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Receptor–receptor interactions involving adenosine A_1 or dopamine D_1 receptors and accessory proteins

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Summary The molecular basis for the known intramembrane receptor–receptor interactions among heptahelical receptors (G protein coupled receptors, GPCR) was postulated to be heteromerization based on receptor subtype specific interactions between different types of homomers of GPCR. Adenosine and dopamine receptors in the basal ganglia have been fundamental to demonstrate the existence of receptor heteromers and the functional consequences of such molecular interactions. The heterodimer is only one type of heteromeric complex and the evidence is equally compatible with the existence of higher order heteromeric complexes, where also adapter proteins such as homer proteins and scaffolding proteins can exist, assisting in the process of linking the GPCR and ion channel receptors together in a receptor mosaic that may have special integrative value and may constitute the molecular basis for learning and memory. Heteromerization of D_2 dopamine and A_{2A} adenosine receptors is reviewed by Fuxe in another article in this special issue. Here, heteromerization between D_1 dopamine and A_1 adenosine receptors is reviewed. Heteromers formed by dopamine D_1 and D_2 receptors and by adenosine A_1 and A_{2A} receptors also occur in striatal cells and open new perspectives to understand why two receptors with apparently opposite effects are expressed in the same neuron and in the nerve terminals. The role of accessory proteins also capable of interacting with receptor–receptor heteromers in regulating the traffic and the molecular physiology of these receptors is also discussed. Overall, the knowledge of the reason why such complex networks of receptor–receptor and receptor–protein interactions occur in striatal cells is crucial to develop new strategies to combat neurological and neuropsychiatric diseases.

Keywords: Adenosine deaminase, hsc73, clustering parkinson, caveolin

Introduction

The striatum is the main input structure of the basal ganglia and is functionally subdivided in dorsal and ventral striatum. The dorsal striatum (caudate and putamen nucleus) is involved in the performance and learning of complex motor acts. The ventral striatum (nucleus accumbens) forms part of brain circuits involved in the conversion of motivation into action. Medium-sized striatal GABAergic efferent neurons constitute more that 90% of the striatal neuronal population and receive two main inputs that converge in their dendritic spines: a dopaminergic input from the mesencephalon (substantia nigra and ventral tegmental area) and a glutamatergic input from cortical, thalamic and limbic (hippocampus and amygdala) areas. There are two subtypes of striatal GABAergic efferent neurons, projecting to the thalamus across two different pathways: the striatopallidal neurons (indirect pathway) and the striatonigrostriatoentopeduncular neurons (direct pathway).

The two types of striatal GABAergic neurons can be distinguished neuroanatomically. The striatopallidal neurons contain the peptide enkephalin and dopamine receptors predominantly of the D_2 subtype (D_2R). The striatonigrostriaoentopeduncular neurons contain dynorphin and substance P and dopamine receptors predominantly of the D_1 subtype (D_1R). Drs. Fuxe and Ferré and other investigators have shown that the neuromodulator adenosine plays an important role in the function of striatal GABAergic neurons (Ferré et al., 2004). Their effects are mediated by adenosine receptors; four subtypes of them (A_1, A_{2A}, A_{2B} and A_3 receptors) have been cloned and pharmacologically characterized (Fredholm et al., 1994). From these receptors A_1 and A_{2A} are the main targets of the behavioural effects appearing in experimental animals after the administration

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of adenosinergic agonists and antagonists (Ferré et al., 1992; 1997; Fredholm, 1995). The A_1 and A_{2A} receptors (A_1R, $A_{2A}R$) present in the basal ganglia and, in particular, in the striatum, are the responsible of the motor depressant effects of adenosinergic agonists and of the motor stimulating effects of adenosine receptor antagonists (Ferré et al., 1992; 1997). The majority of striatal adenosinic receptors are found in GABAergic efferent neurons (Schiffmann et al., 1991; Rivkees et al., 1995). Both striatopallidal neurons and striatonigro-striatoentopeduncular neurons contain A_1R whereas $A_{2A}R$ are only located in the striatopallidal neurons of the indirect pathway (Schiffmann et al., 1991; Gerfen, 1992; Parent and Hazrati, 1995; Ferré et al., 1996). As a consequence of these anatomical locations, there is a specific codistribution of $A_{2A}R$ and dopaminergic D_2R whereas the A_1R can codistribute with both D_2R and D_1R.

D_2R and $A_{2A}R$, coexpressed in the indirect pathway, and D_1R and A_1R, coexpressed in the direct pathway, can form heteromers. Our research group demonstrated A_1R/D_1R or $A_{2A}R/D_2R$ heteromerizations by coimmunoprecipitation experiments and by BRET (Bioluminiscence Resonance Energy Transfer) and FRET (Fluorescence Resonance Energy Transfer) techniques in transfected living cells and in the striatum of lamb and rat (Ginés et al., 2000; Hillion et al., 2002; Torvinen et al., 2002; Canals et al., 2003; Ciruela et al., 2004). Heteromer formation induces pharmacological changes in the interacting receptors and can also explain some characteristics of the antagonistic functional cross-talk that, in the striatum, takes place between adenosine and dopamine. The dopamine and adenosine functional interactions mediated by A_1R and D_1R and regulated by accessory proteins is here reviewed. The dopamine and adenosine functional interactions mediated by $A_{2A}R$ and D_2R is described in the Fuxe et al. (2006) contribution to this special issue.

Homodimers of D_1 and of A_1 receptors

D_1 receptor homodimers were identified by electrophoresis in insect Sf9 infected cells expressing the human receptor (George et al., 1998).

The first evidence for adenosine A_1 receptor homomerization was obtained by immunoblotting using antibodies that recognized, in samples from pig brain cortical membranes, a specific band of 39 kDa and, in addition, a second band of 74 kDa. This high molecular weight band did not dissociate in a reducing environment or by the treatment at 100° with detergent and did not contain G proteins that could be forming a stable complex with the receptor. Therefore the band likely reflected the existence of dimers in membranes from pig brain. The bands corresponding to the monomer and to the dimer were present in extracts from different pig and rat tissues, the dimer being specially abundant in samples from cortex and striatum (Ciruela et al., 1995).

