0026-895X/06/6901-397–405$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 69:397–405, 2006

Vol. 69, No. 1
16725/3070089
Printed in U.S.A.

The Carboxyl Terminus of the Gα-Subunit Is the Latch for Triggered Activation of Heterotrimeric G Proteins

Christian Nanoff, Romana Koppensteiner, Qiong Yang, Elisabeth Fuerst, Horst Ahorn, and Michael Freissmuth

Institute of Pharmacology, Center of Biomolecular Medicine and Pharmacology, Medical University of Vienna (C.N., R.K., Q.Y., E.F., M.F.); and Boehringer-Ingelheim Austria GmbH, Vienna, Austria (H.A.)

Received July 16, 2005; accepted September 30, 2005

ABSTRACT

The receptor-mimetic peptide D2N, derived from the cytoplasmic domain of the D_2 dopamine receptor, activates G protein α-subunits (G_i and G_o) directly. Using D2N, we tested the current hypotheses on the mechanism of receptor-mediated G protein activation, which differ by the role assigned to the Gβγ-subunit: 1) a receptor-prompted movement of Gβγ is needed to open up the nucleotide exit pathway ("gear-shift" and "lever-arm" model) or 2) the receptor first engages Gβγ and then triggers GDP release by interacting with the carboxyl (C) terminus of Gα (the "sequential-fit" model). Our results with D2N were compatible with the latter hypothesis. D2N bound to the extreme C terminus of the α-subunit and caused a conformational change that was transmitted to the switch regions. Hence, D2N led to a decline in the intrinsic tryptophan fluorescence, increased the guanine nucleotide exchange rate, and modulated the Mg^{2+} control of nucleotide binding. A structural alteration in the outer portion of helix α5 (substitution of an isoleucine by proline) blunted the stimulatory action of D2N. This confirms that helix α5 links the guanine nucleotide binding pocket to the receptor contact site on the G protein. However, neither the α-subunit amino terminus (as a lever-arm) nor Gβγ was required for D2N-mediated activation; conversely, assembly of the Gαβγ heterotrimer stabilized the GDP-bound species and required an increased D2N concentration for activation. We propose that the receptor can engage the C terminus of the α-subunit to destabilize nucleotide binding from the "back side" of the nucleotide binding pocket.

Heterotrimeric G proteins transmit signals from cell surface receptors to intracellular effectors. To activate the G protein, the receptor catalyzes the replacement of Mg-GTP for GDP bound to the G protein α-subunit. Because the guanine nucleotide binding pocket is too remote to be directly contacted by the cytoplasmic peptide loops of the receptor, the receptor has to act at a distance (Iiri et al., 1998); however, the exact molecular mechanism is not well defined. The "lever-arm" and "gear-shift" models have been inferred from a structural analogy of the G protein heterotrimer to that of monomeric GTP binding proteins in a complex with their guanine nucleotide exchange factors (Rondard et al., 2001; Cherfils and Chabre, 2003); they predict that the activated receptor (R*) releases GDP by using the βγ-subunit to directly (Rondard et al., 2001) or indirectly (Cherfils and Chabre, 2003) distort the α-subunit switch regions (I and II).

An alternative hypothesis suggests that R* may use a discrete "latch" on the surface of the α-subunit and thus prompt GDP release from the "back side" of the nucleotide binding pocket (Sprang, 1997). There are several candidate regions, each marked by the receptor "footprint" [the carboxyl (C) terminus, the amino (N)-terminal extension, loops connecting helix α4 to strand β6 and α2 to β4, respectively, as well as side chains linking the N terminus to helix α1; cf. Conklin et al., 1996; Bae et al., 1997; Grishina and Berlot, 2000; Blahos et al., 2001; Herrmann et al., 2004], but which of these regions holds the latch is unclear.

On investigating the mechanism of receptor-catalyzed G protein activation one has to bear in mind that the reaction can be divided in two steps, namely, docking of the receptor to the G protein (coupling) and G protein activation (König et al., 1989; Franke et al., 1990; Onrust et al., 1997). If a structural alteration in any of the α-subunit candidate regions reduced receptor-catalyzed activation, this would reflect uncoupling, and, in addition, might indicate that the activating latch had been undone. Hence, reconstitution with a full-length receptor will not allow to distinguish the acti-

This study was supported by grants P14273 and P16083 from Fonds zur Förderung der Wissenschaftlichen Forschung, the Austrian Science Fund.
Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.105.016725.

ABBREVIATIONS: Ca^{2+}/CaM, Ca^{2+}-calmodulin; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; IGF-II, insulin-like growth factor-II; HEDL, HEPES-NaOH/EDTA/dithiothreitol/Lubrol.

vating latch from docking sites on the surface of the α-subunit, except if they do not overlap (Onrust et al., 1997; Grishina and Berlot, 2000).

Using D2N, a receptor-mimetic peptide derived from the N-terminal portion of the third cytoplasmic loop of the D_2-dopamine receptor, we tested the current hypotheses of receptor-catalyzed G protein activation. D2N directly activates G proteins of the G_i/G_o family and reproduces the G protein selectivity of the D_2 receptor (Voss et al., 1993). There is an additional argument for concluding that the stimulatory activity of D2N is not an accidental property: D2N also represents the receptor segment that binds Ca^{2+}-calmodulin (Ca^{2+}/CaM), and Ca^{2+}/CaM inhibits the regulatory action of the receptor and of D2N with identical affinity (Bofill-Cardona et al., 2000).

Our data suggest that the action of D2N can be integrated into a sequential fit-model where the Gα C-terminal tail interacts with R* to catalyze guanine nucleotide exchange, and this occurs consecutively with the initial encounter between R* and the G protein (Herrmann et al., 2004). Thus, interaction of a single cytoplasmic receptor loop with the α-subunit C terminus is sufficient to enhance the guanine nucleotide exchange reaction; the G protein βγ-subunit, although essential for efficient R/G coupling, is not required to open up the nucleotide exit pathway.

Materials and Methods

Materials. D2N and peptides derived from the same sequence were synthesized by the solid phase method as described previously (Voss et al., 1993). Peptides derived from the C terminus of $G\alpha_{o3}$ (CDIIIADNLRGCGLY; in $G\alpha_{i1}$, TDVIIKNNLKDCGLF) and $G\alpha_q$ (CLQLNLKEYNLV) were gifts from Dr. Bernd Nuernberg (University of Duesseldorf, Duesseldorf, Germany).

The polyclonal antibodies sc-262 and sc-392 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). sc-262 is directed against the C-terminal peptide sequence in $G\alpha_{i3}$ (KNNLKECGLY), which is cross-reactive with $G\alpha_{i1}$ (KNNLKDCGLF); sc-392 is directed against 19 amino acids within the extreme C terminus of $G\alpha_{q/11}$. Radiochemicals were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Restriction enzymes and adenine and guanine nucleotides were from Roche Molecular Biochemicals (Mannheim, Germany).

