Binding of Calmodulin to the D₂-Dopamine Receptor Reduces Receptor Signaling by Arresting the G Protein Activation Switch*

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Signalizing by D₂-dopamine receptors in neurons likely proceeds in the presence of Ca²⁺ oscillations. We describe here the biochemical basis for a cross-talk between intracellular Ca²⁺ and the D₂ receptor. By activation of calmodulin (CaM), Ca²⁺ directly inhibits the D₂ receptor; this conclusion is based on the following observations: (i) The receptor contains a CaM-binding motif in the NH₂-terminal end of the third loop, a domain involved in activating G₁o. A peptide fragment encompassing this domain (D2N) bound dansylated CaM in a Ca²⁺-dependent manner (Kₐ = 0.1 μM). (ii) Activation of purified G₁o₁ by D2N, and D₂ receptor-promoted GTPγS (guanosine 5’-(3-O-thio)triphosphate) binding in membranes was suppressed by Ca²⁺/CaM (IC₅₀ = 0.1 μM). (iii) If Ca²⁺ influx was elicited in D₂ receptor-expressing HEK293 cells, agonist-dependent inhibition of cAMP formation decreased. This effect was not seen with other G-coupled receptors (A₁-adenosine and Mel₁A-melatonin receptor). (iv) The D₂ receptor was retained by immobilized CaM and radiolabeled CaM was co-immuno-precipitated with the receptor. Specifically, inhibition by CaM does not result from uncoupling the D₂ receptor from its cognate G protein(s); rather, CaM directly targets the D₂ receptor to block the receptor-operated G protein activation switch.

Dopamine acts as a neuromodulator (rather than a neurotransmitter) in the central nervous system because dopamine controls the propensity of a neuron to fire action potentials. The receptors for dopamine belong to the class of G protein-coupled receptors. Five receptor subtypes representing two subfamilies have been identified by molecular cloning; D₁/D₅ receptors stimulate adenylyl cyclase activity, whereas D₂, D₃, and D₄ receptors couple to G proteins of the Gi/o class to inhibit adenylyl cyclase. Gi/o-mediated signal transduction in excitable cells proceeds in the presence of oscillating intracellular Ca²⁺ concentrations and there is reason to assume that the signaling mechanism is interrelated with the intracellular Ca²⁺ level.

Calmodulin (CaM), a small acidic protein, can be considered the primary decoder of Ca²⁺ information in the cell. CaM has a Ca²⁺ affinity of 10⁻⁶ M and thus acts as a switch when the concentration rises from a resting value of ~10⁻⁷ M to 10⁻⁵ M. Calmodulin can be activated by persistent elevation of intracellular Ca²⁺ and by Ca²⁺ oscillations, as they occur on repeated depolarizations of nerve cells. It has long been known that major effectors regulated by the D₂-dopamine receptor can be regulated by Ca²⁺ and that these effector molecules are enriched in striatal neurons. In these instances, increases in Ca²⁺ levels elicit effects similar to D₂ receptor activation. For example, Ca²⁺ reduces the intracellular cAMP levels by inhibiting adenylyl cyclase type V (and type VI) and by activating CaM-sensitive phosphodiesterases, which break down cAMP; both type V adenylyl cyclase (5, 6) and a 63-kDa isoform of phosphodiesterase (PDE1B1) are expressed in striatal neurons (7, 8). Another example for the cross-talk between D₂ receptor signaling and Ca²⁺/CaM is the target protein DARPP-32, an inhibitor of protein phosphatase 1. DARPP-32 is dephosphorylated on D₂-dopamine receptor activation and thus becomes active; this effect is strongly enhanced by Ca²⁺/CaM through activation of calcineurin (9). These examples suggest that the signal transduced by Ca²⁺/CaM and signaling initiated by the intracellular D₂ receptor overlap and may add to each other.

We have found in the primary peptide sequence of the human D₂-dopamine receptor a CaM-binding motif, which is located in the NH₂-terminus of the third cytoplasmic loop of the receptor. In the present work, we report that CaM can convey on presynaptic nerve terminals of nigrostriatal projections and, postsynaptically, on the medium spiny neuron, the predominant nerve cell of the neostriatum (2). The excitatory drive for the medium spiny neuron is provided by glutamatergic afferents which through NMDA receptors trigger Ca²⁺ influx (3). Hence, neuronal signal transduction by dopamine receptors proceeds in the presence of oscillating intracellular Ca²⁺ concentrations and there is reason to assume that the signaling mechanism is interrelated with the intracellular Ca²⁺ level.

