INTERACTIONS BETWEEN CALMODULIN, ADENOSINE A2A AND DOPAMINE D2 RECEPTORS*
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Runnig title: Interactions between CaM, D2 and A2A receptors
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Abbreviations: BRET, Bioluminescence Resonance Energy Transfer; CaM, calmodulin; Fluorescence Resonance Energy Transfer, FRET; GPCR, G-protein-coupled receptor; SRET, Sequential Resonance Energy Transfer.

The Ca2+-binding protein calmodulin (CaM) has been shown to bind directly to cytoplasmic domains of some G protein-coupled receptors (GPCRs), including the dopamine D2 receptor. CaM binds to the N-terminal portion of the long third intracellular loop of the D2 receptor, within an Arg-rich epitope that is also involved in the binding to Gi/o proteins and to the adenosine A2A receptor, with the formation of A2A-D2 receptor heteromers. In the present work, by using proteomics and Bioluminescence Resonance Energy Transfer (BRET) techniques, we provide evidence for the binding of CaM to the A2A receptor. By using BRET and sequential resonance energy transfer (SRET) techniques, evidence was obtained for CaM-A2A-D2 receptor oligomerization. BRET competition experiments indicated that, in the A2A-D2 receptor heteromer, CaM binds preferentially to a proximal C-terminus epitope of the A2A receptor. Furthermore, Ca2+ was found to induce conformational changes in the CaM-A2A-D2 receptor oligomer and to selectively modulate A2A and D2 receptor-mediated MAPK signaling in the A2A-D2 receptor heteromer. These results may have implications for basal ganglia disorders since A2A-D2 receptor heteromers are being considered as a target for anti-parkinsonian agents.

G-protein-coupled receptors are able to form homo- and hetero-oligomers with unique biochemical and functional characteristics (1-7) and they are easily detected in vitro by using biophysical techniques (8-10). Heteromers of adenosine A2A and dopamine D2 receptors were one of the first G-protein-coupled receptor (GPCR) heteromers to be described (11). A close physical interaction between both receptors was shown using co-immunoprecipitation and co-localization assays (11), and Fluorescence and Bioluminescence Resonance Energy Transfer (FRET or BRET) techniques (12-14). At the biochemical level, two types of antagonistic A2A-D2 receptor interactions have been discovered that may explain the A2A-D2 receptor interactions described.
both at the neuronal and behavioral level (11, 15-18). First, by means of an allosteric interaction in the receptor heteromer, stimulation of A$_{2A}$ receptor decreases the affinity of D$_2$ receptor for their agonists (12). Second, the stimulation of the G$\text{i/o}$-protein-coupled D$_2$ receptor inhibits the cAMP accumulation induced by the stimulation of the G$s/olf$-protein-coupled A$_{2A}$ receptor (11, 17, 18).

In view of the well-known role of dopamine in Parkinson’s disease, schizophrenia and drug addiction, it has been suggested that the A$_{2A}$-D$_2$ receptor interactions in the central nervous system may provide new therapeutic approaches to combat these disorders (16, 19).

An epitope-epitope electrostatic interaction between an Arg-rich epitope of the N-terminus of the third intracellular loop (3IL) of the D$_2$ receptor and an epitope containing a phosphorylated Ser localized in the distal part of the C-terminus of the A$_{2A}$ receptor is involved in A$_{2A}$-D$_2$ receptor heteromer interface (14, 20, 21). The same Arg-rich epitope of the D$_2$ receptor is able to interact with CaM (22-25). In the absence of phosphorylated residues, adjacent aspartates or glutamates, which are abundant in CaM, may also form non-covalent complexes with Arg-rich epitopes (26). Therefore, CaM can potentially convey a Ca$^{2+}$ signal to the D$_2$ receptor through a direct binding to the 3IL of the D$_2$ receptor (22). Mass spectrometry data have shown that bovine CaM can form multiple non-covalent complexes with an Arg-rich peptide corresponding to the N-terminal region of the 3IL of the D$_2$ receptor [VLRRKRRKRVN] (24), as well as a peptide from the proximal C-terminus of the A$_{2A}$ receptor (24). This epitope, whose sequence is $^{291}\text{RIREFRTFR}^{300}$ in the human A$_{2A}$ receptor, also contains several Arg residues. Since the suspected interaction between the A$_{2A}$ receptor and CaM was awaiting confirmation by assays using complete proteins, the present study was undertaken to demonstrate the existence of interactions between the A$_{2A}$ receptor and CaM both in a recombinant protein expression cell system and in the brain. A proteomics approach was used for the discovery of protein-protein interactions between the A$_{2A}$ receptor and CaM in rat brain, whereas BRET in transfected cells demonstrated a direct interaction between CaM and this receptor. Furthermore, by using BRET and sequential resonance energy transfer (SRET) techniques and analyzing MAPK signaling in transfected cells, evidence was obtained for CaM-A$_{2A}$-D$_2$ receptor oligomerization and a selective Ca$^{2+}$-mediated modulation of A$_{2A}$ and D$_2$ receptor function in the A$_{2A}$-D$_2$ receptor heteromer.

**EXPERIMENTAL PROCEDURES**

**Animals-** Male Sprague-Dawley rats (250 g) were obtained from Harlan (Barcelona, Spain). Procedures involving animals were in accordance with the guidelines established by the normative of the European Council (86/609/EEC). The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra (Ref. 060/07).

