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Differential signalling of both wild-type and Thr^{343}Arg dopamine D_{2short} receptor by partial agonists in a G-protein-dependent manner

Petrus J. Pauwels*, Stéphanie Tardif, Françis C. Colpaert

Department of Cellular and Molecular Biology, Centre de Recherche Pierre Fabre, 17 Avenue Jean Moulin, F-81106 Castres, France

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Abstract

G-protein activation and Ca^{2+} responses by the wild-type D_{2short} receptor and a mutation Thr^{343}Arg, in the distal BBX*X*B motif of its third intracellular loop, were investigated in CHO-K1 cells in terms of ligand:receptor:G-protein interactions. No evidence was obtained for constitutive, agonist-independent receptor activation, but differences in the ligand-mediated activation profiles of both the wild-type and mutant Thr^{343}Arg D_{2short} receptor were observed. Most of the partial agonists, but not bromocriptine, displayed an enhanced response at the mutant D_{2short} receptor, suggesting that the mutation brings the receptor in a more active state. This enhancement was apparent both at the level of G-protein activation ([^{35}S]GTPγS binding) and at the effector (Ca^{2+} response) and occurred with different G_{α}-proteins. Partial agonists were also found to act differently via the wild-type D_{2short} receptor depending on the involved G_{α}-protein. Compared with higher efficacy agonists, partial agonists displayed Ca^{2+} responses with slower and dissimilar kinetic properties. Lisuride and in particular bromocriptine produced a more potent response in the co-presence of a $G_{\alpha o}$ protein instead of a chimeric $G_{\alpha q/o}$- or a promiscuous $G_{\alpha 15}$-protein. S(+)-propylnorapomorphine showed a similar partial response irrespective of the combined G_{α}-protein. Bromerguride and (+)-UH 232 induced weak (16 to 21% versus dopamine) intrinsic activity in the co-presence of a $G_{\alpha q/o}$-protein in contrast to their silent properties with a $G_{\alpha 15}$- or a $G_{\alpha o}$Cys^{351}Ile-protein. In conclusion, the present data strongly suggest that multiple activation binding sites are involved with these ligands at the D_{2short} receptor, and that their activation may be unravelled by either the mutation or co-expressed G_{α}-proteins being investigated.

Keywords: Recombinant human dopamine D_{2short} receptor; G_{α}-protein; [^{35}S]GTPγS binding and Ca^{2+} response; Intrinsic ligand activity; Differential signalling

1. Introduction

A widely accepted model used to describe the activation of G-protein-coupled receptors by agonists is the ternary complex model, which accounts for the co-operative interactions between agonist, receptor, and G-protein [1]. This model has recently been extended to accommodate the observation that several receptors can activate G-proteins in the absence of agonist and that mutations in different structural domains of the G-protein-coupled receptors can enhance this agonist-independent activity [2,3]. The extended ternary complex model also accounts for the effects of different classes of ligands (efficacious agonists, partial agonists, neutral antagonists, and partial to efficacious inverse agonists) on receptor signalling [4]. The C-terminal portion of the third intracellular loop has been suggested to be involved in constraining the G-protein-coupled receptors in an inactive (G-protein-uncoupled) conformation [5]. Mutagenesis studies of the distal BBXXB motif (in which B represents a basic residue and X a non-basic residue) in the third intracellular loop of α_{1B}-adrenergic, α_{2A}-adrenergic, 5-hydroxytryptamine$_{1B}$ (5-HT$_{1B}$), 5-HT$_{2A}$, and 5-HT$_{2C}$ receptors demonstrated constitutively active mutants [5–11]. Whereas the degree of constitutive receptor activation varies between these receptor systems, two common features are observed at these mutant receptors: an enhanced basal response that can be reversed by an inverse agonist, and an increased affinity and potency for agonists. In contrast, analogous mutations in the 5-HT$_{1A}$ receptor did not produce constitutive activation. The 5-HT$_{1A}$ receptor mutations also seem to alter receptor: G-protein coupling, allowing ligand-

* Corresponding author. Tel.: +33 563 71 42 65; fax: +33 563 71 43 63.

E-mail address: peter.pauwels@pierre-fabre.com (P.J. Pauwels).

Abbreviations: NPA, propylnorapomorphine; PTX, *Bordetella pertussis* toxin; 7-OH-DPAT, 7-(hydroxy-2-(di-*n*-propylamino)tetralin; and (+)-UH 232, cis-(+)-5-methoxy-1-methyl-2-(di-n-propylamino)tetralin.

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dependent coupling of receptor to G_s in addition to G_i/G_o-proteins [12]. The role of the distal BBXXB motif in the third intracellular loop with regard to activation of the dopamine D_2 receptor subtypes has to our knowledge not been precisely defined.

Measuring forskolin-stimulated cAMP accumulation in transfected CHO-K1 cells, Hall and Strange [13] suggested that most antipsychotic drugs act as inverse agonists at both wild-type D_{2short} and D_{2long} receptors. Also, these antagonists only weakly (<12%) inhibited basal [^{35}S]GTPγS binding to membranes expressing either the short or long isoform of the D_2 receptor [14–16]. One strategy to overcome this weak amplitude of inverse agonist activity may be by co-expression of the D_{2short} receptor with G_α protein subunits. We have previously reported on facilitation of constitutive α_{2A}-adrenergic receptor activity by both a single amino acid mutation (Thr^{373}Lys in the BBX*X*B motif) and co-expression of a pertussis toxin (PTX)-resistant $G_{\alpha o}$Cys^{351}Ile-protein [10].

