Binding of calmodulin to the D₂-dopamine receptor reduces receptor signaling by arresting the G protein activation switch

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Summary
Signaling by D2-dopamine receptors in neurons likely proceeds in the presence of Ca\textsuperscript{2+} oscillations. We here describe the biochemical basis for a cross-talk between intracellular Ca\textsuperscript{2+} and the D2-receptor. By activation of calmodulin (CaM), Ca\textsuperscript{2+} directly inhibits the D2-receptor; this conclusion is based on the following observations: (i) The receptor contains a CaM-binding motif in the N-terminal end of the 3\textsuperscript{rd} loop, a domain involved in activating G\textsubscript{i/o}. A peptide fragment encompassing this domain (D2N) bound dansylated CaM in a Ca\textsuperscript{2+}-dependent manner (K\textsubscript{D} \approx 0.1 \mu M). (ii) Activation of purified G\alpha\textsubscript{i-1} by D2N, and D2-receptor-promoted GTP\gammaS-binding in membranes was suppressed by Ca\textsuperscript{2+}/CaM (IC\textsubscript{50} \approx 0.1 \mu M). (iii) If Ca\textsuperscript{2+}-influx was elicited in D2-receptor expressing HEK293 cells, agonist-dependent inhibition of cAMP formation decreased. This effect was not seen with other G\textsubscript{i}-coupled receptors (A\textsubscript{1}-adenosine and Mel\textsubscript{1A}-melatonin receptor). (iv) The D2-receptor was retained by immobilized CaM and radiolabeled CaM was co-immunoprecipitated 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.
**Introduction**

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 G_{i/o} class to inhibit adenylyl cyclase. G_{i/o}-mediated signal transduction in excitable cells is also known to inhibit voltage activated N-type Ca^{2+}-channels and to gate inwardly rectifying K^+-channels (GIRK) via the release of βγ-subunit; the former effect was also demonstrated for D2-dopamine receptors (for a review, see 1).

D1- and D2-receptors, the ”classical” dopamine receptor subtypes, are abundantly expressed in the basal ganglia and are important targets in pharmacotherapy, yet the basis for their neuromodulatory effects is not well understood at the cellular level. The D2-dopamine receptor is found (as an autoreceptor) 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 Ca^{2+} influx (3). Hence, neuronal signal transduction by dopamine receptors proceeds in the presence of oscillating intracellular Ca^{2+} concentrations and there is reason to assume that the signaling mechanism is interrelated with the intracellular Ca^{2+} level.

Calmodulin (CaM), a small acidic protein, can be considered the primary decoder of Ca^{2+}-information in the cell. CaM has a Ca^{2+} affinity of 10^{-6} M and thus acts as a switch when the concentration rises from a resting value of \sim 10^{-7} M to 10^{-5} M. Calmodulin can be activated by persistent elevation of intracellular Ca^{2+} and by Ca^{2+} oscillations such as they occur on repeated depolarizations of nerve cells (4). It has long been known that major effectors regulated by the D2-dopamine receptor can be regulated by Ca^{2+} and that these effector molecules are enriched in striatal neurons. In these instances, increases in Ca^{2+} levels elicit effects similar to D2-receptor activation. For example, Ca^{2+} 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 Ca^{2+}/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 N-terminus of the third cytoplasmic loop of the receptor. In the present work we report that CaM can convey a Ca²⁺-signal directly to the receptor through binding to this receptor domain. When Ca²⁺/CaM binds to the receptor, it antagonizes signaling by the receptor at the level of receptor-mediated G protein turnover. Based on these observations, we propose to extend the concept of a cross-talk between CaM activation and D₂-receptor signaling; by binding to the receptor CaM exerts feed-back inhibition to down-tone the signaling efficiency of the D₂-receptor. In the presence of repetitive Ca²⁺ oscillations, i.e. when the neuron is actively firing action potentials, CaM is suggested to suppress overt enhancement of dopamine receptor signal transduction in striatal nerve cells.

**Experimental Procedures**

**Materials**

[^3H]adenine, [α⁻³²P]ATP (adenosine triphosphate), [³⁵S]GTPγS (guanosine 5′-(3-O-thio)triphosphate), [¹²⁵I]OH-PIPAT ((+)‐trans‐7‐hydroxy‐2‐(N‐propyl‐N‐3‐[¹²⁵I]‐iodo‐2′‐propenyl)aminotetralin) and [¹²⁵I]calmodulin were purchased from NEN (Boston, MA). [¹²⁵I]epideprid was obtained from the Austrian Research Centre (Seibersdorf, Austria). Peptides derived from the amino acid sequence of the NH₂-terminal and of the COOH-terminal part of the third intracellular loop of the human D₂-dopamine receptor were synthesized by solid-phase peptide synthesis as described (10).

D2N:  aa 208-226 = VYIKIYIVLRERKRVRNTK
D2N':  aa 214-232 = IVLRRRRKRVNTKRSRAFA
D2N'': aa 217-235 = RRRKVRNTKRSRAFRAH
D2C:  aa 360-377 = RRKLSSQKEKKATQMALI

Dulbecco’s modified Eagle medium (DMEM), non-essential amino acids, β-mercaptoethanol, G418 (geneticin) and materials for cultivating bacteria were obtained from GIBCO-BRL (Grand Island, NY). L-glutamine, penicillin G, streptomycin and A23187
Epitope-tagging of the $D_2$-dopamine receptor

Complementary DNA encoding for the human $D_2$-dopamine receptor (short isoform) was amplified from the $D_2$-receptor cDNA inserted into the pCMV-5 expression vector as a template. Extension of the coding sequence on the NH$_2$-terminus of the receptor was performed using a 5'-sense primer in which the sequence for one copy of the c-myc (EQKLISEEDLN) or the hemagglutinin epitope (YPYDVPDYA) was flanked by the sequence for the HindIII restriction site (5'-end) and by the first 24 nucleotides of the receptor coding sequence following the start codon (3'-end). The 3'-antisense primer encoded for a unique BstE restriction site present in the sequence of the $D_2$-dopamine receptor followed by the up-stream 5'-nucleotide sequence. The appropriate fragment was generated and amplified in 37 amplification cycles using Ex-Taq Polymerase at 58° annealing temperature in the first two and at 72°C in the consecutive amplification cycles. The amplified product was purified and re-ligated into the pCMV-5 vector using HindIII/BstE. The identity of the extended receptor sequence was confirmed by DNA sequencing.

