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Calcium-mediated modulation of the quaternary structure and function of adenosine A_{2A}-dopamine D_2 receptor heteromers

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Abstract

The adenosine A_{2A}-dopamine D_2 receptor heteromer is one of the most studied receptor heteromers. It has important implications for basal ganglia function and pathology. Recent studies using Bioluminescence and Sequential Resonance Energy Transfer techniques shed light on the role of Ca^{2+} in the modulation of the quaternary structure of the A_{2A}-D_2 receptor heteromer, which was found to depend on the binding of calmodulin (CaM) to the carboxy terminus of the A_{2A} receptor in the A_{2A}-D_2 receptor heteromer. Importantly, the changes in quaternary structure correlate with changes in function. A Ca^{2+}/CaM-dependent modulation of MAPK signaling upon agonist treatment could only be observed in cells expressing A_{2A}-D_2 receptor heteromers. These studies provide a first example of a Ca^{2+}-mediated modulation of the quaternary structure and function of a receptor heteromer.

Introduction

Ca^{2+} plays an important role in the physiology of higher order organisms and is involved in the regulation of many cellular events. Various stimuli, such as membrane depolarization or binding of ligands to plasma transmembrane receptors trigger Ca^{2+}-channel opening, which results in a significant influx of Ca^{2+} into the cytosol. Then, Ca^{2+}-binding proteins act as sensors and mediators of the initial Ca^{2+} signal. CaM is a small (17 kDa), highly conserved, soluble, intracellular, acidic Ca^{2+}-binding protein that is considered to be a major transducer of Ca^{2+}-mediated signals in mammalian cells [1]. A series of recent studies have revealed that CaM can directly interact with intracellular domains of G protein-coupled receptors (GPCRs). CaM's roles in the interaction with GPCR function are very diverse. It was initially found that CaM binds to the carboxy-terminus of metabotropic glutamate receptors ($mGlu_5$ and $mGlu_7$ receptors) altering their phosphorylation [2,3]. CaM has also been shown to bind to the third intracellular loop (3IL) of the μ opioid receptor and the dopamine D_2 receptor at overlapping

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regions required for $G_{i/o}$ protein activation, thereby compromising the receptor $G_{i/o}$ protein-mediated activation [4–6•]. Furthermore, CaM can be directly involved in receptor signaling, as in the recently described G protein-independent and arrestin-dependent MAPK activation by the 5-HT_{2C} receptor, which depends on the direct interaction of CaM with the carboxy-terminus of the receptor [7].

The adenosine A_{2A}-dopamine D_2 receptor heteromer is one of the most studied receptor heteromers [8•]. In the brain, A_{2A} and D_2 receptors are highly expressed in one type of striatal neuron, the GABAergic enkephalinergic neuron [9,10]. This type of neuron constitutes almost half the neuronal population in the striatum and its malfunction plays a key role in the pathogenesis of basal ganglia disorders (such as Parkinson's disease and Huntington's chorea) and most probably in obsessive-compulsive disorders, schizophrenia and drug addiction [11]. The A_{2A}-D_2 receptor heteromer plays an important role in the modulation of the activity of the GABAergic enkephalinergic neuron [9–12••] and therefore could be targeted in the development of drugs for these neuropsychiatric disorders. Here we review recent data that demonstrates that calmodulin (CaM) oligomerizes with the A_{2A}-D_2 receptor heteromer and modulates its function in a Ca^{2+}-dependent manner.