In the present study, we investigated G-protein activation by both the wild-type and Thr^{343}Arg (mutated in the distal Lys-Lys-Ala-*Thr*-Gln motif of its third intracellular loop) D_{2short} receptor in the co-presence of a $G_{\alpha o}$Cys^{351}Ile-protein. We found no evidence for constitutive Thr^{343}Arg D_{2short} receptor activation. However, most partial agonists displayed an enhanced response, suggesting that the mutant receptor is in a more active conformation. The partial agonist bromocriptine failed to demonstrate an enhanced response at the mutant receptor. This may suggest that activation of the Thr^{343}Arg D_{2short} receptor by partial agonists may occur via multiple molecular mechanisms. The latter hypothesis was further explored by monitoring dynamic agonist: D_{2short} receptor interactions following activation of either a chimeric $G_{\alpha q/o}$- [17] or the promiscuous $G_{\alpha 15}$-protein [18]. These G_α-proteins have been shown to couple G-protein-coupled receptors efficaciously to the cellular Ca^{2+} signalling pathway [19,20]. The herein described agonist: D_{2short} receptor: G-protein kinetic Ca^{2+} data strongly suggest that the partial agonists activate the D_{2short} receptor via multiple ligand activation binding sites.

2. Materials and methods

2.1. Construction of human wild-type and mutant Thr^{343}Arg dopamine D_{2short} receptor, wild-type, and chimeric G_α-protein genes

The short splice variant of the human dopamine D_2 receptor cDNA (RC: 2.1.DA.02) was cloned by PCR using oligonucleotide primers designed according to the sequence deposited in the Genbank database (accession number: S69899). The PCR mixture (50 μL) consisted of 250 ng of reverse-transcribed poly (A^+) RNA from human whole brain, 350 μM of each dNTP, 400 nM of each primer, and 1 μL of Expand long-template DNA polymerase mix in PCR buffer [16 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 50 mM Tris–HCl (pH 9.2)]. The PCR program consisted of 30 repetitive cycles with a strand separation step at 96° for 30 sec, an annealing step at 60° for 1 min, and an elongation step at 68° for 1.5 min. Site-directed mutagenesis of the Thr^{343} position (ACT codon) into an Arg residue (AGA codon) was performed using a Quick Change site-directed mutagenesis kit, according to Stratagene's instructions. Wild-type rat $G_{\alpha o}$ (M17526), mouse $G_{\alpha q}$ (M55412), and mouse $G_{\alpha 15}$ (M80632) protein cDNA were PCR-amplified under similar experimental conditions using gene-specific primers. The chimeric $G_{\alpha q/o}$-protein was constructed by exchanging the last five amino acids (Glu^{355}-Tyr-Asn-Leu-Val) of a mouse $G_{\alpha q}$-protein by those corresponding to a rat $G_{\alpha o}$ (Gly-Cys-Gly-Leu-Tyr) protein. This has been performed by inserting the respective nucleotide sequence on the reverse oligonucleotide primer used in a PCR reaction on cloned wild-type $G_{\alpha q}$ protein cDNA [21]. Receptor and G_α-protein constructions were cloned into a pCR3.1 mammalian expression vector and the nucleotide sequences were fully verified by DNA sequencing and confirmed the respective sequences.

2.2. Measurement of intracellular Ca^{2+} responses

Subconfluent CHO-K1 cells were transiently transfected with either a wild-type or mutant Thr^{343}Arg D_{2short} receptor and a $G_{\alpha q/o}$- or $G_{\alpha 15}$-protein plasmid (unless indicated) in an equimolecular amount (10 μg) by electroporation [19]. Cells were assayed between 24 and 48 hr upon transfection for intracellular Ca^{2+} responses upon 1-hr pulse with 2 μM Fluo-3 fluorescent calcium indicator dye as described [19]. Either dopamine or other dopaminergic ligands were assayed for their Ca^{2+} response. Data for Ca^{2+} responses were obtained in arbitrary fluorescence units and were not translated into Ca^{2+} concentrations. Fluorescent readings were made every 2 sec for the first 3 min using a fluorometric imaging plate reader (FLIPR, Molecular Devices). E_{max} values were defined as the ligand's maximal high-magnitude response in percentage versus that obtained with 10 μM dopamine. pEC_{50} values correspond to a ligand concentration at which 50% of its own maximal high-magnitude Ca^{2+} response was measured. Two kinetic parameters were deduced from the agonist-mediated Ca^{2+} responses: the onset-time (T) to yield maximal activation by a given agonist and the attenuation of the Ca^{2+} signal upon maximal agonist activation. This latter was determined by the residual activity (%) upon 1 min of its maximal activation.

2.3. Guanosine 5′-O-(3-[^{35}S]thiotriphosphate ([^{35}S]GTPγS) binding responses

CHO-K1 cells grown to 60–80% confluency in Petri dishes (50 cm^2) with nutrient mixture Ham's F-12 supplemented with 10% heat-inactivated foetal bovine serum were used for transfection using a Lipofectamine Plus kit [21].

pCR3.1 plasmid (0.6 microgram) containing either the wild-type or mutant $Thr^{343}Arg$ D_{2short} receptor cDNA supplemented with 0.6 micrograms of the mutant $G_{\alpha o}Cys^{351}Ile$ protein cDNA was mixed with 10 μL of Lipofectamine Plus reagent in 0.2 mL of Opti-Mem and incubated at room temperature for 15 min. Subsequently, 20 μL of Lipofectamine reagent diluted in 0.2 mL of Opti-Mem was added for 15 min and exposed with 5 mL of Opti-Mem to CHO-K1 cells for 3 hr at 37°. Thereafter, cells were incubated further with 10 mL of complete growth medium and harvested 48 hr after transfection. Treatment with PTX (20 ng/mL) was performed overnight before membranes were prepared. Basal and agonist-dependent [^{35}S]GTPγS binding [22] to the above-mentioned membrane preparation was performed in 20 mM HEPES (pH 7.4) supplemented with 30 μM GDP, 100 mM KCl, 3 mM $MgCl_2$, and 0.2 mM ascorbic acid. Maximal stimulation of [^{35}S]GTPγS binding was defined in the presence of 10 μM dopamine and calculated versus basal [^{35}S]GTPγS binding.

2.4. Radioligand binding to D_{2short} receptor

[^{3}H]Nemonapride binding (0.12 nM) was assayed to membrane preparations of transfected CHO-K1 cells as previously described [23]. [^{3}H]Sulpiride binding (2.0 nM) was determined on intact transfected CHO-K1 cells as described [19]. Non-specific binding was determined in the presence of 10 μM (+)-butaclamol.

2.5. Protein content

Membrane and cellular protein levels were estimated with a dye-binding assay using a Bio-Rad kit; bovine serum albumin was used as standard [24].

