or the 34 of the A_3R. Thus, by taking advantage of the yeast two-hybrid (YTH) screening methodology and by using this long $A_{2A}R$ C-terminal tail as bait, several binding partners for the receptor have been found. Accordingly, the $A_{2A}R$ C-terminal tail has been shown to function as a binding site for several proteins, namely α-actinin, Arf nucleotide site opener (ARNO)/cytohesin-2, ubiquiting-specific processing protease (USP4), translin-associated protein-X (TRAX) and Neuronal Calcium-Binding Protein 2 (NECAB2) (Fig. 2).

3.2.1. α-actinin

It has been recently demonstrated that $A_{2A}R$ anchors to the actin cytoskeleton through a direct interaction of the receptor C-terminal tail with α-actinin (Fig. 2) [61]. α-actinin, discovered over more than 40 years ago as a component of the skeletal muscle [62], is a major F-actin cross-linking protein composed of two identical anti-parallel rod shaped peptides that bind actin filaments in a parallel fashion (Fig. 2), thus playing a key role in bundling actin filaments in multiple cell-type and cytoskeleton frameworks. Each α-actinin monomer has three functionally distinct domains: the N-terminus, containing two calponin homology domains (CH) that mediate the interaction with actin, a central region composed of four spectrin-like motifs (SPEC) and the C-terminus which contains EF-hand domains (EFH), thus making the protein sensible to calcium ions (for