Since the description of the existence of homodimers for serotonin 5HT1B, dopamine receptors (Ng et al., 1993; 1994a, b; 1996) and A_1R (Ciruela et al., 1995) a number of reports have described the occurrence of homodimers for a variety of GPCRs. In fact it now seems that any member of the GPCR superfamily can be present in form of dimers in the plasma membrane.

Heteromers of D_1 and A_1 receptors

A_1R is a prototypic G-protein-coupled receptor able to for heterotypic interactions with other members of the superfamily of receptors. Heteromerizations described for A_1R include those established with D_1R, metabotropic purinergic P2Y1 receptors (Yoshioka et al., 2001), and with metabotropic glutamate receptors (Ciruela et al., 2001).

Due to the dopamine/adenosine antagonism in the central nervous system it was suspected that adenosine and dopamine receptors could form heteromers in the surface of the neuron. Working with mouse fibroblast Ltk2 cells cotransfected with human A_1R and D_1R cDNAs it was found that A_1R and D_1R, did coimmunoprecipitate. In constrast, coimmunoprecipitation was not posible in fibroblasts cotransfected with human A_1R and D_2R (Fig. 1, Ginés et al., 2000). Fibroblast cell pretreatment with D_1R agonist reduces the intensity of D_1R immunoreactive band in immunoprecipitates with A_1R antibody and this effect was no longer seen after combined treatment with A_1R and D_1R agonists. This suggests that coactivation induces heteromer stabilization. On the other hand, coimmunoprecipitation of A_1R and D_1R can be achieved in samples from rat nucelus accumbens and interestingly, coimmunoprecipitation of D_1R and A_1R is reduced in accumbens from rats treated with cocaine (Toda et al., 2003). Moreover, a high degree of A_1R and D_1R colocalization, demonstrated in double immunofluorescence experiments with confocal laser microscopy, was found in both cotransfected fibroblast cells and cortical neurons in culture, whereas, a low degree of A_1R and D_2R colocalization was observed in cotransfected fibroblasts. Pretreatment with the A_1R agonist caused coclustering (coaggregation) of A_1R and D_1R, which was blocked by combined pretreatment with the D_1R and A_1R agonists in both fibroblast cells and in cortical neurons in culture. This indicated that the movement of

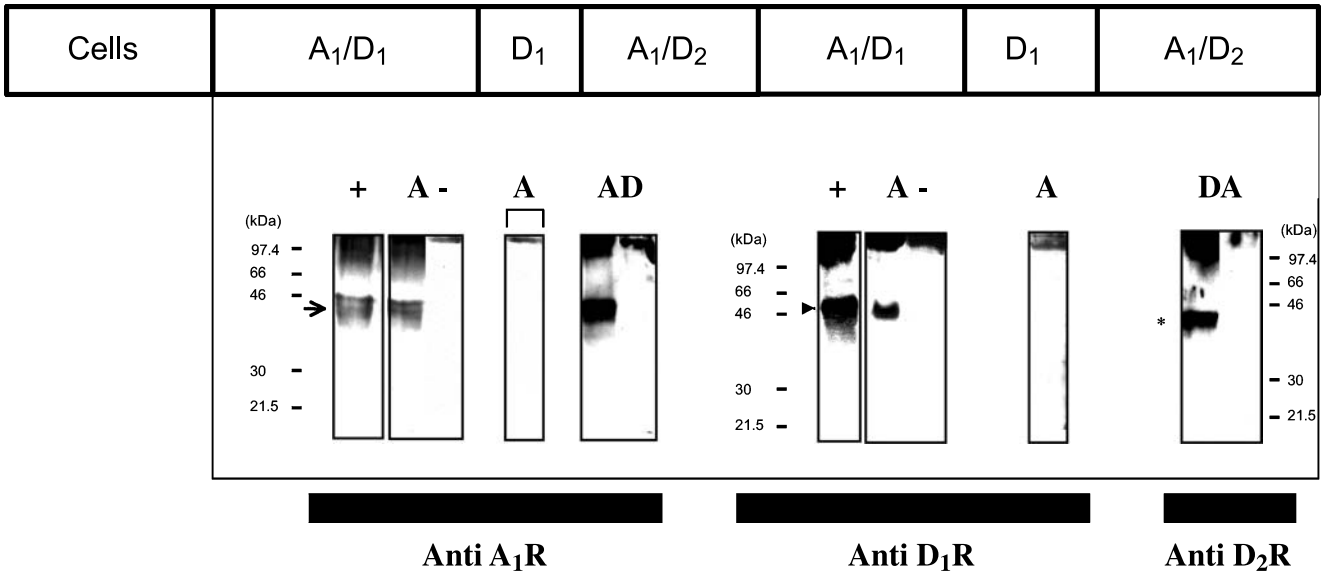

Fig. 1. Coimmunoprecipitation of D_1R and A_1R in cells coexpressing the two receptors. As a control cells expressing A_1R and D_2R or only D_1R were also assayed. Coimmunoprecipiation was performed using specific anti-A_1R (A) or anti-D_1R (D) antibodies and coimmunoprecipitates were blotted using anti-D_1R, anti-A_1R or anti-D_2R antibodies (adapted from Ginés et al., 2000)

heterodimers and/or clusters of heterodimers in the plasma membrane can be agonist-regulated. It is still not known whether the agonist effects on clustering are consequence of changes in the percentage of heteromers. Combined pretreatment with D_1R and A_1R agonists, but not with either one alone, substantially reduced the D_1R agonist-induced accumulation of cAMP (Fig. 2, Ginés et al., 2000), which means that desensitization is only possible after coactivation. It seems that, for many receptors including D_1R and A_1R, dimer formation occurs in the endoplasmic reticulum and that once formed dimers cannot be disassembled (Bouvier, 2001). In the case of A_1R and D_1R heteromers, simultaneous activation allows the antagonistic intramembrane receptor–receptor interaction to take place, namely the G protein uncoupling with the disappearance of the D_1R high affinity state. One functional meaning of this intramembrane receptor–receptor interaction is therefore uncoupling of D_1R from Gs protein.