Plasmids were purified with the use of kits from QIAGEN (Valencia, CA), hexa-histidine-tagged proteins with a cobalt-chelating affinity matrix (Talon; BD Biosciences, San Jose, CA). Oligonucleotides were obtained from GenXpress (Maria Wörth, Austria); the integrity of the DNA constructs was verified by fluorescent sequencing. Pertussis toxin, calmodulin, and 5-(dimethylamino)-1-naphtalenesulfonyl (dansyl) chloride were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of the highest purity grade available.

Production and Purification of G Protein Subunits. Recombinant G protein α-subunits were produced in *Escherichia coli* and purified from bacterial lysates. Myristoylated $G\alpha_{i1}$ was generated in the B-MT strain and purified from lysates essentially as described previously (Mumby and Linder, 1994). N-Terminally truncated versions of $G\alpha_{i1}$ were created by primer-directed amplification of the rat $G\alpha_{i1}$ cDNA; a hexa-histidine tag was appended to the C terminus by subcloning into a pQE-60 vector, and the *E. coli* strain BL21(DE3) was transformed with the respective constructs.

To generate N-terminally truncated versions of full-length $G\alpha_{i1}$, a KpnI restriction site was inserted 5′ upstream to the ATG start codon (oligonucleotide sequences available on request); the N-terminal peptide sequence is rather divergent between subtypes up to position 35 in $G\alpha_{i1}$ from where a conserved sequence links the N-terminal extension to the G1 region. The reverse primer covered a BamHI restriction site present in the sequence of α_{i1} (codons 211–213).

The truncated constructs $\alpha_i\Delta17$ and $\alpha_i\Delta30$ could be expressed in *E. coli* to substantial amounts of protein (expression of proteins corresponding to $\alpha_i\Delta27$ and $\alpha_i\Delta34$ was not detected). Site-directed mutagenesis to introduce a proline in position 343 was performed on $\alpha_i\Delta17$ using the QuikChange kit from Stratagene (La Jolla, CA). Specific activity of Gα preparations was assessed by binding of [^{35}S]GTPγS in the presence of 10 mM $MgSO_4$. Purification of Gβγ from porcine brain extracts was carried out essentially as described previously (Nanoff et al., 1997).

Binding of [^{35}S]GTPγS, [α-^{32}P]GDP, and Hydrolysis of [γ-^{32}P]GTP. For measuring concentration-dependent effects of D2N and D2N-derived peptides, G protein α-subunit (1–10 pmol/assay, alone or mixed with purified brain Gβγ) was taken up in HEDL [50 mM HEPES-NaOH, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Lubrol (detergent, formula not disclosed)], and a Mg^{2+} salt was added as indicated. After a short preincubation of G protein with peptide on ice, the binding reaction was started by the addition of warmed radioligand solution containing [^{35}S]GTPγS (30 nM-1 μM final concentration; specific activity 1000 cpm/fmol at low and 8000 cpm/pmol at high GTPγS concentrations) and terminated after 2 min (stop solution: 25 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, and 1 μM GTPγS). Bound and free radioactivities were separated by filtration over nitrocellulose filters (BA 85; Millipore Corporation, Billerica, MA). Association and dissociation time courses were performed on 0.1 to 0.5 pmol of purified α-subunit, and GTPγS (1 μM) was used as the trapping ligand.

Release of prebound GDP and steady-state GTPase activity were determined essentially as described by Ross and Higashijima (1994). When measuring the concentration-dependent effect of D2N, dissociation of [α-^{32}P]GDP was initiated by diluting [α-^{32}P]GDP-loaded α-subunit in HEDL buffer containing D2N at the indicated concentrations plus $MgCl_2$ (2 mM) and 100 μM GDP. The reaction was terminated after 3 min at 28°C with ice-cold stop buffer. Steady-state GTPase activity was measured using purified G protein α-subunit (5–10 pmol/reaction), the indicated concentrations of D2N, and buffer (HEDL plus 2 mM $MgCl_2$). The reaction was started by the addition of substrate GTP (0.5 μM [γ-^{32}P]GTP, specific activity ~100,000 cpm/pmol) and carried out for 2 min at 28°C in a volume of 50 μl. For the determination of concentration-dependent GTP turnover rates, between 0.2 (at a concentration of 30 or 100 nM GTP) and 1 pmol (at 300 or 1000 nM GTP) of α-subunit was used. The assay volumes were adjusted to between 40 and 120 μl to allow for substantial excess of substrate over enzyme. The specific activity of [γ-^{32}P]GTP was between 20 and 200 cpm/fmol, and initial turnover rates were assessed by five determinations over a time course of 15 min.

Fluorescence Spectroscopy. Fluorescence emission was recorded in a Hitachi F4500 spectrofluorometer at 18°C. The assay volume amounted to 0.4 ml, composed of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, and 0.01% Lubrol; $G\alpha_{i1}$ was included at a concentration of 0.3 μM. For recording changes in intrinsic tryptophan fluorescence, the excitation wavelength was 290 nm; five emission wavelength scans were recorded (scanning time 6 s) successively and averaged. Fluorescence changes of dansylated calmodulin were measured as described by Bofill-Cardona et al. (2000).

Miscellaneous Procedures. Native gel electrophoresis of calmodulin in complex with D2N or a D2N analog was carried out as described in Bofill-Cardona et al. (2000). Pertussis toxin-catalyzed ADP-ribosylation of G_i was done as in Nanoff et al. (1995). The final concentration of pertussis toxin was 10 μg/ml (~0.13 μM).

Results

Activation of Gα by D2N. The 19-amino acid peptide D2N derived from the third cytoplasmic loop of the D_2-dopamine receptor regulates the activity of the G protein α-subunit. Addition of the peptide enhances the release of GDP from the α-subunit of G_i (Fig. 1A) and G_o (Fig. 1B). At a concentration of 1 μM D2N, the increment in the GDP dissociation rate was greater with $G\alpha_{i1}$ (from 0.024 ± 0.004 to 0.167 ± 0.031/min) than with $G\alpha_o$ (from 0.10 ± 0.01 to 0.21 ± 0.04/min), but the stimulated release rates were comparable between the two subtypes.