**Membrane protein purification-** Animals were killed by decapitation and the brains were rapidly removed. To obtain P2 membrane proteins, the striatum was dissected out and homogenized with a glass-teflon homogenizer in ice-cold 0.32 M sucrose, 10 mM Hepes (pH 7.4), 2 mM EDTA and complete protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). After spinning at 1,000 g for 15 min, the supernatant was removed and centrifuged at 200,000 g for 15 min to yield the crude membrane pellet (P2). The pellet was resuspended in homogenization buffer and spun again at 200,000 g to yield washed crude membrane pellet (P2’).
**Immunoprecipitation** - The pellet containing membrane proteins (P2') was solubilised in immunoprecipitation buffer: PBS (pH 7.4), complete protease inhibitor cocktail and 1% Igepal Ca-630 (Sigma, St. Louis MO) for 1 h at 4°C. Protein concentration was determined by the BCA method (Pierce Chemical Co., Rockford, IL, USA). A total amount of 0.5 mg of protein was used for immunoprecipitation. Samples were pretreated with 30 µl of protein A/G agarose beads (Santa Cruz Biotechnology, Heidelberg, Germany) with gentle rocking for 1 h at 4°C and centrifuged at 10,000 g to remove the beads. Membrane proteins were incubated with 5 µg of polyclonal anti A2A receptor (Affinity Bioreagents, bioNova Científica, Madrid, Spain) or with 5 µg of normal rabbit IgG (Upstate, Charlovilles, VA) for 90 min and then 30 µl of Protein A/G beads were added, and left for 16 h at 4°C with gentle rocking. Immunoprecipitated proteins were washed three times with immunoprecipitation buffer.

**Proteomic analysis** - Immunoprecipitated proteins were analyzed in the Proteomic Laboratory of CIMA. Briefly, proteins were eluted by incubation with 100 mM Glycine-HCl (pH 2.5) for 10 min at room temperature with gentle rocking. The samples were centrifuged at 10,000 g for 1 min and the supernatant content precipitated with 25% TCA for 20 min at 4°C. After spinning at 10,000 g for 15 min, the pellet was washed twice with precooled acetone, completely dried in a SpeedVac and resuspended in 30 µl of 100 mM ammonium bicarbonate. Proteins were reduced with 10 mM DTT for 15 min in darkness and finally digested with trypsin 1:50 (v:v) for 16 h at 37°C. Peptide mass fingerprinting was obtained from tryptic digests by LC-ESI-MS/MS analysis (27). A peak list was generated by ProteinLynx Global Server 2.1 (Waters, Milford, MA). Database search was done with ProteinLynx Global Server 2.1 (Waters) and Phenyx for raw data and mzXML data respectively. The database used in this study was SwissProt.

**Cell culture** - HEK-293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) heat inactivated Foetal Bovine Serum (FBS) (all supplements were from Invitrogen, Paisley, Scotland, UK). Cells were maintained at 37°C in an atmosphere of 5% CO2, and were passaged when they were 80-90% confluent, i.e. approximately twice a week.

**Mutant A2A receptors, fusion proteins and expression vectors** - The sequence R291IREFR296QTFR300 in the C-terminal domain of the human A2A receptor was mutated to A291IREFA296QTFA300 by site-directed mutagenesis (Cellogenetics, Ijamsville, MD, USA). The human cDNAs for A2A, mutant A2A, which is denoted as A2A_RAAA, D2 and A1 receptors, cloned into pcDNA3.1, were amplified without their stop codons using sense and antisense primers harboring unique EcoRI and BamHI sites to clone A2A receptor, mutant A2A receptor or A1 receptor in Rluc vector and EcoRI and KpnI to clone D2 receptor in Rluc or EYFP vectors. The amplified fragments were subcloned to be in-frame into restriction sites of pcDNA3.1Rluc and pEYFP-N1 (enhanced yellow variant of GFP; Clontech, Heidelberg, Germany) vectors resulting in the plasmids A2A_Rluc, A2A_RAAA_Rluc, A1_Rluc, D2_Rluc, and D2_RYFP. The cDNA encoding the human EYFP-CaM (CaMYFP) fusion protein was kindly provided by Dr. Carles Enrich (Hospital Clinic de Barcelona, Spain). CaM was subsequently subcloned into the pGFP2-C1 (BioSignal Packard, Montreal, Canada) using EcoRI and BamHI, resulting in the GFP2-CaM plasmid. The
cDNA encoding the 5HT<sub>2B</sub>R-YFP fusion protein was kindly provided by Dr. Irma Nardi (University of Pisa, Italy). Expression of constructs was tested by confocal microscopy (see results) and the receptor functionality by second messengers, ERK1/2 phosphorylation and cAMP production as described previously (12, 14; data not show).

**Transient transfection and protein determination**- HEK-293T cells growing in 6-well dishes were transiently transfected with the corresponding fusion protein cDNA by the PEI (PolyEthylendImine, Sigma) method. Cells were incubated (4 h) with the corresponding cDNA together with PEI (5 µl/µg cDNA of 10 µM PEI) and 150 mM NaCl in a serum-free medium. After 4 hours, the medium was changed to a fresh complete culture medium. Forty-eight hours after transfection, cells were washed twice in quick succession in HBSS with 10 mM glucose, detached by scraping, and resuspended in the same buffer unless otherwise indicated. To control the cell number, sample protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin as standard protein. Cell suspensions (20 µg of protein) in HBSS buffer containing 1.26 mM CaCl<sub>2</sub> or, when indicated, in Ca<sup>2+</sup>-free HBSS buffer, were distributed into 96-well microplates; black plates with a transparent bottom were used for fluorescence determinations, while white plates were used for BRET and SRET experiments.

**BRET assays**- HEK-293T cells were transiently co-transfected with the indicated amounts of plasmid cDNAs corresponding to the indicated fusion proteins (see figure legends). To quantify fluorescence proteins, cells (20 µg protein) were distributed in 96-well microplates (black plates with a transparent bottom) and fluorescence was read at 400 nm in a Fluo Star Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter. Receptor-fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing protein-Rluc alone. For BRET measurements, the equivalent of 20 µg of cell protein were distributed in 96-well microplates (Corning 3600, white plates; Sigma) and 5 µM coelenterazine H (Molecular Probes, Eugene, OR) was added. After 1 minute of adding coelenterazine H, the readings were collected using a Mithras LB 940, which allows the integration of the signals detected in the 485 nm-short-(440-500 nm) and the 530 nm-long-(510-590 nm) wavelength filters. To quantify receptor-Rluc expression luminescence readings were performed after 10 minutes of adding 5 µM coelenterazine H. The same protocol was used to determine BRET with membranes obtained from cells (48 h post-transfection) disrupted using a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica, Basel, Switzerland). Disruption was performed for three 5 s-periods in 10 volumes of free Ca<sup>2+</sup> HBSS, pH 7.4 containing a proteinase inhibitor cocktail (Sigma, St. Louis, MO, USA). Cell debris were eliminated and membranes were obtained by centrifugation at 105,000 g (40 min, 4°C), and the pellet was resuspended and recentrifuged under the same conditions. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the Rluc construct expressed alone in the same experiment.

