British Journal of Pharmacology (2001) 134, 88–97

www.nature.com/bjp

Real-time analysis of dopamine: antagonist interactions at recombinant human D_{2long} receptor upon modulation of its activation state

*,[1]Petrus J. Pauwels, [1]Stéphanie Tardif, [1]Thierry Wurch & [1]Francis C. Colpaert

[1]Department of Cellular and Molecular Biology, Centre de Recherche Pierre Fabre, 17, avenue Jean Moulin, 81106 Castres Cédex, France

1 Antipsychotic drugs may mediate their therapeutic effects not only by preventing the binding of dopamine but also by decreasing the propensity of the dopamine receptor to assume an active R* state. Ligand-mediated activation and blockade of the recombinant human D_{2long} receptor was investigated in CHO-K1 cells upon modulation of its R* state.

2 Both the Ala^{371}Lys (A371K) and Thr^{372}Arg (T372R) D_{2long} receptor mutants could be activated in a ligand-dependent manner *via* a chimeric $G_{\alpha q/o}$ protein, and more efficaciously so than with the promiscuous $G_{\alpha 15}$ protein.

3 Dopamine and partial agonists (E_{max}: lisuride $>>$ (+)-UH 232 $\simeq$ bromerguride) displayed dissimilar Ca^{2+} kinetic properties at wild-type and mutant receptors. A371K and T372R D_{2long} receptor mutants demonstrated an attenuated and enhanced maximal response to these partial agonists, respectively.

4 Dopamine antagonists were unable to block the transient high-magnitude Ca^{2+} phase at the wild-type D_{2long} receptor upon simultaneous exposure to antagonist and dopamine, while full blockade of the low-magnitude Ca^{2+} phase did occur at a later time (onset-time: haloperidol $<$ bromerguride $<$ (+)-butaclamol). A similar, though more efficacious, antagonist profile was also found at the A371K mutant receptor. Conversely, the blockade of the low-magnitude Ca^{2+} phase was attenuated (haloperidol) or almost absent [(+)-butaclamol and bromerguride] at the T372R mutant receptor.

5 In conclusion, mutagenesis of the Ala^{371} and Thr^{372} positions affects in an opposite way the ligand-dependent activation and blockade of the D_{2long} receptor. The observed attenuation of dopamine-mediated Ca^{2+} signal generation with different decay-times may underlie distinct properties of the dopaminergic ligands.

British Journal of Pharmacology (2001) **134,** 88–97

Keywords: Dopamine D_{2long} receptor; agonist: antagonist interactions; [^{35}S]-GTPγS binding and Ca^{2+} response; antagonist capacity; mutagenesis of BBXXB motif

Abbreviations: 5-HT, 5-hydroxytryptamine; PTX, Bordetella pertussis toxin; (+)-UH 232, cis-(+)-5-methoxy-1-methyl-2-(di-n-propylamino)tetralin

Introduction

Early models for drug-receptor interactions considered that competitive antagonists share with agonists their ability to bind a common site on the receptor protein. Antagonists differ from agonists in that they cannot trigger the stimulus, including a conformational change of the receptor, that evokes the cellular response. Thus, antagonists lack intrinsic activity. This view has been challenged, considering that an antagonist may have biological effects opposite to that of an agonist, due to an ability to stabilize an antipodal conformation of the receptor (Daeffler & Landry, 2000). This was first established for the action of β-carbolines on the $GABA_A$-benzodiazepine receptor complex (Braestrup *et al*., 1982). The concept of inverse agonism of G protein-coupled receptors arose from studies by Costa & Hertz (1989) who showed that some δ-opioid receptor antagonists inhibit basal GTP hydrolysis. Two types of antagonists were found, those with null intrinsic activity (neutral antagonists) and those with negative intrinsic activity (inverse agonists, also called negative antagonists; see de Ligt *et al*., 2000).