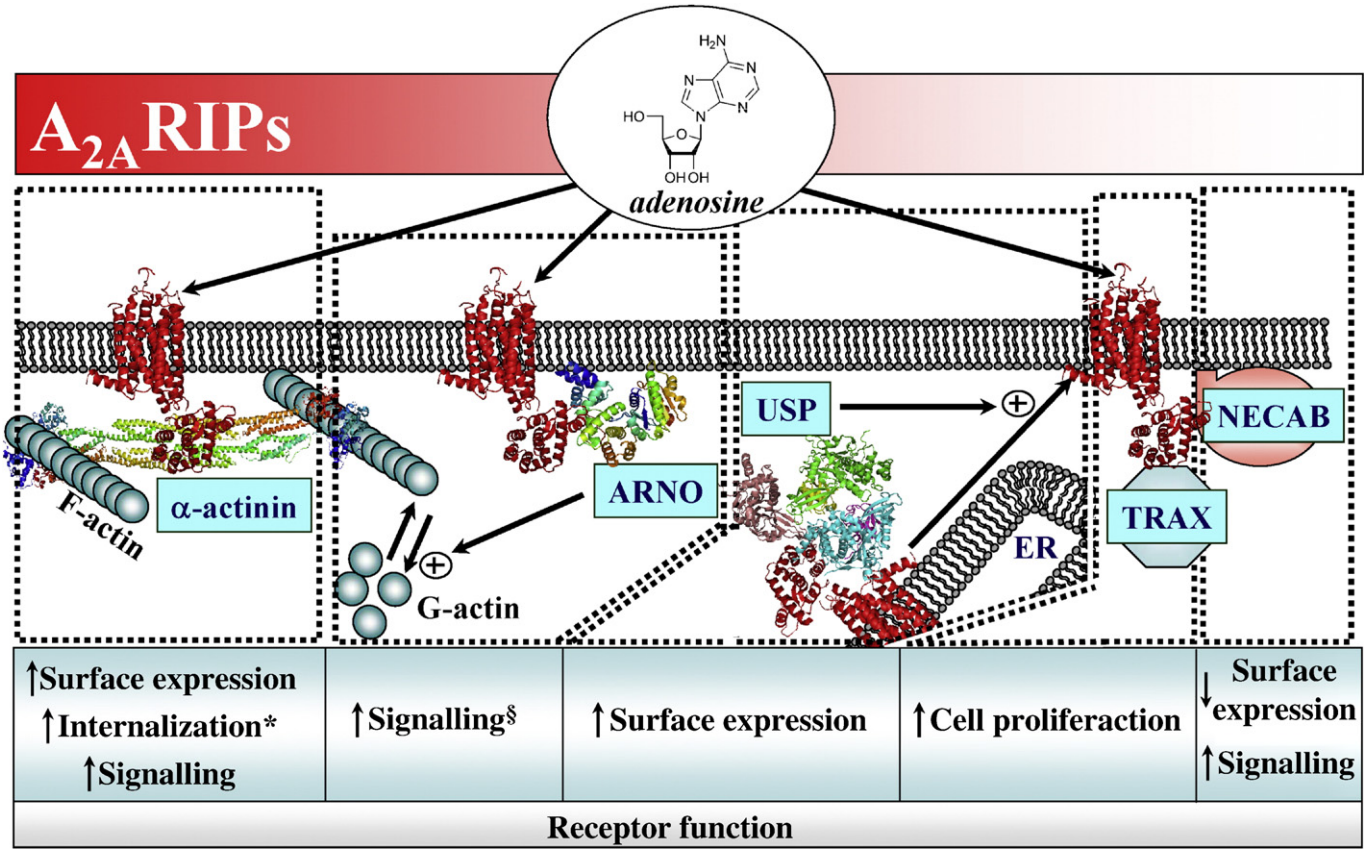

Fig. 2. Schematic representation of $A_{2A}R$ interaction with a-actinin, ARNO, TRAX, USP and NECAB. The schematic cartoon of the $A_{2A}R$ structure parallel to the plasma membrane is adapted from [128] (PDB ID: 3eml). Other cartoon diagrams from the RCSB are: Actinin (PDB ID: 1sjj); ARNO (PDB ID: 1r8s); USP/UBP (PDB ID: 1nbf); USP/UBP (PDB ID: 1nbf). The schematic diagrams are prepared has described in Fig. 1. §Promote $A_{2A}R$ long lasting ERK1/2 activation.

review see [63]). It is important to mention here that while non-muscle isoforms (α-actinin-1 and α-actinin-4) can bind calcium ions, the muscle isoforms (α-actinin-2 and α-actinin-3) do not [64]. Thus, the only described functional difference between these α-actinin isoforms is the fact that the binding of the non-muscle isoforms to F-actin is calcium sensible whereas the muscle isoforms are calcium insensitive [65]. Interestingly, α-actinin has emerged as a major multivalent platform for a large number of protein–protein interactions, thus apart from its interaction with actin filaments it has been described to interact with several cytoskeletal and regulatory proteins [64,66]. On the other hand, while the $A_{2A}R$ was the first GPCR shown to bind α-actinin [61], other receptors and ion channels have also been found to interact with α-actinin, namely Kv1-type potassium channels [67], ATP-gated ion channels $P2X_7$ [68], glutamate NMDA receptors [69], L-type calcium channel [70], policystin-2 [71] and metabotropic glutamate receptor type 5 [72]. Thus, although the precise regulatory role of these interactions remains to be established, it is tempting to assume that the existence of such protein complexes is involved either in the anchoring of those transmembrane proteins to specific subcellular locations or in the targeting of α-actinin to specific functional plasma membrane domains. For instance, it has been described that under resting cell conditions, α-actinin is bound to NMDA receptor causing a decrease of the receptor channel shut time, which results in an increased open probability (P_{open}). When the intracellular calcium concentration increases during neuronal excitation, α-actinin dissociates and calmodulin binds to the receptor, ultimately resulting in a reduction in P_{open} [73]. Interestingly, the NMDA/α-actinin-2 interaction was reported in rat striatum [69], precisely where the $A_{2A}R$ is particularly concentrated. In these rat striatal neurons, the stimulation of $A_{2A}R$ produced an inhibition of NMDA currents, and since the treatment with an actin depolymerizing agent (e.g. cytochalasin B) precluded this $A_{2A}R$-mediated inhibitory effect [74], it could be concluded that this mechanism of action was dependent of an intact α-actinin/actin cytoskeleton. Hence, it is likely that in the rat striatum the actin cytoskeleton in general and α-actinin particularly play a key role in the $A_{2A}R$-mediated functional regulation of NMDA action. Future work will be needed to elucidate the potential role of these protein–protein interactions in specific pathological conditions were these two receptors might be involved, for instance in Parkinson's disease. Furthermore, the $A_{2A}R$ structural dependence of actin cytoskeleton was also corroborated by the fact that a truncated version of the receptor, lacking the α-actinin binding site, was unable to either internalize or to cluster upon agonist challenge, an effect that was reproduced when the actin cytoskeleton was depolymerized by incubating the cells with cytochalasin D [61]. From here, we can assume that the α-actinin-mediated $A_{2A}R$ attachment to the actin cytoskeleton is a prerequisite for the agonist-induced plasma membrane clustering and β-arrestin-mediated internalization. Overall, these data underlie the importance of this physical interaction for the $A_{2A}R$ trafficking and function.