Three assays were used to measure the concentration dependence and the magnitude of the D2N effect, namely, release of [α-^{32}P]GDP, binding of [^{35}S]GTPγS, and release of [^{32}P]phosphate upon GTP hydrolysis. The data obtained by measuring the release of [α-^{32}P]GDP at an early time point could be fitted to a monophasic curve (Fig. 2, ●) and gave a half-maximal D2N concentration of ~1 μM for both $G\alpha_{i1}$ and $G\alpha_o$ (Table 1). In the two assays that relied on the binding of GTP, however, the curves were bell-shaped (Fig. 2, □, ▼). If assessed at early time points, the magnitude of D2N stimulation was pronounced, typically leading to a 10-fold increase in the binding of GTPγS and somewhat less in the GTP turnover number. As can be seen in Fig. 2, the maximum was reached between 3 and 10 μM D2N in the biphasic curves, indicating that the apparent potency was lower than in the GDP release experiment. The bell-shaped curves obtained in the GTPγS binding and GTP turnover experiments suggest that at high concentrations, D2N impeded the binding of GTP, a property shared with several other peptides and receptor-mimetic lipoamines (Okamoto and Nishimoto, 1992; Ross and Higashijima, 1994; Breitweg-Lehmann et al., 2002). We made sure in GTPγS uptake experiments that the binding of D2N was revertible and did not denature the protein (data not shown). The bell shape of the concentration-response curves has been attributed to the hydrophobic character of the compounds (Taylor et al., 1994); an alternative explanation may be related to the Mg^{2+} dependence of peptide- and receptor-catalyzed G protein activation that was found to exhibit a similar biphasic pattern (Higashijima et al., 1990; Senogles et al., 1990).

Mg^{2+} exerts multiple effects on G protein activity. Very low (approximately nanomolar) concentrations are required for GTP hydrolysis; at higher concentrations Mg^{2+} supports receptor-catalyzed G protein activation and, in addition, may reduce the spontaneous GDP off rate (in $G\alpha_o$; Higashijima et al., 1987). We examined the way in which Mg^{2+} modulates D2N-mediated G protein activation. The results are compatible with a receptor-type mode of action and similar to those obtained with mastoparan (Higashijima et al., 1990).

If the guanine nucleotide exchange reaction was followed over time by measuring the association of GTPγS (Fig. 3A), Mg^{2+} led to decreased association rates. These were observed both in the presence of D2N (k_{app} = 0.23 ± 0.05/min with $MgCl_2$ versus 0.48 ± 0.08/min without $MgCl_2$) and in its absence (0.035 ± 0.003/min versus 0.1 ± 0.03/min; n = 3 experiments carried out in parallel). However, inclusion of 1 mM $MgCl_2$ did not affect the -fold increase because of D2N. A second finding is that the addition of both D2N and Mg^{2+} enhanced the amount of GTPγS binding (Fig. 3A, ▼). It is most likely that D2N—just as the activated receptor—reduced the Mg^{2+} concentration required for GTPγS binding (Senogles et al., 1990).

D2N caused a global decrease in guanine nucleotide affinity, and this, too, is in keeping with its receptor-mimetic action (Posner et al., 1998). As can be seen in Fig. 3B, D2N also accelerated the dissociation of prebound GTPγS in the absence of Mg^{2+}. The dissociation of GTPγS proceeded at a rate that was similar to the association rate (0.6/min; Fig. 3B, ▽). If Mg^{2+} was present when the dissociation was initiated, no significant release of GTPγS occurred (▼).

The inhibitory Mg^{2+} effect on guanine nucleotide exchange was further assessed in short-term (2 min) GTPγS binding experiments. Figure 4 shows that activation by D2N was gradually reduced by increasing Mg^{2+}; in the lower concentration range (≤10 mM), the inhibition was overcome by increasing D2N (Fig. 4A). If the data were plotted versus the Mg^{2+} concentration, it became evident that the apparent

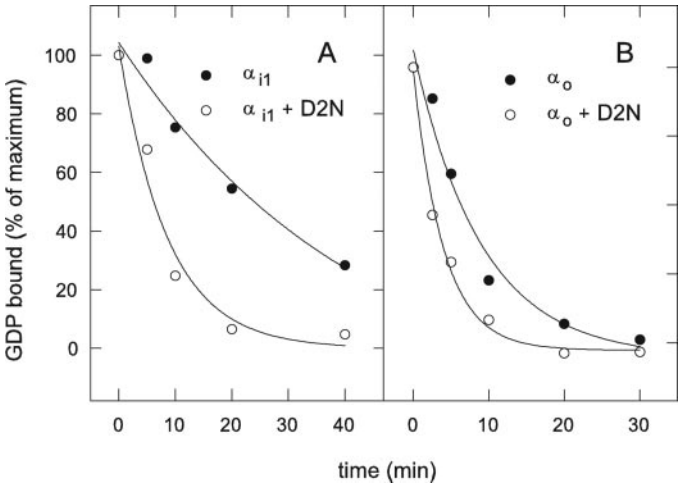

Fig. 1. D2N stimulates the release of [α-^{32}P]GDP from $G\alpha_{i1}$ (A) and $G\alpha_o$ (B). Recombinant myristoylated α-subunit (10 pmol) was incubated with [α-^{32}P]GTP (1 μM) for 30 min; dissociation of nucleotide was initiated by a 60-fold dilution in buffer with (○) or without (●) 3 μM D2N. The reaction was stopped at the indicated time points by the addition of ice-cold stop buffer with a fluoroalumino complex.

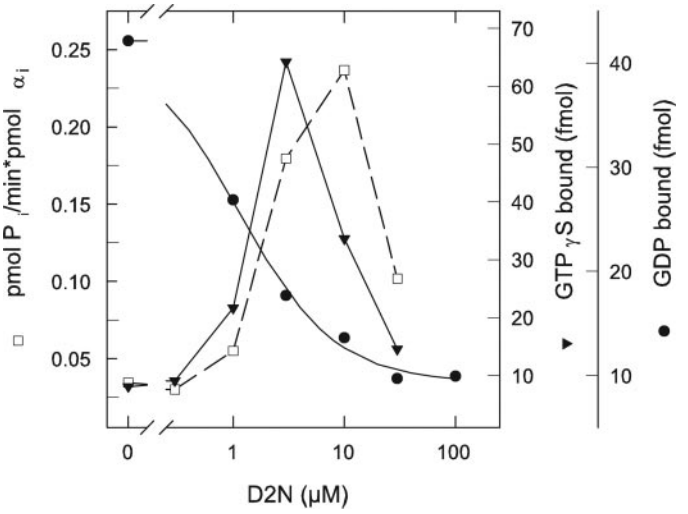

Fig. 2. Concentration dependence of the effect of D2N. The GDP/GTP exchange reaction was assessed by measuring in parallel the dissociation of [α-^{32}P]GDP (○; 3 min), the binding of [^{35}S]GTPγS (▼; 2 min), and the release of [^{32}P]phosphate upon hydrolysis of [γ-^{32}P]GTP (□; 2 min). The experiments were carried out in the presence of 1 mM free $MgCl_2$.

potency of Mg^{2+} was lower at higher D2N concentrations (Fig. 4B). In the absence of D2N, the inhibition occurred at less than 1 mM Mg^{2+}, but in the presence of D2N higher concentrations were needed; inhibition was maximal at 10 mM Mg^{2+} (Fig. 4B). Thus, D2N reduced 1) the Mg^{2+}-dependent inhibition of GDP release from Gα (see also Fig. 5B) and 2) the Mg^{2+} requirement for the binding of GTPγS. Our data confirm what had been observed with mastoparan on Gα_o (Higashijima et al., 1990): Mg^{2+} is not necessary for the triggered release of nucleotide, but it is necessary for the conversion to the active GTP-bound form.