The receptor counterparts of inverse agonist properties for ligands at G protein-coupled receptors are the presence of two conformational states of the receptor, a resting state R and an active state R*, and the existence of some constitutive activity of R* receptors, i.e. activity in the absence of bound agonist but related to spontaneously bound G protein (Daeffler & Landry, 2000; de Ligt *et al*., 2000). Notwithstanding the disorders that may be due to spontaneous constitutively active mutant receptors (Parma *et al*., 1994), a relative excess of R* may result from wild-type receptor overexpression, inducing a constitutively active phenotype (Milano *et al*., 1994; Bond *et al*., 1995; Zhou *et al*., 1999). Such a phenotype may be unresponsive to neutral antagonists and, in this case, inverse agonists may represent an important and specific therapeutic approach. Alternatively, antagonists

*Author for correspondence;
E-mail: peter.pauwels@pierre-fabre.com

currently used in human disease may mediate their therapeutic effects not only by preventing the binding of agonists but also by decreasing the propensity of the receptor to assume an active R* state. Such may perhaps be the case with β-adrenergic antagonists in cardiovascular diseases or dopamine antagonists in schizophrenia. Measuring forskolin-stimulated cyclic AMP accumulation in transfected CHO-K1 cells, Hall & Strange (1997) 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 (Malmberg *et al*., 1996; Backlund Höök *et al*., 1999; Choi *et al*., 2000). One strategy to overcome this apparently weak amplitude of inverse agonist activity may be by introducing a facilitating mutation in the receptor sequence. 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 (Kjelsberg *et al*., 1992). Mutagenesis studies of the distal BBXXB motif (in which B and X represent a basic and a non-basic residue, respectively) 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 (Kjelsberg *et al*., 1992; Ren *et al*., 1993; Egan *et al*., 1998; Herrick-Davis *et al*., 1997; Pauwels *et al*., 1999; 2000a; Rossier *et al*., 1999). The role of the distal BBXXB motif in the third intracellular loop with regard to activation of the dopamine receptor subtypes has to our knowledge not been accurately defined.

In the present study, we investigated G protein activation by the wild-type D_{2long} receptor and a series of three mutations in the distal Lys^{369}Lys**AlaThrGln**^{373} motif (see Table 1) of its third intracellular loop by co-expression with a pertussis toxin (PTX)-resistant $G_{\alpha o}$Cys^{351}Ile protein (Dupuis *et al*., 1999). 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 BB**X**X**B** motif) and co-expression of a $G_{\alpha o}$Cys^{351}Ile protein (Pauwels *et al*., 2000a). However, this combined approach did not result in constitutive D_{2long} receptor activation. Activation of these receptor mutants was further investigated by real-time analysis of Ca^{2+} responses in the co-presence of either a chimeric $G_{\alpha q/o}$ protein (Conklin *et al*., 1993) or the promiscuous $G_{\alpha 15}$ protein (Wilkie *et al*., 1991). These G_α proteins have been shown to couple G protein-coupled receptors efficaciously to the cellular Ca^{2+} signalling pathway (Pauwels *et al*., 2000b; Pauwels & Colpaert, 2000). Ca^{2+} mobilization by dopamine D_2 receptors has previously been demonstrated in CHO-K1 and Ltk$^-$ fibroblast cells stably expressing the receptor (Hayes *et al*., 1992; Liu *et al*., 1992). Attention was given to the partial agonist lisuride and the blockade of the dopamine-mediated response by the putative antagonists haloperidol, (+)-butaclamol and its inactive (−)-enantiomer, as well as by (+)-UH 232 and bromerguride. The Ca^{2+} data demonstrate that the B^{369}BXXB^{373} motif is critically involved both in the enhancement and attenuation of ligand-mediated activation and in the blockade of the dopamine D_{2long} receptor. The present data also strongly suggest that the dopamine D_{2long} receptor can be blocked *via* multiple molecular mechanisms.

Methods

Construction of wild-type and mutant human dopamine D_{2long} receptors and G_α protein subtypes

The long 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: S62137). PCR mixtures (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 $(NH_4)_2SO_4$, 1.75 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°C for 30 s, an annealing step at 60°C for 1 min and an elongation step at 68°C for 1.5 min. The mutant dopamine D_{2long} receptors [Ala^{371}Lys (A371K), Thr^{372}Arg (T372R) and Gln^{373}Lys (Q373K)] were constructed by using a PCR-based overlap extension procedure with the mutagenic oligonucleotide primer pairs indicated in Table 1 and amplification conditions previously described (Dupuis *et al*., 1999). A 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 (Genbank accession number: M55412) by those corresponding to a $G_{\alpha o}$ protein (Gly-Ile-Gly-Leu-Tyr) and subsequent mutation of the fourth last residue into an Ile. This was realized 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 (Pauwels & Colpaert, 2000). The pertussis-toxin resistant mutant $G_{\alpha o}$Cys^{351}Ile protein and $G_{\alpha 15}$ protein were obtained as described (Dupuis *et al*., 1999). Wild-type and mutant D_{2long} receptors and G_α protein constructions were inserted into a pCR3.1 mammalian expression vector