3.2.2. Arf nucleotide site opener (ARNO)/cytohesin-2

Another confirmed $A_{2A}R$ interacting protein, through the C-terminal tail at the inner leaflet of the plasma membrane, is ARNO (Arf nucleotide site opener)/cytohesin-2, a nucleotide exchange factor for the small (monomeric) G proteins of the Arf (ADP-ribosylation factor) family (Fig. 2) [75]. ARNO/cytohesin-2 is thought to act as the guanine nucleotide exchange factor of Arf6, a member of the Arf family required for the internalization of many GPCRs [76] and distinct from other members of this family because of its location primarily at the cell membrane (Fig. 2). Here, Arf6 regulates

endocytosis through both the clathrin/dynamin-dependent and a less well-understood clathrin/caveolae-independent pathway. Interestingly, it was shown that the A_{2A}R-mediated adenylyl cyclase activation was neither affected by ARNO nor by its dominant-negative [75]. These results suggest that ARNO/cytohesin-2 is neither involved in the G_s-mediated cAMP increase induced after A_{2A}R stimulation nor in the agonist-mediated receptor desensitization mechanism. However, as previously mentioned, the A_{2A}R can also signal through the ERK/MAP kinase pathway in a G_s-independent fashion, in which activation of A_{2A}Rs promotes an ERK1/2 biphasic phosphorylation. Firstly, there is an initial fast and robust phosphorylation, that is next followed by a swift decline that rapidly rises again, in a slower and slight phosphorylation with a longer time-span [25]. Interestingly, it was demonstrated by means of the co-expression of A_{2A}R with an ARNO dominant-negative mutant, that ARNO/cytohesin-2 was essential in the A_{2A}R-mediated ERK1/2 phosphorylation second phase [75]. Consequently, the binding of ARNO/cytohesin-2 to the proximal portion of the C-terminal tail of the receptor might be a prerequisite that allows the A_{2A}R to signal in a G_s-independent mode. Overall, it can be concluded that different mechanisms must be involved in the A_{2A}R-mediated biphasic phosphorylation of ERK1/2, which brings a greater diversity regarding the signaling features of this adenosine receptor subtype and presumably of other GPCRs. Another fact worth mentioning is that although the C-terminal tail of the A_{2A}R is relatively long, both α-actinin and ARNO/cytohesin-2 interact with a similar amino acidic region of the receptor: α-actinin binds specifically to a region between amino acids 293 and 321 [61], while ARNO/cytohesin-2 interacts with the region comprised between amino acids 290 and 311 [75]. Thus, the close vicinity showed by both interacting molecules opens up the question of the existence of a fine-tuning regulatory mechanism driving the binding of α-actinin and ARNO/cytohesin-2 to the C-terminal tail of A_{2A}R. Further efforts will be needed to find out the basis of those regulatory mechanisms and we envisage that this interacting dichotomy might be behind the regulation of the actin cytoskeleton remodeling under certain circumstances. Indeed, ARNO/cytohesin-2 is recruited to the plasma membrane by ARL4D where it efficiently activates Arf6. As a consequence, Arf6-GTP induces membrane ruffling by decreasing the number of actin stress fibers, thus modulating actin remodeling [77]. On the other hand, α-actinin is necessary for F-actin bundle formation, hence having the opposite effect than ARNO/cytohesin-2. Therefore, given that both α-actinin and ARNO/cytohesin-2 proteins compete for the interaction with the A_{2A}R, it would seem adequate to prospect if this receptor plays a role in such fine-tuning regulation.

3.2.3. Ubiquitin-specific protease 4 (USP4)

In a different way from the previously described A_{2A}R-binding proteins, the ubiquitin-specific protease 4 (USP4) has been shown to bind to this receptor within the last 50 amino acids of the C-terminal tail (Fig. 2) [78], thus it does not compete with α-actinin nor with ARNO/cytohesin-2 for the C-terminal domain of the receptor.