1 The abbreviations used are: CaM, calmodulin; HEK, human embryonic kidney; D₁R, human D₁-dopamine receptor, short splice variant; Mel₁,R, human Mel₁₅, melatonin receptor; A₁R, human A₁-adenosine receptor; GTPγS, guanosine 5’-3-(O-thio)triphosphate; HA, amino acids(s); D₂N, 19 amino acid-peptide fragment from the amino-terminal part of the third cytoplasmic loop of the human D₂-dopamine receptor; D₂C, 18 amino acid-peptide fragment from the carboxy-terminal part of the third cytoplasmic loop of the human D₂-dopamine receptor; DSS, disuccinimidyl suberate; [125I]OH-PIPAT, (++)-trans-7-hydroxy-2-(N-propyl-N-3-[125I]iodo-2-propenylamino)tetralatin; HA, hemagglutinin; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; CaM kinase IV, Ca²⁺/calmodulin-dependent protein kinase II; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; HIPA, N²-(4-hydroxyphenylisopropyl)adenosine.
had been described (D2-dopamine receptor and the human A1-adenosine receptor in Ref. 11; Mel1a-melanotin receptor in Ref. 12). The D2-dopamine receptor tagged with the hemagglutinin epitope was transiently expressed in COS-7 cells using the Ca2+-phosphate precipitation procedure; after 60 h, the cells were harvested for the preparation of membranes.

**Determination of cAMP Formation—**HEK293 cells were grown to confluence in six-well plates. The adenine nucleotide pool was labeled by incubating the cells for 16 h with [3H]adenine (2 μCi/ml). After that the medium was replaced, and the cells were pre-incubated for 1 h with 100 μM of the phosphodiesterase inhibitor rolipram. The production of cAMP was translated by the addition of 25 μM forskolin, a cAMP-activated inhibition of cAMP formation was assessed in the absence and presence of the Ca2+ ionophore A23187 (calcinemycin) at a concentration of 3 μM (Ca2+ concentration in the assay medium = 1.8 mM) and of the receptor agonists at the indicated concentrations. Accumulation of cAMP was allowed to proceed for 15 min at room temperature, and the reaction was stopped by adding 2.5% perchloric acid with 100 μM cAMP (1 ml/dish). The supernatant (0.9 ml) was aspirated, neutralized with 100 μl of 0.4 M KOH, and diluted with 1.5 ml 50 mM Tris-HCl, pH 8.0. [3H]cAMP was isolated by sequential chromatography on Dowex AG 50WX-4 and neutral alumina columns (13).

**Membrane Preparation and Protein Purification—**Membranes from HEK293 cells were prepared as described in Ref. 11. For some experiments, membranes were resuspended in HME buffer (25 mM Hepes-NaOH, pH 7.5, 7.2 mM MgCl2, and 1 mM EDTA) at a protein concentration of 8–10 mg/ml and were stored in aliquots at −80 °C. Recombinant, myristoylated Gαi1α was prepared in Escherichia coli and purified from bacterial lysates as described in Ref. 11.

**Radioligand Binding Experiments—**Receptor-promoted G protein activation was determined by measuring the association rate of [125I]GTP·S in HeK293 membranes expressing the D2-dopamine and the A1-adenosine receptor as described (11); the A1-adenosine receptor was used as a control instead of the Mel1a receptor. The latter only weakly stimulates GTP·S binding because of its tight association with G proteins (15). EGF·A was washed cells membranes (10 μg) were suspended in an assay volume of 30 μl of buffer containing 25 mM Hepes-NaOH, pH 7.5, 1.5 mM MgCl2, 100 mM NaCl, 1 mM EDTA, and 0.01 g/ml GDP. When indicated 0.1 mM CaCl2 was added. Following preincubation of the membranes (10 min at 25 °C) with a receptor agonist or receptor antagonist, the reaction was initiated by adding [35S]GTP·S to a final concentration of 3 nM (specific activity = 2400 cpm/nmol). Quinpirol (1 μM) and sulpiride (10 μM) were used as agonist and antagonist, respectively, for the D2-dopamine receptor, N-cyclopentyladenosine (1 μM) was used as an agonist and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (1 μM) as an antagonist for the A1-adenosine receptor. When indicated, calmodulin (CaM) (1 μM) was included in the preincubation mixture. After the indicated reaction times, 0.9 ml of ice-cold stop buffer containing (mass): 10 Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl2, 0.5 mM EGTA, and 0.5 mM free radioactive ligand was separated by filtration over glass fiber filters. Binding of [35S]GTP·S to purified Gαi1 was carried out in an assay volume of 40 μl comprising buffer (50 mM Hepes-NaOH, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.01% Lubrol, 10 mM MgSO4, and 2 mM CaCl2), 1.3 pM of protein, and 1 μM [35S]GTP·S (specific activity, 30 cpm/nmol) in the absence or presence of 1 μM CaM. The reaction proceeded at 30 °C and was terminated by the addition of ice-cold stop buffer at the indicated time points. Bound and free radioactivity were separated by filtration over BA-85 nitrocellulose filters. Acceleration of [35S]GTP·S binding to Gαi1 by a peptide derived from the third intracellular loop of the D2-dopamine receptor (D2N) and its inhibition by calmodulin was performed on 0.8 pmol of Gαi1 in the absence or presence of 0.3 or 1 μM CaM. The binding reaction was carried out over a period of 15 min in the assay buffer described above.