**SRET Experiments**- HEK-293T cells were transiently co-transfected with the indicated amounts of plasmid cDNAs corresponding to the indicated fusion proteins (see figure legends). Using aliquots of transfected cells (20 µg of protein), three different determinations were performed in parallel. First,
quantification of protein-YFP expression by determination of the fluorescence due to protein-YFP; cells distributed into 96-well microplates (black plates with a transparent bottom) were read in a Fluostar Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using an excitation filter at 485 nm, and 10 nm bandwidth emission filters corresponding to 510 nm (506-515 nm) (Ch1) and 530 nm (527-536 nm) (Ch2); the contribution of the GFP and YFP proteins alone, to the two detection channels (spectral signature, 28) was measured in experiments with cells expressing only one of these proteins and normalized to the sum of the signal obtained in the two detection channels; for protein-YFP expression quantification, linear un-mixing was done taking into account the spectral signature as described by Zimmermann et al., (28, 29) to separate the two emission spectra; the sample fluorescence is the fluorescence calculated as described minus the fluorescence of cells expressing only protein-Rluc and protein-GFP. Second, quantification of protein-Rluc expression by determination of the luminescence due to protein-Rluc; cells were distributed in 96-well microplates (Corning 3600) and luminescence was determined 10 minutes after addition of 5 µM coelenterazine H in a Mithras LB 940 multimode reader (Berthold Technologies, DLReady, Germany). Third, SRET measurement; cells were distributed in 96-well microplates (Corning 3600) and 5 µM DeepBlueC (Molecular Probes, Eugene, OR) was added; the SRET signal was collected using a Mithras LB 940 reader with detection filters for short-wavelength (400 nm (370-450 nm)) and long-wavelength (530 nm (510-590 nm)); by analogy with BRET, net SRET is defined as [(long-wavelength emission)/(short-wavelength emission)]--Cf where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for cells expressing protein-Rluc, protein-GFP and the other protein partner not fused to a fluorescence protein (similar values were obtained measuring Cf in cells expressing protein-Rluc only and protein-GFP); Linear un-mixing was done for SRET quantification, taking into account the spectral signature (28) to separate the two fluorescence emission spectra.

Immunostaining- For immunocytochemistry, transiently transfected HEK-293T cells were fixed in 4% paraformaldehyde for 15 min and washed with PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. Then, after permeabilization with buffer A containing 0.2% Triton X-100 for 5 min, cells were treated with PBS containing 1% bovine serum albumin. After 1 h at room temperature, protein-Rluc was labeled with the primary mouse monoclonal anti-Rluc antibody (1/100, Chemicon, Billerica, MA) for 1 h, washed, and stained with the secondary antibody Cy3 Donkey anti-mouse (1/100, Jackson Immunoresearch Laboratories, West Grove, PA, USA). CaMYFP was detected by its fluorescence properties. Samples were rinsed and observed in a Leica SP2 confocal microscope (Leica Microsystems, Mannheim, Germany).

ERK phosphorylation assay- Cells were grown in 25 cm² flasks to 80% confluence and cultured in serum-free medium for 16 h before the addition of any agent. Cells resuspended in HBSS buffer containing 1.26 mM CaCl₂ were treated or not with 1 µM ionomycin for 3 min before the addition of the agonists A2A receptor agonist CGS2168 (200 nM), the D₂ receptor agonist quinpirole (1 µM) or a mixture of both ligands for 5 min. Cells were rinsed with ice-cold phosphate-buffered saline and lysed by
the addition of 500 µL of ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM β-glycerophosphate, 1% Triton X-100, 20 µM phenyl-arsine oxide, 0.4 mM NaVO₄ and protease inhibitor cocktail). The cellular debris was removed by centrifugation at 13,000 g for 5 min at 4°C and the protein was quantified by the bicinchoninic acid method using bovine serum albumin dilutions as standard. To determine the level of ERK1/2 phosphorylation, equivalent amounts of protein (10 µg) were separated by electrophoresis on a denaturing 7.5% SDS-polyacrylamide gel and transferred onto PVDF membranes. The membranes were then probed with a mouse anti-phospho-ERK1/2 antibody (Sigma, 1:2500). In order to rule out that the differences observed were due to the application of unequal amounts of lysates, PVDF blots were stripped and probed with a rabbit anti-ERK1/2 antibody that recognizes both, phosphorylated and non-phosphorylated ERK1/2 (Sigma, 1:40,000). Bands were visualized by the addition of anti-mouse HRP conjugated (Dako, Glostrup, Denmark) or anti-rabbit HRP conjugated (Sigma) secondary antibodies, respectively, and SuperSignal West Pico Chemiluminescent Substrate (Pierce). Bands densities were quantified with a LAS-3000 (Fujifilm) and the level of phosphorylated ERK1/2 isoforms was normalized for differences in loading using the total ERK protein band intensities. Quantitative analysis of detected bands was performed by Image Gauge V4.0 software. One-way ANOVA and Student’s t test for unpaired samples were used for statistical comparisons.