Electrostatic interactions involved in CaM binding to A_{2A} and D_2 receptors

It has been suggested that the binding sites of CaM in different proteins are often formed by amphiphilic domains, with hydrophobic residues interspersed with several positively charged ones [1]. Based on these characteristics, the introduction of the human A_{2A} receptor sequence in the Internet-based search program "Calmodulin Target Data Base" (Cancer Institute, Ontario, 2002; http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html) disclosed the existence of a very likely binding site in the proximal portion of the carboxy-terminus of the A_{2A} receptor. The sequence, $_{291}$RIREFRQTFR$_{300}$, contains several arginines (Arg; positively charged residues), 1 Isoleucine and 2 Phenylalanine residues (hydrophobic residues). In fact, in vitro, this A_{2A} receptor epitope formed multiple stable non-covalent complexes with CaM [13•]. However, a more detailed analysis indicated the particular involvement of electrostatic interactions. A close look at the amino acid sequence of CaM shows that 32.5% of its residues contain a negative charge [13•]. Furthermore, CaM has several serine (Ser) and threonine (Thr) residues susceptible of phosphorylation by casein kinases located in the vicinity of acidic residues [13•]. A mass spectrometric analysis of an enzymatic digest of the CaM-A_{2A} receptor epitope non-covalent complexes indicated the involvement of strong electrostatic interactions between the acid motifs of CaM and the Arg residues (particularly the RIR sequence) of the A_{2A} receptor epitope [13•]. A proteomics approach demonstrated the existence of CaM-A_{2A} receptor interactions in rat brain tissue and a direct interaction between both proteins was demonstrated by BRET experiments in transfected mammalian cells [14•]. An important negative control was that the A_{2A} receptor mutated in its putative CaM binding motif located in the carboxi-terminus (with the sequence $_{291}$AIREFAQTFA$_{300}$) did not interact with CaM [14•].

Electrostatic interactions also play a key role in the binding of CaM to the D_2 receptor. A mass spectrometric analysis indicated that CaM binds to an Arg-rich epitope from the amino-terminal part of the long 3IL of the D_2 receptor [13•]. Importantly, the Arg-rich D_2 receptor motif is also involved with the activation of Gi/o protein and with the heteromerization with adenosine A_{2A} receptor (see below). Binding of CaM to the Arg-rich motif does not disturb $G_{i/o}$ recognition but impedes the D_2 receptor-induced activation of Gα and this effect is Ca^{+} dependent [6•]. Thus, Ca^{2+}/CaM blocks $G_{i/o}$ protein activation by D_2 receptor in a non-competitive manner [6•]. But since the Arg-rich motif of the D_2 receptor is also involved in A_{2A}-D_2 receptor heteromerization, it was interesting to know if CaM could also bind to the D_2 receptor in the A_{2A}-D_2 receptor heteromer.

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Intermolecular interactions in the A_{2A}-D_2 receptor heteromer

A_{2A}-D_2 receptor heteromerization has been demonstrated in mammalian transfected cells with co-immunoprecipitation and fluorescence and bioluminescence resonance energy transfer techniques (FRET and BRET, respectively) (reviewed in ref. [8•]). By using computerized modeling, pull-down techniques and mass spectrometric analysis, it was shown that A_{2A}-D_2 receptor heteromerization depends on an electrostatic interaction between the Arg-rich epitope located in the amino-terminal portion of the 3IL of the D_2 receptor and a single phosphate group from a casein kinase phosphorylable Ser localized in the distal portion of the carboxy-terminus of the A_{2A} receptor [15]. Studies in vitro with peptides corresponding to both epitopes demonstrated that the Arg-phosphate interaction possesses a "covalent-like" stability. Hence, these bonds could withstand fragmentation by mass spectrometric collision-induced dissociation at energies similar to those that fragment covalent bonds [16]. The Arg-phosphate electrostatic interaction between epitopes located in intracellular domains is obviously not the only interaction responsible for A_{2A}-D_2 receptor heteromerization. Thus, a significant but not complete reduction of BRET is observed when transfected cells express mutated D_2 receptors that lack the key amino acids involved in the Arg-phosphate interaction [15], indicating that other receptor domains are also involved. Most probably, intramembrane domains play an important role in A_{2A}-D_2 receptor heteromerization, as it has been demonstrated for other GPCR homomers and heteromers [17,18]. Nevertheless, the significant modification of BRET with mutated receptors indicates that the Arg-phosphate interaction is necessary to provide the final quaternary structure of the heteromer, which in fact determines its function. Patch-clamp experiments in identified GABAergic enkephalinergic neurons demonstrated that disruption of the Arg-phosphate interaction in A_{2A}-D_2 receptor heteromers (by intracellular addition of small peptides with the same sequence than the receptor epitopes involved in the Arg-phosphate interaction) completely eliminates the ability of the A_{2A} receptor to antagonistically modulate the D_2 receptor-mediated inhibition of neuronal excitability [12••].