Cell Culture

HEK293 cells were maintained in culture at 37°C and under 5% CO$_2$ in DMEM medium supplemented with 10% fetal calf serum, 2mM L-glutamine, 50mM $\beta$-mercaptoethanol, non-essential amino acids, 100 units/ml penicillin G, and 100 $\mu$g/ml streptomycin. For
transfection, HEK293 cells were plated at a density of ~2.5*10^6 cells/10 cm dish. The cells were transfected using the Ca^{2+} phosphate precipitation method with 7.5 µg of the plasmid encoding the c-myc-labeled human D_2S-dopamine receptor (c-myc-D_2R) and 0.75 µg of the pEGFP-C1 vector (Clontech, Palo Alto, CA) which carries the geneticin resistance cassette. Positive clones were selected in the presence of G418 (0.8 mg/ml). A positive cell clone which expressed the D_2-dopamine receptor to high density (~2 pmol/mg of membrane protein) was propagated in the presence of 0.2 mg/ml G418. The generation of stable HEK293 cell lines stably expressing receptors has been described (D_2-dopamine receptor and the human A_1-adenosine receptor in ref. 11; Mel_1a-melatonin receptor in ref. 12). The D_2-dopamine receptor tagged with the hemagglutinin epitope was transiently expressed in COS-7 cells using the Ca^{2+} phosphate precipitation procedure; after 60 hours the cells were harvested for the preparation of membranes.

**Determination of cAMP formation**

HEK293 cells were grown to confluence in 6-well plates. The adenine nucleotide pool was labeled by incubating the cells for 16 h with [³H]adenine (2 µCi/well). After that the medium was replaced and the cells were pre-incubated for 1h with 100 µM of the phosphodiesterase inhibitor rolipram. The production of cAMP was stimulated by the addition of 25 µM forskolin; receptor-mediated inhibition of cAMP formation was assessed in the absence and presence of the Ca^{2+}-ionophore A23187 (calcimycin) at a concentration of 3 µM (Ca^{2+} 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 0.4M KOH and diluted with 1.5 ml 50 mM Tris-HCl, pH 8.0. [³H]cAMP was isolated by sequential chromatography on Dowex AG 50W-X4 and neutral alumina columns (13).

**Membrane preparation and protein purification**

Membranes from HEK293 cells were prepared as described in (11). For some experiments the membranes were washed three times with 10 mM EGTA in Hepes pH 7.5, to chelate free Ca^{2+} and thus deplete membrane-bound CaM. Subsequently, membranes were resuspended in HME buffer (25 mM Hepes.NaOH pH 7.5, 2 mM MgCl_2 and 1 mM EDTA) at a protein concentration of 8 to 10 mg/ml and were stored in aliquots at -80°C.
Recombinant, myristoylated Gαᵢ₁ was produced in Escherichia coli and purified from bacterial lysates as described in (14).

**Radioligand binding experiments**

Receptor-promoted G protein activation was determined by measuring the association rate of [³⁵S]GTPγS 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 Mel₁₇-receptor. The latter only weakly stimulates GTPγS-binding because of its tight association with G proteins (15). EGTA-washed cell membranes (~10 µg) were suspended in an assay volume of 30 µl buffer containing 25 mM Hepes.NaOH, (pH 7.5), 1 mM MgCl₂, 100 mM NaCl, 1 mM EDTA and 0.01 mM 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]GTPγS to a final concentration of ~3 nM (specific activity = 2400 cpm/fmol). Quinpirol (1µM) and sulpirid (10µM) were used as agonist and antagonist, respectively, for the D₂-dopamine receptor, N⁶-cyclopentyladenosine (CPA) (1µM) was used as an agonist and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (1µM) as an antagonist for the A₁-adenosine receptor. When indicated calmodulin (CaM) (1µM) was included in the preincubation mixture. After the indicated reaction times, 0.9 ml of ice-cold stop buffer containing (mM) 10 Tris-HCl, pH 8.0, 100 NaCl, 10 MgCl₂ and 0.1 GTP were added. Bound and free radioactivity were separated by filtration over glassfiber filters. Binding of [³⁵S]GTPγS 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 pmol protein and 1 µM [³⁵S]GTPγS (specific activity 30 cpm/fmol) 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]GTPγS 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 Gαᵢ₁ in the absence or presence of 0.3µM 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 [¹²⁵I]OH-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 90 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 glassfiber filters using a cell harvester (Skatron, Lier, Norway). Non-specific binding of [¹²⁵I]OH-PIPAT and of [¹²⁵I]HPIA was determined in the presence of 10 µM sulpirid and of 1 µM DPCPX, respectively, and amounted to less than 10% of the total binding of either radioligand in the Kᵅ-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 0.01% lubrol for 30 min at 22°C. Subsequently, the sepharose-beads were sedimented by centrifugation and washed once to remove excess D2N. The D2N-loaded CaM-sepharose was 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 buffer each 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 Gαᵢ₁ specific antiserum (I1C, directed against the residues 160-169 of Gαᵢ₁, 16). The immunoreactive bands were visualized by enhanced chemiluminiscence using horseradish peroxidase-conjugated anti-rabbit immunoglobulin.