RESULTS

Identification of an interaction between CaM and the A₂A receptor—According to the CaM target database (http://calcium.uhnres.utoronto.ca/ctdb), the sequence RIREFRQTFR, which is present in the proximal part of the C-terminus of the A₂A receptor, has a high probability to bind to CaM. In fact, we recently demonstrated that a synthetic peptide with the same sequence forms non-covalent complexes with bovine CaM (24). A proteomics approach was then used to find in situ evidence of direct interactions between the A₂A receptor and CaM, in the brain. Samples from rat striatal membranes were immunoprecipitated using an antibody directed against a synthetic peptide of the A₂A receptor C-terminus, SHGDMGLPDVELLSHEL, which does not overlap with the putative CaM-binding motif. Co-immunoprecipitates were digested with trypsin and the resulting peptides were analyzed by mass spectrometry as described in the Experimental Procedures section. Among other peptides, two corresponding to the sequence of rat CaM (P62161) were detected after co-immunoprecipitation with the specific polyclonal antibody but not with rabbit IgG (Table 1). The significance of the hits was confirmed by the low p values for both the 13-mer and the 9-mer peptides, which collectively covered 15% of the rat CaM sequence (Table 1). These results suggest that CaM and the A₂A receptor may interact, but the existence of a third bridging protein cannot be ruled out. For this purpose, BRET assays were performed in cells transfected with the cDNAs for the A₂A_RRluc and CaMYFP fusion proteins. The hyperbola obtained upon increasing the YFP/Rluc ratio (Figure 1A) indicates that a specific interaction between CaM and the A₂A receptor can occur in living cells. Similar experiments were performed using D₂RRluc and CaMYFP to confirm that these two proteins may establish direct molecular interactions (Figure 1B). The specificity of the CaM and A₂A receptor or CaM and D₂ receptor interactions in HEK cells was confirmed by the unspecific (linear) BRET signal obtained when assaying A₁RRluc and
CaMYFP (Figure 1). To demonstrate that the A2A receptor sequence RlREFRQTFR acts as a CaM binding domain, BRET competition experiments were performed in cells transfected with the cDNAs for the A2ARRluc and CaMYFP fusion proteins (amounts adjusted to give values around the BRET50 and increasing amounts of cDNA corresponding to the wild type A2AR or to the A2ARRAAA (RlREFRQTFR mutated to AIREFAQTFA). As expected, the A2A receptor did decrease BRET values by competing with A2ARRluc for its binding to CaMYFP, while A2ARRAAA did not (Figure 2A). Accordingly, in cells expressing A2ARRluc and CaMYFP a linear and negligible BRET was detected in the absence or in the presence of ionomycin (Figure 2B).

CaM interacts with A2A and D2 receptors at the plasma membrane-Immunolocalization of receptors and CaM were performed in co-transfected HEK cells. When expressed in the absence of receptors, CaM showed a cytosolic localization (Figure 3). Co-expression of CaM and any of the two receptors did not modify the localization of the receptor. On the other hand, a significant membrane localization of CaM was only observed when the protein was co-expressed with either A2A or D2 receptors (Figure 3). The translocation of CaM to the plasma membrane did not take place when co-expressed with the A1 receptor (Figure 3), which is not able to establish molecular interactions with the protein (see above). Treatment of cells with a Ca2+ free buffer and EDTA or treatment with ionomycin did not modify colocalization of CaM and A2A or D2 receptors in cell membranes (Figure 3). Effect of Ca2+ levels on the interaction between CaM and A2A or D2 receptors- BRET experiments were performed to give some insight about the role of Ca2+ in the interaction of CaM with either A2A or D2 receptors. In cells co-expressing D2RRluc and CaMYFP, resuspended in a medium containing 1.26 mM CaCl2, ionomycin treatment led to significant decreases in BRET measurements obtained after periods of incubation of 2, 5 and 10 min (Figure 4B). In cells co-expressing A2ARRluc and CaMYFP, ionomycin treatment also led to significant decreases in BRET measurements at 2 and 5 min of preincubation. On the other hand, with a longer period of incubation (10 min), the BRET signal for A2ARRluc and CaMYFP increased significantly above that found in untreated cells (Figure 4A). These data indicate that Ca2+ is not needed for CaM being able to interact with A2A or D2 receptors.

The ionophore-induced increase in intracellular Ca2+ concentration does not disrupt the oligomerization of CaM with the receptors but leads to conformational changes in the CaM molecule and/or the receptors that alter the distance between Rluc (fused to the C-terminus of the D2 or A2A receptors) and YFP (fused to CaM). The observed effects are not due to the Ca2+ toxicity or changes in the membrane structure since they were also observed in a cell-free system (Figure 4D). Moreover, ionomycin did not induce significant changes in BRET measurements in cells co-expressing A1RRluc and CaMYFP (Figure 4C).

Interactions of CaM with the A2A-D2 receptor heteromer- By means of BRET competition experiments performed with cell expressing A2ARRluc and CaMYFP (with adequate expression levels to give a suboptimal BRET value), the increase in D2 receptor expression (tested by Western blot experiments) did not modify the BRET signal due to A2ARRluc and CaMYFP (Figure 5A). Analogously, increasing the expression of CaM did not modify the BRET signal due to A2ARRluc and D2RYFP (Figure 5B). As indicated in the Introduction, CaM seems to interact with the Arg-rich epitope of the 3IL of the D2 receptor that is also...
involved in A2A-D2 receptor heteromerization (see Introduction). In fact, competition assays demonstrated that increasing amounts of A2A receptor led to significant reduction in the BRET signal due to the interaction between the D2 receptor and CaM (Figure 5C). However, a differential effect was obtained depending on the isoform of the D2 receptor (D2S or D2L). On the one hand, the BRET signal between D2LRRluc and CaMYFP increased with low quantities of A2A receptor but decreased dose-dependently with higher quantities of A2A receptor. On the other hand, using the pair D2SRRluc-CaMYFP, a dose-dependent decrease in BRET was found. These findings correlate with the potential ability of CaM and A2A receptors to bind to any of the two Arg-rich epitopes of the 5IL of the D2L receptor (one of which is not present in the D2S structure; see Discussion). The specificity of these effects was demonstrated by the independence of the BRET signal with increasing expression of the A1 receptor (Figure 5C).