Mutation of the D2N Sequence. The action of D2N cannot be accounted for by nonspecific stimulation because of the polybasic composition of the peptide. First, D2N did not stimulate Gα_s at concentrations up to 100 μM (data not shown). Second, the D2N-peptide sequence comprises a CaM-binding motif that conforms to a reversed 1-8-14 pattern; binding of CaM blocked the action of D2N and likewise that of the receptor (Bofill-Cardona et al., 2000). To differentiate binding to CaM from that to Gα, we mutated the motif-flanking residues in D2N (Table 1, positions 1 and 14 shaded) to alanine. In addition, neighboring basic residues were also replaced by alanine. Reducing the hydrophobicity invariably lowered the affinity for Gα, both Gα_{i1} and Gα_o (peptide 2A in Table 1) but not for CaM (data not shown). Likewise, arginine in position 2 was crucial with peptide 3A displaying a further 3-fold reduction in apparent G protein affinity. In contrast, substituting the lysine residue in position 13 (peptide 4A) or lysine in position 3 (peptide 4A′) did not result in an additional decrease in affinity (cf. peptide 2A). The affinity of all D2N-derived peptides for Ca^{2+}/CaM (assessed by fluorescence change of dansyl-CaM, Table 1; and by native gel electrophoresis of peptide CaM complexes, data not shown) was comparable with—or even higher than—that of the parent peptide (Table 1). The affinity for CaM only dropped by >10-fold when an aspartate residue had been introduced to replace leucine in position 8 (data not shown).

Monitoring the D2N-Induced Conformational Switch by Fluorescence Spectroscopy. Gα_{i1} contains three tryptophan residues. One is located in the switch II region (W^{211}), the fluorescence of which is sensitive to the conformational state (summarized by Sprang, 1997). Fluorescence emission increases upon binding of Mg-GTP and thus reflects the active state. Conversely, the release of prebound GDP is expected to affect the conformation of the switch II region. As shown in Fig. 5, the action of D2N was detectable by recording the intrinsic tryptophan fluorescence of Gα_{i1}. Addition of D2N led to a concentration-dependent decline in fluorescence emission, indicating that the tryptophan residue became more exposed to the aqueous solvent (Fig. 5, A and B). The emission data could be fitted to a monophasic curve where the half-maximal decrement was reached at a concentration of ~0.5 μM (Fig. 5B, ○). The decrease in tryptophan fluorescence is likely to result from

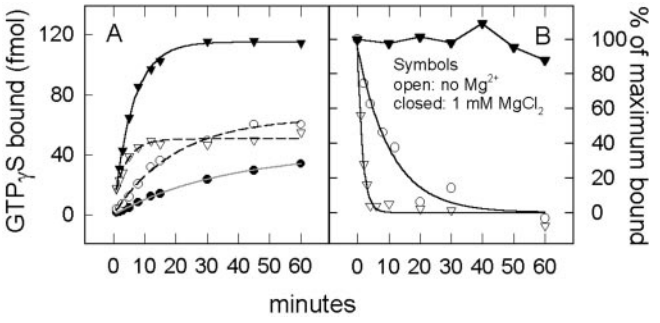

Fig. 3. Interfering effects of Mg^{2+} and D2N on the binding GTPγS. Association of GTPγS with (A) and dissociation of GTPγS from (B) Gα_{i1} in the absence (open symbols) or presence of $MgCl_2$ (1 mM free concentration, closed symbols). The experiments were performed with the addition of D2N (▼, ▽; 1 μM) or without (○, ●); in the presence of Mg^{2+}, GTPγS was not released (data obtained without D2N not shown). The experiments shown are representative of three performed.

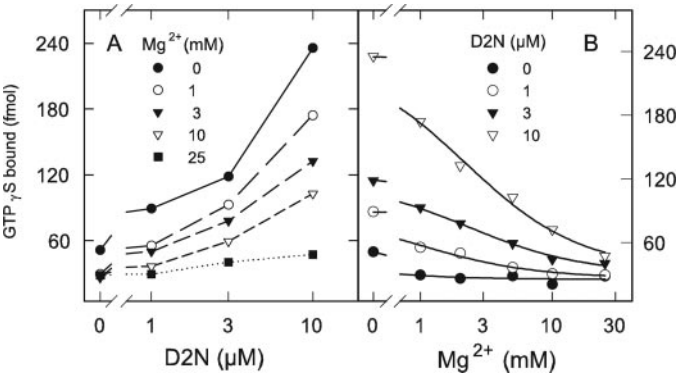

Fig. 4. Short-term GTPγS binding (2 min) to assess the inhibitory effect of Mg^{2+} on guanine nucleotide exchange. GTPγS binding was determined at increasing concentrations of D2N and of Mg^{2+}. The GTPγS binding data are plotted versus the concentration of D2N (A) or versus the concentration of Mg^{2+} (B). The Mg^{2+} counter ion was SO_4^{2-}, which was held constant at a concentration of 50 mM. The Mg^{2+} effects could not be reproduced with Ca^{2+} or Mn^{2+}.

TABLE 1

Sequence and potency of D2N and D2N-derived peptides

Sequence of D2N and peptides obtained by D2N sequence permutation. Arrows and asterisks (*) indicate the position of amino acid variation; asterisks mark the flanking residues of the putative CaM-binding motif, the amino acid positions of which are numbered in the top row. Right-hand columns give affinity estimates for the interaction with Gα_{i1}, Gα_o, and with Ca^{2+}-activated calmodulin. The EC_{50} values ± S.E.M.) were estimated from concentration-response curves in GDP release experiments (Gα_{i1} and Gα_o) or by fluorescence changes of dansyl-CaM.

			14	13	12	11	10	9	8	7	6	5	4	3	2	1				EC_{50}		
																				$G\alpha_{I1}$	$G\alpha_o$	d-CaM
																				μM		
Pept.			*	↓										↓	↓	*						
D2N	V	Y	I	K	I	Y	I	V	L	R	R	R	R	K	R	V	N	T	K	1.0 ± 0.1	0.8 ± 0.1	0.7 ± 0.2
2A	V	Y	A	K	I	Y	I	V	L	R	R	R	R	K	R	A	N	T	K	16.9 ± 15.2	4.8 ± 1.0	0.38 ± 0.09
3A	V	Y	A	K	I	Y	I	V	L	R	R	R	R	K	A	A	N	T	K	48.8 ± 18.0	10.2 ± 5.9	0.27 ± 0.04
4A	V	Y	A	A	I	Y	I	V	L	R	R	R	R	K	A	A	N	T	K	7.7 ± 3.4	3.0 ± 0.8	0.13 ± 0.11
4A′	V	Y	A	K	I	Y	I	V	L	R	R	R	R	A	A	A	N	T	K	51.8 ± 14.6	4.5 ± 1.5	0.24 ± 0.03

Pept., peptide.

the conformational change that favors GDP release. Therefore, the D2N effect was abolished by the inclusion of a high concentration of GDP (Fig. 5B, bottom inset). As predicted by the experiments shown in Figs. 3 and 4, the addition of Mg^{2+} resulted in a right shift of the concentration-response curve (Fig. 5B, ●). When titrating the effect of D2N, we made sure that fluorescence emission was stable before each recording. We can also rule out that longer incubation times in the presence of D2N led to denaturing of the protein because a 1-h preincubation with D2N at 18°C followed by the addition of GTPγS gave a robust increase in tryptophan fluorescence (data not shown).