**Fluorescence measurements**

The interaction between D2N and dansyl-CaM were 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 detected at a wavelength of 510 nm in the absence and at 495 nm in the presence of dansyl-CaM ligands (i.e. Ca²⁺ or receptor peptides). Dansyl-CaM and the D₂-dopamine receptor-derived peptides (D2N or D2C) were diluted in assay buffer (20 mM Tris-HCl, pH 8.0, 1mM EDTA, 150 mM NaCl and – as indicated – 2 mM CaCl₂). The concentrations of dansyl-CaM were varied from 0.1 µM 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 vs. 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 disuccinimidyl suberate (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 non-denaturing gel electrophoresis. D2N (aa 208-226) and the related D₂-receptor peptides with overlapping sequences, D2N’ (aa 214-232) and D2N’’(aa 217-235) were incubated with CaM in the presence of 0.1 mM Ca²⁺ for 30 min at room temperature. The incubation mixture was taken up in SDS-less sample buffer and applied to an 8% acrylamide gel which contained 0.1 mM Ca²⁺. Electrophoresis was performed at a current of 10-15 mA with the addition of 0.1 mM Ca²⁺ in the electrophoresis buffer. CaM and CaM-peptide complexes were visualized by Coomassie blue staining.

Immunoprecipitation of the epitope-tagged D₂-dopamine receptor

Membranes prepared from the cells stably expressing c-myc-D₂R were washed with EGTA as described above; subsequently the membranes (2 mg) were solubilized with 0.6 % cholate (the ratio of detergent to membrane protein was 3:1) in HME buffer containing 750 mM NaCl and protease inhibitors (Pefabloc, Boehringer Mannheim). The insoluble material was collected by centrifugation at 35,000*g for 20 min. The supernatant was concentrated over a porous polycarbonate membrane (Amicon, cut off size = 30kDa) and subsequently, 0.1% digitonin was added, the concentration of cholate adjusted to 0.12% and the concentration of NaCl to 150 mM. In a volume of 0.25 ml, the soluble extract was incubated for 2 h at 4°C with 10 µg of a monoclonal c-myc antibody (clone 9E10) and 50 µl of a 50% slurry of protein-G sepharose (Amersham Pharmacia). The sepharose was collected by centrifugation at 500 rpm, washed three times with 200 µl ice-cold detergent-containing buffer. To determine the efficiency of the immunoprecipitation 30 µl aliquots were diluted in SDS-sample buffer which contained 6 M urea and 40 mM DTT. The samples were brought to 30°C for 30 min,
and were applied to a SDS-polyacrylamide gel containing 6 M urea. The supernatant, the last out of three washes and the immunoprecipitate were analyzed. The c-myc-tagged D₂R was detected by immunoblotting using a polyclonal antiserum directed against c-myc; in addition, the blot was probed with a G protein β-subunit-specific rabbit antiserum (17). Alternatively, the immunoprecipitate that had been prepared as described above was resuspended and gently mixed with 5 nM of [¹²⁵I]CaM (corresponding to 0.3 µCi) in the detergent containing buffer to which 2 mM CaCl₂ was added for 2 h at 4°C. The sepharose was then washed three times with the buffer containing CaCl₂ and detergents and was taken up in 40 µl of sample buffer which included 6 M urea and 40 mM DTT. After separating the samples on a 13% SDS-polyacrylamide gel [¹²⁵I]CaM was visualized by autoradiography.

**Binding of the D₂-dopamine receptor to calmodulin sepharose**

Membranes from HEK293 cells expressing the D₂-dopamine receptor (Bₘₐₓ ~ 2 pmol/mg) or the A₁ adenosine receptor (Bₘₐₓ ~ 2 pmol/mg) were washed free of Ca²⁺ and were incubated with saturating concentrations of the high-affinity antagonist radioligands [¹²⁵I]epideprid (for the D₂-receptor) and [³H]DPCPX (for the A₁-adenosine receptor) for 90 min at 25°C. Matched amounts of receptor (~ 0.3 pmol) were solubilized with 0.6% cholate in HME buffer containing 750 mM NaCl and 1 mM PMSF at 4°C for 60 min (detergent: protein = 3 : 1). This mixture was then centrifuged at 35,000*g for 20 min; 0.1% digitonin and 2 mM CaCl₂ were added to the supernatant and the concentration of cholate and of NaCl was adjusted to 0.06% and 150 mM, respectively. Of this an aliquot was removed and kept at 4°C. The solubilized receptors were then incubated with CaM immobilized on a sepharose matrix (packed matrix = 1/10 of the volume of the soluble extract) which had been equilibrated in matrix-buffer (HME, 0.06% cholate, 0.1% digitonin, 150 mM NaCl, 2 mM CaCl₂) for 2 h at 4°C. Subsequently the CaM-sepharose was washed three times in 200 µl matrix-buffer and bound receptors were eluted with 200 µl matrix-buffer which contained 10 mM EGTA instead of CaCl₂. The aliquots of the soluble extract, of the supernatant, the wash-fractions and the eluate were then filtrated through glass-fiber filters pre-treated with 1% polyethyleneimine. Radioactivity that failed to elute from the CaM-sepharose was recovered by boiling in 2% SDS. The filter-bound radioactivity and the radioactivity recovered by boiling the matrix were measured. Alternatively, CaM immobilized on an agarose-resin was utilized to assay the retention of solubilized D₂-dopamine receptors following the same procedure. Non-specific radioligand binding to the CaM-matrix was determined with matched amounts of [¹²⁵I] epideprid and [³H]DPCPX incubated with membrane protein from non-transfected cells; the
obtained values amounted to 5-15% of the values for receptor-bound radioactivity from which they were subtracted. In order to control for ligand dissociation from the solubilized receptors the decrease in binding was quantified for each of the 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 min and 20 minutes, respectively. In the case of the A₁-adenosine receptor, 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 receptors) and was incubated with equilibrated CaM-sepharose (150 µl packed matrix) under the conditions described above for 2 h. Thereafter, the CaM-sepharose was washed with 750 µl matrix buffer and finally taken up in 150 µl 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 Gᵢ/ₒ subfamily and mediates inhibition of adenyl 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 adenyl cyclase inhibition was not seen in HEK293 cells expressing a different Gᵢ/ₒ coupled receptor, the melatonin Mel₁a receptor (Fig. 1B). In all cell lines, stimulation by forskolin was moderately decreased (to ~75%) by calcimycin.