Binding of the D2-dopamine receptor agonist radioligand [3H]IOH-PIPAT and of the A1-adenosine receptor agonist radioligand [125I]HPIA to membranes from stable HEK293 cell lines was carried out as described (11). Before the binding assay, cell membranes were washed with 10 mM EGTA as described above. The binding reaction was carried out for 60 min at 25 °C in the absence or presence of 0.1 mM CaCl2, and 1 μM CaM as indicated. The reaction was terminated by filtration over glass fiber filters using a cell harvester (Skatron, Lier, Norway). Non-specific binding of [3H]IOH-PIPAT and of [125I]HPIA was determined in the presence of 10 μM sulpiride and of 1 μM DPCPX, respectively, and amounted to less than 10% of the total binding of either radioligand in the Kd concentration range.
Immobilization of Gαi1 on CaM-Sepharose—D2N (10 μM) was incubated with 40 μl of a 50% slurry of calmodulin-Sepharose in buffer consisting of 20 mM Hepes-NaOH, pH 8.0, 100 μM CaCl₂, 2 mM MgSO₄, 0.1 mM GTP, and 0.1% Lubrol for 30 min at 22 °C. Subsequently, the Sepharose beads were sedimented by centrifugation and washed once to remove the detergent. The D2N-loaded CaM-Sepharose was then resuspended in 100 μl of the same buffer including 1.5 μg of purified Gαi1. After 45 min at 22 °C, the CaM-Sepharose was washed three times with 100 μl of buffer each time and finally resuspended in 100 μl of SDS sample buffer; 30-μl aliquots were boiled and separated on an SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, and the blot was probed with a G protein-specific antiserum raised against the residues 160–169 of Gαi1 (Ref. 16). The immunoreactive bands were visualized by enhanced chemiluminescence using horseradish peroxidase-conjugated anti-rabbit immunoglobulin.

Fluorescence Measurements—The interaction between D2N and dan-syl-CaM was examined by measuring peptide-induced changes in the fluorescence intensity of dansyl-CaM on excitation with UV light at a wavelength of 340 nm. The fluorescence emission spectrum of dansyl-CaM was recorded using a Hitachi F-4500 fluorescence spectrophotometer in a cuvette at a volume of 0.24 ml. The fluorescence maximum was determined at 510 nm in the absence and at 495 nm in the presence of dansyl-CaM ligands (i.e., CaM- and the D2-dopamine receptor-derived peptides (D2N or D2C)) at various concentrations of cholate and of NaCl, and (as indicated) 2 mM CaCl₂. The concentrations of dansyl-CaM were varied from 0.1 to 0.5 μM, and concentration-response curves for fluorescence enhancement by D2N were generated at each dansyl-CaM concentration. EC₅₀ estimates were derived by fitting the concentration-response curves to the Hill equation and replotted versus the concentration of dansyl-CaM in the cuvette.

Detection of Receptor Peptide–CaM Complexes—Binding of D2N to CaM was examined by cross-linking. CaM (6.6 μM) and D2N (3 μM) were incubated in 50 mM Hepes-NaOH buffer, pH 8.0, containing 0.1 mM CaCl₂ in a volume of 30 μl. The bifunctional amine-reactive cross-linking reagent DSS was added at a concentration of 1 mM if indicated. After 30 min at room temperature, SDS-sample buffer was added to the reaction mixture followed by heating to 90 °C for 5 min. The samples were resolved on a 13% SDS-polyacrylamide gel and stained with Coomassie Blue. To verify the presence of calmodulin in cross-linked complexes, the proteins were transferred to PVDF membranes, cross-linked with glutaraldehyde, and visualized with a monoclonal antibody raised against calmodulin.

Alternatively, the formation of receptor peptide–CaM complexes was evaluated by nondenaturing gel electrophoresis. The respective time points at which a wash or elution step was performed. At 4 °C the recovery of labeled D2 receptors was 98% and 85% after 15 and 20 min, respectively. In the case of the A₁-adenosine receptor, the melatonin Mel 1a receptor (Fig. 1B), the recovery amounted to 70% and 56% at the respective intervals. The values obtained were used to correct for the proportion of radioligand that dissociated during the wash and elution procedure. A soluble extract was also prepared from COS-7 cell membranes expressing the HA-tagged D₂ dopamine receptor (0.14 mg containing about 0.12 pmol of receptors) and was incubated with equilibrated CaM-Sepharose (150 μl of packed matrix) under the conditions described above for 2 h. Thereafter, the CaM-Sepharose was washed with 750 μl of matrix buffer and finally taken up in 150 μl of SDS-sample buffer. An aliquot was electrophoretically resolved and immunoblotted using a monoclonal anti-HA antibody (clone 16B12).