Table 1 Sequence characteristics of the BBXXB motif in the distal portion of the third intracellular loop of the human dopamine D_{2long} receptor

Dopamine D_{2long} receptor	*Amino acids in the $B^{369}BXXB^{373}$ motif*	*Mutagenic oligonucleotide sense primer*
Wild-type	KKATQ	
A371K	KK**K**TQ	5′-CAGCAGAAGGAGAAGAAA**AAA**ACTCAGATGCTCGCCATTGTTCT-3′
T372R	KKA**R**Q	5′-CAGCAGAAGGAGAAGAAAGCC**AGA**CAGATGCTCGCCATTGTTCT-3′
Q373K	KKAT**K**	5′-CAGCAGAAGGAGAAGAAAGCCACT**AAG**ATGCTCGCCATTGTTCT-3′

The mutated amino acid positions in the BBXXB motif in the distal portion of the third intracellular loop of the dopamine D_{2long} receptor are indicated in bold. The mutated codons highlighted in bold are indicated for the sense primers of the complementary mutagenic primer pairs used in the overlap extension PCR procedure.

and DNA sequencing confirmed the respective nucleotide sequences.

Radioligand binding to D_{2long} receptor

[^{3}H]-Nemonapride binding (0.12 nM; K_d: 0.05±0.01 nM) was assayed to membrane preparations of transfected CHO-K1 cells as previously described (Pauwels *et al*., 2000b). [^{3}H]-Sulpiride binding (2.0 nM, K_d: 1.17±0.08 nM) was determined on intact transfected CHO-K1 cells as described (Pauwels *et al*., 2000b). Non-specific binding was determined in the presence of 10 μM (+)-butaclamol.

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

CHO-K1 cells grown to 60–80% confluence in Petri dishes (50 cm^2) with nutrient mixture Ham's F-12 supplemented with 10% heat-inactivated foetal calf serum were used for transfection using a Lipofectamine Plus kit (Dupuis *et al*., 1999). 0.6 microgram of pCR3.1 plasmid containing either the wild-type or a mutant D_{2long} receptor cDNA supplemented with 0.6 μg of the mutant $G_{\alpha o}Cys^{351}Ile$ protein cDNA plasmid 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 h at 37°C. Thereafter, cells were incubated further with 10 ml of complete growth medium and harvested 48 h after transfection. Treatment with PTX (20 ng ml^{-1}) was performed overnight before membranes were prepared. Basal and ligand-dependent [^{35}S]-GTPγS binding (Pauwels *et al*., 1999) 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.

Measurement of intracellular Ca^{2+} responses

Subconfluent CHO-K1 cells were transiently transfected with either a wild-type or mutant D_{2long} receptor and a $G_{\alpha q/o}$ or $G_{\alpha 15}$ protein plasmid in an equimolecular amount (10 μg) by electroporation (Pauwels *et al*., 2000b). Cells were assayed between 24 and 48 h upon transfection for intracellular Ca^{2+} responses upon 1 h pulse with 2 μM Fluo-3 fluorescent calcium indicator dye as described (Pauwels *et al*., 2000b). 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 s 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 Ca^{2+} 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. The onset-time to yield maximal activation (T_{max}, s) by a given agonist was also derived. Antagonists were either pre-incubated for 10 min (T_{-10}) before dopamine to prevent the Ca^{2+} response in the antagonist-bound receptor state, or added simultaneously (T_0) with dopamine for a period of 10 min. Fluorescent readings were made for the pre-incubation period with antagonist every 3 s for the first 3 min, every 15 s for the next 7 min, followed by every 3 s for the 10 min period in the presence of dopamine. Antagonist capacity (%) of dopamine-mediated Ca^{2+} response was defined as the property of the ligand (1 μM) to antagonize the dopamine-mediated Ca^{2+} response. This was calculated as the surface area between the dopamine and ligand condition for a period of 10 min upon addition of dopamine. A typical dopamine-mediated Ca^{2+} response displayed two phases: the Ca^{2+} concentration increased to a maximum during the first, high-magnitude phase, and decreased to an apparent asymptote during the second, low-magnitude phase. These biphasic time-response data could be described adequately by an equation consisting of the sum of four exponentials (i.e., response = $(\Sigma A_i * e^{-k_i} * \text{time}) + C$, I = 1 to 4, $A_4 = -A_3$, C = asymptote), which was fitted to the data by the solver function of Microsoft Excel. From this equation, the following measures were calculated for the Ca^{2+} response at the wild-type D_{2long} receptor in case antagonist was added simultaneously (T_0) with dopamine for a period of 10 min: maximum response (E_{max}), time (in s) at which half-maximum response was reached ($T^{1/2}$ E_{max}), asymptote (E_{asym}), and time (in s) at which half-asymptote was reached ($T^{1/2}$ E_{asym}) (calculated from the time at which E_{max} occurred). Newman-Keuls multiple comparison test was applied after significant randomized block ANOVA to calculate the significance between $T^{1/2}$ E_{asym} values.