Ubiquitination plays a key role in the quality control mechanism of cells and ensures a correct folding of newly synthesized membrane proteins before they leave the endoplasmic reticulum (ER). Membrane proteins have to be inserted cotranslationally into the endoplasmic reticulum via the translocon, a channel formed by the Sec61 subunit [79]. Incorrect folding, detected by chaperons in the ER, leads to the activation of ubiquitinating enzymes and the consequent retrotranslocation of the misfolded protein and degradation by the 26S proteasome [80]. Deubiquitinating enzymes are divided into three different subfamilies: the smaller ubiquitin C-terminal hydrolase (UCH) subfamily, the larger ubiquitin-specific processing protease (USP/UBP) subfamily and the subfamily of OUT-domain containing proteins. Thus, USP/UBPs are large proteins and represent the vast majority of deubiquitinating enzymes [81]. It was Milojevic and collaborators that identified the ubiquitin-specific protease USP4 as a protein partner that played a pivotal role in controlling cell surface trafficking of newly synthesized A_{2A}Rs (Fig. 2). In such way, several evidences for the specificity of the interaction between USP4 and A_{2A}Rs were provided. Namely, when compared to the wild-type receptor, the overexpression of USP4 failed to promote the cell surface expression of a C-terminal deleted A_{2A}R version which lacked the USP4 interacting domain. On the other hand, another protein from the USP family, namely the USP14, failed to mimic the USP4 effects (e.g. to rescue A_{2A}R to the cell surface). And finally, USP4 did not have any effect on cell surface expression of another GPCR, as the metabotropic glutamate receptor type 5, thus confirming the specificity of the A_{2A}R/USP4 interaction [78]. Collectively, all the existing data indicate that by its interaction with USP4, the A_{2A}R relaxes its ER quality control, thus favoring its ER exit and the subsequent plasma membrane trafficking (Fig. 2). For instance, an increment in the expression of USP4 may increase the subsequent deubiquitination of A_{2A}Rs, thus leading to an increase in A_{2A}R cell surface expression (Fig. 2). Interestingly, there are several human pathologies that are the result of an exaggerated intervention of these ER quality control mechanisms which are designated to guaranty that misfolded proteins are not delivered to the cell surface, even in the case where they may still be functional [80,82]; for instance, such is the case of cystic fibrosis, typically caused by a mutation on the CFTR transporter [83], and diabetes insipidus, where there is a defect of the V2-vasopressin receptor [84]. Thus, in a similar manner, this might have special relevance in the pathofisiological conditions where A_{2A}Rs have been proved to be involved (e.g. Parkinson's disease), and where its hiper- or hipofunction might be controlled by the USP4-mediated receptor cell surface expression.

3.2.4. Translin-associated protein X (TRAX)

TRAX (Translin-associated protein X) is another protein that binds to the C-terminal tail of the A_{2A}R (Fig. 2). This interaction was identified in a yeast two-hybrid screen and later confirmed by pull-down assays, co-immunoprecipitation and co-localization experiments in the brain [85]. TRAX is a 33-kDa cytoplasmatic protein that was first described as an interacting partner for Translin [86]. Translin is an RNA and single-stranded DNA-binding protein, involved in DNA rearrangement and repair, mitotic cell division, mRNA transport and translational regulation [87]. For instance, one of the TRAX-mediated effects on Translin consists in reducing the binding affinity for RNA but not to DNA [88]. The TRAX/Translin protein complexes are located in centrosomes, where they play an important role in cell cycle control and proliferation [89], and in neuronal dendrites, where a role in RNA processing is the most plausible function [90]. TRAX may be implicated as well in DNA repair via binding to the nuclear matrix protein, C1D, an activator of the DNA-dependent protein kinase essential for DNA double-strand repair and V(D)J recombination [91]. Also, it was recently shown that TRAX regulated GAP-43 transcription and regeneration-promoting effects during the postnatal maturation period [92]. Nevertheless, very little is known about TRAX biological functions, even when, in addition, four other TRAX interacting proteins with a cytosolic location have been described: snaxip1, MEA-2, Akap9, and Sun-1 [93].

Interestingly, it was recently proved that the ability of A_{2A}R to regulate proliferation and neurite outgrowth was TRAX-dependent (Fig. 2) [85]: first, a truncated version of the receptor missing its C-terminal tail, where the potential binding site of TRAX is located, registered a decline of the rescue effect; and secondly, a markedly reduction was achieved with the transfection of an antisense construct of TRAX in PC-12 cells. Moreover, TRAX overexpression in these cells rescued by itself the reduced neurite outgrowth caused by a p53 impairment. Overall, under the light of all these evidences, the interaction between the A_{2A}R and TRAX might represent a new GPCR signal transduction pathway where extracellular signals are transmitted to the nucleus or to the translational machinery.