2.6. Statistics

Statistical analysis was performed on E_{max} and T values using either a one-way repeated measure analysis of variance, followed by all pairwise multiple comparison procedures (Student–Newman–Keuls method) or a one-way analysis of variance, followed by all pairwise multiple comparison procedures (Tukey test).

2.7. Materials

All molecular biology reagents were purchased from In Vitrogen, Roche Diagnostics, Stratagene, and PE Biosystems. CHO-K1 cells were obtained from ATCC. [*N*-methyl-^{3}H]Nemonapride (85 Ci/mmol) and (−)-[methoxy-^{3}H]sulpiride (60–87 Ci/mmol) were obtained from NEN. [^{35}S]GTPγS (1000–1200 Ci/mmol) was from Amersham. Fluo-3 was obtained from Molecular Probes. Dopamine hydrochloride, pergolide mesylate, and haloperidol were obtained from Sigma. Bromocripine mesylate, 7-OH-DPAT hydrobromide, apomorphine hydrochloride, S(+)-propyl-norapomorphine hydrochloride [(+)-NPA], R(−)-NPA, and (+)-butaclamol were from RBI. Lisuride maleate and bromerguride were from Schering. (+)-UH 232 was from Tocris.

Table 1
Basal and dopamine-mediated [^{35}S]GTPγS binding responses by wild-type and mutant $Thr^{343}Arg$ D_{2short} receptor in the co-presence of a pertussis toxin-resistant $G_{\alpha o}Cys^{351}Ile$-protein

	[^{35}S]GTPγS binding response (fmol/mg protein)	
	wild-type D_{2short}	$Thr^{343}Arg$ D_{2short}
Basal	167 ± 28	168 ± 38
10 μM dopamine	412 ± 47*	370 ± 76*

CHO-K1 cells were transfected with 0.6 μg receptor and 0.6 μg $G_{\alpha o}Cys^{351}Ile$ plasmid and treated with pertussis toxin (20 ng/mL) overnight before membranes were prepared as described in Methods. [^{35}S]GTPγS binding responses were performed with 0.5 nM [^{35}S]GTPγS. Specific [^{3}H]nemonapride binding represented 1.17 ± 0.30 and 0.64 ± 0.15 pmol/mg protein for the wild-type and mutant receptor preparation, respectively. Values represent means ± SEM of 6 independent transfection experiments.

* $P < 0.05$ for comparison with basal value (one-way repeated measures analysis of variance, followed by all pairwise multiple comparison procedures [Student–Newman–Keuls method]).

3. Results

3.1. [^{35}S]GTPγS binding responses mediated by wild-type and mutant $Thr^{343}Arg$ dopamine D_{2short} receptor

Transfection of the wild-type and $Thr^{343}Arg$ D_{2short} receptor in CHO-K1 cells was performed in the co-presence of a PTX-resistant $G_{\alpha o}Cys^{351}Ile$-protein [21] in order to compare their activation under similar experimental conditions. Consequently, PTX treatment (20 ng/mL) of the transfected cells blocked the D_{2short} receptor coupling to endogenous PTX-sensitive $G_{i/o}$-proteins. Also, dopamine (10 μM) stimulated [^{35}S]GTPγS binding via the $G_{\alpha o}Cys^{351}Ile$-protein by 147 and 120% for the wild-type and $Thr^{343}Arg$ D_{2short} receptor, respectively (Table 1). Assessment of the receptor amount as measured with [^{3}H]nemonapride binding suggested a 45% decrease in binding sites at the mutant receptor preparation (Table 1) in accordance with that observed at similar receptor mutants [6,25]. It cannot be excluded that the decreased number of binding sites at the receptor mutant is due to a decreased stability of the binding site.

A comparison of the intrinsic activity of a series of dopaminergic ligands at both the wild-type and $Thr^{343}Arg$ D_{2short} receptor in the co-presence of the PTX-resistant $G_{\alpha o}Cys^{351}Ile$-protein is illustrated in Fig. 1. Table 2 summarises the corresponding ligands' E_{max} and pEC_{50} values. Except for (−)-NPA, each of the compounds being investigated displayed a significantly smaller maximal response than dopamine at the wild-type D_{2short} receptor. The ampli-

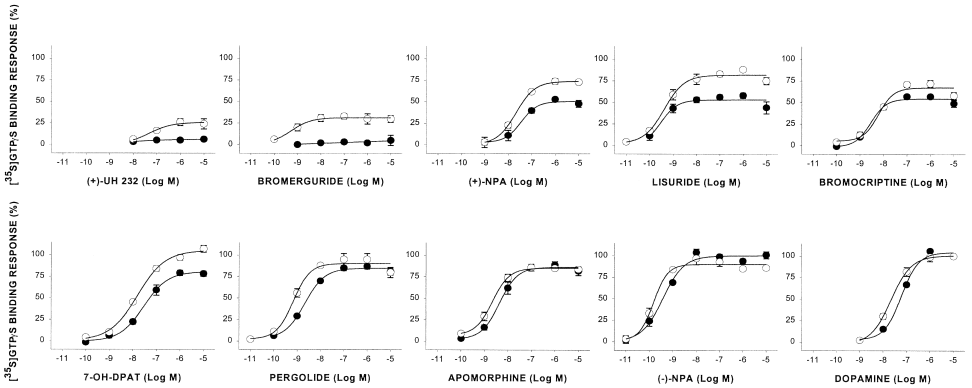

Fig. 1. Concentration-[^{35}S]GTPγS binding curves of dopaminergic ligands to membrane preparations of CHO-K1 cells transfected with either a wild-type or Thr^{343}Arg dopamine D_{2short} receptor in the co-presence of a pertussis toxin-resistant $G_{\alpha o}$ Cys^{351}Ile-protein. [^{35}S]GTPγS binding was measured as described in Methods. Maximal stimulation of [^{35}S]GTPγS binding was determined in the presence of 10 μM dopamine. Stimulation of [^{35}S]GTPγS binding is expressed as a percentage of that obtained with 10 μM dopamine. Curves are constructed using mean values ± SEM from 3 to 6 independent transfection experiments. Mean E_{max} and pEC_{50} values ± SEM are summarized in Table 2. ●——●: wild-type D_{2short} receptor, ○——○: Thr^{343}Arg D_{2short} receptor.