The just mentioned antagonistic interaction between A_{2A} and D_2 receptors is most probably related to the existence of an allosteric modulation in the A_{2A}-D_2 receptor heteromer. This kind of allosteric modulation, in which binding of a ligand to one of the receptor units in the receptor heteromer changes the binding properties of another receptor unit seems to be a common biochemical characteristic of receptor heteromers [19,20•]. An antagonistic A_{2A}-D_2 receptor interaction has been demonstrated in many different membrane preparations from different transfected mammalian cells and from rat and human striatal tissues and implies the ability of A_{2A} receptor stimulation to change the binding characteristics (decrease the affinity) of the D_2 receptor for agonists [8•,10,19]. In experiments with chimeric D_1/D_2 receptors, the A_{2A} receptor could still modulate the binding characteristics of a D_2 receptor with the 6th transmembrane domains of the D_1 receptor, but the modulation disappeared with a D_2 receptor with the 3IL and 5th and 6th transmembrane domains of the D_1 receptor [21]. These results indicate that the epitope (or epitopes) of the D_2 receptor involved in the antagonistic A_{2A}-D_2 receptor interaction might be located somewhere in the 3IL or 5th transmembrane. Experiments using more detailed mutants are in progress to demonstrate that the epitope, in fact, corresponds to the Arg-rich domain of the 3IL.

In addition to the allosteric modulation in the A_{2A}-D_2 receptor heteromer, A_{2A} and D_2 receptors can also interact at the second messenger level, and this has been well demonstrated both in cell culture and in the brain. In this case, however, it is the stimulation of D_2 receptor that counteracts the effects of A_{2A} receptor stimulation. A_{2A} receptor, through its coupling to $G_{s/olf}$ proteins, can potentially stimulate adenyl-cyclase, with phosphorylation of several PKA substrates, such as DARPP-32, CREB and AMPA receptors and the consequent increase in the expression of different genes, such as *c-fos* or *preproenkephalin* in the GABAergic enkephalinergic neuron [8•,9,10,19]. D_2 receptor, on the other hand, can potentially couple to

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$G_{i/o}$ proteins and counteract the ability of A_{2A} receptor stimulation to signal through the cAMP/PKA cascade [8•,9,10,19]. It might sound counterintuitive, but both types of antagonistic A_{2A}-D_2 receptor interactions coexist in the same cells and, in fact, they do coexist in the GABAergic enkephalinergic neurons [8•,9,10,19]. Thus, co-stimulation of A_{2A} and D_2 receptors implies a simultaneous A_{2A} receptor-mediated inhibition of the D_2 receptor-mediated modulation of neuronal excitability and a D_2 receptor-mediated inhibition of the A_{2A} receptor-mediated modulation of gene expression, which provides a clear example of a functional dissociation between neuronal excitability and gene expression. This apparently incompatible coexistence of reciprocal antagonistic A_{2A}-D_2 receptor interactions could be explained by the presence in the same cell of A_{2A} and D_2 receptors forming and not forming A_{2A}-D_2 receptor heteromers. The antagonistic A_{2A}-D_2 receptor interaction at the second messenger level depends on the activation of Gi/o proteins, which requires the Arg-rich domain not being interacting with either CaM or the A_{2A} receptor.

Role of CaM in A_{2A}-D_2 receptor heteromer function

The existence of CaM-A_{2A}-D_2 receptor oligomerization has been detected by the recently introduced Sequential-BRET-FRET (SRET) technique, which allows the identification of oligomers formed by three different proteins [22••,23]. In $SRET^2$, the oxidation of a *Renilla Luciferase* (RLuc) substrate by an RLuc fusion protein triggers the excitation of the acceptor GFP^2 by $BRET^2$ and subsequent energy transfer to the acceptor YFP by FRET [22••]. $SRET^2$ was obtained with the fusion proteins A_{2A}Rluc, Ca$MGFP^2$ and D_2YFP when adding DeepBlueC as Rluc substrate, indicating that the two acceptor/donor pairs, A_{2A}Rluc-Ca$MGFP^2$ and Ca$MGFP^2$-D_2YFP were well oriented at distances of less than 10 nM [14•] (Figure 1). But, how are these three proteins arranged? We know that CaM binds to A_{2A} and D_2 receptors when not forming heteromers. Does CaM bind to both receptors in the A_{2A}-D_2 receptor heteromer? Does CaM disrupt the heteromerization or does it change the quaternary structure of the A_{2A}-D_2 receptor heteromer and therefore its function? Which Ca^{2+}-dependend functional changes does CaM convey in the A_{2A}-D_2 receptor heteromer?

