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# On the Existence of a Possible $A_{2A}$–$D_2$–β-Arrestin2 Complex: $A_{2A}$ Agonist Modulation of $D_2$ Agonist-Induced β-Arrestin2 Recruitment

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Given that coactivation of adenosine $A_{2A}$ ($A_{2A}R$) and dopamine $D_2$ ($D_2R$) receptors results in the coaggregation, cointernalization, and codesensitization of the $A_{2A}R$ and $D_2R$ and the role of scaffolding protein β-arrestin2 in the desensitization, internalization, and signaling of G-protein-coupled receptors, in this study we explored the ability of the $A_{2A}R$ agonist CGS21680 in $A_{2A}R$–$D_2R$-coexpressing cells to modulate the $D_2R$ agonist-induced recruitment of β-arrestin2 to the $D_2R$ by means of proximity-based bioluminescence resonance energy transfer ($BRET^2$) and co-trafficking analysis. We found evidence that CGS21680 can increase the maximal $BRET^2$ signal between β-arrestin2$^{RLuc}$ and $D_{2L}R^{GFP2}$ upon $D_2R$ activation, by increasing the potency of the $D_2R$ agonist to exert this action. In addition, this change was associated with an increased formation of cytoplasmic clusters containing β-arrestin2$^{GFP2}$ and $D_{2L}R^{YFP}$ as seen from the co-trafficking analysis. Furthermore, the $A_{2A}R$ agonist advanced the time for the increase in Akt phosphorylation obtained with the $D_2R$ agonist. Finally, using a novel bioinformatics approach to predict the protein–protein interface, we have also found that amino acid pro-triplets TNY, LLS, RAF, and VSR may be crucial for the -induced β-arrestin2 recruitment by $A_{2A}R$–$D_2R$ heteromers. Taken together, the results indicate that the antagonistic $A_{2A}R$–$D_2R$ allosteric receptor–receptor interaction in $A_{2A}R$–$D_2R$ heteromers favors β-arrestin2 recruitment to the $D_{2L}R$ protomer with subsequent cointernalization associated with a reduced time onset of Akt phosphorylation followed by a rapid dephosphorylation. Thus, β-arrestin2 action becomes more rapid and short-lasting and, in this way, mimics G-protein-mediated signaling.



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Abbreviations used: $A_{2A}R$, adenosine $A_{2A}$ receptor; $D_2R$, dopamine $D_2$ receptor; BRET, bioluminescence resonance energy transfer; PBS, phosphate-buffered saline.

## Introduction

Adenosine $A_{2A}$ receptor ($A_{2A}R$)–dopamine $D_2$ receptor ($D_2R$) heteromerization has been demonstrated by means of biochemical and biophysical methods [co-immunoprecipitation, bioluminescence resonance energy transfer (BRET), and fluorescence resonance energy transfer analyses] to occur upon transient cotransfection of the two receptors in cell lines including HEK293 T cells.[1–3] Antagonistic allosteric $A_{2A}R$–$D_2R$ receptor–receptor interactions have been shown to exist in $A_{2A}R$–$D_2R$ heteromers, reducing the affinity of the $D_2R$ agonist binding sites, Gi/o coupling, and signaling via adenylate cyclase, phospholipase C, and mitogen-activated protein kinases.[2,4–6]

In $D_2R$-cotransfected neuroblastoma cells, coactivation of $A_{2A}R$ and $D_2R$ results in the coaggregation, cointernalization, and codesensitization of the $A_{2A}R$ and $D_2R$.[7] However, it is unknown how the scaffolding protein β-arrestin2, being recognized to participate in the desensitization, internalization, and signaling of G-protein-coupled receptors, is involved in these events.[8] The sequence amino acid 212-IYIV-215 in the N-terminal part of the third intracellular loop (IC-3) of the $D_2R$ appears critical for β-arrestin2 binding.[9,10] Furthermore, Lys149 in the second intracellular loop (IC-2) in the $D_2R$ gives preferential β-arrestin2 binding to $D_2R$ IC-2 *versus* $D_3R$ IC-2, as shown in studies with glutathione *S*-transferase fusion proteins of $D_2R$ and $D_3R$ IC-2.[10] β-Arrestin2 is also involved in mediating the Akt/GSK3 signaling of $D_2Rs$.[11,12]