Oligomerization of CaM, A2A and D2 receptors- To test the possible existence of CaM-A2A-D2 receptor oligomerization, the recently described sequential BRET-FRET (SRET) technique was used (29). In SRET2, the oxidation of an Rluc substrate triggers GFP2 acceptor excitation by BRET2 and subsequent energy transfer to a YFP FRET acceptor. SRET2 was attempted using A2ARRluc, CaMGFP2, D2RYFP, and DeepBlueC as Rluc substrate. SRET2 would only occur if the two acceptor/donor pairs, A2ARRluc/CaMGFP2 and CaMGFP2/D2RYFP, are well oriented at a distance of less than 10 nm. A SRET saturation curve obtained by augmenting D2RYFP expression while maintaining the same A2ARRluc/CaMGFP2 ratio is shown in Figure 6. To obtain optimal results (see ref. 29), cells were transfected with an amount of cDNA (0.6 µg) for A2ARRluc giving readouts of about 100,000 luminescent units, an amount of cDNA (0.4 µg) for CaMGFP2 giving about 6,000 fluorescent units and increasing amounts of cDNA (0.5 to 6 µg) for to D2RYFP (giving a range of 500-8,000 fluorescence units). From the saturation curve (Figure 6) apparent SRETmax (0.29 ± 0.03) and apparent SRET50 (0.010 ± 0.006) values were calculated. To prove whether SRET was possible when substituting A2A receptors by A1 receptors, cells were transfected with 0.7 µg of cDNA for A1RRluc (100,000 luminescent units), 0.4 µg of cDNA for CaMGFP2 (6,000 fluorescent units) and 0-5 to 6 µg of cDNA for D2RYFP (500-9,000 fluorescence units). The possible formation of a trimer in which the D2 receptor was substituted by the serotonin 5-HT2B receptor was tested using cells transfected with 0.6 µg of cDNA for A2ARRluc, 0.4 µg of cDNA for CaMGFP2 and 0.5 to 6 µg of cDNA for the 5-HT2B receptor (500 to 9,000 fluorescence units). Using these two combinations of fusion proteins, a very low and non saturating (linear) SRET was observed, which indicates that oligomerization of CaM, A1 and D2 receptors or CaM, A2A and 5-HT2B receptors does not occur.

Effects of intracellular Ca^{2+} on CaM-A2A-D2 receptor oligomerization-BRET was measured in the presence and in the absence of ionomycin in cells co-transfected with A2ARRluc and D2RYFP. The calcium ionophore led to similar qualitative changes in the BRET curves (Figure 7A) than those in the experiments with cells co-expressing A2ARRluc and CaMYFP (see Figure 4A). Thus, the BRET signal for A2ARRluc and D2RYFP decreased at shorter times of incubation with ionomycin whereas it increased at longer times (Figure 7A). These results suggest that Ca^{2+} binding to CaM induces conformational changes in the A2A receptor such that the distance between the donor Rluc (fused to the C-terminus
of the $A_{2A}$) and the acceptor YFP is modified. Taking all the results into account, the most probable scenario is the existence of a CaM-$A_{2A}$-$D_2$ receptor oligomeric complex, Ca$^{2+}$ binding to CaM being unable to disrupt $A_{2A}$-$D_2$ receptor heteromers. To further test this possibility, SRET$^2$ for $A_{2A}$RRluc,-CaMGFP$^2$-$D_2$RYFP was measured in cells treated with ionomycin. As shown in Figure 6B a positive SRET$^2$ was detected in the presence of ionomycin with an apparent SRET$_{max}$ of 0.24 ± 0.08 and an apparent SRET$_{50}$ of 0.051 ± 0.003. Therefore, calcium binding to CaM led to a decrease in the apparent SRET$_{max}$ and an increase in the apparent SRET$_{50}$ values. CaM-$A_{2A}$-$D_2$ receptor oligomers seem to be stable complexes that are not disrupted by calcium binding to CaM.

**Effects of intracellular Ca$^{2+}$ on the function of $A_{2A}$-$D_2$ receptor heteromers**

To test whether calcium binding to CaM-$A_{2A}$-$D_2$ receptor oligomers modifies receptor functionality, MAP kinase pathway signaling in cells expressing CaM and $A_{2A}$R, $D_2$R or both receptors was analyzed. ERK1/2 phosphorylation was determined in cells treated with 200 nM of the $A_{2A}$ receptor agonist CGS21680, and/or with 1 µM of the $D_2$ receptor agonist quinpirole in the presence and in the absence of 1 µM ionomycin. As shown in Figure 8A, activation of $A_{2A}$ or $D_2$ receptors, in cells transfected with low amounts of cDNA for CaM (0.3 µg) and expressing $A_{2A}$ or $D_2$ receptors, induced similar ERK1/2 phosphorylation in the absence or in the presence of ionomycin. On the other hand, in cells transfected with 0.3 µg of cDNA for CaM and the two receptors, a different effect was obtained in the absence or in the presence of ionomycin. In this case, ionomycin significantly reduced or potentiated ERK1/2 phosphorylation induced by, respectively, activation of $A_{2A}$ or $D_2$ receptors (Fig. 8B). The well known antagonistic interaction between $A_{2A}$ and $D_2$ receptors was enhanced upon treatment with ionomycin. Results similar to those in Figure 8A and 8B were obtained if cells were expressing higher amounts of CaM (1 µg of cDNA, results not shown), indicating that moderate amounts of exogenous CaM are already able to modulate receptor operation. In contrast, the effects of ionomycin were not observed in cells expressing only endogenous CaM (Figure 8C). Therefore, the effects of ionomycin were dependent on the intracellular levels of CaM. Results similar to those in Figure 8A and 8B were also obtained using cells in suspension (not adherent cells), i.e. in the same conditions than those used for BRET and SRET experiments (results not shown). Overall, the results suggest a correlation between structural changes in the oligomer and the modulation of its functionality exerted by Ca$^{2+}$ binding.

**DISCUSSION**

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 highly conserved, soluble, intracellular 17kDa Ca$^{2+}$-binding protein, regarded as a major transducer of Ca$^{2+}$ signals in mammalian cells (30). CaM has been shown to bind directly to cytoplasmic domains of plasma membrane proteins, including GPCRs, such as opioid $\mu$, serotonin 5-HT$_{1A}$, acetylcholine muscarinic and dopamine $D_2$ receptors (21, 31-33). In the present work we also provide clear evidence for the binding of CaM to adenosine $A_{2A}$ receptors. It was demonstrated that the sequence
RIREFRQTFR, which is present in the proximal part of the C-terminus of the A2A receptor, is the CaM binding domain in these receptors. Furthermore, we provide evidence for CaM-A2A-D2 receptor oligomerization and for a specific Ca2+ dependent-modulation of A2A-D2 receptor heteromer function.