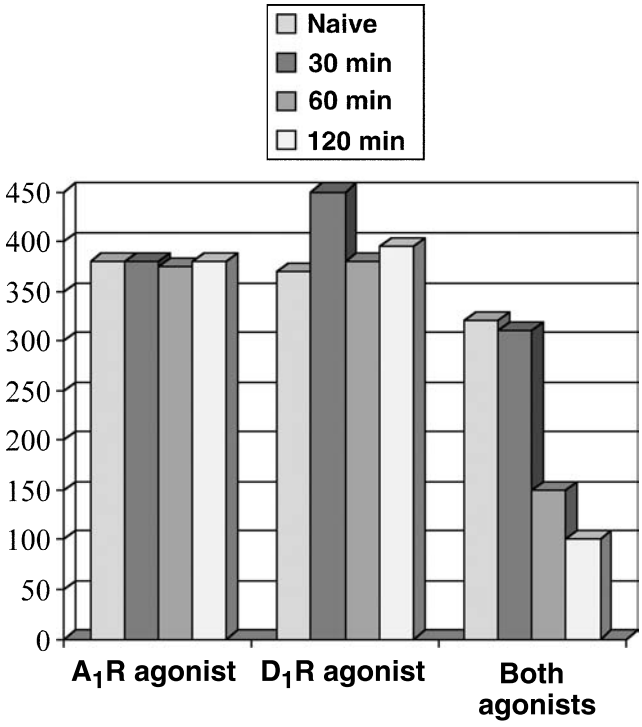

Fig. 2. Desensitization of D_1R only after coactivation of D_1R and A_1R. cAMP production via D_1R after pretreatment with agonists of A_1R and/or D_1R in A_1R/D_1R cotransfected mouse Ltk- fibroblasts (adapted from Ginés et al., 2000)

The A_1R/D_1R heteromeric complex may therefore give the molecular basis for the well documented antagonistic A_1/D_1 receptor–receptor interactions found in the neuronal networks of the brain (Ferré et al., 1997; Fuxe et al., 1998; 2002; Franco et al., 2000; 2001). The A_1R/D_1R heteromerization also appears to have an impact on receptor trafficking (Giés et al., 2000). Thus, an A_1R agonist after a 3 h exposure produced a coaggregation of A_1 and D_1 receptors, while a D_1R agonist after an exposure of 3 h only produced an aggregation of D_1R immunoreactivity with a lack of coaggregation in agreement with the ability of the D_1R agonist to disrupt A_1R/D_1R heteromerization (see above). The D_1R signalling remained unaffected by the formation of D_1R alone or A_1R/D_1R clusters, as seen in terms of an

unchanged D_1R stimulated cAMP accumulation and thus with no signs of D_1R desensitization. In contrast, combined A_1R and D_1R agonist treatments under the same conditions did not result in the formation of A_1R/D_1R receptor clusters but the diffuse A_1R/D_1R colocalization was maintained and now signs of D_1R desensitization developed as seen from reductions in D_1R induced increases of cAMP levels. Thus, essential features of D_1 receptor desensitization may be maintained heteromerization with no A_1R/D_1R coaggregates formed and a prolonged combined exposure to A_1R and D_1R agonists with no indications of receptor internalization. It seems possible that the D_1R desensitization may be mainly caused by a prolonged allosteric change in the D_1 receptor brought about by the A_1R/D_1R receptor–receptor interaction within the heteromeric complex and related also to subsequent phosphorylation changes and/or association with betaarrestin-like molecules (Lefkowitz, 2000; McDonald and Lefkowitz, 2001), leading overall to a reduced D_1R/Gs coupling. Thus, it may be suggested that the intramembrane A_1/D_1 receptor–receptor interaction in this heteromeric complex is relevant not only for acute antagonism of D_1R signalling but also for a persistent long term antagonism of D_1R signalling to the G_s protein. The details of the composition and stoichiometry of the A_1R/D_1R heteromeric complex are unknown and A_1 and D_1 receptors are known to exist as monomers and homomers (Ciruela et al., 1995; Franco et al., 2000; see Lee et al., 2000). It is unknown if heteromers are preferred when A_1 and D_1 receptors are coexpressed in the same cells.

Heteromerization between D_1 and D_2 and between A_1 and A_{2A} receptors

Both, A_1R and D_1R can form heteromers with other membrane receptors of the GPCR family. Recently BRET, FRET and co-immunoprecipitation assays have allowed the interesting finding that A_1R can form heteromers with $A_{2A}R$ (Ciruela et al., 2006). The existence of heterodimers for two fully functional receptor subtypes for the same neurotransmitter/neuromodulator has been also recently described for dopamine D_1 and D_2 receptor subtypes (Lee et al., 2004; So et al., 2005). There is a nice parallelism between the structural characteristics of the heteromers involving dopamine and adenosine receptors.

Previous studies have provided evidence for functional antagonistic interactions between A_1R and $A_{2A}R$ that modulate glutamate release in the striatum and hippocampus (O'Kane and Stone, 1998; Lopes et al., 2002; Quarta et al., 2004a, b). The coexistence of both facilitatory $A_{2A}R$ and inhibitory A_1R in the same terminal is intriguing, particularly in view of their opposite functional effects. A_1R- and $A_{2A}R$-immunoreactivity can be detected in single glutamatergic nerve terminals, identified by immunostaining using antibodies against vesicular glutamate transporters type 1 and 2 (vGluT1 and vGluT2), which are expressed in glutamatergic neurons (Ciruela et al., 2006). Also it has been possible to co immunoprecipitate $A_{2A}R$ and A_1R from solubilized rat striatal nerve terminals. These results demonstrate that A_1R and $A_{2A}R$ can form heteromeric receptor complexes in the striatal nerve terminals.