3.2.5. Neuronal Calcium-Binding Protein 2 (NECAB2)

So far, the last protein shown to interact with the C-terminal tail of the $A_{2A}R$ is NECAB2 (Neuronal Calcium-Binding Protein 2) [94]. NECAB2 belongs to a family of neuronal Ca^{2+}-binding proteins that share common structural features. Briefly, at the N-terminus, NECAB proteins contain an EF-hand domain with a single Ca^{2+}-binding site which is responsible for the calcium binding, thus differing from many other neuronal EF-hand Ca^{2+}-binding proteins [95]. Next to the EF-hand domain, there is a central unique and highly conserved region also called NHR (*N*ECAB *h*omology *r*egion) domain which is characterized by the presence of a coiled-coil domain. Finally, at the C-terminal part of the NECAB molecule a DUF176 or ABM motif is found, a bacterial domain of unknown function in mammalians that was previously found in monooxygenases involved in the biosynthesis of several antibiotics in *Streptomyces* species. NECAB's family is composed by three members that are expressed in rat either primarily in brain (NECAB1 and 2) or in both brain and muscle (NECAB3) [96]. The NECAB protein function still remains largely unknown. Regarding NECAB1, which is also called EFCBP1, it was isolated using affinity chromatography on the C2A domain of synaptotagmin 1 (Syt 1) [97]. This subtype of NECAB protein is mainly expressed in the temporal and fontal lobes, the occipital pole and the putamen in the brain [98], thus making feasible the potential implication of this protein in the sensory processing and cognitive functions, although the mechanisms behind its function remain unravelled. About NECAB3, also called XB51/NIP1, it was isolated as an interacting partner of the neuron-specific X11-like protein (X11L) [99] and it has been suggested that it may play an important role in the regulatory system of amyloid precursor protein (APP) metabolism and β-amyloid generation [99,100]. Also, it has been shown that NECAB3 is a potential substrate of the mammalian protein kinase Nek2, a protein that share homology with the Aspergillus NIMA kinase which plays a key role in controlling entrance into mitosis and required for centrosome assembly and maintenance [101]. Concerning NECAB2, it was demonstrated both heterologous in systems and in native tissue the existence of a short isoform, NECAB2S, generated by the presence of an extra putative translation initiation codon within the cDNA sequence of NECAB2, thus generating a shorter protein (39 kDa) when compared to the full length NECAB2 protein (43 kDa) [94,96]. Interestingly, both long and short isoforms of NECAB2 were recently identified as binding partners for the C-terminal domain of $A_{2A}R$ [94]. By means of immunoelectron microscopy detection of NECAB2 and $A_{2A}R$ in the rat striatopallidal structures, it was shown that both proteins were co-distributed in the same glutamatergic nerve terminals, thus this interaction might be physiologically relevant *in vivo* [94]. Indeed, besides NECAB2 reduced (~25%) cell surface expression of $A_{2A}R$, the agonist-promoted signaling of the receptor was enhanced (Fig. 2) [94]. In addition, calcium ions markedly inhibited in a dose-dependent manner the binding of NECAB2 to $A_{2A}R$ *in vitro*, suggesting that the degree of interaction between these two proteins was determined by the physiological concentration of intracellular calcium ions. Therefore, signals that raise the intracellular calcium concentration will, in theory, promote dissociation of NECAB2 from $A_{2A}R$, thus affecting the receptor-mediated signal transduction and cell surface expression of the receptor. NECAB2 and α-actinin share in common the fact that they interact with $A_{2A}R$ and that they both contain EF-hand domains, thus making the interaction with $A_{2A}R$ potentially dependent of intracellular calcium ions. In contrast, they differ in their ability to modulate cell surface expression of $A_{2A}R$.

Overall, it is important to mention here that although the $A_{2A}R$ C-terminal tail may exist in an extended conformation [102], hence providing room enough for direct protein–protein interactions, the size of the individual binding partners (see Fig. 2) makes it unlikely that the C terminus can accommodate several partners simultaneously. These interactions probably occur in a more subtle way, since the binding site for some of the interacting molecules is pretty close or even overlap (e.g. α-actinin and ARNO). Thus, individual interactions are likely to be only transient, a feature that will allow the $A_{2A}R$ C-terminal tail to become a signal integrator where interacting proteins will associate or dissociate depending on cellular inputs.

3.3. Adenosine A_{2B} receptor interacting proteins (A_{2B}RIPs)

The knowledge of $A_{2B}Rs$ molecular pharmacology and physiological relevance has been always one step behind the other adenosine receptor subtypes. Several reasons might account for this issue but classically the lack of selective drugs together with the fact that $A_{2B}Rs$ have lower affinity for adenosine when compared to A_1R and $A_{2A}R$ (Table 1), has led to the false assumption that these receptors are of lesser physiological relevance [26]. Interestingly, this adenosine receptor subtype couples to different intracellular pathways from those described for $A_{2A}R$, a fact that may account for their distinct physiological role. Thus, $A_{2B}R$ stimulation leads to an increase in either cAMP or IP_3 levels, so it has been proposed that it can activate both Gs, through which they couple to calcium channels [26], and Gq proteins (Table 1). As far as the intracellular pathways are concerned, these receptors have as much in common with A_1R (activation of phospholipase C) as with $A_{2A}R$ receptors (activation of adenylyl cyclase).