Previous studies have shown that an Arg-rich domain of the N-terminal portion of the 3IL of the D2 receptor is involved in the binding to Gi/o proteins, CaM, and the A2A receptor (14, 20, 22, 24, 34). The binding of CaM to the D2 receptor disrupts Gi/o coupling and, therefore, its ability to inhibit the activity of adenyl cyclase (22). We here report competition of CaM and A2A receptors for the same domain in the D2 receptor. This competition is particularly evident when the short isoform of the D2 receptor was assayed. The two isoforms of the D2 receptor (short and long, D2S and D2L, respectively) are generated by alternative splicing (35). D2L, the isoform used in most of the experiments of the present study, contains 29 additional amino acid residues within the middle portion of the 3IL. The Arg-rich epitope of the D2 receptor [VLRRRKRKVNR] that is able to interact with the A2A receptor is common to both D2 receptor isoforms and is localized in the N-terminal portion of their long 3IL (14, 20). However, there is an additional Arg-rich epitope in the 3IL of D2L [VNRRRVEEAA], which is not present in D2S and which can potentially interact with CaM or with the A2A receptor (21). The VNRRRVEEAA peptide interacts in vitro with CaM and with a phosphorylated Ser-containing epitope of the C-terminus of the A2A receptor that is involved in A2A-D2 receptor heteromerization (data not shown). In BRET competition experiments using D2SRRluc-CaMYFP the increase of A2A receptor expression produced a significant decrease of the BRET signal. On the other hand, when co-transfecting the A2A receptor, the BRET signal between D2RRluc and CaMYFP first increased, while higher expression levels led to a reduced BRET signal. It is possible that, in the D2L receptor, CaM binds preferentially to the [VLRRRKRKVNR] epitope and that increasing quantities of transfected A2A receptor induce, first, translocation of CaM to the [VNRRRVEEAA] epitope, thus leading to an enhancement of the BRET signal. Upon further increase of receptor expression A2A-R may also bind to the [VNRRRVEEAA] epitope, with the consequent displacement of CaM from the D2L receptor. The results from BRET competition experiments demonstrating that increasing expression of D2 receptor did not modify the BRET signal due to A2A-RRluc and CaMYFP, and that increasing expression of CaM did not modify the BRET signal due to A2RRluc and D2RYFP, were also compatible with the possibility of CaM-A2A-D2 receptor oligomerization with A2A receptors able to bind simultaneously to CaM and to the D2 receptor. This was demonstrated with the use of the recently introduced sequential SRET techniques, which allow the demonstration of direct interaction of three protein molecules (29).

The results not only indicated that CaM may interact with A2A receptors in cells which express A2A-D2 receptor heteromers, but also, that the conformation and function of A2A-D2 receptor heteromers are modulated by Ca2+. In fact ionomycin, an ionophore able to increase intracellular Ca2+, did affect the energy transfer between A2A-RRluc and D2RYFP. Agonist-induced conformational modifications in a receptor heteromer can be demonstrated by using resonance energy transfer techniques (36). In case of the A2A-D2 receptor heteromer and by means of the same techniques used in the present study, no energy transfer variations were found after exposure to A2A or D2 receptor agonists (12). Therefore, the ability of BRET techniques to detect
Ca\textsuperscript{2+}-triggered conformational changes within the CaM-A\textsubscript{2A} receptor, CaM-D\textsubscript{2} receptor and CaM-A\textsubscript{2A}-D\textsubscript{2} receptor oligomers suggests that Ca\textsuperscript{2+} exerts a strong CaM-dependent control of the function of A\textsubscript{2A} and D\textsubscript{2} receptors and A\textsubscript{2A}-D\textsubscript{2} receptor heteromers. Interestingly, Ca\textsuperscript{2+} exerted a selective modulation of A\textsubscript{2A}-D\textsubscript{2} receptor heteromer-mediated activation of MAPK pathway. On the other hand, since Ca\textsuperscript{2+} binding to CaM did not individually modulate the receptor-mediated signalling towards the MAPK pathway. On the other hand, Ca\textsuperscript{2+} significantly modified ERK1/2 phosphorylation induced by activation of A\textsubscript{2A} or of D\textsubscript{2} receptors in cells co-expressing the two receptors. Furthermore, the previously described (18) antagonistic interaction between A\textsubscript{2A} and D\textsubscript{2} receptors was regulated by ionomycin-mediated variation in the intracellular calcium concentration.

The present study demonstrates the existence of oligomeric intermolecular interactions between CaM, A\textsubscript{2A} and D\textsubscript{2} receptors. When not expressed together A\textsubscript{2A} or D\textsubscript{2} receptors may potentially bind CaM, but in cells expressing the A\textsubscript{2A}-D\textsubscript{2} receptor heteromer CaM binds preferentially to the A\textsubscript{2A} receptor. Furthermore, CaM transduces Ca\textsuperscript{2+}-dependent changes of MAPK signaling in the CaM-A\textsubscript{2A}-D\textsubscript{2} receptor oligomer.