RESULTS

The Ca²⁺ Ionophore Calcimycin Impedes the Inhibition of cAMP Production Mediated by the D₂-Dopamine Receptor in HEK293 Cells—The D₂ dopamine receptor couples to G proteins of the Gαi subfamily and mediates inhibition of adenylyl cyclase. In HEK293 cells stably transfected with the human D₂ receptor, quinpirol completely inhibited forskolin-stimulated cAMP formation (Fig. 1A). If the cells were incubated with the Ca²⁺ ionophore calcimycin (3 μM), the inhibition elicited by quinpirol was attenuated; quinpirol failed to completely reverse the forskolin-induced cAMP production and the concentration-response curve shifted to higher agonist concentrations. The calcimycin effect on adenylyl cyclase inhibition was also seen on HEK293 cells expressing a different Gαi-coupled receptor, the melatonin Mel 1a receptor (Fig. 1B). In all cell lines, stimulation by forskolin was moderately decreased (to ~75%) by calcimycin.

Caldumulin Blocks G Protein Activation by the D₂-Dopamine Receptor—The data shown in Fig. 1 suggest that the ionophore-induced Ca²⁺ influx interfered with the signaling pathway.
activated by the D₂-dopamine receptor but not by other G-protein-coupled receptors. The Ca²⁺-sensing protein calmodulin is a mediator of Ca²⁺ signals, which it conveys to the target either by direct protein-protein interaction or indirectly by activating protein kinases that phosphorylate the target protein. In isolated membranes that had been washed with EGTA to chelate Ca²⁺ ions, the presence (●) or absence (○) of calcium (5 μM). After 15 min the reaction was stopped with 2.5% perchloric acid, and cAMP was isolated by column chromatography. Shown are the means ± S.E. from four experiments.

domains. In addition to the flanking residues and the core hydrophobic residue in position 8, congruent substitutions were found at variant positions. Apparently, the best possible alignment of the receptor peptide sequence with the known CaM-binding motifs was given if the sequence were read from the COOH- to NH₂-terminal end; there was also an appreciable but lesser degree of similarity in the conventional NH₂-to-COOH orientation (see Fig. 3A).

A peptide encompassing amino acids 208–226 of the receptor that comprised this motif (D2N) as well as peptides representing amino acids 214–232 (D2N') and 217–235 (D2N") were produced. To test if D2N was indeed capable of combining with CaM, it was subjected to a cross-linking experiment, which showed that D2N was covalently bound to CaM in the presence of DSS (Fig. 3B, lane 5). In contrast, the cross-linker did not affect the migration of CaM (Fig. 3B, lane 2) and no cross-linking product was observed if D2N was added to DSS in the absence of Ca²⁺/CaM. The presence of CaM in the complex with D2N was verified by immunoblotting on PVDF membranes with the use of a calmodulin-specific monoclonal antibody. Similar results were obtained with an alternative cross-linker (Tris-succinimidyl aminotriacetate; data not shown).

Formation of a single peptide-CaM complex was confirmed on a nondenaturing gel when CaM was applied together with D2N in the presence of Ca²⁺ (Fig. 3C). In order to define the extent of the CaM-docking site, we have also used two other 19-amino acid peptides derived from the same receptor domain but with their peptide sequences shifted 6 (D2N') and 9 (D2N") amino acids toward the COOH terminus. As opposed to D2N, both control peptides, which lack the first amino acids of the CaM-binding motif, entirely failed to combine with Ca²⁺/CaM, underlining the importance of the motif-flanking residues.

**Binding Affinity of Ca²⁺/CaM for D2N**—Because it is difficult to obtain reliable affinity estimates in cross-linking experiments, we determined the affinity of CaM for the D2N peptide by recording the changes in fluorescence emission of dansyl-CaM. The conformational change associated with binding of a ligand to CaM causes an enhancement in fluorescence emission. In addition, the emission peak is blue-shifted to a lower wavelength. This is illustrated by the original tracings shown in Fig. 4A. In the absence of Ca²⁺, dansyl-CaM displayed only weak fluorescence with a maximum at 510 nm (Fig. 4A). Addition of Ca²⁺ augmented the fluorescence intensity and blue-shifted the maximum to 495 nm. In the presence of D2N, there was an additional increase in fluorescence emission. The control peptides D2N', D2N", and D2C, the latter derived from the COOH-terminal end of the third loop of the D₂ receptor, were completely ineffective (data not shown). Concentration-dependent binding of D2N to dansyl-CaM was then examined. Fluorescence cannot be reliably measured at concentrations below 0.1 μM dansyl-CaM; hence, concentrations of dansyl-CaM in excess of 0.1 μM were employed. It is evident from Fig. 4B that, under these conditions, D2N and dansyl-CaM were present in equimolar amounts; this resulted in depletion, where the total and the free concentration of D2N differed substantially, in particular at low concentrations. Hence, with increasing concentrations of dansyl-CaM (Fig. 4B, at 0.1, 0.3, and 0.5 μM CaM), the concentrations of D2N required to induce fluorescence enhancement also increased. The apparent EC₅₀ values derived from binding curves obtained at various concentrations of dansyl-CaM fell onto a straight line with a slope that was reasonably close to 1 (Fig. 4C). The true affinity was approximated by extrapolating to infinitely low concentrations of dansyl-CaM, i.e., to the y axis intercept. This calculation gave a Kₐ of 80 nM, a value similar to the IC₅₀ for CaM observed in membranes (see Fig. 2C). In the absence of Ca²⁺, addition of