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 (Bradford, 1976).

Materials

Conventional molecular biology reagents were purchased from either In Vitrogen (San Diego, U.S.A.), Roche Diagnostics (Indianapolis, U.S.A.) or PE Biosystems (Forster City, U.S.A.). CHO-K1 cells were obtained from ATCC (Rockville, U.S.A.). [N-methyl-^{3}H]-Nemonapride (85 Ci mmol^{-1}) and (−)-[methoxy-^{3}H]-sulpiride (60–87 Ci mmol^{-1}) were obtained from NEN (Les Ulis, France). [^{35}S]-GTPγS (1000–1200 Ci mmol^{-1}) was from Amersham (Les Ulis, France). Fluo-3 was obtained from Molecular Probes (Oregon, U.S.A.). Dopamine chlorhydrate and haloperidol were obtained from Sigma (St. Louis, MO, U.S.A.). (+)- and (−)-Butaclamol were from RBI (Natick, MA, U.S.A.). Lisuride maleate and bromerguride were from Schering (Berlin, Germany). (+)-UH 232 was from Tocris (Ballwin, MO, U.S.A.).

Results

[^{35}S]-GTPγS binding responses by wild-type and mutant dopamine D_{2long} receptors

Transient transfection of the wild-type dopamine D_{2long} receptor with a PTX-resistant $G_{\alpha o}Cys^{351}Ile$ protein in CHO-K1 cells displayed a dopamine-dependent [^{35}S]-GTPγS binding response (Figure 1A). Basal [^{35}S]-GTPγS binding