From the pharmacological point of view, $A_{2B}Rs$ have been historically orphans of selective agonists and its affinity for nonselective agonists (e.g. 5′-Nethylcarboxamidoadenosine, NECA) is notably lower when compared to the other adenosine receptors. It was not until very recently that high affinity agonists and antagonists for $A_{2B}R$ were developed (for review see [103]). Interestingly, $A_{2B}R$ were implicated in mast cell activation and asthma, vasodilation, regulation of cell growth, intestinal function and modulation of neurosecretion [26]. As a consequence, this subtype of adenosine receptors would have a broad potential of therapeutic action; in fact, as we speak, some $A_{2B}R$ agonists are under preclinical scrutiny for potential treatment of cardiac ischemia [2,104].

Also, when compared with the previously described adenosine receptor subtypes, the search for $A_{2B}R$ interacting partners has been always one step behind, thus the use of massive proteomic (e.g. pull-down or protein–protein affinity chromatography) and genomic (e.g. yeast two-hybrid screening) experimental approaches has not been commonly applied in the study of this receptor subtype. Therefore, only sporadic descriptions of interacting proteins have been shown, namely adenosine deaminase (ADA), SNARE complex and NHERF-2. Also, another particular interaction involving $A_{2B}R$ and the deleted colorectal cancer protein (DCC) has been described, but it have been met with some controversy. Briefly, DCC, which has been postulated to be a netrin receptor, was found to be a binding partner of $A_{2B}R$ in a yeast two-hybrid screen [105]. These authors found that $A_{2B}R$ was a netrin-1 receptor and induced cAMP accumulation on binding netrin-1, thus proposing that the growth-promoting function of netrin-1 would require a receptor complex containing DCC and $A_{2B}R$ [105]. On the other hand, Stein and collaborators demonstrated that DCC, apart to bind directly netrin-1, played a central role in netrin signaling of axon growth and guidance and this was independent of $A_{2B}R$ activation [106]. Thus, the $A_{2B}R$ activation, which was initially proposed to contribute to netrin effects on axons, was not required for rat commissural axon outgrowth or *Xenopus* spinal axon attraction to netrin-1 [106]. Therefore, the initially described $A_{2B}R$/DCC interaction might have not relevance *in vivo*.

Trafficking of the $A_{2B}R$ involves different processes and interacting proteins which are necessary for triggering and sustaining receptor movement, as it is generally described for GPCRs. Interestingly, it was observed that, in cells with reduced levels of arrestin or when its expression was depleted, endogenous $A_{2B}R$ resensitization was affected, while overexpression of either arrestin-2 or arrestin-3 reversed this impairment. Furthermore, agonist activation of $A_{2B}Rs$

promoted arrestin-2^{GFP} translocation from cytosol to cell membrane and subsequently after long agonist treatment both proteins, $A_{2A}R$ and arrestin-2^{GFP}, underwent internalization to compartments which co-localized with the endosomal markers transferrin and rab-5 [107]. Also, it was demonstrated that the $A_{2B}R$ internalized in an arrestin- and dynamin-sensitive fashion [108]. It has been extensively described that arrestins bind to GPCRs upon GRK-mediated receptor phosphorylation [109]; for $A_{2B}R$, it was described that GRK2 was the kinase involved in the process [110] and a serine residue (Ser^{329}) close to the end of the COOH terminus of $A_{2B}R$ was critical for rapid agonist-induced desensitization and internalization of the receptor [111]. Overall, it is generally accepted that $A_{2B}R$ undergoes desensitization and internalization in a GRK- and arrestin-dependent manner. Interestingly, it was demonstrated that after transient (5 min) $A_{2B}R$ agonist challenge the receptor co-immunoprecipitated as part of a multiprotein complex containing SNAP-23 and VAMP-2 [112]. In polarized epithelial cells, it was proposed that under resting conditions $A_{2B}R$ accumulated in intracellular compartments, thus after apical or basolateral agonist challenge the receptor was recruited to the apical membrane where the SNARE proteins, VAMP-2 and SNAP-23, were in charge of this recruitment, therefore the SNARE-mediated $A_{2B}R$-plasma membrane recruitment might be required for receptor signaling (Fig. 3) [112].

Once the receptor is recruited to the plasma membrane by the SNARE proteins, then it is anchored there with its signaling complex via its interaction with NHERF-2 and ezrin proteins (Fig. 3) [113]. Ezrin is known to act as a protein kinase A (PKA) anchoring protein [114] in addition to associate with the actin cytoskeleton (Fig. 3) (reviewed in [115]). The interaction between NHERF, ezrin, and PKA has been shown to be critical for the functional response of transporters including CFTR [114] and NHE-3 [116]. Interestingly, it was shown that $A_{2B}R$ co-immunoprecipitated with both ezrin and NHERF-2 proteins [113], a fact that seemed to indicate that this complex would act as a stabilizing scaffold that may enhance signaling after the receptor is stimulated.