The occurrence of $A_1R/A_{2A}R$ heteromers provide a rationale to understand how adenosine might facilitate or inhibit glutamatergic transmission depending on the concentration of adenosine. In fact, the functional characteristics of the $A_1R/A_{2A}R$ heteromers are different from A_1 or A_{2A} receptors, due to an $A_{2A}R$-mediated reduction in the affinity of A_1R for agonists. In fact, adenosine, trough adenosine receptors in the $A_1R/A_{2A}R$ heteromers provides a biphasic effect on glutamate release. At low concentrations adenosine inhibits glutamate release via A_1R; in contrast at higher concentrations activation of $A_{2A}R$ shuts down the A_1R-mediated signalling thus resulting in stimulation of glutamate release from striatal nerve terminals (see Fig. 3).

Interestingly, $A_1R/A_{2A}R$ heteromers also provides a rationale to understand some of the pysiological effects of caffeine. In agreement with previous studies (Fredholm et al., 2001), $A_{2A}R$ display higher affinity for caffeine than A_1R when studied in single transfected cells. However, $A_1R/A_{2A}R$ heteromerization, but not heteromerization of

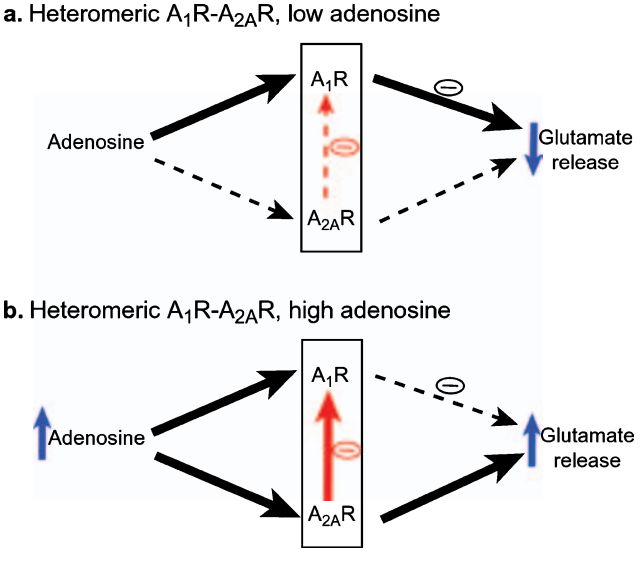

Fig. 3. Scheme of $A_1R/A_{2A}R$-mediated adenosine effects on striatal glutamate release (adapted from Ciruela et al., 2006)

adenosine receptors with other receptors, is associated with a prominent decrease in the affinity of $A_{2A}R$ for caffeine, with a K_D value comparable to that observed in striatal tissue. Heteromerization and modification in the affinity of adenosine receptors for caffeine after chronic caffeine treament helps to elucidate the molecular mechanisms underlying caffeine tolerance (see Ciruela et al., 2006).

Heteromerization between D_1 and D_2 dopamine receptors, which as A_1R and $A_{2A}R$ mediate apparently opposite responses, has been also demonstrated recently in HEK transfected cells by the group of Susan George (Lee et al., 2004). The close proximity of the receptors allows cross-phosphorylation of the D_2R receptor by selective activation of the D_1R. Also D_1R/D_2R oligomers cointernalize when one or the two receptors are activated. Interestingly the D_2R do not internalize when expressed without the D_1R partner. Although further studies are needed to know where D_1R/D_2R heteromers are expressed in brain and their exact physiological role, D_1R/D_2R heteromers may represent an important drug target for diseases related to the dopaminergic system.

Heteromeric receptor–protein interactions. Regulatory role of accessory proteins

In an attempt to understand how D_1R can couple to multiple G proteins, the groups around Goldman-Rakic and Bergson began searching for D_1 receptor interacting proteins with a yeast two-hybrid screen, using the C-terminal part of the human D_1R as a bait (Lezcano et al., 2000). They found a 24 kDa single transmembrane protein, named calcyon, that could interact with the D_1 receptor. Immunocytochemistry demonstrated that the D_1R and calcyon co-located in the same population of pyramidal cells of the cerebral cortex and in a subpopulation of D_1R containing striatal neurons. It is of interest that both calcyon and D_1R were located perisynaptically in dendritic spines at a post-synaptic location. The formation of the D_1R/calcyon heteromeric complex resulted in a marked change in D_1R signalling in the HEK-293 cells. After priming by activation of ATP P2Y receptors but not otherwise, the D_1R agonist SKF 81297 produced a rapid increase in Ca^{2+} signalling dependent on release from intracellular Ca^{2+} stores provided that transfection with calcyon had been performed. This Ca^{2+} response was similar in size to that produced by the P2Y receptor linked to Gq/11. Further experimental effort is needed to elucidate the physiological relevance of this interaction in brain.

A_1R has been also used as a model to identify interacting proteins other than the G proteins and the scaffolding proteins and kinases (clathrin, beta-arrestin, GRKs, etc) described for the members of the superfamily of G-protein-coupled receptors. The first, and unexpected, direct molecular receptor–protein interaction involving A_1R was with adenosine deaminase (ADA). The ADA/A_1R interaction occurs with the extracellular loops of the receptor and therefore ADA can there deaminate extracellular adenosine, which is the physiological agonist of A_1R. Apart from being an enzyme capable of converting adenosine into inosine, ADA is also a multifunctional protein appearing on the cell surface anchored to different proteins (Kameoka et al., 1993; Lluis et al., 1998; Mirabet et al., 1999; Herrera et al., 2001). It can therefore act enzymatically but also extraenzymatically (Franco et al., 1997; 1999). Formation of A_1R/ADA complexes were demonstrated in experiments involving confocal laser microscopy, coimmunoprecipitation and affinity chromatography. Thus, (i) ADA and A_1R coimmunoprecipitated; (ii) A_1R were retained in a matrix of ADA-sepharose and (iii) A_1R colocalized with ADA on cell membranes, including cell surface cortical neurons (primary cultures, Ruiz et al., 2000). Using the same A_1R/D_1R fibroblast cell line as described above, evidence was obtained that ADA in A_1R/D_1R cells exists on the plasma membrane, while in cells expressing D_1R alone ADA was not present on the plasma membrane (Torvinen et al., 2002). These results indicated that ADA can be targeted to the membrane by A_1R but not by D_1R. The binding of ADA to the A_1R appears to be essential for the high affinity agonist binding to the A_1R, giving a functional role of this physical interaction in A_1R-mediated transmission (Ciruela et al., 1996; Saura et al., 1996; 1998). The appearance of this high-affinity site is essential for the antagonistic modulation of D_1R signalling by adenosine.