As mentioned above, the Arg-rich domain of the amino-terminal portion of the 3IL of the D_2 receptor is a binding site for both CaM and for a phosphorylated Ser localized in the distal portion of the carboxy-terminus of the A_{2A} receptor. BRET competition experiments demonstrated that increasing the expression of the D_2 receptor does not modify the binding of CaM to the A_{2A} receptor. Also, increasing the expression of CaM does not modify A_{2A}-D_2 receptor heteromerization. In contrast, increasing amounts of A_{2A} receptor led to significant reduction in the BRET signal due to the interaction between the D_2 receptor and CaM [14•]. Overall these results indicate that CaM cannot compete with the A_{2A} receptor for its binding to the D_2 receptor but that binds to the A_{2A} receptor in the A_{2A}-D_2 receptor heteromer.

The increase in intracellular Ca^{2+} (with ionomycin treatment) in cells expressing A_{2A} and D_2 receptors produced modifications in the BRET signal due to the interaction between A_{2A} and D_2 receptors [14•], which implies alterations in the quaternary structure of the A_{2A}-D_2 receptor heteromer. Importantly, Ca^{2+} exerted a selective modulation of A_{2A}-D_2 receptor heteromer-mediated activation of MAPK pathway. First, in cells transfected with either A_{2A} or D_2 receptors, agonist-induced activation of MAPK pathway (ERK1/2 phosphorylation) was not modified by increasing intracellular Ca^{2+} (Figure 2a,b). At least for the D_2 receptor, this suggests that MAPK signaling, in HEK-293T cells [6•,14•], is not dependent on the activation of Gi/o protein, since it has been previously shown that the Gi/o protein-dependent signaling efficiency of the D2 receptor is regulated (decreased) by a rise in Ca2+ via CaM [6•]. Second, in cells expressing both receptors, Ca^{2+} significantly decreased A_{2A} receptor agonist-induced ERK1/2 phosphorylation (Figure 2c) whereas it significantly increased D_2 receptor agonist-induced ERK1/2 phosphorylation [14•] (Figure 2d). Third, co-activation of A_{2A} and D_2

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receptors in cells co-expressing both receptors led to the same qualitative response than activation of A_{2A} receptors alone, with a significant Ca^{2+}-dependent decrease of agonist-induced ERK1/2 phosphorylation [14•] (Figure 2e). The effects of Ca^{2+} were dependent on the intracellular levels of CaM [14•], indicating that CaM selectively transduces Ca^{2+}-dependent changes of MAPK signaling in the A_{2A}-D_2 receptor heteromer. In summary, the results indicate that in the absence of Ca^{2+} the A_{2A}-D_2 receptor heteromer produces very similar effects on MAPK signaling upon stimulation of one of both receptor units, while binding of Ca^{2+} to CaM produces a very different qualitative response, reducing and enhancing the effects of the stimulation of A_{2A} and D_2 receptors, respectively. Additionally, with co-activation of the A_{2A} receptor, the ability of Ca^{2+} to increase the effect of D_2 receptor stimulation on ERK1/2 phosphorylation is lost. This could be related to the allosteric interaction in the A_{2A}-D_2 receptor heteromer, with A_{2A} receptor stimulation antagonizing the effects of D_2 receptor stimulation (Figure 2e).