**Fig. 1. Inhibition of cAMP accumulation in HEK293 cells expressing the D₂-dopamine (A) or the Mel₁₇-melatonin receptor (B).** Stably transfected cells were plated in six-well dishes, grown close to confluence, and labeled with [³H]adenine (2 μCi/well). The formation of cAMP was stimulated by forskolin (25 μM) in the presence of rolipram (0.1 mM) for 15 min. The receptor-mediated effect was assessed by adding increasing concentrations of quinpirol (A) or melatonin (B) in the presence (●) or absence (○) of calcium (5 μM). After 15 min the reaction was stopped with 2.5% perchloric acid, and cAMP was isolated by column chromatography. Shown are the means ± S.E. from four experiments.
Binding of Calmodulin to the D₂-Dopamine Receptor

**Fig. 2.** Ca²⁺/CaM inhibits D₂-dopamine receptor promoted GTP-γ-S binding. A and B, time course of [³⁵S]GTP-γ-S binding to membranes from cells expressing the D₂-dopamine receptor (A) or the A₁-adenosine receptor (B). EGTA-washed membranes (~10 μg) were suspended in 30 μl of buffer (25 mM Hepes-NaOH, pH 7.5, 1 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, and 0.01 mM GDP, in the absence (●) or presence of 0.1 mM CaCl₂ (■) or in the presence of 0.1 mM CaCl₂ plus 1 μM CaM (▲). Following preincubation of the membranes (10 min at 25 °C) with agonists (1 μM quinpirol in A, 1 μM N⁶-cyclopentyladenosine in B) or antagonists (10 μM sulpirid in A, 1 μM DPCPX in B), the reaction was initiated by adding [³⁵S]GTP-γ-S to a final concentration of ~3 nM and continued for the time periods indicated. After stopping with ice-cold stop solution, free and bound radioactivity were separated by filtration over glass-fiber membranes. Shown are the means of duplicate determinations from a representative experiment, which was reproduced three times. C, effect of increasing the concentrations of calmodulin (in the presence of 100 μM CaCl₂) on [³⁵S]GTP-γ-S binding to membranes containing D₂-receptor in the presence of quinpirol (●) or sulpirid (○). Assay conditions were as in A. The reaction period was 5 min. Data represent means ± S.E. from three experiments carried out in duplicate.

**Fig. 3.** The third intracellular loop of the D₂-dopamine receptor contains in its NH₂-terminal part a CaM binding motif and binds to CaM. A, the peptide sequence (read from the COOH-terminal end) corresponding to amino acid residues 208–226 from the D₂-dopamine receptor (D₂N) is juxtaposed to the peptide sequences from mastoparan, murine inducible nitric oxide synthase (iNos), CaM kinase IV, from a CaM kinase inhibitor (35), the plasma membrane Ca²⁺-ATPase, and smooth muscle myosin light chain kinase (smMLCK). The motif found in CaM kinase IV is aligned with D₂N also in the N-to-C orientation. All of these motifs correspond to the type 1 binding motif, which carries hydrophobic residues in position 1, 8, and 14 according to Ref. 18. Congruent substitutions found in two or more of the aligned peptides are highlighted in bold or in italics. B, binding of the D₂-dopamine receptor peptide D₂N to CaM, visualized by cross-linking. Purified D₂N (6.6 μM) and CaM (3 μM) were incubated separately or in combination for 30 min in the absence or presence of DSS (1 mM) as indicated in the boxes on top of the graph. 30 μl of the reaction mixture was separated on a 13% polyacrylamide gel and stained with Coomassie Blue. C, visualization of a receptor peptide-CaM complex on a non-denaturing gel stained with Coomassie Blue. CaM (4 μM) was incubated together with the D₂ receptor-derived peptides D₂N, D₂N⁺, and D₂N⁻ (concentrations as indicated) under the conditions given above. CONT, control.