It seemed rather illogical that the enzyme that degrades adenosine was coupled to adenosine receptors on the cell surface (neurons included, Ruiz et al., 2000). Based on the experience of the interactions of ADA and proteins on the surface of T cells (Franco et al., 1997; 1998; Martin et al., 1995; Pacheco et al., 2005), we suspected that the ADA/A_1R interaction would be important for transducing signals delivered via A_1R. Also, the finding of the high-affinity binding component of the receptor only when ADA was present reassured our idea. The hypothesis was confirmed when substantial calcium mobilization or phosphoinositide hydrolysis in response to A_1R agonists was only possible after addition of ADA (Ciruela et al., 1996). To be sure that ADA was not simply degrading endogenous adenosine, we searched for a compound able to inhibit enzyme activity without affecting its stimulatory role in signal transduction. In fact low Hg^{2+} concentrations led to inhibition of the

enzyme without affecting the effect of ADA on A_1R-mediated signalling (Saura et al., 1996; Ciruela et al., 2006). These data show clearly that a heterotypic interaction ADA/A_1R is necessary for high-affinity binding of agonists and subsequently for allowing efficient signal transduction.

One of the most interesting identified interaction involves a heat shock cognate protein, hsc73 (Sarrió et al., 2000). This protein, hsc73, not only affects the binding of agonists to A_1R but it also affects the binding of adenosine deaminase to A_1R. Regulation of receptor operation by protein–receptor interactions has many facets. For instance, it should be noted that ADA and hsc73 compete for binding to A_1R (Sarrió et al., 2000). Although this is surprising since ADA interacts extracellularly and hsc73 interacts in the cytoplasmic side of A_1R, the activity of the receptor depends on both interactions. When hsc73 binds to purified A_1R, there is a marked loss of affinity for agonists (Sarrió et al., 2000). In contrast, ADA is necessary for high affinity binding and for efficient signalling (Ciruela et al., 1996). It should be noted that all these interactions play a role in ligand binding and in signalling but also in traffic and downregulation of the receptors.

Finally there are interactions which are relevant for receptor traffic and complement the function of GRKs and of ß-arrestin. One such interaction, which has a key role for receptor internalization (see below) takes place between the A_1R and caveolin. This direct interaction of the C-terminal domain of A_1R with caveolin-1 was demonstrated by pull down experiments and colocalization assays (Ginés et al., 2001; Saura et al., 1998). Recently another interaction involving a scaffolfing protein has been described by the group of Rivkees (Lu et al., 2004). The authors have reported that cytoskeletal protein 4.1G binds to the third intracellular loop of A_1R. All of these interactions are imporatnt for traffic and down-regulation of the receptor.

Physiological role of the interactions

Cooperativity on ligand binding

Quite often Scatchard plots of ligand binding to receptors are nonlinear. These plots, which reflect an apparent negative cooperativity in binding, can be explained assuming that the receptor exists in two affinity states which are independent, i.e. not interconvertible. The existence of dimers may explain non-linear Scatchard plots without assuming the existence of these two independent affinity sites. If receptor dimers occur, negative cooperativity can be explained in the classical enzymological way, i.e. the binding of the second ligand to the dimer is more difficult than the binding of the first ligand molecule. Conformational changes occurring when a ligand binds to a receptor in a dimer can be transmitted to the second receptor in the dimer, promoting a lower affinity for the second molecule. Experimentally it has been proved for adenosine/dopamine

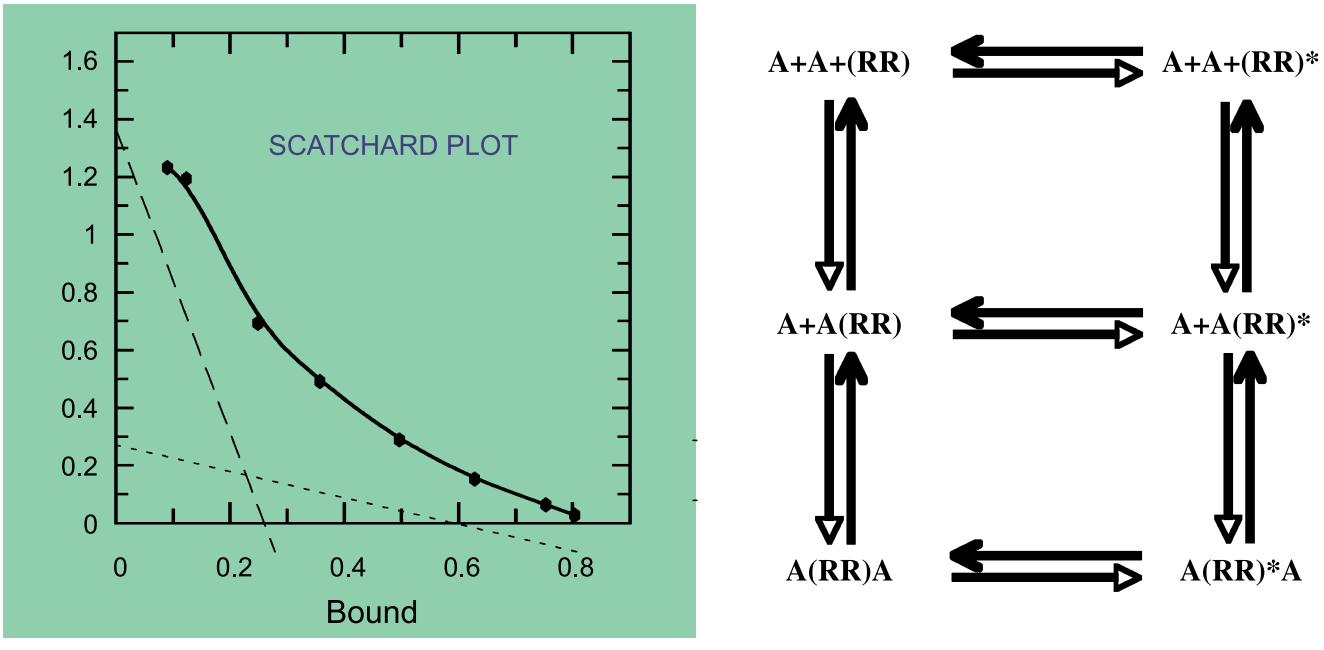

Fig. 4. Dimer model of receptor operation (adapted from Franco et al., 2005). This model can, among other features, explain cooperativity on agonist binding (non-linear Scatchard plots)