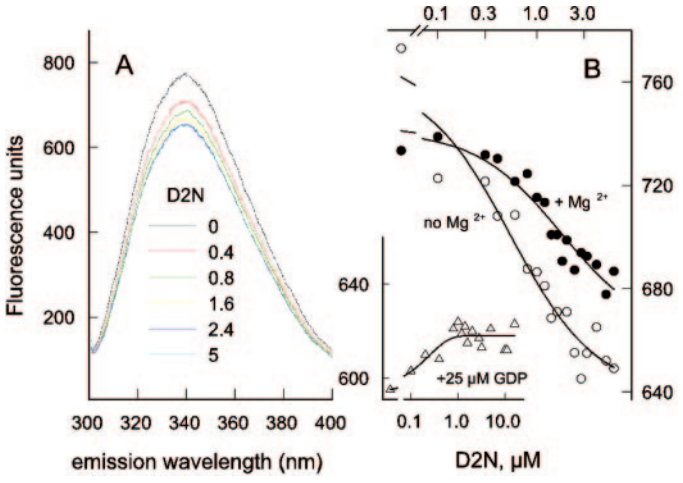

Fig. 5. Intrinsic tryptophan fluorescence of $G\alpha_{i1}$ recorded in the presence of increasing D2N. D2N-peptide was mixed in stepwise, in volumes of 0.1% of the final volume, and recordings were performed in 3-min intervals. A, emission traces from wavelength scans recorded upon the addition of D2N at the indicated concentrations (in micromolar). B, maximum emission (at ~340 nm) from wavelength scans recorded with (●) or without (○) $MgCl_2$ (10 mM). For the experiment depicted in the bottom inset, GDP was added to a final concentration of 25 μM (△). The lower emission in the presence of GDP was due to UV absorbance by the guanine nucleotide.

Role of the α-Subunit N Terminus and of Gβγ in D2N-Mediated G Protein Activation. The N terminus of the α-subunit is thought to have an important part in receptor interaction and has been implicated in triggered G protein activation. Both a receptor-mimetic peptide isolated from the third loop of the α_2-adrenergic receptor (α_2i3c in Tables 2 and 3; Taylor et al., 1994) and mastoparan (Higashijima and Ross, 1991) were cross-linked to the N terminus of the α-subunit; moreover, for activation by mastoparan the N terminus was essential. Because of its flexibility, the N-terminal extension has also been integrated as a lever-arm into the model of receptor-mediated G protein activation (Iiri et al., 1998; Cherfils and Chabre, 2003). We have therefore tested whether the α-N terminus was required for the D2N-mediated guanine nucleotide exchange. The experiments depicted in Fig. 6 compared $G\alpha_{i1}$ to an N-terminally truncated protein ($\alpha_i\Delta30$) in which the entire subtype-specific N terminus had been deleted; in addition, $\alpha_i\Delta30$ lacked the N-terminal myristate and was engineered to include a C-terminal hexa-histidine tag. The truncated protein demonstrated a basal exchange rate virtually identical (0.041/min) to that of full-length myristoylated $G\alpha_{i1}$. As shown by two representative experiments using GTP turnover (Fig. 6A) and fluorescence emission (compare Fig. 6B with 5B), $\alpha_i\Delta30$ was activated by D2N in a manner indistinguishable from that of the full-length protein. Average maximal activation of GTP turnover was similar in $\alpha_i\Delta30$ (8.9 ± 1.3-fold over basal, means ± S.E., three experiments performed in parallel) and full-length $G\alpha_{i1}$ (6.9 ± 1.3). Thus, we did not find any appreciable defect upon N-terminal truncation of the protein.

According to the lever-arm and gear-shift models, the receptor uses Gβγ to open the guanine nucleotide binding pocket and to release GDP. D2N, however, can act on Gα alone, and, in fact, when Gβγ is added to the reaction, it suppresses the stimulatory effect of D2N. Figure 7 shows GTPγS binding experiments performed on the heterotrimer. To rule out that an effect by Gβγ is caused by scavenging of

TABLE 2

Alignment of peptide activator sequences

Sequences of peptide G protein activators derived from the third (i3) or second (i2) cytoplasmic loop of the α2-adrenergic, D_2 dopamine, and M_4 muscarinic receptor of mastoparan (MP) and of a peptide fragment of the insulin-like growth factor II receptor (IGF II-R). Segments taken from the C-terminal loop portion are given in the inverse orientation (c→n); similarity to D2N (D_2i3n) is remote for the amino acids indicated by bold text.

α_2i2				**R**	**Y**	**W**	**S**	**I**	**T**	**Q**	**A**	**I**	**E**	**Y**	**N**	**L**	**K**	**R**	**T**	**P**	**R**	**R**
M_4i2				**R**	**Y**	**F**	**C**	**V**	**T**	**K**	**P**	**L**	**T**	**Y**	**P**	**A**	**R**	**R**	**T**	**T**	**K**	
α_2i3n				R	I	Y	Q	I	A	K	R	R	T	R								
D_2i3n	V	Y	I	K	I	Y	I	V	L	R	R	R	R	K	R	V	N	I	K			
M_4i3c c→n	**R**	**T**	**V**	**K**	**R**	**E**	**R**	**A**	**A**	**M**	**Q**	R	K	K	R	V	Q	N	R			
α_2i3c c→n						**F**	**T**	**F**	**R**	**K**	**E**	R	N	Q	R	G	R	W	R			
IGF II-R				**R**	**V**	**G**	**L**	**V**	**R**	**G**	**E**	**K**	**A**	**R**	**K**	**G**	**K**					
MP				**I**	**N**	**L**	**K**	**A**	**L**	**A**	**A**	**L**	**A**	**K**	**K**	**I**	**L**					

TABLE 3

Activation characteristics of activator peptides

G protein activation characteristics of peptide activator sequences. "Gβγ required" indicates whether addition of Gβγ was needed for full stimulation by the peptide.