Calmodulin 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 Gᵢ-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 which phosphorylate the target protein. In isolated membranes which had been washed with EGTA to chelate free Ca²⁺ and to deplete membrane-bound CaM, we examined
whether CaM directly inhibited the receptor-mediated G protein activation. The addition of 1 µM Ca²⁺/CaM (▲) but not of 100 µM Ca²⁺ alone (■) slowed the receptor-enhanced association of [³⁵S]GTPγS as compared to the time course observed in the absence of Ca²⁺/CaM (Fig. 2A). No effect of Ca²⁺/CaM was found on agonist-promoted GTPγS binding if elicited by the A₁-adenosine receptor expressed in HEK293 cells (Fig. 2B). The inhibitory effect of Ca²⁺/CaM was increased by increasing the concentration of CaM. Figure 2C shows that Ca²⁺/CaM suppressed quinpirol-stimulated GTPγS binding with an IC₅₀ of ~ 0.1 µM. Binding of GTPγS in the presence of the receptor antagonist was only slightly affected. This selective inhibition of the D₂-receptor mediated G protein activation is indicative of a direct interaction of CaM with the membrane-bound receptor. An antagonism by CaM was also observed when inhibition of adenylyl cyclase was measured in membranes; Ca²⁺/CaM reversed the inhibition by quinpirol and reduced forskolin-stimulated adenylyl cyclase activity to a similar extent as did calcimycin in intact cells (not shown). We therefore tested if CaM added exogenously to isolated membranes blocked the ligand binding pocket of the D₂-dopamine receptor. This was not the case as saturation isotherms with the radioligand [¹²⁵I]epideprid revealed no significant impairment by CaM (data not shown, see also Fig. 5D for agonist binding to the D₂-receptor).

Ca²⁺/CaM binds to a peptide derived from the aminoterminal end of the third intracellular loop of the human D₂-dopamine receptor (D2N)

Analysis of the intracellular domains of the receptor peptide sequence revealed a CaM binding motif in the N-terminal part of the third intracellular loop (aa 210-223 Fig. 3A). As a general rule the hallmark of a CaM-binding motif is the presence of several hydrophobic residues interspersed with a number of positively charged residues (lysine and arginine) in a stretch of 14 amino acids. The sequence of the motif was classified as a type 1B motif according to (ref. 18) and was aligned with well-defined CaM-binding domains. In addition to the flanking 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 C- to N-terminal end; there was also an appreciable but lesser degree of similarity in the conventional N-to-C orientation (see Fig. 3A).

A peptide encompassing amino acids 208-226 of the receptor which 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 (lane 5 in Fig. 3B). In contrast, the cross-linker did not affect the migration of CaM (lane 2 in Fig. 3B) and no cross-linking product was observed if D2N was added DSS in the absence of Ca\(^{2+}\)/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 (TSAT, 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 Ca\(^{2+}\) (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 C-terminus. As opposed to D2N both control peptides which lack the first amino acids of the CaM-binding motif entirely failed to combine with Ca\(^{2+}/\)CaM, underlining the importance of the motif-flanking residues.

**Binding affinity of Ca\(^{2+}/\)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 Ca\(^{2+}\), dansyl-CaM displayed only weak fluorescence with a maximum at 510 nM (Fig. 4A). Addition of Ca\(^{2+}\), 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 C-terminal end of the third loop of the D\(_2\)-receptor were completely ineffective (not shown). Then, concentration-dependent binding of D2N to dansyl-CaM was 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 EC\(_{50}\) 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 $K_D$ of 80 nM, a value similar to the $IC_{50}$ for CaM observed in membranes (see Fig. 2C). In the absence of Ca$^{2+}$, addition of D2N also induced an increment in the fluorescence of dansyl-CaM; however, the affinity was substantially lower than in the presence of Ca$^{2+}$ (Fig. 2C, □).