In this study, we explored the ability of the $A_{2A}R$ agonist CGS21680 in $A_{2A}R$–$D_2R$-cotransfected cells to modulate the $D_2R$-like agonist-induced recruitment of β-arrestin2 to the $D_2R$ by means of $BRET^2$ and co-trafficking analyses. In parallel, the $A_{2A}R$ agonist modulation of the $D_2R$-induced changes in the temporal dynamics of protein kinase B (Akt) phosphorylation was studied to link changes in signaling to the $BRET^2$ and co-trafficking analyses. Therefore, according to the experimental results and using a novel bioinformatics approach to predict the protein–protein interface, the amino acid pro-triplets TNY, LLS, RAF, and VSR may be crucial for the agonist-induced β-arrestin2 recruitment by $A_{2A}R$–$D_2R$ heteromers.

## Results

### $D_2R$ agonist-mediated internalization is increased upon combined $A_{2A}R$ and $D_2R$ agonist treatment

$A_{2A}R$–$D_2R$ heteromers are known to be formed after transient coexpression of the two receptors in HEK cells,[2,3] resulting in co-trafficking of the $D_2R$ and $A_{2A}R$.[13] We investigated whether $A_{2A}R$ affects the $D_2R$ agonist-mediated $D_2R$ internalization in transiently cotransfected HEK293T cells using a cell surface receptor expression assay.

HEK293T cells coexpressing 3xHA-$D_{2L}R$ and $A_{2A}R$ were incubated in the presence of the $D_{2L}R$ agonist quinpirole (0.25 μM) with or without the $A_{2A}R$ agonist CGS21680 (1 μM) at 37 °C for 30 min to monitor internalization (Fig. 1a). Notably, quinpirole induced a rapid internalization of 3xHA-$D_{2L}R$, resulting in a cell surface expression of approximately 56% of control. However, combined treatment with CGS21680 (1 μM) and quinpirole (0.25 μM) further enhanced 3xHA-$D_{2L}R$ internalization, reducing the level of cell surface expression to 28%. Such an increased internalization of 3xHA-$D_{2L}R$ was abolished by specific $A_{2A}R$ and $D_2R$ antagonist treatment (data not shown), suggesting that this effect is mediated through the coactivation of $D_{2L}R$ and $A_{2A}R$. As also seen in Fig. 1a, cotransfected cells preincubated with 0.45 M sucrose for 30 min prior to agonist treatment resulted in a marked reduction of 3xHA-$D_{2L}R$ internalization after combined treatment as well as $D_2R$ agonist treatment alone. This supports the involvement of clathrin-coated pits in the 3xHA-$D_{2L}R$ internalization process.

Internalization may target the $D_2R$ and $D_2R$–$A_{2A}R$ heteromers to either a degradative pathway, leading to prolonged attenuation of cell signaling, or to a cell surface recycling pathway, facilitating receptor resensitization. We therefore evaluated the time course of 3xHA-$D_{2L}R$ recycling to the plasma membrane. Cells were treated with quinpirole (0.25 μM) alone or together with CGS21680 (1 μM) during a time course of 0 to 45 min to promote 3xHA-$D_{2L}R$ internalization. The agonists were then removed by extensive washing with phosphate-buffered saline (PBS), and the reappearance of the 3xHA-$D_{2L}R$ at the cell surface was monitored over time (from 45 min to 120 min) by checking its expression level in the surface membrane. As shown in Fig. 1b, exposure of transiently cotransfected cells to quinpirole (0.25 μM) for 45 min reduced 3xHA-$D_{2L}R$ cell surface expression by approximately 52%. Thirty minutes after agonist removal, a highly significant proportion of 3xHA-$D_{2L}R$ returned to the plasma membrane (90%). In addition, Fig. 1b shows that combined $A_{2A}R$ and $D_2R$ stimulation of the cells resulted in a significant further reduction by 23% of the cell surface expression of 3xHA-$D_{2L}R$ after 45 min of incubation compared to the cell surface expression following quinpirole treatment alone. Furthermore, the time course and rate of return of internalized 3xHA-$D_{2L}R$ (i.e., resensitization) to the plasma membrane after agonist removal were significantly