Peptide	Selectivity	Docking Site	Gβγ Required	Reference
α_2i2	Gi>>Go,Gs	N.D.	N.D.	Okamoto and Nishimoto (1992)
M_4i2	Gi,Go>Gs	N.D.	N.D.	
α_2i3n	Gi,Go,Gs	N.D.	N.D.	
M_4i3c	Go,Gi>Gs	N.D.	N.D.	
D_2i3n	Go,Gi>>Gs	C terminus	No	This report
α_2i3c c→n	Gi,Go	N terminus	Yes	Taylor et al. (1994)
IGF-II	Gi	C terminus	Yes	Okamoto and Nishimoto (1991)
Mastoparan	Go>Gi >>Gs	N and C terminus	Yes	Weingarten et al. (1990), Higashijima and Ross (1991)

N.D., not determined.

the peptide by $\beta\gamma$ (or contaminants), we examined the D2N effect on the heterotrimer composed of full-length $G\alpha_{i1}$ (Fig. 7A) and on the heterotrimer composed of an N-terminally truncated version ($\alpha_i\Delta17$; Fig. 7B). By gauging the effect of $G\beta\gamma$ on pertussis toxin-mediated ADP-ribosylation, we found that the affinity of $\alpha_i\Delta17$ for $G\beta\gamma$ was lower than that of the full-length protein by approximately an order of magnitude but sufficient to support heterotrimer formation (data not shown; cf. Graf et al., 1992). If purified $G\beta\gamma$ was included, activation by D2N at low concentrations was reduced. With full-length $G\alpha_{i1}$, this inhibition was observed at an excess (□) and a substoichiometric amount of $G\beta\gamma$ (△). The concentration-response curve shifted to the right even when the concentration of $\beta\gamma$ was low. If instead truncated $\alpha_i\Delta17$ was used, $G\beta\gamma$ caused a shift only at an excess (□) but not a substoichiometric amount (Fig. 7B, △). Hence, the affinity of the α-subunit for $G\beta\gamma$ determined the decrease in sensitivity to D2N; stabilization of the inactive state was overcome by increasing D2N.

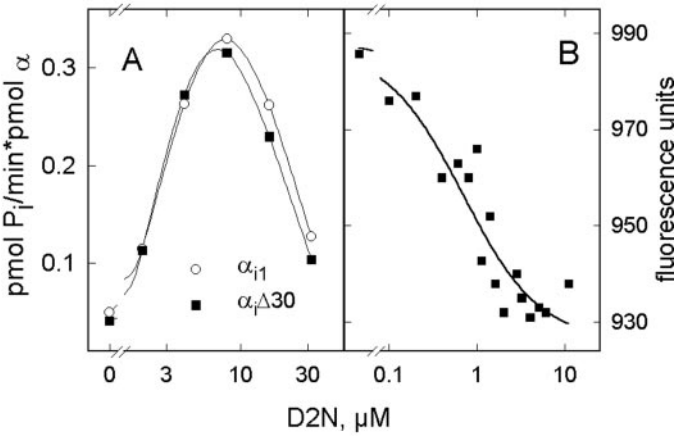

Fig. 6. Truncation of the $G\alpha_{i1}$ N terminus by 30 amino acids. A, D2N-mediated activation of the guanine nucleotide exchange in the truncated α-subunit (■), compared with full-length myristoylated $G\alpha_{i1}$ (○). Shown are typical activation patterns determined by [^{32}P]phosphate release. B, D2N-induced decrease of the intrinsic tryptophan fluorescence of $\alpha_i\Delta30$, determined as in Fig. 5 and in the absence of Mg^{2+}. The experiment shown was repeated once with similar results.

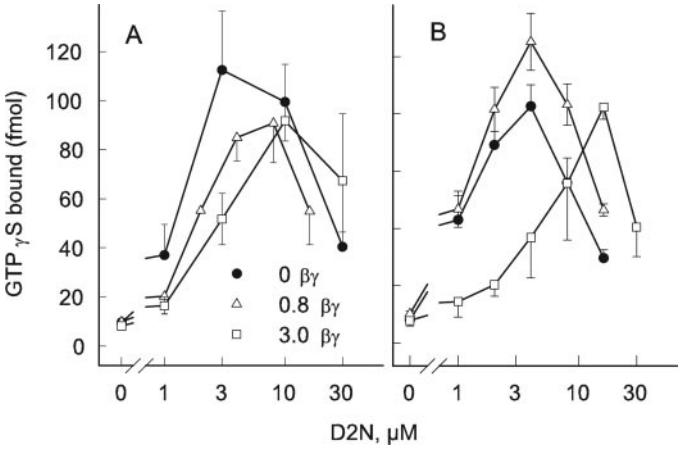

Fig. 7. $G\beta\gamma$ inhibits D2N activation of $G\alpha$. $G\alpha_i$ (A, $G\alpha_{i1}$; B, $\alpha_i\Delta17$) was combined with a buffer control (HEDL plus 2 mM $MgCl_2$; ●) and a substoichiometric (0.8; △) and an excess (3-fold; □) molar amount of $G\beta\gamma$, and D2N-stimulated [^{35}S]GTPγS binding was measured for 2 min. Mean values (± S.E.M.) are representative of four experiments.

Mapping the D2N Binding Site. The C-terminal end—at least the last five amino acids—of $G\alpha_i$ are required for recognition by pertussis toxin (Scheuring et al., 1998), which uses the cysteine residue at position −4 as the substrate for ADP-ribosylation. We exploited the ADP-ribosylation reaction to examine whether in the heterotrimer, D2N bound to the $G\alpha$ C terminus. If so, D2N should diminish ADP-ribosylation by pertussis toxin; Fig. 8A shows that this was indeed the case. D2N decreased ADP-ribosylation—in the presence of excess $G\beta\gamma$—of both full-length and truncated $\alpha i\Delta17$.

To confirm the C terminus as the D2N binding site, we used an antibody directed against the last 10 amino acids of $G\alpha_i$. Inclusion of the antibody in the GTPγS-binding assay caused the activation shift to higher concentrations of D2N; increasing the antibody concentration led to a further rightward shift (Fig. 8B). The antibody alone did not affect the basal guanine nucleotide exchange rate. In the reaction volume, the IgG concentration was kept constant to avoid effects due to adsorption of D2N. Because the extent of activation and the shape of the curve were not affected, we conclude that the antibody blocked D2N in a competitive manner. Likewise, a 15-mer peptide derived from the extreme C terminus of α_o antagonized D2N (Fig. 8C). Inclusion of the peptide led to a rightward shift of the D2N activation curve (not shown). By contrast, a C-terminal peptide from α_q (Fig. 8C) and an antibody directed to the C terminus of α_q (data not shown) had very little effect. Thus, the C-terminal end is likely to be the D2N docking site; however, we cannot rule out that D2N also attaches to surface patches in the vicinity of the C terminus.