Calmodulin inhibits the activation of $\alpha_{i-1}$ by D2N
Neither Ca$^{2+}$ nor Ca$^{2+}$/CaM had any appreciable effect on the rate of GTP$\gamma$S-binding to purified (recombinant) $\alpha_{i-1}$ (Fig. 5A), a reaction limited by the release of prebound GDP. D2N directly stimulates the guanine nucleotide exchange reaction of Gi and Go purified from bovine brain (10). This stimulation does not require the presence of G protein $\beta\gamma$-dimers; thus, D2N potently stimulated the guanine nucleotide exchange reaction of $\alpha_{i-1}$ (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$\mu$M) enhanced the binding of $[^{35}\text{S}]$GTP$\gamma$S to $\alpha_{i-1}$ (Fig. 5B, ●) and the stimulation was suppressed by the inclusion of Ca$^{2+}$/CaM. CaM was tested at two concentrations (0.3 $\mu$M, ■ and 1$\mu$M, ▲); in each case, the inhibition was not overcome by peptide concentrations in large excess of CaM. For instance, at a concentration of 1 $\mu$M CaM, 3$\mu$M D2N failed to increase the binding of GTP$\gamma$S above the binding level achieved by 0.3 $\mu$M D2N in the absence of CaM. This finding is inconsistent with a competitive type of inhibition due to a bimolecular reaction. Non-competitive inhibition, in contrast, implies an alternative hypothesis where CaM and D2N are simultaneously bound to $\alpha_{i-1}$; this was tested using CaM cross-linked to a sepharose-matrix.

Immobilization of $\alpha_{i-1}$ on a calmodulin-sepharose requires the presence of D2N
Since Ca$^{2+}$/CaM did not affect the spontaneous activation of $\alpha_{i-1}$ it was unlikely that CaM per se bound to $\alpha_{i-1}$; accordingly, recombinant $\alpha_{i-1}$ was not retained on CaM-sepharose. However, a significant proportion of the $\alpha$-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) $\alpha_{i-1}$ that was recovered in the supernatant clearly decreased in the presence of D2N. Conversely, elution with SDS-sample buffer released a marked amount of $\alpha_{i-1}$ as compared to that released in the absence of D2N. This observation indicates that the receptor peptide
simultaneously bound to CaM and to G\(\alpha_{i-1}\). 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 Ca\(^{2+}\)/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 \([^{125}\text{I}]\text{OH-PIPAT}\) was similar in EGTA-treated membranes incubated with or without Ca\(^{2+}\)/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-liganded 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 D\(_2\)-dopamine receptor and CaM

In order to demonstrate a physical interaction of the D\(_2\)-receptor with CaM we tagged the N-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 non-transfected 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 D\(_2\)-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 D\(_2\)-receptor but not from control extracts. The bands were diffuse, a finding typical of posttranslationally modified receptors and migrated to a Mr 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 D\(_2\)-dopamine receptor formed (SDS-resistant) oligomeric aggregates; alternatively, the multiple bands may represent different states of glycosylation, a finding previously reported by others (19, 20). In addition, it is also evident that the antibody precipitated the \(~70-90\) kDa form more efficiently than the large-size complexes. To directly demonstrate binding of CaM to the receptor, the immunoprecipitate (from control and from D\(_2\)-receptor containing extracts) was incubated with 5 nM \([^{125}\text{I}]\text{CaM}\) in the presence of Ca\(^{2+}\). Fig. 6B shows that \([^{125}\text{I}]\text{CaM}\) was retained by the immunoprecipitated D\(_2\)-dopamine receptor but not by the extract prepared from control cells. Thus, Ca\(^{2+}\)/CaM specifically interacted with the D\(_2\)-dopamine receptor. The experiment was carried out with trace amounts of \([^{125}\text{I}]\text{CaM}\) (5 nM, i.e. well below the \(K_D\) or IC\(_{50}\)). Furthermore, in detergent
solution the affinity of CaM for the 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 \(^{125}\text{I}\)CaM added was recovered by immunoprecipitation. CaM has also been reported to interact with the (conserved) N-terminus of Gβ-subunits (21). Gβγ-dimers contribute to the receptor G protein interface and some receptors directly bind Gβγ in vitro in the absence of Gα (22, 23, 24); thus, the precipitation of \(^{125}\text{I}\)CaM could have occurred by virtue of βγ-dimer tightly bound to the receptor. To control for this possibility, the level of Gβ was assessed in the immunoprecipitate with a β-specific antiserum; immunoreactivity for Gβ was very low (<<1% of the total amount added) and was comparable in the precipitates from D2-receptor containing extracts and from control extracts (data not shown). This finding presumably reflected non-specific adsorption of βγ-dimers to the protein G-sepharose and, more importantly, did not account for the specific retention of \(^{125}\text{I}\)CaM by the D2-receptor.

**Binding of the solubilized D2-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 \(^{125}\text{I}\)CaM with the D2-receptor reflected binding to a functional receptor. To this end, membrane-bound receptors were labeled with \(^{125}\text{I}\)epideprid 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 non-specific 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 grey 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 radiolabeled 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 D2-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 non-specific binding of \(^{125}\text{I}\)epideprid to CaM-sepharose using a matched amount of radioactivity; this non-specific 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 \(^{3}\text{H}\)DPCPX were retained on the CaM-sepharose in the absence or presence of the receptors (1.0% vs. 1.2% of total, 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. Secondly, 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 (inset in Fig. 6C). We stress that the immune reactive bands were probed with an anti-HA antiserum 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\(_2\)-dopamine receptor and these were independent of the epitope-tag and of the cellular source.