We still need to determine which molecular mechanisms are involved in the Ca^{2+}/CaM-modulated, A_{2A}-D_2 receptor heteromer-mediated MAPK signaling. Which are the G proteins involved? Recent studies strongly suggest that the minimal signal unit for class A GPCRs is composed of two receptors and a single G protein and that probably applies to both GPCR homomers and heteromers [24••]. We then need to figure out which is the G protein that binds to the A_{2A}-D_2 receptor heteromer and if it changes in the presence of CaM. We already mentioned that D_2 receptor signals through MAPK by a $G_{i/o}$ protein-independent mechanism in cells only expressing D_2 receptors and, most probably, $G_{i/o}$ proteins are not involved either in MAPK signaling by the A_{2A}-D_2 receptor heteromer. Thus, the Arg-rich domain of the D_2 receptor required for $G_{i/o}$ protein activation is bound to the C-terminus of the A_{2A} receptor in the A_{2A}-D_2 receptor heteromer (see above). Also, we have preliminary data suggesting that the $G_{s/olf}$/PKA pathway, commonly used by the A_{2A} receptor, is not involved either. One open possibility is the involvement of G_q, as it has been shown for the D_1-D_2 receptor heteromer [25]. But it is also probable we are dealing with a CaM-dependent, G protein-independent MAPK activation, as recently reported for the 5-HT_{2c} receptor [7].

Conclusions

Receptor heteromers constitute an expanding new area of research which is changing previous concepts of receptor pharmacology. A receptor heteromer is defined as a macromolecular complex composed of at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components [20•]. This definition underscores the fact that the receptor heteromer constitutes a new functional unit and a previously unforeseen number of emerging properties have already been described for several different receptor heteromers. Those properties include changes in ligand recognition, allosteric interactions, G-protein coupling switching and changes in the pattern of activation of signaling pathways and receptor internalization [20•].

Here we reviewed a new biochemical property of the receptor heteromer formed by adenosine A_{2A} and dopamine D_2 receptors, which has important implications for basal ganglia function and dysfunction [9–11]. This property consists of a specific Ca^{2+}-mediated modulation of MAPK signaling, which depends on the binding of CaM to the proximal portion of the carboxy-terminus of the A_{2A} receptor in the A_{2A}-D_2 receptor heteromer. This is associated with changes in the quaternary structure of the A_{2A}-D_2 receptor heteromer, as demonstrated by RET techniques. Experiments with mutated receptors are in progress to determine which receptor domains are key determinants for the establishment of the appropriate Ca^{2+}/CaM-dependent functional and structural changes of the A_{2A}-D_2 receptor heteromers.

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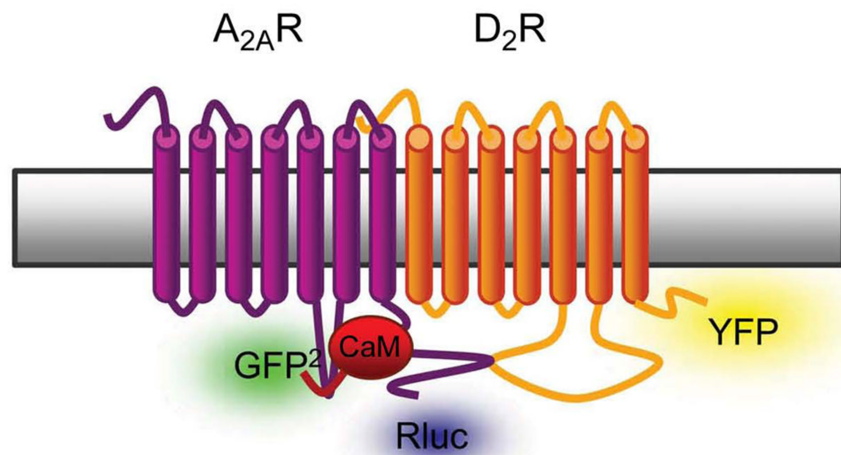