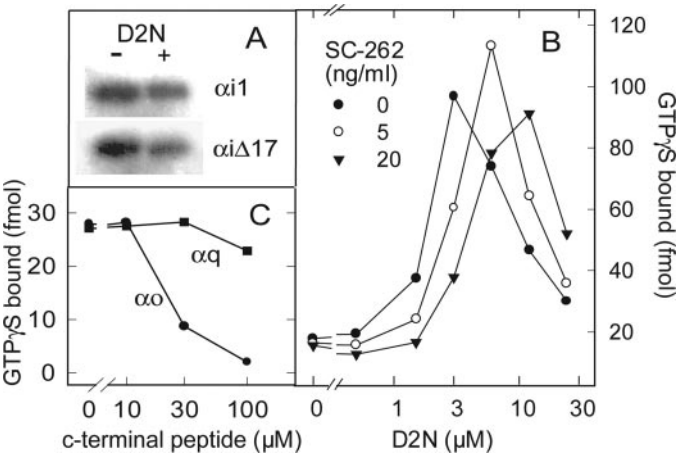

Fig. 8. D2N binds to the α-subunit C terminus. A, D2N inhibits pertussis toxin-mediated ADP-ribosylation. Incubation with pertussis toxin (10 μg/ml) was carried out with $G\alpha_{i1}$ (0.6 pmol; A) or $\alpha_i\Delta17$ (2 pmol; B) combined with $G\beta\gamma$, which was in excess over α ($\beta\gamma/\alpha_{i1}$, ~3; $\beta\gamma/\alpha_i\Delta17$, ~5). The + indicates inclusion of D2N (3 μM). Band representation was electronically adjusted—according to the staining obtained in the absence of D2N—to approximately comparable levels with α_{i1} and $\alpha_i\Delta17$. The experiment was repeated once with similar results. B, antibody blockage of the C terminus. GTPγS binding was determined in the absence (●) or presence of a polyclonal antibody (○, ▼) directed against the $G\alpha_i$ C terminus (for the epitope, see *Materials and Methods*). The antibody was added at two concentrations, and the amount of IgG was held constant throughout at a concentration of 20 ng/ml by complementation with irrelevant IgG. The experiment shown is representative of three performed. C, inhibition of D2N by a C-terminal peptide derived from $G\alpha_{o3}$ (●) and from $G\alpha_q$ (■). Short-term GTPγS binding to $G\alpha_{i1}$ (0.5 pmol) was assayed in the presence of 1 μM D2N and of the C-terminal peptides at the indicated concentrations. The experiment was repeated once.

Helix α5 connects the Gα C terminus to the guanine nucleotide binding pocket. When bound to the receptor, the extreme C-terminal residues adopt an α-helical conformation (Kisselev et al., 1998). Thus, they can be viewed as a continuous extension of helix α5. A kink engineered in the outer portion of helix α5 (by substituting proline for an isoleucine) disrupted rhodopsin activation of transducin (Marin et al., 2002). This suggests that the C-terminal helix is a key structure in receptor-dependent activation.

To test whether D2N—upon binding to the extreme C terminus—prompts guanine nucleotide exchange by a rhodopsin-related mechanism, we mutated an analogous isoleucine (at position 343) in Gα_{i1}. As can be seen in Fig. 9A, the mutant Gα_{i1} was less sensitive to D2N, but the affinity was unchanged compared with an α-subunit bearing the wild-type sequence. Maximal activation of the turnover rate (determined by GTPase assays) was 6.2-fold over basal (95% confidence interval = 5.1 to 7.3) with the original sequence and 3.9-fold with the mutant (95% confidence interval = 3.1 to 4.7; n = 3 experiments performed in parallel). Thus, the proline-induced structural alteration in α5 reduced activation by D2N in a manner that could not be overcome by increasing D2N.

Compared with Gα_{i1}, intrinsic activation of transducin α is sluggish. It has been proposed that this is partly because of the isoleucine at issue (339; see Muradov and Artemyev, 2000). However, substitution by proline of the corresponding isoleucine in Gα_{i1} did not affect the spontaneous exchange rate (0.22 ± 0.01 with 1 mM free $MgCl_2$). However, when the intrinsic GTP turnover rate was determined at lower substrate concentrations (≤1 μM), it became apparent that the mutation resulted in a reduced affinity for GTP. Figure 9B shows a Lineweaver-Burk transformation of the initial GTP turnover rates determined at various GTP concentrations in the absence of D2N. The apparent affinity for GTP was reduced by approximately 1 order of magnitude compared with $\alpha_i\Delta17$, whereas the results with the latter were indistinguishable from that obtained with full-length myristoylated Gα_{i1} (data not shown). To overcome the affinity difference, the experiment shown in Fig. 9A was carried out at a high GTPγS concentration (1 μM) at which the spontaneous exchange rate was comparable with the protein with the wild-type sequence. Thus, the structural derangement of helix α5 in Gα_{i1} reduced the activity of D2N and, in addition, the affinity for GTP, confirming that helix α5 impinges on the conformation of the guanine nucleotide pocket.

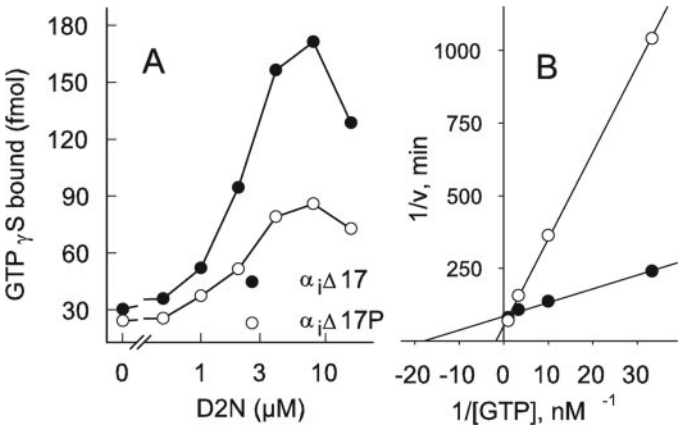

Fig. 9. D2N engages helix α5 to stimulate the guanine nucleotide exchange. A, effect of a mutation in helix α5 (I343P) on D2N activation. Shown are typical D2N concentration-dependent GTPγS binding patterns for $\alpha_i\Delta17$ (●) and $\alpha_i\Delta17P^{343}$ (○); the experiment was carried out for 2 min at a final concentration of 1 μM GTPγS and 1 mM free Mg^{2+}. B, double reciprocal transformation of the GTP hydrolysis rates obtained with $\alpha_i\Delta17$ and $\alpha_i\Delta17P^{343}$ in the absence of D2N. Initial-rate constants were determined at four GTP concentrations in two independent experiments. The K_m value for GTP is estimated from the regression line intercept with the x-axis.

Discussion

The α-Subunit C-Terminal Region Transmits the Activating Impulse. In the present report, we show that D2N, a peptide from the N-terminal portion of the third loop of the D_2 dopamine receptor, directly activated G protein α-subunits by engaging the C terminus of Gα and thereby triggering the conformational switch. D2N promoted the guanine nucleotide exchange reaction and led to a decline in the intrinsic tryptophan fluorescence of Gα_i. The D2N binding site was delineated by antibody and peptide competition experiments; in addition, a mutation introduced into the C-terminal helix (α5) blunted D2N stimulation. The mutation was 12 positions removed from the end of the α-polypeptide, and it did not affect the apparent potency of D2N. From this, we infer that D2N only bound to the extreme C terminus, which was consistent with the inhibition of pertussis toxin and the G protein subtype selectivity retained in the peptide (Conklin et al., 1996). Our conclusion is that the C terminus of Gα_{i1}—in addition to representing a key to selective G protein recognition—exposes an activation latch; this is supported by the observation that also peptides with divergent sequences (Table 2, mastoparan and IGF-II receptor) and nonpeptide G protein activators (Breitweg-Lehmann et al., 2002) require the α-C terminus for activation. However, we refrained from testing a C-terminally truncated version of Gα_{i1} because the effect of C-terminal truncation on nucleotide binding and protein stability is uncertain but may be deleterious (see below).