This adds new information about the function of A\textsubscript{2A}-D\textsubscript{2} receptor heteromers, which are considered as a target for the development of anti-parkinsonian agents (37).
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**FOOTNOTES**

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Table 1
Peptides identical to the rat calmodulin sequence identified by LC-ESI-MS/MS

| Sequence   | Position | z  | d m/z | z-score | p-value   |
|------------|----------|----|-------|---------|-----------|
| ADQLTEEQIAEFK | 2-14     | 2  | -0.014| 11.76   | 1.45E-30  |
| DTDSEE1EIR | 79-87    | 2  | -0.035| 4.16    | 4.8E-4    |
FIGURE LEGENDS

Figure 1. Identification of CaM-A2A and CaM-D2 receptor oligomers by BRET experiments. BRET saturation curves were performed using HEK-293 cells co-expressing (A) A2ARRluc and CaMYFP (triangles) or (B) D2RRluc and CaMYFP (inverted triangles) or A1RRluc and CaMYFP (squares). Co-transfections were performed with increasing amounts of plasmid-YFP (0.1 to 1 μg cDNA), whereas the plasmid-RLuc construct was maintained constant (0.6 μg cDNA for A2ARRluc, 1 μg cDNA for D2RRluc 0.7 μg cDNA for A1RRluc). Both fluorescence and luminescence for each sample were measured before every experiment to confirm similar donor expressions (about 100,000 luminescent units) while monitoring the increase in acceptor expression. The relative amount of acceptor is given as the ratio between the fluorescence of the acceptor (YFP) and the luciferase activity of the donor (RLuc). BRET data are expressed as means ± S.D. of four to eight different experiments grouped as a function of the amount of BRET acceptor.

Figure 2. Identification of the A2A receptor CaM binding site. (A) BRET competition experiments were performed with HEK-293 cells transfected with A2ARRluc (0.6 μg cDNA), CaMYFP (0.6 μg cDNA) and increasing amount of cDNA corresponding to A2AR (white bars) or A2ARAAA (R291IREFR296QTFR300 mutated to A291IREFA296QTFA300, black bars). Both fluorescence and luminescence for each sample were checked to confirm similar donor and acceptor expression (about 100,000 luminescent units and 10,000 fluorescence units). A2AR and A2ARAAA receptor expression was monitored by Western blot (not shown). (B) BRET saturation curves were performed with HEK-293 cells co-expressing A2ARRAAAARRluc (0.6 μg cDNA) and increasing amounts of CaMYFP (0.1 to 1 μg cDNA), and untreated (white symbols) or treated (10 min, black symbols) with 1 μM ionomycin in HBSS buffer containing 1.26 mM CaCl2. Both fluorescence and luminescence of each sample were checked to confirm similar donor expressions (about 100,000 luminescent units) while monitoring the increase in acceptor expression. The relative amount of acceptor is given as the ratio between the fluorescence of the acceptor (YFP) and the luciferase activity of the donor (RLuc). BRET data are expressed as means ± S.D. of four to six different experiments grouped as a function of the amount of BRET acceptor. Results are compared with the curve obtained for A2ARRluc and CaMYFP (dotted line, see Fig.1A).

Figure 3. Membrane colocalization of CaM with A2A or D2 receptors. Co-transfected HEK-293 cells were washed and resuspended for 2.5 h in HBSS buffer containing 1.26 mM CaCl2 (A-D and I-L) or in a Ca2+-free HBSS buffer containing 1 mM EDTA (E-H). (A, E and I) confocal microscopy images of HEK-293T cells expressing (from left to right, in each panel) CaMYFP (0.6 μg of cDNA), A2ARRluc (1 μg of cDNA) or D2RRluc (1 μg of cDNA). (B-D, F-H and J-L) confocal microscopy images of HEK-293T cells co-transfected with the above-described amounts of cDNA for CaMYFP and A2ARRluc (B, F and J), CaMYFP and D2RRluc (C, G and K) or CaMYFP and A1RRluc (1 μg of cDNA) (D, H and L). Ionomycin-treated-cells (10 min prior to fixation) are shown in I-L. CaM was identified by YFP fluorescence (green images) and receptor-Rluc constructs were identified by immunocytochemistry (using a monoclonal anti-Rluc primary antibody and a cyanine-3-conjugated secondary antibody). Colocalization (yellow) is shown in right panels.
Figure 4. Effect of intracellular Ca\(^{2+}\) on the interaction between CaM and A\(_{2A}\) or D\(_2\) receptors detected by BRET experiments. (A-C) BRET saturation curves were performed using HEK-293 cells co-expressing A\(_{2A}\)Rluc and CaMYFP (A), D\(_2\)RRluc and CaMYFP (B) or, A\(_1\)Rluc and CaMYFP as negative control (C) – see legend to Figure 1.-. In all cases cells were treated with HBSS buffer containing 1.26 mM CaCl\(_2\) and 1 \(\mu\)M ionomycin at 10 (blue), 5 (green) or 2 (red) min before BRET determination. BRET saturation curves were compared with the one obtained in the absence of ionomycin (dotted line, see Figure 1). The relative amount of acceptor is given as the ratio between the fluorescence of the acceptor (YFP) and the luciferase activity of the donor (Rluc). BRET data are expressed as means ± S.D. of four to eight different experiments grouped as a function of the amount of BRET acceptor. In (D), HEK-293 cells were transfected with 0.6 \(\mu\)g cDNA for A\(_{2A}\)RRluc (A\(_{2A}\)R) or 1 \(\mu\)g cDNA for D\(_2\)RRluc (D\(_2\)R) and 1 \(\mu\)g cDNA of CaMYFP and membranes were obtained 48 h after transfection. Membranes were resuspended in free Ca\(^{2+}\) HBSS buffer and 1.26 mM CaCl\(_2\) was added for the indicated times before BRET determination. BRET data are expressed as means ± S.E.M. of five different experiments. Significant differences over ionomycin non-treated cells were calculated by one way ANOVA and Bonferroni test (* \(p<0.05\), ** \(p<0.01\) and *** \(p<0.005\)).