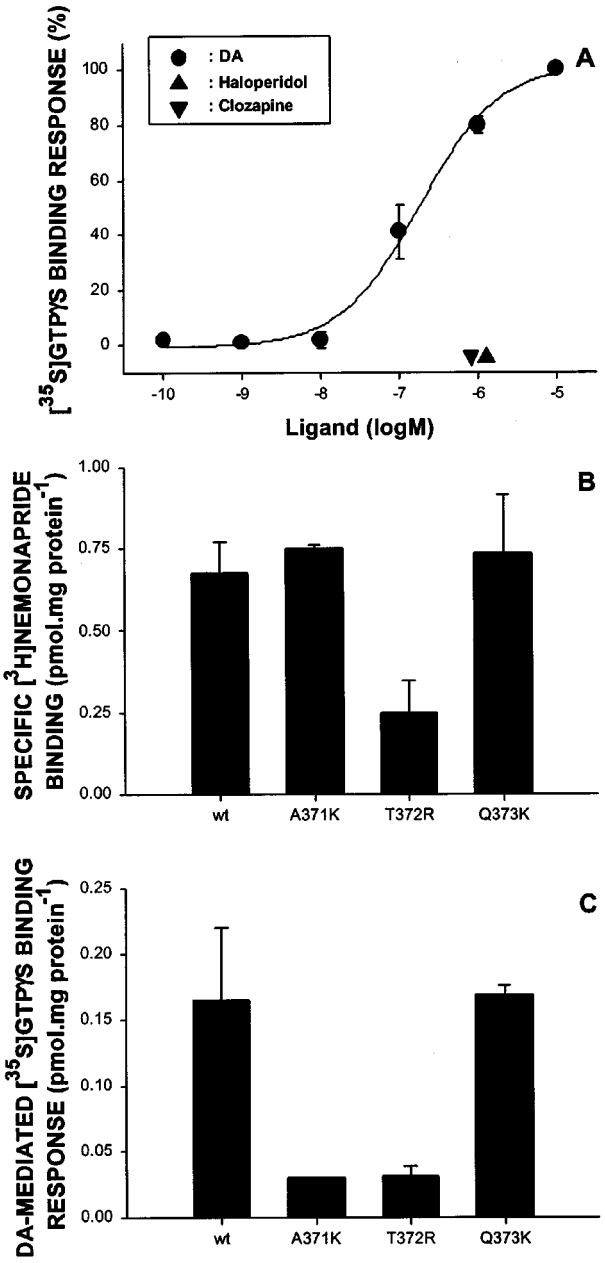

Figure 1 [^{35}S]-GTPγS binding responses and amount of binding sites for wild-type and mutant D_{2long} receptors in CHO-K1 cells. [^{35}S]-GTPγS binding responses were performed to membrane preparations of transfected CHO-K1 cells with 0.5 nM [^{35}S]-GTPγS as described in Methods. The amount of binding sites to these membrane preparations was determined with 0.12 nM [^{3}H]-nemonapride as described in Methods. (A) Wild-type dopamine D_{2long} receptor, concentration [^{35}S]-GTPγS binding response of dopamine, 1 μM haloperidol, and 1 μM clozapine. (B) Amount of [^{3}H]-nemonapride binding sites at wild-type and mutant D_{2long} receptors. (C) Dopamine (10 μM)-mediated [^{35}S]-GTPγS binding response by wild-type and mutant D_{2long} receptors. Basal [^{35}S]-GTPγS binding values varied between 0.17 and 0.18 pmol mg protein^{-1}. Data correspond to mean values $\pm$ s.e.mean of a minimum of three independent transfection experiments. DA: dopamine; wt: wild-type.

was only slightly affected (maximally -4%) in the presence of 1 μM of the putative antagonists haloperidol and clozapine. In analogy with reported mutants for other G protein-coupled receptors (Daeffler & Landry, 2000) that have been shown to display constitutive receptor activity, three mutations were introduced in the distal Lys^{369}Lys-AlaThrGln^{373} motif of the third intracellular loop of the dopamine D_{2long} receptor: Ala^{371}Lys (A371K), Thr^{372}Arg (T372R) and Gln^{373}Lys (Q373K). The T372R receptor mutant displayed a 60% decrease in specific [^{3}H]-nemonapride binding, whereas the amount of specific [^{3}H]-nemonapride binding with the two other mutants was not affected (Figure 1B). Further assay of dopaminergic ligands (dopamine, lisuride, (+)-UH 232, bromerguride, (+)-butaclamol, haloperidol and clozapine) at the wild-type and mutant D_{2long} receptors indicated a similar binding profile with the radioligand [^{3}H]-nemonapride (0.12 nM) (not shown). The magnitude of the dopamine-mediated [^{35}S]-GTPγS binding was attenuated by about 80% with the A371K and T372R receptor mutants in contrast to the Q373K receptor mutant which displayed a similar amount of dopamine-mediated response as the wild-type D_{2long} receptor (Figure 1C). Basal [^{35}S]-GTPγS binding for these dopamine receptor mutants was either not or weakly attenuated (maximally -7%) by the presence of either 1 μM haloperidol or clozapine (not shown).