tude of the responses mediated by 7-OH-DPAT, pergolide, apomorphine, and (−)-NPA were not significantly different from that of dopamine at the Thr^{343}Arg D_{2short} receptor, and their potencies were increased 2- to 3-fold. (+)-UH 232, bromerguride, (+)-NPA, and lisuride also displayed an enhanced response but with an amplitude smaller than that of dopamine. Having an E_{max} value similar to that of (+)-NPA and lisuride at the wild-type D_{2short} receptor, bromocriptine did not display a significantly enhanced [^{35}S]GTPγS binding response at the Thr^{343}Arg D_{2short} receptor: neither its E_{max} nor its pEC_{50} value was significantly different as compared to the wild-type receptor. The putative antagonist haloperidol (1 μM) did not significantly inhibit basal [^{35}S]GTPγS binding (Table 2).

3.2. Ca^{2+} responses mediated by wild-type and mutant Thr^{343}Arg dopamine D_{2short} receptors

In contrast to its lack of effect in non-transfected cells, dopamine produced a time- and concentration-dependent increase in the intracellular Ca^{2+} concentration in CHO-K1 cells transiently co-transfected with a wild-type D_{2short} receptor and either a chimeric $G_{\alpha q/o}$ or $G_{\alpha 15}$ protein (Fig. 2A). The magnitude of the dopamine-mediated Ca^{2+} response was greater with a $G_{\alpha q/o}$- than with a $G_{\alpha 15}$-protein, but both Ca^{2+} responses showed a similar potency for dopamine (Table 4). In addition, the high-magnitude Ca^{2+} peak response occurred significantly more rapidly in the co-presence of a $G_{\alpha q/o}$-protein (11 sec) than $G_{\alpha 15}$-protein (25 sec)

Table 2
E_{max} and pEC_{50} values of [^{35}S]GTPγS binding responses as mediated by dopaminergic ligands at wild-type and Thr^{343}Arg dopamine D_{2short} receptor

Ligand	[^{35}S]GTPγS binding response			
	wild-type D_{2short} receptor plus $G_{\alpha o}$Cys^{351}Ile		Thr^{343}Arg D_{2short} receptor plus $G_{\alpha o}$Cys^{351}Ile	
	E_{max}	pEC_{50}	E_{max}	pEC_{50}
Haloperidol	−2 ± 4		−6 ± 5	
(+)-UH 232	6 ± 1		25 ± 3**	7.35 ± 0.12
Bromerguride	7 ± 1		31 ± 4**	9.09 ± 0.07
(+)-NPA	48 ± 4	7.39 ± 0.13	73 ± 3**	7.67 ± 0.07
Lisuride	51 ± 4	9.40 ± 0.10	77 ± 4**	9.28 ± 0.11
Bromocriptine	53 ± 4	8.42 ± 0.04	63 ± 5	8.20 ± 0.06
7-OH-DPAT	78 ± 3	7.45 ± 0.13	95 ± 4*	7.89 ± 0.04
Pergolide	83 ± 4	8.71 ± 0.04	84 ± 6*	9.12 ± 0.07
Apomorphine	83 ± 4	8.35 ± 0.10	86 ± 1*	8.71 ± 0.10
Dopamine	102 ± 1	7.30 ± 0.00	101 ± 1	7.65 ± 0.10
(−)-NPA	103 ± 2*	9.36 ± 0.03	91 ± 4*	9.80 ± 0.08

CHO-K1 cells were transfected with 0.6 μg $G_{\alpha o}$Cys^{351}Ile plasmid and either 0.6 μg wild-type D_{2short} receptor or Thr^{343}Arg D_{2short} receptor plasmid and treated with pertussis toxin (20 ng/mL) as described in Methods. [^{35}S]GTPγS binding experiments were performed with 0.5 nM [^{35}S]GTPγS as described in Methods. E_{max} values are expressed in percentage versus the stimulation as obtained with 10 μM dopamine. A dose–response curve was performed when a ligand-mediated maximal response was >20% versus that of 10 μM dopamine. E_{max} values correspond to mean values ± SEM of 4 to 6 independent transfection experiments. pEC_{50} values ± SEM are obtained in 3 independent transfection experiments. Statistical analysis was performed by one-way analysis of variance, followed by all pairwise multiple comparison procedures (Tukey test).

* P = NS versus dopamine of either wild-type or Thr^{343}Arg D_{2short} receptor.