receptors that the ligand for one receptor affects the affinity for the ligand to the other receptor; there is a shift from a ''high to a low affinity binding state''. This is likely due to receptor–receptor communication within the heteromers (Salim et al., 2000). A dimer model for heptaspanning receptors has been reported (Franco et al., 1996; 2005; 2006). As shown in Fig. 4, the homodimer can be in inactive form (RR) or in active form $(RR)^*$. This ''two-state dimer receptor model'' is good for fitting data of binding to dimeric receptors displaying either linear, concave upward or concave downward Scatchard plots. In fact, the existence of dimers makes possible the cooperativity on agonist binding, which is highly relevant from a physiological and pharmacological point of view.

Intramembrane cross-talk in heteromers and its modulation

The A_1R/D_1R heteromerization may be one molecular basis for the demonstrated antagonistic modulation of A_1R of D_1R receptor signaling in the brain. Some features of the well documented dopamine-adenosine antagonism (see Agnati et al., 2003; Ferré et al., 2003) can be explained by cross talk at the level of second messengers. However, changes in the pharmacological profile of receptors in heteromers are a consequence of the intramembrane cross-talk and a negative cooperativity coming from receptor–receptor interactions. Activation of A_1R leads to changes in the binding characteristics of dopamine to D_1R. In the absence of cross-modulation, the binding of dopamine analogs to D_1R can be fitted to two affinity sites. Tipically 80–90% of the binding of dopamine analogs is to a low affinity form of the receptor and the remaining 10–20% is to a high affinity form of the receptor (Table 1, Ferré et al., 1998). The high affinity form is completely lost in the presence of agonists activating adenosine receptors (Ferré et al., 1998). If instead of high and low affinity forms, the concept of negative cooperativity in the binding of dopamine analogs to dopamine receptors is taken, the effect of adenosine would be simply due to negative cooperativity.

Since ADA has a role not only as a degradative ectoenzyme but also as an A_1R activity modulating protein, it became of interest to study a possible role of ADA in the A_1R/D_1R heteromeric complex. Interestingly enough, the A_1R-mediated effect upon D_1R requires the binding of ADA to A_1R. As indicated in Table 1 the modulation is lost in the presence of deoxycoformycin, which disrupts the ADA/A_1R interactions. This counteraction is unrelated to the rise of endogenous adenosine levels (see Torvinen et al., 2002). On the other hand, A_1 receptor agonist (R-PIA) preincubation of the above described A_1R/D_1R fibroblast cell line results in coaggregations of both A_1R and ADA and D_1R and ADA in the A_1R/D_1R fibroblast cells. These results suggest that after A_1R agonist treatment with maintained A_1R/D_1R heteromerization coaggregates are formed that contain high-order molecular structures (Torvinen et al., 2002) involving $ADA/A_1R/D_1R$ heteromeric complexes and other interacting proteins that have a special functional role, especially in receptor trafficking. However, ADA does not seem to be linked directly to D_1R. In line with this view, ADA/D_1R aggregates are no longer present after D_1R receptor agonist pretreatment (SKF 38593) which indicates a disruption of A_1R/D_1R heteromerization. In fact, agonist treatment leads to aggregation of D_1R alone, while ADA/A_1R receptor immunoreactivity remains diffusely colocalized (Torvinen et al., 2002). Thus, ADA is part of the A_1R/D_1R heteromeric complex but directly linked only to the A_1R, where it makes possible the high affinity state of the A_1R, allowing it to modulate the operation of the D_1R. Therefore, it seems that a functional unit existing in the surface of the neuron is that formed by ADA, A_1R and D_1R (Torvinen et al., 2002). ADA would be then necessary for high affinity binding of adenosine to A_1R and this would be necessary for intramembrane regulation of binding of dopamine to D_1R in the heteromers.

Interestingly enough the occurrence in striatum of heteromers formed by two subtypes of adenosine receptors, A_1R and $A_{2A}R$, has been recently reported (Ciruela et al., 2006). A negative cross-talk among the receptor in the heteomer and, in particular a shut-down of the A_1R-mediated signalling when $A_{2A}R$ is activated, allows adenosine to exert a fine-tuning modulation of glutamatergic neurotransmission in striatum. In fact at low adenosine concentrations adenosine inhibits glutamate release in striatum whereas the contrary occurs at higher adenosine concentrations. Furthermore, it is also shown that A_1R-$A_{2A}R$ heteromers constitute a unique target for caffeine and that chronic caffeine treatment leads to modifications in the function of the A_1R-$A_{2A}R$ heteromer that could underlie the tolerance to caffeine's psychomotor effects (Ciruela et al., 2006).