Ca^{2+} responses by wild-type and mutant dopamine D_{2long} receptors

Since the magnitude of the observed dopamine-mediated [^{35}S]-GTPγS binding responses *via* a $G_{\alpha o}$Cys^{351}Ile protein was small, the effect of the respective mutations on the intrinsic activity of dopaminergic partial agonists was evaluated by measuring Ca^{2+} responses. Figure 2 illustrates that both wild-type and mutant dopamine receptors display robust dopamine-mediated Ca^{2+} responses in the co-presence of a chimeric $G_{\alpha q/o}$ protein as compared to the promiscuous $G_{\alpha 15}$ protein. A high-magnitude Ca^{2+} peak occurred within 12–19 s after dopamine addition *via* a $G_{\alpha q/o}$ protein; the Ca^{2+} signal decayed to a low-magnitude phase which continued for the recorded time period (10 min) in contrast to a $G_{\alpha 15}$ protein (not shown). Though no differences were apparent between the onset-times of maximal activation by dopamine at D_{2long} receptor mutants *via* a $G_{\alpha 15}$ protein (T_{max}: 22–26 s), it was slower for the T372R mutant receptor *via* a $G_{\alpha q/o}$ protein (T_{max}: 19 ± 1 s) as compared to the other D_{2long} receptor mutants (T_{max}: 12–14 s). Dopamine showed the following rank order of maximal Ca^{2+} response *via* a $G_{\alpha q/o}$ protein: Q373K = wild-type > A371K = T372R (Table 2). The T372R receptor expression as estimated with [^{3}H]-sulpiride binding on intact transfected CHO-K1 cells was only 16% (Table 2) in line with the observation at the membrane preparation (Figure 1). Transfection of the D_{2long} receptor mutants with a $G_{\alpha 15}$ protein instead of a $G_{\alpha q/o}$ protein displayed a similar profile of [^{3}H]-sulpiride binding and gradient of dopamine-mediated Ca^{2+} responses (Table 2). Time-dependent Ca^{2+} responses for a series of dopaminergic ligands as assayed at the wild-type and mutant D_{2long} receptors with a $G_{\alpha q/o}$ protein are exemplified in Figure 3. The corresponding ligand-mediated onset-times of maximal activation, as well as the E_{max} and pEC_{50} values are summarized in Tables 3 and 4. The ligand-mediated Ca^{2+} responses as obtained with the Q373K receptor were virtually similar to the wild-type D_{2long} receptor. Each of the ligands displayed a different onset-time of maximal activa-