D₂N also induced an increment in the fluorescence of dansyl-CaM; however, the affinity was substantially lower than in the presence of Ca²⁺ (Fig. 2C, ○).

Calmodulin Inhibits the Activation of Gαᵢ, by D₂N—Neither Ca²⁺ nor Ca²⁺/CaM had any appreciable effect on the rate of GTP-γ-S-binding to purified (recombinant) Gαᵢ (Fig. 5A), a reaction limited by the release of prebound GDP. D₂N directly stimulates the guanine nucleotide exchange reaction of Gᵢ and Gᵢ, purified from bovine brain (10). This stimulation does not require the presence of G protein βγ-dimers; thus, D₂N potently stimulated the guanine nucleotide exchange reaction of Gαᵢ (Fig. 5B). We then tested if the D₂N peptide faithfully reproduced the CaM-sensitive interaction of the receptor with Gᵢ. In the absence of CaM, D₂N (in a concentration range between 0.1 and 3 μM) enhanced the binding of [³⁵S]GTP-γ-S to Gαᵢ (Fig. 5B, ●) and the stimulation was suppressed by the inclusion of Ca²⁺/CaM. CaM was tested at two concentrations (0.3 μM, ■, 1 μM, ▲); in each case, the inhibition was not overcome by peptide concentrations in large excess of CaM. For instance, at a concentration of 1 μM CaM, 3 μM D₂N failed to increase the binding of GTP-γ-S above the binding level achieved by 0.3 μM D₂N in the absence of CaM. This finding is inconsistent with a competitive type of inhibition due to a bimolecular reaction. Noncompetitive inhibition, in contrast, implies an alternative hypothesis where CaM and D₂N are simultaneously bound to Gαᵢ; this was tested using CaM cross-linked to a Sepharose matrix.

Immobilization of Gαᵢ on a Calmodulin-Sepharose Requires the Presence of D₂N—Since Ca²⁺/CaM did not affect the spontaneous activation of Gαᵢ, it was unlikely that CaM per se bound to Gαᵢ; accordingly, recombinant Gαᵢ was not retained on CaM-Sepharose. However, a significant proportion of the α-subunit was immobilized on the matrix when it had been pre-incubated with D₂N. As can be seen from Fig. 5C, the amount of (unbound) Gαᵢ that was recovered in the supernatant clearly decreased in the presence of D₂N. Conversely, elution with SDS-sample buffer released a marked amount of Gαᵢ, as compared with that released in the absence of D₂N. This observation indicates that the receptor peptide simultaneously bound to CaM and to Gαᵢ. It also predicts that CaM interacts with the intact receptor in a fashion that is compatible with receptor/G protein coupling. We therefore assessed the effect of Ca²⁺/CaM on high affinity agonist binding (i.e. formation of the high affinity ternary complex composed of agonist, receptor, and G protein). Binding of the agonist [¹²⁵I]OH-PIPAT was similar in EGTA-treated membranes incubated with or without Ca²⁺/CaM (Fig. 5D). In contrast to the marked inhibition of G protein activation (see Fig. 2), CaM only
very modestly reduced the number (but not the affinity) of high affinity agonist binding sites. Thus, CaM did not interfere with the ability of the agonist-ligated receptor to form a complex with its cognate G protein(s) but selectively blocked the subsequent reaction step in signal transduction, i.e. the G protein turnover catalyzed by the active receptor.

Co-immunoprecipitation of the Epitope-tagged D2-Dopamine Receptor and CaM—In order to demonstrate a physical interaction of the D2 receptor with CaM, we tagged the NH2 terminus with the c-Myc epitope and expressed the epitope-tagged receptor in HEK293 cells. EGTA-pretreated membranes were solubilized, and the receptor was immunoprecipitated with a monoclonal antibody directed against the c-Myc epitope. As a control we solubilized membranes from nontransfected cells and subjected the extract (in parallel) to the immunoprecipitation procedure. The results are shown in Fig. 6A. Immunoreactive bands corresponding to the IgG heavy and light chain were visible in the precipitate from both D2 receptor transfected and from control cells. Receptor-specific immunoreactive bands were detected exclusively in the supernatant (SN) and in the precipitate (IP) from extracts containing D2 receptor but not from control extracts. The bands were diffuse, a finding typical of posttranslationally modified receptors and migrated to a molecular mass position of ~70–90 kDa and to other, larger size positions; a minor band was also visualized at ~55 kDa in the supernatant. The pattern of immunoreactivity suggested that the D2-dopamine receptor formed (SDS-resistant) oligomeric aggregates; alternatively, the multiple bands may...
receptor may be smaller than in the absence of detergent. These two facts presumably accounted for the observation that only a minor fraction of the $^{125}$I-CaM added was recovered by immunoprecipitation. CaM also has been reported to interact with the (conserved) NH$_2$ terminus of G$\beta$-subunits (21). G$\beta$-dimers contribute to the receptor G protein interface and some receptors directly bind G$\beta$ in vitro in the absence of Go (22–24); thus, the precipitation of $^{125}$I-CaM could have occurred by virtue of $\beta\gamma$-dimer tightly bound to the receptor. To control for this possibility, the level of G$\beta$ was assessed in the immunoprecipitate with a $\beta$-specific antisera; immunoreactivity for G$\beta$ was very low (<1% of the total amount added) and was comparable in the precipitates from D$_2$ receptor-containing extracts and from control extracts (data not shown). This finding presumably reflected nonspecific adsorption of $\beta\gamma$-dimers to the protein G-Sepharose and, more importantly, did not account for the specific retention of $^{125}$I-CaM by the D$_2$ receptor.