As it has been described for A_1R, adenosine deaminase (ADA) also interacts with the $A_{2B}R$ subtype. ADA has been shown to be expressed in the surface of lymphocytes where it interacts with the T-cell activation antigen CD26. Interestingly, the interaction of ADA with $A_{2B}R$ in CHO and Jurkat cells, an immortalized line of T lymphocyte cells, increased the affinity of the receptor for the non-selective agonist NECA and also potentiated receptor-mediated cAMP production [117]. Therefore, cell surface ADA, apart from degrading extracellular adenosine, would regulate the adenosine actions that are mediated via $A_{2B}R$ subtype, in lymphocytes. Finally, the interaction between $A_{2B}R$ and the enzyme was also observed in cultured dendritic cells [118]. Dendritic cells are the most potent antigen-presenting cells (APC) specialized in the initiation of immune responses by directing the activation and differentiation of naïve T lymphocytes [119]. In this context, ADA anchored to the surface of dendritic cells by means of an $A_{2B}R$-mediated attachment would interact with the CD26 which is expressed on the surface of the T cells, thus triggering costimulation. This costimulatory signal would promote an augmented T cell activation with a Th1 pattern and proinflammatory cytokine production, therefore enabling an enhanced immune response [118].

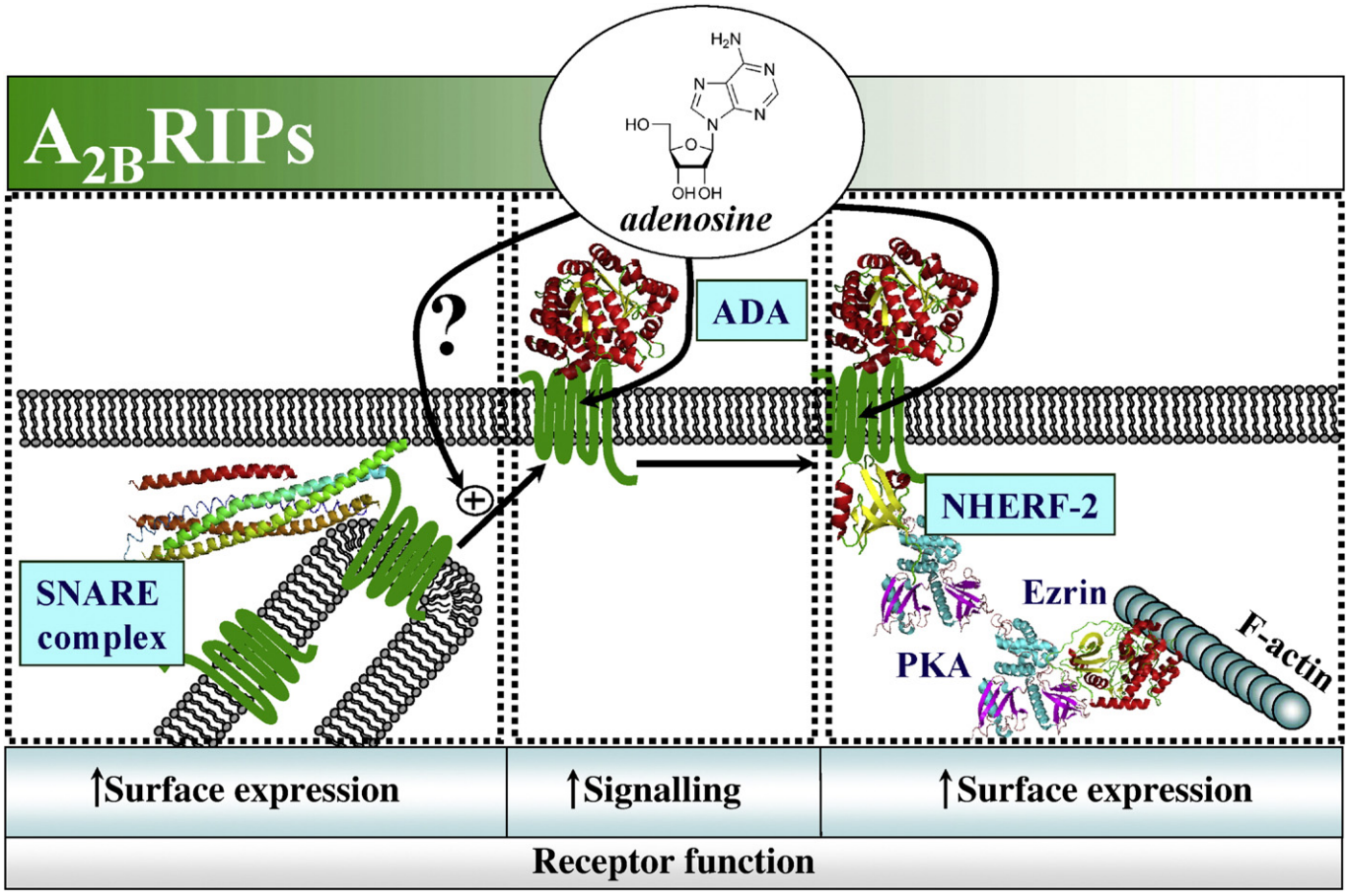

Fig.3. Schematic representation of $A_{2B}R$ interaction with ADA, NHERF-2 and SNARE proteins. Proposed interacting model where ADA interacts with the extracellular domain and NHERF-2 with the intracellular domains of the receptor. Ezrin (PDB ID: 1ni2), NHERF-2 (PDB ID: 2ocs), PKA (PDB ID: 2uzt), SNARE complex (PDB ID: 1kil). The schematic diagrams are prepared has described in Fig. 1.

3.4. Adenosine A_3 receptor interacting proteins (A_3RIPs)

The A_3R was the last adenosine receptor subtype cloned [120], and it was quickly demonstrated that this adenosine receptor subtype inhibited cAMP accumulation after agonist challenge [121]. Therefore, A_3R is coupled to $G_{i2,3}$ and $G_{q/11}$ proteins, leading to adenylyl cyclase inhibition and stimulation of phospholipase C (PLC), thus reducing cAMP levels and increasing IP_3, intracellular calcium and DAG (Table 1). These main signaling pathways, together with other secondary routes, regulate several functions, such as neuroprotection and neurodegeneration, cardioprotection, inflammatory signals, immunostimulation and tumorigenic effects [122]. For instance, adenosine released during cardiac ischemia exerts a potent, protective effect in the heart, an effect that might be mediated via the activation of A_3R, which is expressed on cardiac ventricular cells. Furthermore, A_3R has been shown to be involved in the inhibition of neutrophil degranulation in neutrophil-mediated tissue injury. Also, it has both neuroprotective and neurodegenerative effects, and it may also mediate both cell proliferation and cell death. In consequence, given that the A_3R has emerged as a new adenosine-based therapeutic target, the pharmacology around this receptor has quickly