Receptor heteromers and receptor clusters in neuronal function and neural plasticity

Adenosine A_1 and dopamine D_1 G-protein-coupled receptors form homomers and heteromers in transfected cells and in striatal neurons. Agonist-induced conformational changes within interacting receptors of the oligomer modify their pharmacology, signalling and/or trafficking. We

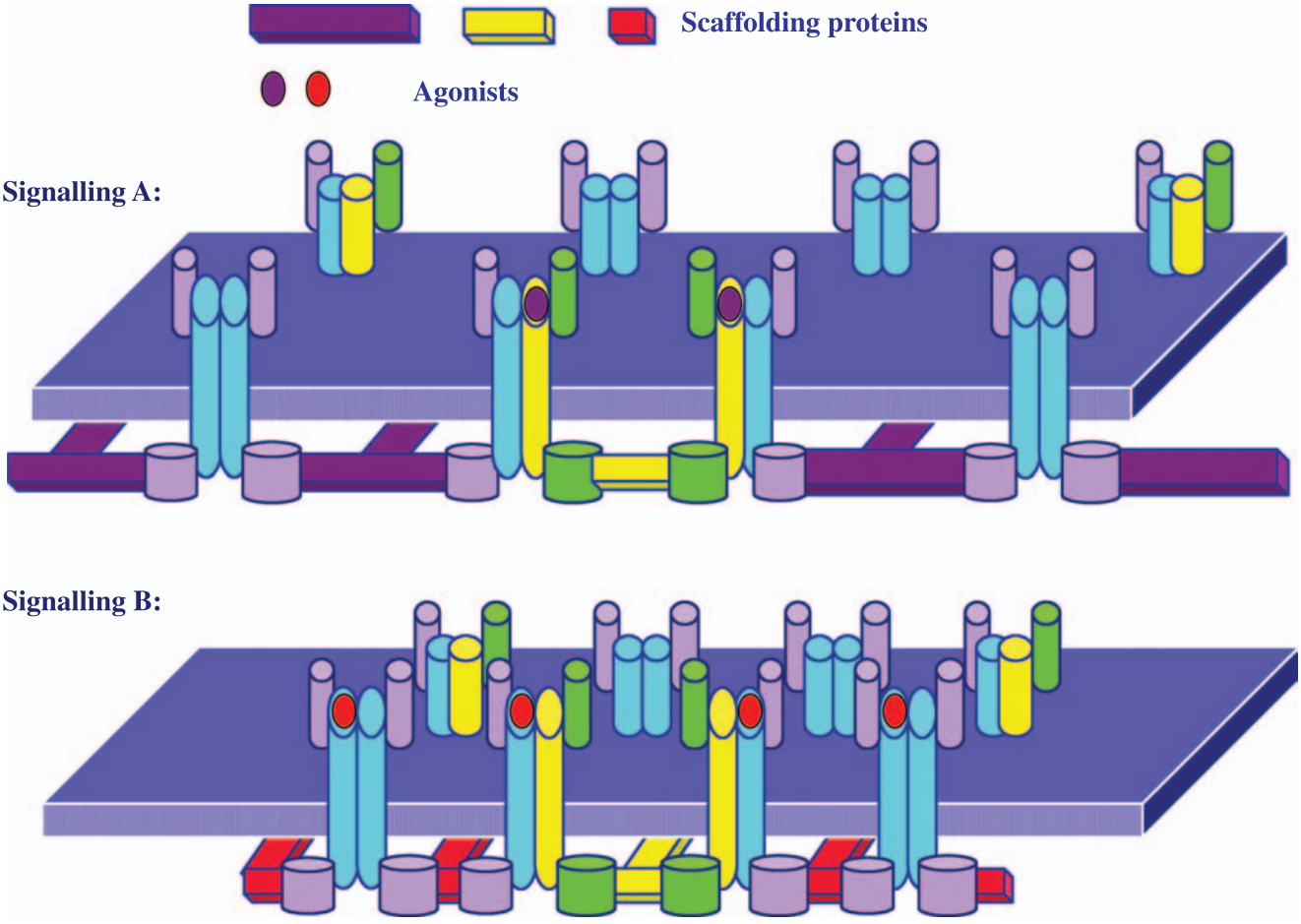

Fig. 5. Scheme of two different signalling pathways triggered by a single pair of heteromeric receptors

have described that this is a first level of regulation of G-protein-coupled receptors that is complemented by a second level of regulation derived from receptor clustering (Franco et al., 2003). In fact when A_1R, D_1R or D_1R/A_1R are activated, the oligomers rearrange and cluster and a novel mechanisms of receptor-operation regulation by oligomer intercommunication is possible (Fig. 5). This intercommunication would be assisted by components of the plasma membrane and by scaffolding proteins (Fig. 6). Receptor cross-sensitization, cross-desensitization and novel, integrated receptor responses can then develop between oligomeric complexes of the cluster even without direct contact between them. The variety of molecular networks in the clusters might be described as several systems of ''crystallization'' in the patch. Different processes of freezing the receptors within those molecular networks might lead to the formation of memory traces in the membrane (Franco et al., 2003).

In a system of interconnected binary elements (Fig. 5), some of the elements might freeze in fixed states of activity (either active or inactive). According to this hypothesis, a cluster of frozen elements in a receptor mosaic might represent a mechanism for the maintenance of a constant input of a neurone and, hence, might play a role in the learning process (where the input of a neurone is likely to remain constant for a period of time), which sometimes is simply represented by a phenomenon of sensitization and desensitization. Even for a single cell, the arrangement of the receptors in the cluster might depend on the type of receptor that ''guides'' the clustering, that is, arrangement is dependent on the type of receptor activated first, which receptor is activated second, and so on. It seems evident that the location of the receptors in the membrane after activation and the clustering influences the physiological effect. Thus, the concentration, the degree of activation and the arrangement in this case of D_1R and A_1R in the clusters could condition signalling in such a way that signalling might differ from cell to cell and even within the same neuron (among distinct locations on a given neuron) depending on the spatial-temporal course of activation. This