Binding of the Solubilized D$_2$-Dopamine Receptor to Immobilized CaM—G protein-coupled receptors are notoriously unstable when removed from the membrane environment. It was, therefore, important to verify that the interaction of $^{125}$I-CaM with the D$_2$ receptor reflected binding to a functional receptor. To this end, membrane-bound receptors were labeled with $^{125}$I-leupeptide and unbound ligand was removed by centrifugation; subsequently, the membranes were solubilized and the extract was incubated with immobilized CaM. After several wash steps the receptor was eluted with EGTA. In order to avoid fallacious results because of nonspecific binding to the resin, we utilized two different types of matrix (Sepharose and agarose) to which CaM was linked. As can be seen from the bar diagram shown in Fig. 6C, the experiment yielded similar results with both resins, although there were differences in the proportion of receptors that bound; CaM-agarose (light gray bars) retained less receptor-bound radioactivity than CaM-Sepharose (black bars). After incubation with immobilized CaM and subsequent washing, the majority of the receptors was recovered in the supernatant while 15–40% of the radio-labeled receptors remained on the matrix (Fig. 6C). For illustrative purposes we depicted the last wash step; thereafter, the addition of EGTA released 10% and 25% of the radiolabeled D$_2$ receptors added to CaM-agarose and CaM-Sepharose, respectively.

Thus, on CaM-Sepharose, more than on CaM-agarose, a significant amount of radioactivity remained bound even after chelation of free Ca$^{2+}$. This radioactivity was released by boiling in SDS and nominally amounted to ~15% of the total receptor-bound radioactivity (Fig. 6C). In order to prove that this fraction was liganded to the receptor, we chose two approaches. First, we determined the nonspecific binding of $^{125}$I-leupeptide to CaM-Sepharose, using a matched amount of radioactivity; this nonspecific binding was negligible. As a control, the same experiment was also performed with the A$_1$-adenosine receptor expressed in HEK293 cells where similar amounts of the ligand [H]DPCPX were retained on the CaM-Sepharose in the absence or presence of the receptors (1.0% versus 1.2% of total; data not shown). This confirms that binding of CaM is a property specific to the D$_2$ receptor, which is not shared by the A$_1$-adenosine receptor. Second, the CaM-Sepharose was loaded with an epitope-tagged D$_2$ receptor. After carrying out the wash steps, receptor-specific immunoreactivity was recovered by boiling the matrix in SDS (Fig. 6C, inset). We stress that the immune reactive bands were probed with an anti-HA antisera and that the HA-tagged receptor had been transiently expressed in COS-7 cells. Thus, immunoprecipitation (Fig. 6A) as well as immobilization on CaM-Sepharose (Fig. 6C, inset) yielded similar migration patterns of the D$_2$-
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dopamine receptor, and these were independent of the epitope tag and of the cellular source.

**DISCUSSION**

In the present work, we show that Ca<sup>2+</sup>/CaM impairs the efficiency of signaling by the D<sub>2</sub>-dopamine receptor through a direct interaction with the receptor. The inhibition is caused by the binding of CaM to the NH<sub>2</sub>-terminal end of the third intracellular loop of the D<sub>2</sub> receptor. This domain contains a CaM binding motif, which conforms to one of the classified recognition sequences of which have been shifted toward the COOH terminus of the third loop, thereby curtailing the putative binding of CaM to the NH<sub>2</sub>-terminal end of the third intracellular loop of the D<sub>2</sub> receptor. This motif has to be completely represented (i.e., including the flanking NH<sub>2</sub>-terminal residues) for CaM binding; the control peptides do not bind to Ca<sup>2+</sup>/CaM (Fig. 3C) and do not activate G<sub>i</sub> either (data not shown). Thus, functionally important residues are found in the third cytoplasmic loop/α-helix boundary and this is also true for many other receptors (27). The current view holds that receptor activation results in an enhanced tertiary interaction of these residues (28, 29). This conformational change activates the cognate G protein and may similarly facilitate the docking of CaM, which makes it a ligand-regulated process.