An involvement of the entire C-terminal helix (i.e., the C terminus and helix α5) in receptor-catalyzed G protein activation was invoked because of the effect of mutations within the G5-box; these are C325S in Gα_o (Thomas et al., 1993) and A326S in Gα_{i1} (Posner et al., 1998). The G5-box is contained in the loop that precedes helix α5, connecting it to strand β6, and lies adjacent to the guanine ring of the bound nucleotide. The G5 mutations resulted in reduced affinity for GDP but no change in GTPγS binding, thus resembling the receptor-activated state of the G protein. Moreover, helix α5 per se is thought to impinge via lattice forces on nucleotide binding (Denker et al., 1995). Substitution of buried (but not solvent-exposed) residues caused enhanced spontaneous activation of Gt (Muradov and Artemyev, 2000; Marin et al., 2001) as did a truncation by the last 14 amino acids in Gα_o. In Gα_{i2}, the same truncation, however, abolished the GTP-induced conformation (Denker et al., 1995). In keeping with this, the proline substitution in the outer portion of helix α5 in Gα_{i1} caused a decreased affinity for GTP, but it did not affect the intrinsic exchange rate. Because the structure of helix α5 is linked to the conformation of the binding pocket, it seems possible that D2N enhanced guanine nucleotide exchange by displacing the C-terminal helix relative to its surrounding. A straightforward explanation is that this motion led to a re-

arrangement of the G5-box, which mediates the activating effect.

Role of Gβγ in D2N-Mediated G Protein Activation. According to the current models of receptor-catalyzed G protein activation, the receptor has to engage the α- *and* βγ-subunit to release GDP (Kisselev et al., 1999; Rondard et al., 2001; Cherfils and Chabre, 2003). However, the requirement for Gβγ in receptor/G protein coupling is not absolute; we have previously demonstrated that productive coupling can take place—albeit with low efficiency—between a receptor and its cognate α-subunit (Freissmuth et al., 1991; Jockers et al., 1994). Likewise, for activation by D2N Gβγ was dispensable. Among the proposed models, only the sequential fit model states that a specific contact to the α-subunit (i.e., its C terminus) is sufficient for activation; prior contact to Gβγ is thought to make the α-C terminus available to the receptor (Herrmann et al., 2004). Thus, D2N mimics the effect of the activated receptor by forming this specific contact and recapitulates the second step in the reaction sequence. The effect of D2N on either the isolated α-subunit or the holotrimer, however, failed the predictions of the lever-arm and gear-shift models because these unconditionally rely on Gβγ for guanine nucleotide displacement.

A canonical role of Gβγ in the unstimulated heterotrimer is to stabilize the inactive conformation. Therefore, combining Gβγ with $G\alpha_{i1}$ reduced the sensitivity to D2N and required higher D2N concentrations for activation. Our data suggest that, in theory, inhibition by Gβγ would be overcome if the receptor's binding to the C terminus were governed by very high affinity. It is unlikely, however, that receptors have evolved an excessive affinity toward the α-C terminus as this would limit the catalytic efficiency of the receptor (Herrmann et al., 2004). Otherwise, the full-length receptor has to interact with the holotrimer by accessory contacts (on the γ- or α-subunit) in a way that relieves the inhibition.

Alignment of the D2N Sequence. The sequence of D2N compares with some of the receptor-mimetic peptides that directly activate G proteins of the $G_{i/o}$ subfamily (Table 2). The peptides share a length of 10 to 20 amino acids and an amphiphilic, cationic structure. Structural similarity may be detected upon inspection of the sequences present in the i3 loop of the M_4-muscarinic and the α_2-adrenergic receptor (Table 2). The modeled Gα binding site of pertussis toxin also exhibits sequence similarity to the N-terminal half of the D2N. This suggests that D2N forms bonds with the last two amino acids of the α-subunit (Scheuring et al., 1998; data not shown). By contrast, we were unable to find a similarity to peptides derived from the i2 loops (of the M_4- or α_2-receptor), to mastoparan or to the regulatory sequence derived from the IGF-II receptor. All of these peptides preferentially activated Gi and Go with the exception of the short α_2i3n-peptide; α_2i3n, however, is a nonselective activator with low potency, obviously because of the lack of flanking amino acid residues, which are present in D2N, in α_2i3c and in M_4i3c peptides.

Because the alignment is improved, the sequences of M_4i3c and α_2i3c are rendered in the C- to N-terminal orientation (c→n). The similarity is concentrated within eight amino acids in the C-terminal half of D2N where mutations were disruptive to D2N activity (e.g., in 3A, 4A′; Table 1). Conversely, N-terminal truncation of the α_2i3c peptide was systematically more deleterious to its activity than several truncations from the C-terminal end (Wade et al., 1996). This indicates that the congruent portion is oriented away from the plasma membrane, toward the G protein interface when the peptides are inherent to the i3 loop of the receptor.

The properties of D2N could not be predicted in detail from the experimental characteristics of other regulatory sequences (Table 3). The distinctions are as follows. 1) D2N-activated guanine nucleotide exchange independently of Gβγ; if this had been assessed, Gβγ typically was found to assist activation by receptor-mimetic peptides. 2) The α-subunit N terminus was not required for the binding of D2N but was a docking site for the α_2i3c peptide and mastoparan. 3) The activating potency of D2N (D_2i3n) was high, comparable only with that of the M_4-peptides.

In spite of the similarities between D2N and other third cytoplasmic loop peptides in amino acid sequence, subtype-selective action, and orientation, the diversity of activator sequences in general is large (Tables 2 and 3). This is not new if one considers that among G protein-coupled receptors the cytoplasmic domains are unrelated. The question therefore is how different receptor peptides align on the surface of the G protein to operate the C-terminal latch; several configurations must be possible. For example, it has been speculated that those peptides that require Gβγ for G protein activation need to bind to Gβγ for proper alignment (Taylor et al., 1994). However, D2N possibly represents a sequence optimized to bind to the C terminus of $G\alpha_i$ and $G\alpha_o$. If available, the structure of D2N bound to the G protein α-subunit could be used to delineate the molecular coordinates of peptide- and receptor-mediated G protein activation.

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Address correspondence to: Dr. C. Nanoff, Medical University of Vienna, Institute of Pharmacology, Waehringer Straβe 13, A-1090 Vienna, Austria. E-mail: christian.nanoff@meduniwien.ac.at