** $P < 0.05$ for comparison between wild-type and Thr^{343}Arg D_{2short} receptor.

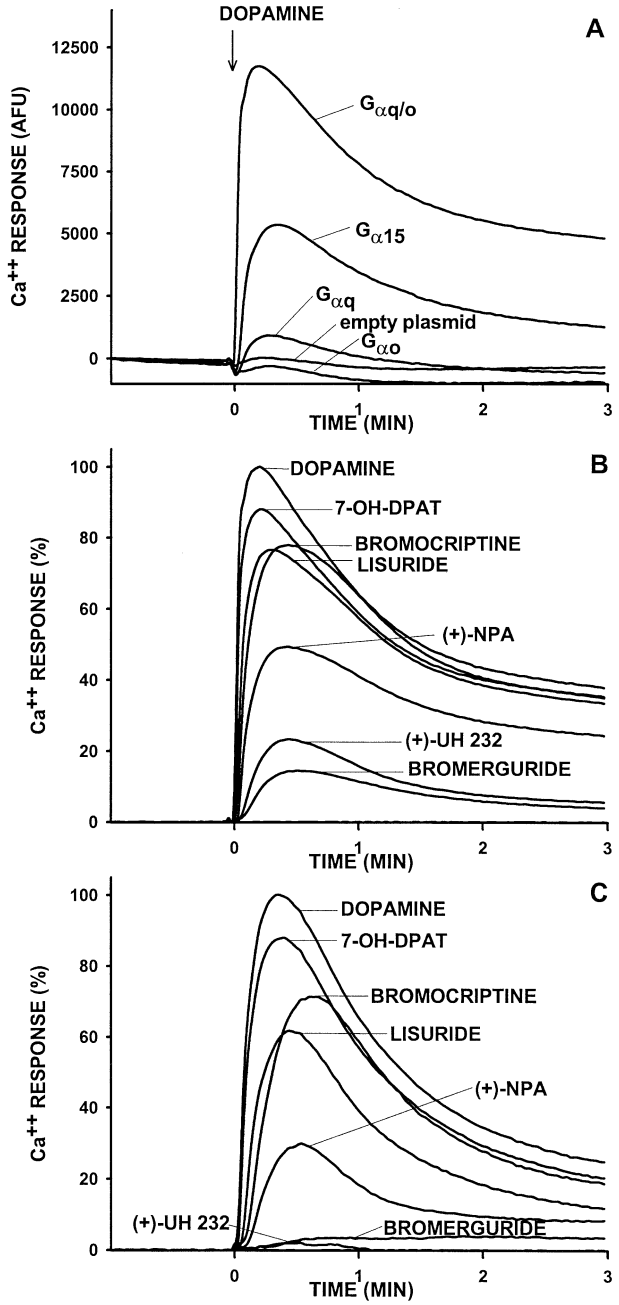

Fig. 2. Ca^{2+} responses obtained with CHO-K1 cells transfected with the wild-type dopamine D_{2short} receptor. Ca^{2+} responses were measured as described in Methods every 2 sec for 3 min. (A) Transfection of the wild-type D_{2short} receptor was performed in the co-presence of empty plasmid, or in combination with $G_{\alpha q/o}$, $G_{\alpha 15}$, $G_{\alpha q}$, or $G_{\alpha o}$-protein, and assayed with 10 μM dopamine. (B) Time-courses of dopaminergic agonist-induced Ca^{2+} responses at wild-type D_{2short} receptor in the co-presence of a $G_{\alpha q/o}$-protein. Ca^{2+} responses were measured at maximally effective ligand concentration (10 μM, except 1 μM for lisuride). Tracings were expressed as a percentage of the respective Ca^{2+} response mediated by 10 μM dopamine and illustrate a representative experiment. The onset time of maximal activation (T) for each ligand is summarized in Table 4. Curves for apomorphine, (−)-NPA, and pergolide (not shown) were similar to that of 7-OH-DPAT and not significantly different from the dopamine-mediated Ca^{2+} response. (C) Similar to B for wild-type D_{2short} receptor in the co-presence of a $G_{\alpha 15}$-protein. Curves for apomorphine, (−)-NPA, and pergolide (not shown) were similar to that of 7-OH-DPAT and not significantly different from the dopamine-mediated Ca^{2+} response.

(Fig. 2, B and C). Assay of the D_{2short} receptor alone or by co-expression with either a wild-type $G_{\alpha q}$- or $G_{\alpha o}$-protein revealed either a small or no dopamine-mediated Ca^{2+} response (Fig. 2A). Activation of the mutant $Thr^{343}Arg$ D_{2short} receptor in the co-presence of a $G_{\alpha q/o}$-protein displayed a smaller and a tendency toward a slower dopamine-mediated Ca^{2+} response (Table 3). An even smaller dopamine-mediated Ca^{2+} response with a kinetic profile similar to that of the wild-type D_{2short} receptor was obtained with the mutant D_{2short} receptor in the co-presence of a $G_{\alpha 15}$-protein (Table 3). Consistent with the observation at the membrane preparation (Table 1), the receptor expression as estimated with [^{3}H]sulpiride binding on the intact transfected CHO-K1 cells demonstrated a 51 to 57% decrease of the mutant receptor irrespective of the co-expressed G_{α}-protein (Table 3).

With the exception of bromerguride and (+)-UH 232, dopaminergic ligands considered as partial agonists at the wild-type D_{2short} receptor in the co-presence of a $G_{\alpha o}Cys^{351}Ile$-protein (Table 2) displayed weaker potencies in the co-presence of a chimeric $G_{\alpha q/o}$-protein (Table 4). This was evident, in particular, for bromocriptine: a 138-fold attenuation in potency was observed. pEC_{50} values of (+)-NPA and lisuride were attenuated 4- and 12-fold, respectively. Each of these partial agonists also displayed a slower onset time of maximal activation as compared to dopamine (Fig. 2B and Table 4). Apomorphine, pergolide, (−)-NPA, and 7-OH-DPAT were not further considered for evaluation at the $Thr^{343}Arg$ D_{2short} receptor as they showed a maximal response with similar kinetic properties to that of dopamine at the wild-type D_{2short} receptor. Fig. 3 compares the Ca^{2+} responses by the wild-type and $Thr^{343}Arg$ D_{2short} receptor in the co-presence of a $G_{\alpha q/o}$ protein. Except for bromocriptine and lisuride, each of the partial agonists induced a significantly enhanced maximal response: this effect was greatest for (+)-UH 232, and was accompanied by a 6-fold increase in potency. Again, each of these compounds displayed a slower onset time of maximal activation versus dopamine (Table 4). With the exception of bromocriptine and lisuride, a comparison between the Ca^{2+} responses for the partial agonists as obtained with a $G_{\alpha 15}$-protein showed a smaller response at the wild-type D_{2short} receptor (Table 4). The onset time for maximal activation by (+)-NPA and bromocriptine was shifted by about 20 sec compared to dopamine (Fig. 2C and Table 4). The ligand activation profile as obtained by the $Thr^{343}Arg$ D_{2short} receptor in the co-presence of a $G_{\alpha 15}$-protein was comparable to that observed with a $G_{\alpha q/o}$-protein (Fig. 4). The intrinsic activity of bromocriptine was not modified, and both bromerguride and (+)-UH 232 displayed a Ca^{2+} response with a smaller amplitude. Onset time of maximal activation for bromocriptine and lisuride was not modified at the mutant $Thr^{343}Arg$ D_{2short} receptor as observed with dopamine (Table 4).