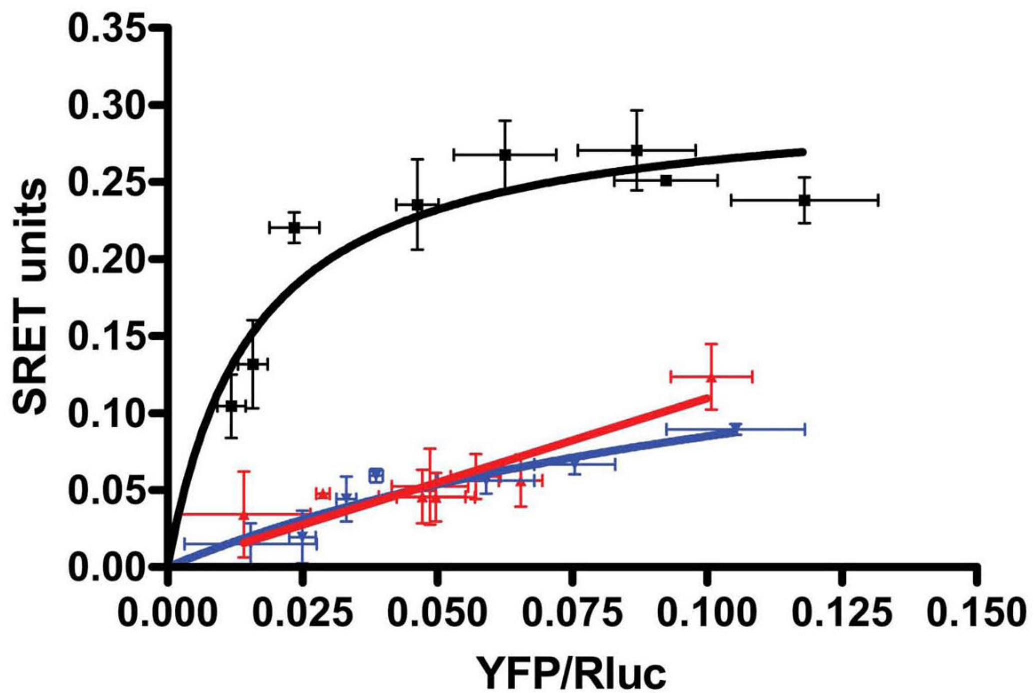

Figure 1. SRET for CaM, A_{2A} receptor and D_2 receptor in living cells

SRET saturation curves performed in HEK-293 cells expressing A_{2A}-Rluc (0.75 μg of cDNA), $CaMGFP^2$ (0.6 μg of cDNA) and increasing amounts of D_2YFP (0.5 to 5 μg of the cDNA). Net SRET was obtained by monitoring the YFP fluorescence emission after DeepBlueC addition, with subtraction of the value obtained with cells expressing the same amount of A_{2A}-Rluc and $CaMGFP^2$. Significant net SRET was detected for A_{2A}Rluc-$CaMGFP^2$-D_2YFP coupling, while negligible or linear net SRET was obtained in cells expressing equivalent amounts (equivalent fluorescence and luminescence units) of A_{2A}Rluc, $CaMGFP^2$ and $5HT_{2B}$YFP (red curve), or A_1RRluc, $CaMGFP^2$ and D_2RYFP (blue curve) as negative controls. Values, expressed as net SRET, represent means ± S.E.M. of 5 independent experiments

performed in triplicate. At the top, scheme depicting the expressed proteins in the SRET assay (modified from ref. [14•]).