**Discussion**

In the present work, we show that Ca\(^{2+}\)/CaM impairs the efficiency of signaling by the D\(_2\)-dopamine receptor through a direct interaction with the receptor. The inhibition is caused by the binding of CaM to the N-terminal end of the third intracellular loop of the D\(_2\)-receptor. This domain contains a CaM binding motif which conforms to one of the classified recognition domains where the hydrophobic residues (val, ile) are located in positions 1-8-14 (18). A peptide (D2N) that comprises this motif binds to CaM with a \(K_D\) of \(\sim 80\) nM in the presence of Ca\(^{2+}\) and the affinity decreases upon chelation of Ca\(^{2+}\). Earlier work has already established that the D2N peptide directly activates G proteins of the G\(_{i/o}\) class and uncouples the D\(_2\)-receptor (10). In this context, it is worth noting that the receptomimetic peptide mastoparan, which directly activates G\(_{i/o}\) proteins (25), also binds CaM with high (nanomolar) affinity, a property shared by other insect venoms (26). Ca\(^{2+}\)/CaM blocks G protein activation by D2N in a non-competitive manner; in addition, Ca\(^{2+}\)/CaM inhibits the guanine nucleotide-exchange reaction promoted by the D\(_2\)-receptor in membranes. This effect and the binding of Ca\(^{2+}\)/CaM to the receptor peptide are governed by the same affinity; we therefore conclude that the N-terminus of the 3\(^{rd}\)-loop actually represents the site of inhibition in the membrane-bound receptor.

This conclusion is further substantiated by the finding that the solubilized D\(_2\)-dopamine receptor associates with CaM; physical interaction of CaM with the intact receptor has been
demonstrated by co-immunoprecipitation of the receptor and $[^{125}\text{I}]\text{CaM}$ and by binding of the solubilized receptor to immobilized CaM. A quantitative assessment using the antagonist-labeled, solubilized receptor indicates that between 15 and 40% of the D$_2$-receptors bind to Ca$^{2+}$/CaM depending on the CaM-matrix employed; given that the type of interaction is in part hydrophobic, however, binding of the solubilized D$_2$-receptor to Ca$^{2+}$/CaM is certainly impaired by the presence of detergent.

In the tertiary structure of the membrane-bound receptor, the CaM interaction site is located adjacent to (the putative) transmembrane domain 5, removed by only two peptide bonds. We have also used as a control the peptides D2N’ and D2N’’, the sequences of which have been shifted toward the C-terminus of the 3$^{rd}$ loop thereby curtailing the putative CaM-binding motif by 4 and 7 N-terminal residues, respectively. From these peptides it is evident that the motif has to be completely represented (i.e. including the flanking N-terminal residues) for CaM binding; the control peptides do not bind to Ca$^{2+}$/CaM (Fig. 3C) and do not activate G$_i$, either (not shown). Thus, functionally important residues are found in the 3$^{rd}$ cytoplasmic loop/$\alpha$-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 cue 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 $\alpha$-subunit to the receptor peptide is not mutually exclusive; the receptor-peptide combines simultaneously with G$\alpha_{i-1}$ 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 (G$_{\text{t}}$) 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 except if all possible contacts have formed (30). Secondly, 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\(^{2+}\) leads to suppression of D\(_2\)-receptor-dependent signaling in intact cells. It has not been possible to directly demonstrate that this effect can be accounted for by Ca\(^{2+}\)/CaM because all available membrane-permeable CaM antagonists (ophiobolin, calmidazolium, and W-7) potently blocked ligand binding to the D\(_2\)-dopamine receptor (not shown). However, the attenuation of cAMP formation by the melatonin Mel\(_{1a}\) receptor in HEK293 cells was not affected by the increase in intracellular Ca\(^{2+}\). Furthermore, the addition of CaM to isolated membranes only impaired G protein activation and cAMP inhibition by the D\(_2\)-receptor (but not by other receptors). We therefore rule out that the selective action of the Ca\(^{2+}\) ionophore on the D\(_2\)-receptor stems from a membrane-delimited inhibition of Gi or of the catalytic domain of adenylyl cyclase.

Our observations indicate that the signaling efficiency of the D\(_2\)-dopamine receptor is regulated by a rise in intracellular Ca\(^{2+}\) via CaM. The modulation of the D\(_2\)-dopamine receptor by CaM has to be placed into a conceptual context with the group III metabotropic glutamate receptor-7 (mGluR7) and the µ–opioid receptor which have recently been identified to bind CaM (24, 34, 35). Like the D\(_2\)-receptor, mGluR7 and µ-opioid receptors couple to G proteins of the Gi/o-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 which 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 carboxy terminus adjacent to a domain through which the receptor accommodates the G protein βγ-dimer (24). Ca\(^{2+}\)-dependent binding of CaM displaces Gβγ; this explains why a rise in Ca\(^{2+}\) does not blunt but enhances effector regulation, e.g. Ca\(^{2+}\)-channel inhibition. In contrast, Ca\(^{2+}\)/CaM is antagonistic to D\(_2\)-receptor signaling and targets a site required for the activation of Ga. Ca\(^{2+}\)/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. While the µ-opioid receptor is uncoupled from the G protein, this is not the case with the D\(_2\)-
receptor. The difference is due to different sites of action of CaM (D2-receptor = 3rd loop N-terminus vs. µ-receptor = 3rd loop C-terminus). This interpretation is substantiated by the findings obtained with a peptide derived from the C-terminus of the 3rd loop of the D2-receptor (D2C); D2C does not activate G_i (not shown, see also 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).

Acknowledgment

We appreciate the superb technical assistance of Erich Spielvogel.
References:

The abbreviations used are: CaM, calmodulin; HEK, human embryonic kidney; D_2R, human D_2-dopamine receptor, short splice variant; Mel1aR, human Mel1a-melatonin receptor; A_1R, human A_1-adenosine receptor; cAMP, cyclic adenosine-2':3'-phosphate; GTP_{\gamma S}, guanosine 5'-(3-O-thio)triphosphate; aa, aminoacid; N-terminal, aminoterminal; D2N, 19 aminoacid-peptide fragment from the aminoterminal part of the 3rd cytoplasmic loop of the human D_2-dopamine receptor; D2C, 18 aminoacid-peptide fragment from the carboxyterminal part of the 3rd cytoplasmic loop of the human D_2-dopamine receptor; DSS, disuccinimidyl suberate; [^{125}I]OH-PIPAT, ((+)-trans-7-hydroxy-2-(N-propyl-N-3-[^{125}I]-iodo-2'-propenyl)amino-tetralin); HA, hemagglutinin; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; CaM-kinase IV, Ca^{2+}/calmodulin-dependent protein kinase II.