Table 4
Onset time of maximal activation, E_{max}, and pEC_{50} values of Ca^{2+} responses mediated by dopaminergic agonists at wild-type and $Thr^{343}Arg$ dopamine D_{2short} receptor

G_{α} protein D_{2short} receptor	Ca^{2+} response											
	$G_{\alpha q/o}$ wild-type			$G_{\alpha q/o}$ $Thr^{343}Arg$			$G_{\alpha 15}$ wild-type			$G_{\alpha 15}$ $Thr^{343}Arg$		
	T (sec)	E_{max} (%)	pEC_{50}	T (sec)	E_{max} (%)	pEC_{50}	T (sec)	E_{max} (%)	pEC_{50}	T (sec)	E_{max} (%)	pEC_{50}
Haloperidol	—	0 ± 0	—	—	2 ± 1**	—	—	4 ± 1	—	—	1 ± 4**	
Bromerguride	39.0 ± 4.7*	16 ± 1	6.98 ± 0.09	26.0 ± 1.4*	67 ± 2	7.74 ± 0.17	—	3 ± 1	—	32.0 ± 2.2	39 ± 4****	6.91 ± 0.14
(+)-UH 232	30.3 ± 1.0*	21 ± 2	7.37 ± 0.11	20.0 ± 0.6	79 ± 5	8.16 ± 0.14	—	3 ± 2	—	26.7 ± 1.3	62 ± 6****	7.50 ± 0.26
(+)-NPA	30.3 ± 2.5*	53 ± 3	6.79 ± 0.22	23.0 ± 1.8*	97 ± 4	7.46 ± 0.15	40.3 ± 4.4*	34 ± 8	5.97 ± 0.05	32.3 ± 3.3	98 ± 5	7.13 ± 0.25
Bromocriptine	29.5 ± 2.8*	76 ± 2	6.28 ± 0.07	36.4 ± 2.3*	76 ± 3**	6.24 ± 0.08	42.0 ± 4.0*	68 ± 1***	5.99 ± 0.02	41.7 ± 1.8*	73 ± 3**	5.77 ± 0.14
Lisuride	18.4 ± 0.7	82 ± 6	8.31 ± 0.12	20.7 ± 1.5	101 ± 3**	8.36 ± 0.17	29.2 ± 2.2	65 ± 5***	8.00 ± 0.29	31.7 ± 2.9	94 ± 5	7.73 ± 0.30
Dopamine	11.1 ± 0.5	101 ± 1	8.00 ± 0.10	15.8 ± 0.7	102 ± 2	8.42 ± 0.06	24.8 ± 2.1	101 ± 7	8.07 ± 0.11	25.0 ± 1.6	104 ± 6	8.23 ± 0.10

CHO-K1 cells were transfected with either 10 μg wild-type D_{2short} receptor or $Thr^{343}Arg$ D_{2short} receptor with, respectively, 10 μg $G_{\alpha q/o}$- or $G_{\alpha 15}$-protein as described in Methods. Ca^{2+} responses were measured every 2 sec for 3 min. High-magnitude peak Ca^{2+} values (E_{max}) were expressed versus the peak value obtained with 10 μM dopamine. T (ligand-mediated onset time of maximal activation) values ± SEM were calculated for 6 independent transfection experiments. E_{max} values ± SEM correspond to 4 to 12 independent transfection experiments. pEC_{50} values were obtained in 3 to 8 independent transfection experiments. Apomorphine, pergolide, (−)-NPA, and 7-OH-DPAT (not shown) displayed for the four receptor: G-protein combinations a maximal Ca^{2+} response with kinetic properties not significantly different from the response mediated by 10 μM dopamine. Statistical analysis was performed by one-way analysis of variance, followed by all pairwise multiple comparison procedures (Tukey test).

* $P < 0.05$ versus dopamine value (taken from Table 3).

** P = NS versus wild-type D_{2short} receptor.

*** P = NS versus wild-type D_{2short} receptor plus $G_{\alpha q/o}$-protein.

**** $P < 0.05$ versus $Thr^{343}Arg$ D_{2short} receptor plus $G_{\alpha q/o}$-protein.

Table 3
Receptor amount, magnitude, and kinetic properties of dopamine-mediated Ca^{2+} responses in CHO-K1 cells expressing either wild-type D_{2short} receptor or mutant $Thr^{343}Arg$ D_{2short} receptor in the co-presence of a $G_{\alpha q/o}$- or $G_{\alpha 15}$-protein

	[^{3}H]Sulpiride binding (pmol/mg protein)	Magnitude (arbitrary fluorescence units)	Ca^{2+} response onset time of maximal activation(s)	Residual activity (%) upon 1 min of maximal activation
wild-type D_{2short} receptor				
plus $G_{\alpha q/o}$ protein	1.20 ± 0.14	11076 ± 377	11.1 ± 0.5	68 ± 1
plus $G_{\alpha 15}$ protein	1.53 ± 0.32	4823 ± 772	24.8 ± 2.1*	60 ± 2
$Thr^{343}Arg$ D_{2short} receptor				
plus $G_{\alpha q/o}$ protein	0.59 ± 0.18	6990 ± 889	15.8 ± 0.7	63 ± 3
plus $G_{\alpha 15}$ protein	0.66 ± 0.17	2874 ± 656	25.0 ± 1.6*	54 ± 5

[^{3}H]Sulpiride binding was performed with 2 nM [^{3}H]sulpiride as described in Methods. Ca^{2+} responses to dopamine (10 μM) were monitored as described in Methods. Data represent mean values ± SEM as obtained in 7 to 21 independent transfection experiments.

* $P < 0.05$ versus $G_{\alpha q/o}$-protein [Statistical analysis was performed by one-way analysis of variance, followed by all pairwise multiple comparison procedures (Tukey test)].