Figure 5. CaM interaction with A\(_{2A}\)-D\(_2\) receptor heteromers detected by BRET competition experiments. BRET competition experiments were performed with HEK-293 cells expressing donor and acceptor constructs to give sub-maximal BRET values. In (A), cells were co-transfected with the cDNA corresponding to A\(_{2A}\)RRluc (0.6 \(\mu\)g) and CaMYFP (0.4 \(\mu\)g) and increasing amounts of D\(_2\)R cDNA as competitor (0.3 to 2.5 \(\mu\)g) whose expression was monitored by Western blotting (bottom panel). In (B), cells were cotransfected with the cDNA corresponding to A\(_{2A}\)RRluc (0.6 \(\mu\)g) and D\(_{2L}\)RYFP (1.5 \(\mu\)g) and increasing amounts of cDNA of CaMGFP\(^2\) as competitor (0.2 to 1.5 \(\mu\)g) whose expression was monitored by measuring the GFP\(^2\) fluorescence (1,000 to 20,000 fluorescence units) as described in Methods. To determine the YFP fluorescence in BRET experiments, the spectral signature was considered to control the GFP\(^2\) contribution to the detection channel (28, 29). In (C), cells were cotransfected with the cDNA corresponding to D\(_{2L}\)RRluc (1 \(\mu\)g, top panel) or D\(_{2S}\)RRluc (0.8 \(\mu\)g, bottom panel) and CaMYFP (0.4\(\mu\)g) and increasing amounts of cDNA of A\(_{2A}\)R as competitor (black bars) or A\(_1\)R as negative control (white bars) whose expression was monitored by dot blotting (results not shown). At the top of each panel, a scheme depicts the expressed proteins in BRET competition assays.

Figure 6. SRET for CaM, A\(_{2A}\) receptor and D\(_2\) receptor in living cells. SRET saturation curves were performed in HEK-293 cells expressing A\(_{2A}\)R-Rluc (0.75 \(\mu\)g of cDNA), CaMGFP\(^2\) (0.6 \(\mu\)g of cDNA) and increasing amounts of D\(_2\)RYFP (0.5 to 5 \(\mu\)g of the cDNA). Net SRET was obtained by monitoring the YFP fluorescence emission after coelenterazine H addition, with subtraction of the value obtained with cells expressing the same amount of A\(_{2A}\)R-Rluc and CaMGFP\(^2\). Significant net SRET was detected for A\(_{2A}\)RRluc-CaMGFP\(^2\)-D\(_2\)RYFP coupling, while negligible or linear net SRET was obtained in cells expressing equivalent amounts (equivalent fluorescence and luminescence units) of A\(_{2A}\)RRluc, CaMGFP\(^2\) and 5HT2BYFP, or A\(_1\)RRluc, CaMGFP\(^2\) and D\(_2\)RYFP, which were used as negative controls. Values, expressed as net SRET, represent means ± S.E.M. of 5 independent experiments performed in triplicate. At the top, a scheme depicts the expressed proteins in SRET competition assays.
Figure 7. Effect of intracellular Ca\(^{2+}\) on the molecular interactions between CaM, A\(_{2A}\) and D\(_2\) receptors. (A) Effect of Ca\(^{2+}\) levels on the A\(_{2A}\)-D\(_2\) receptor heteromerization detected by BRET experiments. BRET saturation curves were performed with HEK-293 cells co-expressing A\(_{2A}\)RRluc (0.6 µg cDNA) and increasing amounts of D\(_2\)RYFP (0.3 to 4 µg cDNA). Cells in HBSS buffer containing 1.26 mM CaCl\(_2\) were untreated (black) or treated with 1 µM ionomycin for 2 (red), 5 (green) or 10 (blue) min before BRET determination. Both fluorescence and luminescence for each sample were measured to confirm similar donor expressions (about 100,000 luminescent units) while monitoring the increase acceptor expression (1,000 to 15,000 fluorescent units). The relative amount of acceptor is given as the ratio between the fluorescence of the acceptor (YFP) and the luciferase activity of the donor (Rluc). BRET data are expressed as means ± S.D. of four different experiments grouped as a function of the amount of BRET acceptor. (B) Effect of Ca\(^{2+}\) levels on the CaM-A\(_{2A}\)-D\(_2\) receptor oligomerization detected by SRET experiments. SRET saturation curves were performed in HEK-293 cells expressing A\(_{2A}\)RRluc, CaMGFP\(^2\) and increasing amounts of D\(_2\)RYFP as described in the legend to Figure 6. Cells in HBSS buffer containing 1.26 mM CaCl\(_2\) were treated with 1 µM ionomycin for 10 min before SRET determination as described in Figure 6 legend and the SRET saturation curve was compared with the one obtained in the absence of ionomycin (dotted line, see Figure 6).

Figure 8. Effect of intracellular Ca\(^{2+}\) on A\(_{2A}\) receptor-, D\(_2\) receptor- and A\(_{2A}\)-D\(_2\) receptor heteromer-mediated ERK1/2 phosphorylation. In (A), HEK-293 cells expressing: (A) A\(_{2A}\) receptor (1.2 µg cDNA) or D\(_2\) receptor (1 µg cDNA) and CaM (0.3 µg cDNA); (B) A\(_{2A}\) receptor (1.2 µg cDNA), D\(_2\)R (1 µg cDNA) and CaM (0.3 µg cDNA), or (C) A\(_{2A}\) receptor (1.2 µg cDNA) and D\(_2\)R (1µg cDNA) were placed in HBSS buffer containing 1.26 mM CaCl\(_2\) and treated 3 min with vehicle or with 1 µM ionomycin before the addition of the A\(_{2A}\) receptor agonist CGS21680 (200 nM; CGS), the D\(_2\) receptor agonist quinpirole (1 µM) or both. A representative Western blot from ERK1/2 phosphorylation assays, which were performed as described in Methods, is shown. No significant differences in the basal levels were detected by the presence of CaM or CaM plus ionomycin. The immunoreactive bands from four to six independent experiments were quantified and values represent the mean ± S.E.M. of percentage of increase of phosphorylation over the basal levels (100%) found in ionomycin untreated or treated cells. Significant differences of agonist-treated versus basal (*) or agonist-treated in the presence versus the absence of ionomycin (\&) were calculated by one way ANOVA and Bonferroni test (*\& p<0.05, **\&\& p<0.01 and ***\&\&\& p<0.005).
Figure 2

A

![Graph A showing mBU values with error bars against μg of cDNA levels.]

B

![Graph B showing MBU values against YFP/Rluc ratio.]
Figure 6
Figure 8
Interactions between calmodulin, adenosine A2A and dopamine D2 receptors
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