Figure legends

FIG. 1. Inhibition of cAMP accumulation in HEK293 cells expressing the D_2-dopamine (A) or the Mel1a-melatonin receptor (B). Stably transfected cells were plated in 6-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 calcimycin (3µM). After 15 min reaction was stopped with 2.5% perchloric acid and cAMP was isolated by column chromatography. Shown is the means ± s.e.m from 4 experiments.

FIG. 2. Ca^{2+}/CaM inhibits D_2-dopamine receptor promoted GTP_{\gamma S} binding. A & B, Time course of $[^{35}S]$GTP_{\gamma S} binding to membranes from cells expressing the D_2-dopamine receptor (A) or the A_1-adenosine receptor. B, EGTA-washed membranes (~10 µg) were suspended in 30 µl buffer (25 mM Hapes.NaOH, pH 7.5, 1 mM MgCl_2, 100 mM NaCl, 1 mM EDTA and 0.01 mM GDP in the absence (●) or presence of 0.1 mM CaCl_2 (■) or in the presence of 0.1 mM CaCl_2 plus 1 µM CaM (▲). Following preincubation of the membranes (10 min at 25°C) with agonists (1 µM quinpirol in A, 1µM CPA in B) or antagonists (10 µM sulphirid in A, 1 µM DPCPX in B), the reaction was initiated by adding $[^{35}S]$GTP_{\gamma 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 CaCl_2) on $[^{35}S]$GTP_{\gamma S} binding to membranes containing D_2-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.m. from 3 experiments carried out in duplicate.

**FIG. 3.** The third intracellular loop of the D<sub>2</sub>-dopamine receptor contains in its N-terminal part a CaM binding motif and binds to CaM. A, The peptide sequence (read from the C-terminal end) corresponding to amino-acid residues 208-226 from the D<sub>2</sub>-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 Ca<sup>2+</sup>-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 2 or more of the aligned peptides are highlighted in bold or in italics. B, Binding of the D<sub>2</sub>-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 (1mM) as indicated in the boxes on top of the graph. 30µl 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 nondenaturing gel stained with Coomassie blue. CaM (4µM) was incubated together with the D<sub>2</sub>-receptor derived peptides D2N, D2N' and D2N'' (concentrations as indicated) under the conditions given above.

**FIG. 4.** Binding of D2N to dansylated calmodulin (dansyl-CaM). The fluorescence spectrum of dansyl-CaM was measured on excitation with UV-light (340 nm). Panel A, original traces representing the fluorescence of dansyl-CaM alone (0.4 µM, bottom trace), in the presence of ~1 mM free Ca<sup>2+</sup> (middle trace) or of Ca<sup>2+</sup> plus D2N (1 µM, top trace). Panel B, titration of the effect of D2N on the fluorescence of dansyl-CaM at 495 nm, in the presence of ~1 mM free Ca<sup>2+</sup>. Each set of data shows the fluorescence enhancement at an individual dansyl-CaM concentration present in the cuvette (● = 0.1 µM, ■ = 0.3 µM, ▲ = 0.5 µM). Panel C, the EC<sub>50</sub> values estimated from D2N concentration response curves were plotted against the concentrations at which dansyl-CaM was employed as the fluorescence substrate in the individual experiments (●). Concentration response curves were obtained in the presence of ~1 mM free Ca<sup>2+</sup> except in (○) where no Ca<sup>2+</sup> was added.
**Fig. 5. Inhibition by Ca\(^{2+}/\text{CaM}\) of the D2N-stimulated \[^{35}\text{S}\]GTP\(\gamma\gamma\) binding to G\(\alpha\).** A, the time course of \[^{35}\text{S}\]GTP\(\gamma\gamma\) binding to recombinant G\(\alpha_{i-1}\) (1.3 pmol) was determined in buffer (50 mM Hepes-NaOH, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.01% lubrol and 10 mM MgSO\(_4\)), in the presence of 2 mM CaCl\(_2\) without (●) or with CaM (1µM, ○). B, Binding of \[^{35}\text{S}\]GTP\(\gamma\gamma\) to G\(\alpha_{i-1}\) (0.8 pmol/assay) was determined in the presence of increasing concentrations of D2N in the absence (●) or presence of 0.3 µM (■) or of 1µM CaM (▲); assay conditions were as in A, the reaction was carried out for 15 min. C, Binding of G\(\alpha_{i-1}\) to CaM-sepharose. 20 µl CaM-sepharose pre-loaded with (right) or without 10 µM D2N (left) was incubated with 1.5µg of purified G\(\alpha_{i-1}\) in 100 µl of buffer containing 2 mM MgSO\(_4\), 0.1 mM CaCl\(_2\) and lubrol 0.01%. After 30 min the matrix was collected by centrifugation, washed three times in 100 µl buffer, and finally eluted in SDS-sample buffer. Aliquots of each fraction (supernatant=SN, washes = W and eluate = EL) and 0.5 µg of G\(\alpha_{i-1}\) were analyzed by immunoblotting with a G\(\alpha_{i-1}\)-specific antiserum. In each panel, a typical experiment out of three performed is shown. D, Effect of Ca\(^{2+}/\text{CaM}\) on agonist radioligand binding to the D\(_2\)-dopamine receptor in HEK293 membranes. EGTA-washed membranes (10µg) were incubated with \[^{125}\text{I}\]OH-PIPAT at the indicated concentrations and in the absence (●) or presence of 0.1 mM CaCl\(_2\) (■) or in the presence of 0.1 mM CaCl\(_2\) plus 1 µM CaM (▲). Shown is the specific \[^{125}\text{I}\]OH-PIPAT-binding as defined by sulpirid (10µM). The K\(_D\) values were 1.28 ± 0.54 nM (no CaCl\(_2\)), 1.21 ± 0.37 nM (+CaCl\(_2\)) and 1.14 ± 0.53 nM (+Ca\(^{2+}/\text{CaM}\)). Data are means ± s.e.m. from 3 experiments.