Our evidence suggests that indeed the activated receptor binds CaM even when it is engaged in the high affinity ternary complex (agonist/receptor/G protein complex); based on the following data, we conclude that CaM does not disturb G protein recognition but impedes the receptor-induced activation switch. (i) The binding of CaM and the G protein α-subunit to the receptor peptide is not mutually exclusive; the receptor peptide combines simultaneously with Go<sub>α</sub> and with (immobilized) CaM. (ii) At a concentration where CaM completely blocks G protein activation, the formation of the agonist/receptor/G protein complex, hence, high affinity agonist binding is virtually unaffected. (iii) Inhibition of the receptor peptide by CaM is not competitive, i.e., it cannot be overcome by increasing the peptide concentration. In general, it is conceivable that G protein recognition and high affinity agonist binding requires a different subset of receptor-G protein contact sites than does the “G protein activation-switch,” that is the dissociation of the ternary complex induced by the active receptor and the binding of GTP. Two examples can be given. First, stabilization of the active receptor conformation of rhodopsin (metarhodopsin II) by transducin (Go<sub>α</sub>) is possible with mutant rhodopsins, which lack discrete interface domains of the receptor. However, the process of transducin activation is strongly impaired in these mutants because it cannot occur unless all possible contacts have formed (30). Second, mutations can be introduced into G protein α-subunits, which still support the formation of high affinity ternary complexes (31) but impair efficient activation by the receptor (32, 33).

An elevation of intracellular Ca<sup>2+</sup> leads to suppression of D<sub>2</sub> receptor-dependent signaling in intact cells. It has not been possible to directly demonstrate that this effect can be accounted for by Ca<sup>2+</sup>/CaM because all available membrane-permeable CaM antagonists (ophiobolin, calmidazolium, and W-7) potently blocked ligand binding to the D<sub>2</sub>-dopamine receptor (data not shown). However, the attenuation of cAMP formation by the melatonin Mel<sub>1a</sub> receptor in HEK292 cells was not affected by the increase in intracellular Ca<sup>2+</sup>. Furthermore, the addition of CaM to isolated membranes only impairs G protein activation and cAMP inhibition by the D<sub>2</sub> receptor (but not by other receptors). We therefore rule out that the selective action of the Ca<sup>2+</sup>-ionophore on the D<sub>2</sub> receptor stems from a membrane-delimited inhibition of G<sub>i</sub> or of the catalytic domain of adenylyl cyclase.

Our observations indicate that the signaling efficiency of the D<sub>2</sub>-dopamine receptor is regulated by a rise in intracellular Ca<sup>2+</sup> via CaM. The modulation of the D<sub>2</sub>-dopamine receptor by CaM must be placed into a conceptual context with the group III metabotropic glutamate receptor-7 (mGluR7) and the μ-opioid receptor, which have recently been determined to bind CaM (24, 34, 35). Like the D<sub>2</sub> receptor, mGluR7 and μ-opioid receptors couple to G proteins of the G<sub>α</sub>/subfamily to control neurotransmitter release, and similarly, are under immediate control of CaM. They bind CaM with comparable affinities (60–100 nM), i.e., in a concentration range that is also compatible with the intracellular level of CaM. The observations on each of these receptors, however, infer that CaM has opposing roles through different modes of receptor regulation. In the mGluR7, CaM binds to a motif in the carboxyl terminus adjacent to a domain through which the receptor accommodates the G protein βγ-dimer (24). Ca<sup>2+</sup>-dependent binding of CaM displaces G<sub>βγ</sub>; this explains why a rise in Ca<sup>2+</sup> does not blunt but enhances effector regulation, e.g., Ca<sup>2+</sup>-channel inhibition. In contrast, Ca<sup>2+</sup>/CaM is antagonistic to D<sub>2</sub> receptor signaling and targets a site required for the activation of Go. Go<sub>α</sub>/CaM also inhibits G protein activation by the μ-opioid receptor; however, the mechanisms by which CaM antagonizes the dopamine and the μ-opioid receptor are distinct. Although the μ-opioid receptor is uncoupled from the G protein, this is not the case with the D<sub>2</sub> receptor. The difference is due to different sites of action of CaM (D<sub>2</sub> receptor = third loop NH<sub>2</sub> terminus versus μ receptor = third loop COOH terminus). This interpretation is substantiated by the findings obtained with a peptide derived from the COOH terminus of the third loop of the D<sub>2</sub> receptor (D2C); D2C does not activate G<sub>i</sub> (not shown; see also Ref. 10) and also failed to combine with CaM. Thus, CaM represents an example for an accessory signaling component
which targets distinct cytoplasmic receptor domains to coordinate the cellular response. Even closely related receptors that interact with the same G protein(s) differ substantially in their intracellular loops. These intracellular portions represent binding sites for several other components, which regulate signaling in a discriminative fashion (36–38).

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