grown; for instance, the A_3R selective agonist CP-532,903 has been shown to protect against myocardial ischemia and reperfusion injury in a mouse model of infarction [123], and CF-101, also an A_3R agonist, is in clinical trials for the treatment of rheumatoid arthritis [124]. Overall, nowadays it can be speculated that A_3R receptor selective ligands might show utility in the treatment of ischemic conditions, glaucoma, asthma, arthritis, cancer and other disorders in which inflammation is a feature [122].

It is important to mention here that when compared with the other adenosine receptor subtype members in general and with A_1R and A_{2A}R in particular, the A_3R does not have any specific protein described to interact with, apart from the hypothesized for GPCRs functioning (e.g. G proteins, etc.). Thus, it was shown that GRK2 was involved in the receptor desensitization, although no direct interaction was proven [125]. Furthermore, this trafficking did not seem to be mediated by arrestins, since they did not co-localize with A_3R upon agonist stimulation in neither RBL-2H3 nor CHO cell lines [126,127]. Thus, when compared to the other three adenosine receptor subtypes very little is known about the surrounding A_3R multiprotein environment which might be critical to understand the receptor function under normal and pathological conditions. Therefore, more work providing information about A_3R interacting partners is needed.

4. Concluding remarks

GPCRs in general, and adenosine receptors in particular, have been shown to interact with a large array of accessory proteins different from receptors that in one way or another impinge into receptor function. The ARIPs described here have been discovered by means of genomic and proteomic approaches and validated by using typical biochemical and also fluorescence-based methods (e.g. fluorescence/bioluminescence resonance energy transfer approaches). Surprisingly, despite of the relatively small extracellular/intracellular receptor domains, they have been shown to interact prolifically with a large number and variety of proteins. Therefore, one would expect that these interactions, some of them targeting the same receptor domain, should be regulated in a subtle way in order to allow a fine-tune modulation of receptor function. Now, the challenge would be to functionally/molecularly characterize these interactions in native tissue and to determine how they orchestrate to regulate receptor functioning. At this point, several issues should be taken into account to fully understand the role of these interactions in both normal and pathological conditions: the differential spatio-temporal expression pattern of each independent protein versus the receptor involved, the stoichiometry and relative affinity of these accessory proteins for the receptors, and the functional and molecular cross-talk between the different ARIPs. Overall, the *in vivo* know-how of these issues will be mandatory before the design of any tentative adenosine receptor interactome-based therapeutic strategy.

Acknowledgements

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