4. Discussion

The present study analyses the activation by dopaminergic agonists of the wild-type D_{2short} receptor and a mutation ($Thr^{343}Arg$) in the distal BBXXB motif of its third intracellular loop. This receptor region has previously been documented to be critical in receptor activation [2]. In particular, several mutations [5–11] in this receptor region have been shown to yield a constitutively active receptor with two main features: an enhanced response to partial agonists and a differentiation between ligands, classically defined as antagonists, as either weak partial agonists, neutral antagonists, and partial or full inverse agonists. Under the herein defined experimental conditions, the $Thr^{343}Arg$ mutation in the D_{2short} receptor did not show constitutive activity. The agonist-independent (basal) [^{35}S]GTPγS binding response was not significantly enhanced by the $Thr^{343}Arg$ mutation and could not be attenuated by a dopamine antagonist such as haloperidol. Recently, most of the dopamine antagonists have been proposed as inverse agonists at the dopamine D_2 receptor [13,26], though these compounds, with the exception of (+)-UH 232, could not be differentiated since they share a similar magnitude of inverse agonism. The weak amplitude of constitutive dopamine D_2 receptor activation [14–16] as compared to other G-protein-coupled receptor systems [27] is perhaps one possible explanation for the apparent lack of differentiation between the intrinsic activity of these putative dopaminergic inverse agonists. We found clear differences in the activation profiles of both the wild-type and mutant $Thr^{343}Arg$ D_{2short} receptor by the partial agonists. Most of them displayed an enhanced response at the mutant D_{2short} receptor, suggesting that the receptor mutation facilitates activation by partial agonists. However, bromocriptine did not demonstrate an enhanced response. Note that the amplitude of the response as measured by bromocriptine at the wild-type D_{2short} receptor was of the same magnitude as (+)-NPA and lisuride. These findings were apparent both at the level of the G-protein activation ([^{35}S]GTPγS binding response) and the effector (Ca^{2+} response) and as mediated by different G_α-protein subtypes. We also observed that the responses of the partial agonists by the wild-type D_{2short} receptor were dependent on the G_α-protein involved for its coupling. The degree of receptor activation is highly determined by the coupling efficacy between the receptor and G_α-protein [28,29]. However, the present study shows that the degree of receptor activation versus different G_α-proteins is also dependent on the investigated partial agonist. For instance, lisuride and in particular bromocriptine displayed a more potent response in the co-presence of a $G_{\alpha o}$-protein instead of a $G_{\alpha q/o}$- or a $G_{\alpha 15}$-protein. (+)-NPA displayed a similar partial response at the wild-type D_{2short} receptor irrespective of the combined G_α-protein. Otherwise, bromerguride and (+)-UH 232 displayed some intrinsic activity in the co-presence of a $G_{\alpha q/o}$-protein in contrast to their silent properties with either a $G_{\alpha 15}$- or a $G_{\alpha o}Cys^{351}Ile$-protein. Therefore, the present data strongly suggest that several dopaminergic agonists activate in different ways the D_{2short} receptor, which can be unravelled by either the mutation or co-expressed G_α-proteins described in this study.

Chimeric G_α-proteins constructed by exchanging the C-terminal portion of a given G_α-protein by that of another class of G_α-proteins [28,30] have been introduced to monitor alternative receptor coupling mechanisms. This is useful to measure receptor responses that are otherwise difficult to obtain. Chimeric $G_{\alpha q/i/o}$-proteins have been reported to convert the coupling of $G_{i/o}$-protein-coupled receptors to the phospholipase C pathway [17]; they therefore are suitable to monitor receptor-mediated Ca^{2+} responses. Ca^{2+} mobilisation by dopamine D_2 receptors has previously been demonstrated in CHO-K1 and Ltk^- fibroblast cells stably expressing the receptor; this response was sensitive to the $G_{i/o}$-protein-inactivating agent PTX [30,31]. Hence, this Ca^{2+} response is likely to be mediated by $\beta\gamma$ subunits of $G_{i/o}$ proteins. In the present study, robust dopamine-mediated Ca^{2+} responses were observed in the co-presence of either a $G_{\alpha q/o}$- or $G_{\alpha 15}$-protein; it consisted of rapid, transient responses with a high-magnitude phase. Interestingly,

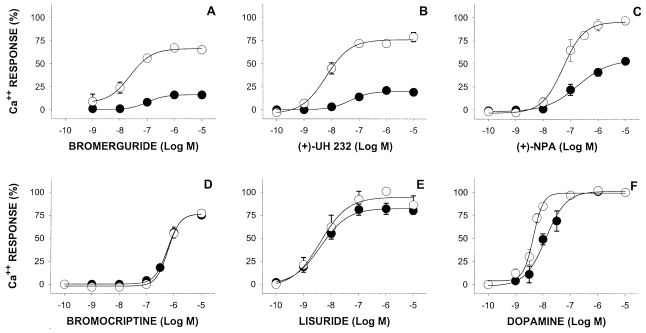

Fig. 3. Comparison between Ca^{2+} responses of dopaminergic ligands at wild-type dopamine and $Thr^{343}Arg$ D_{2short} receptor in the co-presence of a $G_{\alpha q/o}$-protein. Ca^{2+} responses were measured as described in Methods and expressed as a percentage of the respective Ca^{2+} response mediated by 10 μM dopamine. Curves are constructed using mean ± SEM values obtained in 6 to 8 independent transfection experiments. Mean E_{max} and pEC_{50} values ± SEM are summarized in Table 4. ●——●: wild-type D_{2short} receptor, ○——○: $Thr^{343}Arg$ D_{2short} receptor.