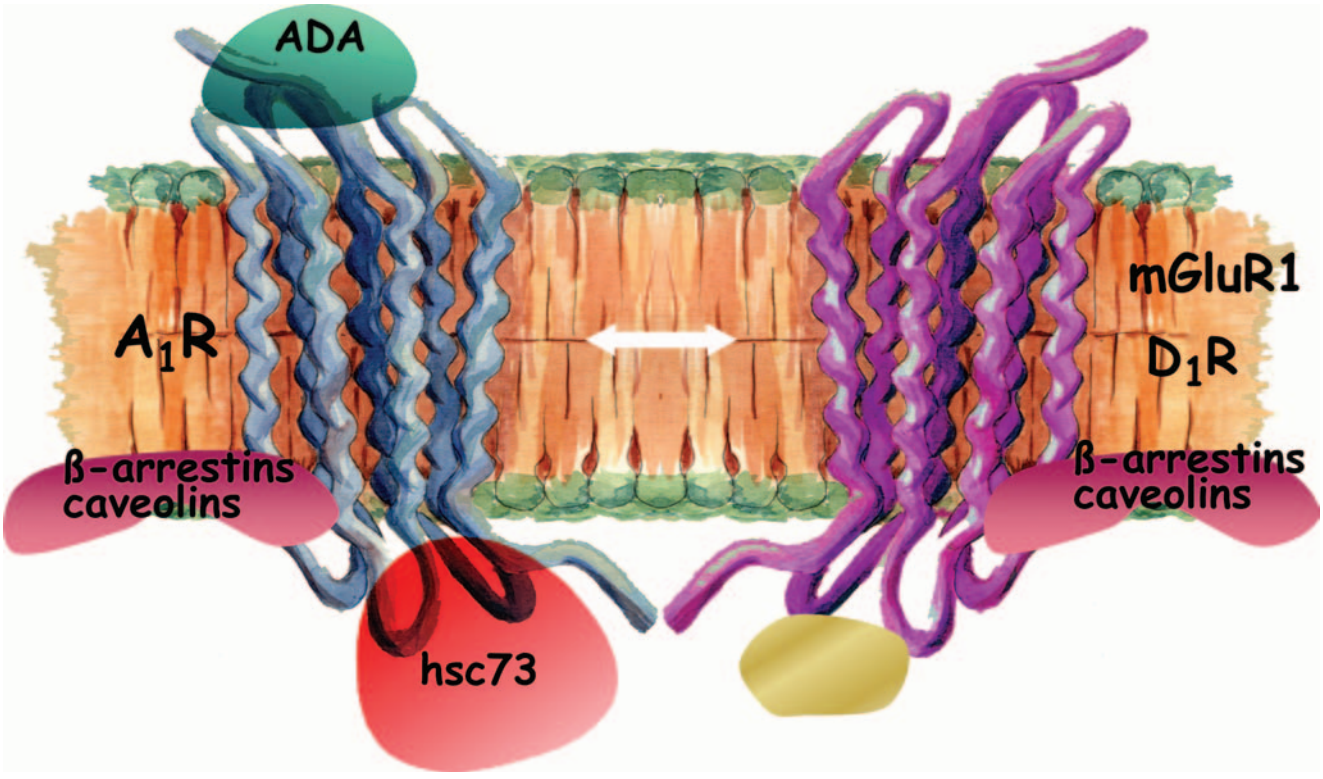

Fig. 6. Scheme of oligomeric heterotypic complexes involving A_1R, D_1R and other receptors and accesory proteins. G proteins and other known interacting proteins such as GRKs are omitted

concept described in detail by Franco et al. (2003) provides a new perspective to the understanding of neurotransmission and neuronal plasticity.

Clinical relevance in Parkinson's disease

Parkinson's disease (PD) is a degenerative process that, generally, appears in a sporadic way. It was reported for the first time by James Parkinson in 1817. Causes are still unknown although several etiological factors are involved, mainly of genetic and environmental nature. The major damage in PD is localized in the pars compacta of the substantia nigra due to a gradual disappearance of the dopaminergic nigrostriatal neurons with depigmentation and gliosis, whereas inclusions named Lewy's bodies appear in surviving neurons. Degeneration of these cells explains the deficit of dopamine in the striatum and the failure in the dopaminergic transmission. PD is characterized by the slow appearance of four major symptoms: tremor in rest, bradikinesia, rigidity and alteration of the postural reflexes. Moreover, there are other alterations including motor, cognitive, autonomous and sensorial disorders.

The most usual strategy is the anti-symptomatic allowing a therapy of substitution with the aim of improving the symptoms. There are different drugs that can be supplied in the symptomatic treatment of PD, being levodopa (L-DOPA), a dopamine precursor that crosses the blood brain barrier, the most commonly used. L-DOPA, that does not stop the evolution of the disease, causes fast initial beneficial effects and it has been shown that extends the life expectancy of parkinsonian patients. The majority of the patients develop belated complications associated with the intermittent administration of dopaminergic drugs of short half-life. After several years (between 5 and 10) of a highly significant efficiency of the L-DOPA treatment, the majority of the patients get worse again. They enter in a new phase of the disease, appearing different changes that require significant modifications in the therapy. The most important long-term disorders of PD are motor (fluctuations and diskinesias) and behavioural alterations. The antagonism between adenosine and dopamine, mediated by receptors A_{2A} and D_2 and also by receptors A_1 and D_1 (Ginés et al., 2001; Torvinen et al., 2002) has made possible the design of a new therapeutic approach for PD. Adenosine receptor antagonists induce in animal models of PD the same type of effect exerted by drugs stimulating dopamine receptors thus enhancing dopamine-mediated effects (Fuxe et al., 2001).

To avoid the side effects due to prolonged treatments with L-DOPA, the development of alternative or complementary therapies is rapidly growing. Among them, the therapy that is in a more advanced clinical phase (phase 3) uses an antagonist ofadenosine receptors, the KW6002 (or istradefylline). The scientific basis that justifies the efficiency of this therapy is the functional antagonism between dopamine and adenosine in the striatum (see below) so that the KW6002 would revert the tonic inhibition upon the striatal dopaminergic system induced by endogenous adenosine. The antiparkinsonian actions of adenosine analogues are to a substantial degree caused by blocking the action of endogenous adenosine receptors of dopamine/adenosine receptor–receptor heteromers (Fuxe et al., 2001; 2003; Ferré et al., 2004).

On the other hand, considering the enhanced corticostriatal glutametergic transmission in animal models of Parkinson's desease, occurrence of presynaptic $A_1R/A_{2A}R$ heteromers migh be important to understand the pathophysiology of Parkinson's and of other neurological and neuropsychiatric diseases affecting the basal ganglia.

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