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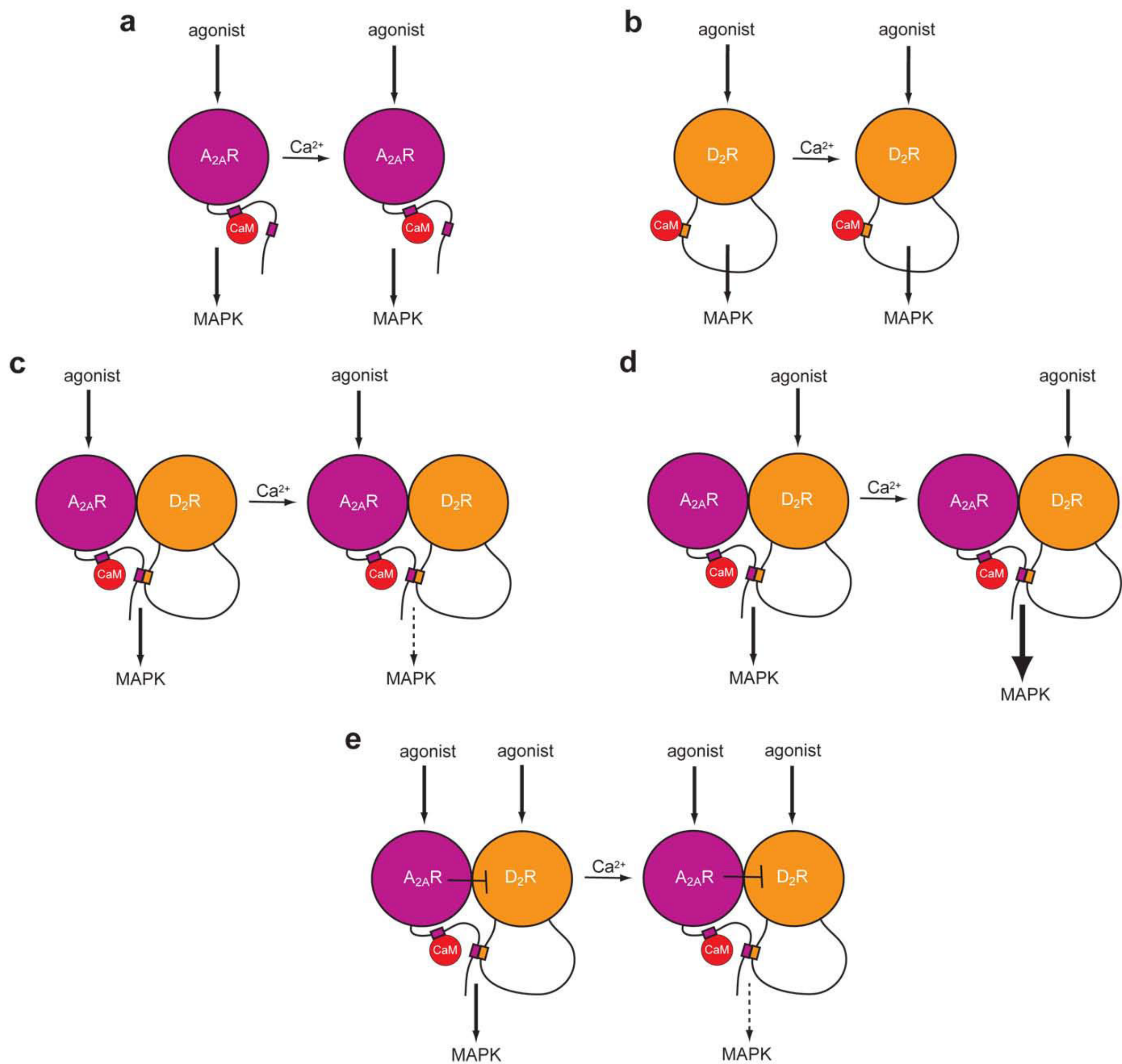

Figure 2. Selective Ca^{2+}/CaM-mediated modulation of A_{2A}-D_2 receptor heteromer function

Scheme showing the motifs of the C-terminus of the A_{2A} receptor and the 3IL of the D_2 receptor involved in the binding of CaM and in A_{2A}-D_2 receptor heteromerizacion, as well as the selective Ca^{2+}/CaM-mediated modulation of MAPK activation in the A_{2A}-D_2 receptor heteromer (see text). **(a, b)** When not forming heteromers, CaM binds to the proximal portion of the carboxy-terminus of the A_{2A} receptor and to the Arg-rich domain localized in the amino-terminus of the 3IL of the D_2 receptor. Under these conditions the effects of the stimulation of either receptor on MAPK activation does not change in the presence or absence of Ca^{2+}. **(c–e)** When forming heteromers, the distal portion of the A_{2A} receptor binds to the Arg-rich domain of the D_2 receptor, which cannot bind to CaM. In the A_{2A}-D_2 receptor heteromer, the binding of Ca^{2+} to CaM modifies its quaternary structure and decreases the effects of stimulation of A_{2A} receptor **(c)** and increases the effects of stimulation of the D_2 receptor **(d)** on MAPK activation. Co-activation of A_{2A} and D_2 receptors in cells co-expressing both receptors led to the same qualitative response than activation of A_{2A} receptors alone, probably due to the ability of A_{2A} receptor stimulation to antagonize allosterically the effects induced by D_2 receptor stimulation in the A_{2A}-D_2 receptor heteromer **(e)**. Receptors are shown as

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monomers for the sake of simplicity, but they are most probably forming homomers when not forming heteromers. Also for schematic purposes, the main portion of each receptor unit is represented by a sphere and only the long 3IL of the D_2 receptor and the long tail of the A_{2A} receptor are explicitly shown.