the dopamine-mediated activation of the D_{2short} receptor in the co-presence of a $G_{\alpha 15}$-protein displayed different properties; the dopamine-mediated magnitude of the response was smaller and also its onset time of action was slower compared to that with a $G_{\alpha q/o}$-protein. These kinetic data probably reflect a different mode of D_{2short} receptor activation by both G_{α}-proteins. A different way of activation by dopamine for the mutant $Thr^{343}Arg$ D_{2short} receptor was also apparent in the co-presence of a $G_{\alpha q/o}$-protein: both its amplitude and onset time of action were reduced. Hence, the kinetic data provided by the Ca^{2+} responses may be an alternative way to discern differences in receptor activation. One series of compounds (i.e., 7-OH-DPAT, pergolide, apomorphine, and (−)-NPA) behaved with similar kinetic properties as dopamine for each of the receptor: G_{α}-protein combinations being investigated. However, the partial agonists (+)-UH 232, bromerguide, (+)-NPA, lisuride, and bromocriptine displayed dissimilar kinetic properties; their onset times of activation varied and could be attenuated by a factor of four, as was the case for bromerguide at the wild-type D_{2short} receptor in combination with a $G_{\alpha q/o}$-protein. The weak or non-existing dopamine-mediated Ca^{2+} response with a $G_{\alpha q}$- and a $G_{\alpha o}$-protein further suggests that replacement of the terminal five amino acids of a $G_{\alpha q}$-protein by that of a $G_{\alpha o}$-protein in the $G_{\alpha q/o}$-protein confers some specificity versus a native coupled $G_{\alpha o}$-protein [28]. The degree of receptor activation by the partial agonists via a $G_{\alpha o}Cys^{351}Ile$-protein was greater than via a $G_{\alpha q/o}$-protein with the exception of bromerguide and (+)-UH 232. The amplitude of the observed responses via the $G_{\alpha o}Cys^{351}Ile$-protein is presumably turned on at a maximal level. The presence of a hydrophobic amino acid (i.e. isoleucine) at position 351 of $G_{\alpha i/o}$-proteins has been shown to increase the amplitude of 5-HT_{1A} and α_{2A}-adrenergic receptor signalling [21,32,33]. The ligand activation profile as observed with the promiscuous $G_{\alpha 15}$-protein [18] indicated weaker responses than with a $G_{\alpha q/o}$-protein, though bromocriptine and lisuride were not affected. Therefore, there appears to be a different pharmacological profile for the three D_{2short} receptor: G-protein combinations. The partial agonists are likely to stabilize or induce different conformations in the receptor leading to differential coupling to G-proteins. $G_{\alpha 15}$- and $G_{\alpha q}$-proteins share 58% identity at the amino acid level, which is mainly located in the effector-interacting portions [34]. Both G_{α}-proteins are divergent in their amino- and carboxy-terminal domains. The amino-terminal extension of the $G_{\alpha q}$-protein is implicated in the receptor recognition specificity, as shown by deletion mutants in this region which can more effectively couple to the D_2 receptor as the wild-type $G_{\alpha q}$-protein [34,35].

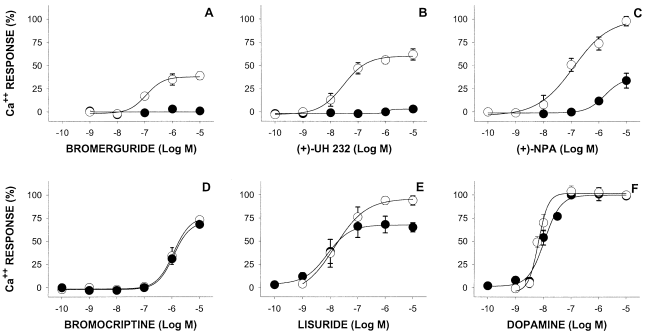

Fig. 4. Comparison between Ca^{2+} responses of dopaminergic ligands at wild-type dopamine and $Thr^{343}Arg$ D_{2short} receptor in the co-presence of a $G_{\alpha 15}$ protein. Ca^{2+} responses were measured as described in Methods and expressed as a percentage of the respective Ca^{2+} response mediated by 10 μM dopamine. Curves are constructed using mean ± SEM values obtained in 3 to 4 independent transfection experiments. Mean E_{max} and pEC_{50} values ± SEM are summarized in Table 4. ●——●: wild-type D_{2short} receptor, ○——○: $Thr^{343}Arg$ D_{2short} receptor.

The point mutation $Thr^{343}Arg$ in the D_{2short} receptor has recently been reported to display increased binding affinities for dopamine and NPA [36]. This mutated receptor may therefore have an increased tendency to adopt the activated R* conformation, leading to the observed increase in agonist affinity. The present functional data support the hypothesis that this mutation enhances the coupling of the D_{2short} receptor to the investigated G-proteins as measured by (+)-UH-232, bromerguide, and (+)-NPA: both their maximal response and potency were enhanced. Lisuride showed a greater maximal response if its amplitude at the wild-type receptor did not exceed 80% relative to that of dopamine; no shift in its potency was observed. Bromocriptine clearly displayed no modification in its intrinsic activity: both its maximal response and its potency were unaltered. Consequently, it can be suggested that these agonists interact in different ways with the mutant D_{2short} receptor. The effect of the $Thr^{343}Arg$ mutation on D_{2short} receptor function is presumably a change in receptor conformation. This modified receptor conformation apparently affects activation by some of the dopaminergic agonists, but not bromocriptine. The mutation $Ser^{197}Ala$ in the fifth transmembrane domain of the D_2 receptor has been reported [37] to strongly inhibit

the binding of dopamine and NPA, whereas bromocriptine is poorly affected by this mutation, in favour of a different receptor binding interface. Heterogeneous responses for adrenergic partial agonists have also been found at the $Asp^{79}Asn$ (located in transmembrane domain II) α_{2A}-adrenergic receptor [38]; the maximal amplitude of activation by *d*-medetomidine and clonidine versus that of (−)-adrenaline was not affected, whereas it was significantly lower for both UK 14304 and oxymetazoline. Each of these adrenergic agonists belong to a same chemical series, the imidazoline derivatives. Therefore, it appears that even structurally related agonists do not necessarily demonstrate the same change in intrinsic activity as induced by a receptor mutation. However, each of these adrenergic ligands displayed an enhanced response at the $Thr^{373}Lys$ (equivalent position to $Thr^{343}Arg$ in the D_{2short} receptor) α_{2A}-adrenergic receptor in the co-presence of either a $G_{\alpha o}Cys^{351}Ile$- or $G_{\alpha 15}$-protein [10,38].

In conclusion, we suggest that multiple ligand binding sites exist at the D_{2short} receptor as illustrated by the dissimilar kinetic data provided by the ligand-mediated Ca^{2+} responses. The activation of these different ligand binding sites may be affected in various ways by either introducing a receptor mutation or by co-expression with several G_{α}-proteins. Colquhoun [39] suggested that each agonist may induce a different receptor conformation or set of conformations, although the available conformational evidence to this concept is sparse. The outcome that multiple molecular mechanisms may activate the D_{2short} receptor opens perspectives for diverse signalling via a single D_{2short} receptor subtype.

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