**Fig. 6. Association of CaM with the solubilized D\(_2\)-dopamine receptor.** A, Immunoprecipitation (IP) of the epitope-tagged D\(_2\)-dopamine receptor. Membranes (2 mg) harboring the c-myc-tagged D\(_2\)-receptor were solubilized, incubated with a monoclonal antibody directed against c-myc and a precipitate was collected after the addition of protein G sepharose. The matrix was washed three times, heated in sample buffer and the eluate (EL), the last of three washes (W) and the supernatant (SN) were analyzed by immunoblotting with an anti-c-myc antiserum. As a control, membranes from untransfected cells were subjected to the same procedure; the blot shows the results from the control IP on the left and from the IP of the c-myc-tagged D\(_2\)-receptor on the right. B, The immunoprecipitates obtained as in A were incubated with \[^{125}\text{I}\]CaM, washed and eluted with sample buffer. \[^{125}\text{I}\]CaM in the corresponding aliquots from each fraction was visualized by autoradiography after separation on a polyacrylamide gel. C, Retention of the D\(_2\)-receptor on immobilized CaM. The D\(_2\)-receptor was labeled with the high-affinity radioligand \[^{125}\text{I}\]epideprid, solubilized and
incubated with either CaM-agarose (light grey) or CaM-sepharose (black). Receptor bound radioactivity was quantified as described in “Experimental Procedures” in the supernatant (SN), the wash fraction (W), the eluate recovered after incubation of the matrix with EGTA (E) and by heating the matrix in SDS (M). The results are means ± s.e.m. from three to five experiments. The inset shows an immunoblot where binding of the receptor to CaM-sepharose was verified using a HA-tagged D2-receptor solubilized from COS-7 cell membranes (right lane) as compared to a detergent extract from untransfected cells (left lane).
Fig. 1

A
D₂R

B
Mel₁αR

cAMP (% of maximum)

Quinpirol (nM)

Melatonin (nM)
Fig. 2

A
D$_2$R

B
A$_1$R

C
D$_2$R

GTP$_y$/S bound (fmol)

GTP$_y$/S bound (fmol)

[Ca$^{2+}$/CaM] μM

time (min)

time (min)

**A**

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</table>

**CaM-kinase IV**  
D2N  
NH₂-V Y I K I Y V L R R R K R V N T K

**B**

<table>
<thead>
<tr>
<th>Ca²⁺/CaM DSS</th>
<th>Ca²⁺/CaM D2N</th>
<th>D2N DSS</th>
<th>Ca²⁺/CaM D2N DSS</th>
</tr>
</thead>
</table>

← CaM + D2N  
← CaM

**C**

<table>
<thead>
<tr>
<th>D2N</th>
<th>D2N⁺</th>
<th>D2N++</th>
<th>CONT</th>
</tr>
</thead>
<tbody>
<tr>
<td>4µM</td>
<td>7µM</td>
<td>4µM</td>
<td>7µM</td>
</tr>
</tbody>
</table>

← CaM + D2N  
← CaM
Fig. 4

A

Fluorescence (AU)

Emission (nm)

B

Fluorescence (AU)

0.0 0.5 1.0 1.5 2.0

D2N (µM)

C

EC₅₀ for D2N (µM)

dansylCaM (µM)
Fig. 5

A  
GTPyS bound (fmol)

0  100  200  300  400  500
0  20  40  60  80  100  120
time (min)

B  
GTPyS bound (fmol)

0  150  250  350  450  550
0  0.1  0.3  1  3
D2N (μM)

C  
Space reserved for Western blot (Fig. 5c)

D  
[\textsuperscript{125}I]OH-PIPAT bound (pmol/mg)

0  0.5  1.0  1.5  2.0  2.5
0  1  2  3  4
[\textsuperscript{125}I]OH-PIPAT (nM)
**Fig. 6**

(A) SDS-PAGE gel showing the separation of proteins based on molecular weight. The gel has markers at 220, 97, 66, 46, and 30 kDa. The gel lanes are labeled as control cells and myc-D2R cells.

(B) autoradiogram showing the binding of 
\[^{125}\text{I}\text{CaM}\] to different fractions of cell lysates. The fractions are labeled as SN, W, IP, IP, W, and SN. The bands are visible for both control and myc-D2R cells.

(C) Bar graph showing the percentage of 
\[^{125}\text{I}\text{epideprid}\] bound to different fractions of cell lysates: SN, W, E, and M. The bars are differentiated by color: grey for CaM-Agarose and black for CaM-Sepharose. The y-axis represents the percentage of total bound radioactivity.
Binding of calmodulin to the D2-dopamine receptor reduces receptor signaling by arresting the G protein activation switch
Elisa Bofill-Cardona, Oliver Kudlacek, Qiong Yang, Horst Ahorn, Michael Freissmuth and Christian Nanoff

*J. Biol. Chem.* published online August 3, 2000

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