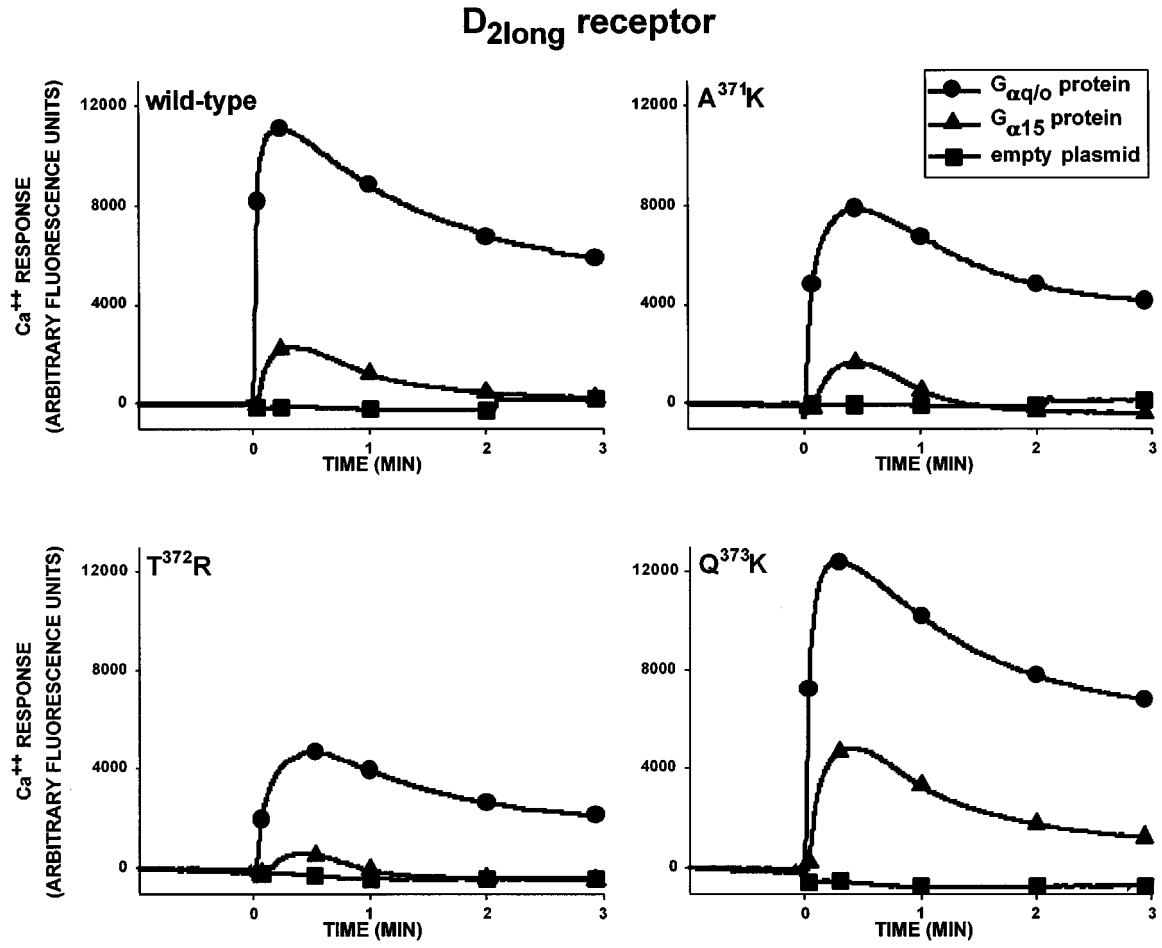

Figure 2 Dopamine-mediated Ca^{2+} responses by wild-type and mutant D_{2long} receptors in either the co-presence of a $G_{\alpha q/o}$ or $G_{\alpha 15}$ protein in CHO-K1 cells. Ca^{2+} responses were measured in the presence of 10 μM dopamine in the co-presence of empty plasmid, $G_{\alpha q/o}$ or $G_{\alpha 15}$ protein as described in Methods. Curves illustrate a representative experiment. The magnitude of the Ca^{2+} responses in mean arbitrary fluorescence units $\pm$ s.e.mean is summarized in Table 2.

Table 2 Magnitude of dopamine-mediated Ca^{2+} responses and amount of [^{3}H]-sulpiride binding sites to wild-type and mutant dopamine D_{2long} receptors in CHO-K1 cells

	$G_{\alpha q/o}$ protein		*$G_{\alpha 15}$ protein*	
	Ca^{2+} response (AFU)	*[^{3}H]-sulpiride binding* (fmol mg protein^{-1})	*Ca^{2+} response (AFU)*	*[^{3}H]-sulpiride binding* (fmol mg protein^{-1})
Wild-type	9437 $\pm$ 692	714 $\pm$ 137	2436 $\pm$ 530	891 $\pm$ 185
A371K	7641 $\pm$ 692	864 $\pm$ 199	1523 $\pm$ 475	1077 $\pm$ 403
T372R	5918 $\pm$ 601	114 $\pm$ 28	608 $\pm$ 265	116 $\pm$ 26
Q373K	13180 $\pm$ 893	781 $\pm$ 144	4328 $\pm$ 885	809 $\pm$ 169

Ca^{2+} responses were measured with 10 μM dopamine as described in Methods. Specific [^{3}H]-sulpiride binding sites were measured on intact CHO-K1 cells as described in Methods. Data are presented as mean values $\pm$ s.e.mean of 10–41 ($G_{\alpha q/o}$) and 6–15 ($G_{\alpha 15}$) independent transfection experiments, each one performed in quadruplicate. AFU: arbitrary fluorescence units.

tion at the wild-type and Q373K D_{2long} receptors (Table 3). In contrast, the ligands with exception of bromerguride behaved with a similar onset-time of activation at the T372R receptor. Both the magnitude and potency of the ligand-mediated Ca^{2+} responses by the A371K mutant receptor were attenuated in contrast to those by the T372R mutant receptor which were enhanced. The potency of dopamine was 8 fold attenuated (pEC_{50}: 7.20 $\pm$ 0.04) at the A371K receptor mutant as compared to the wild-type D_{2long} receptor (pEC_{50}: 8.10 $\pm$ 0.10). The maximal response of the partial agonist lisuride was attenuated by 46% and enhanced by 16% at the A371K and T372R receptor mutant, respectively. Bromerguride and (+)-UH 232 displayed a small but significant amount (<20%) of positive intrinsic activity at the wild-type D_{2long} receptor. This activity was almost fully lost at the A371K receptor mutant, whereas it was largest (53 and 67%) at the T372R receptor mutant. Neither (+)-butaclamol, haloperidol nor clozapine at 1 μM displayed a significant positive or negative response on basal Ca^{2+} signalling (Table 4).

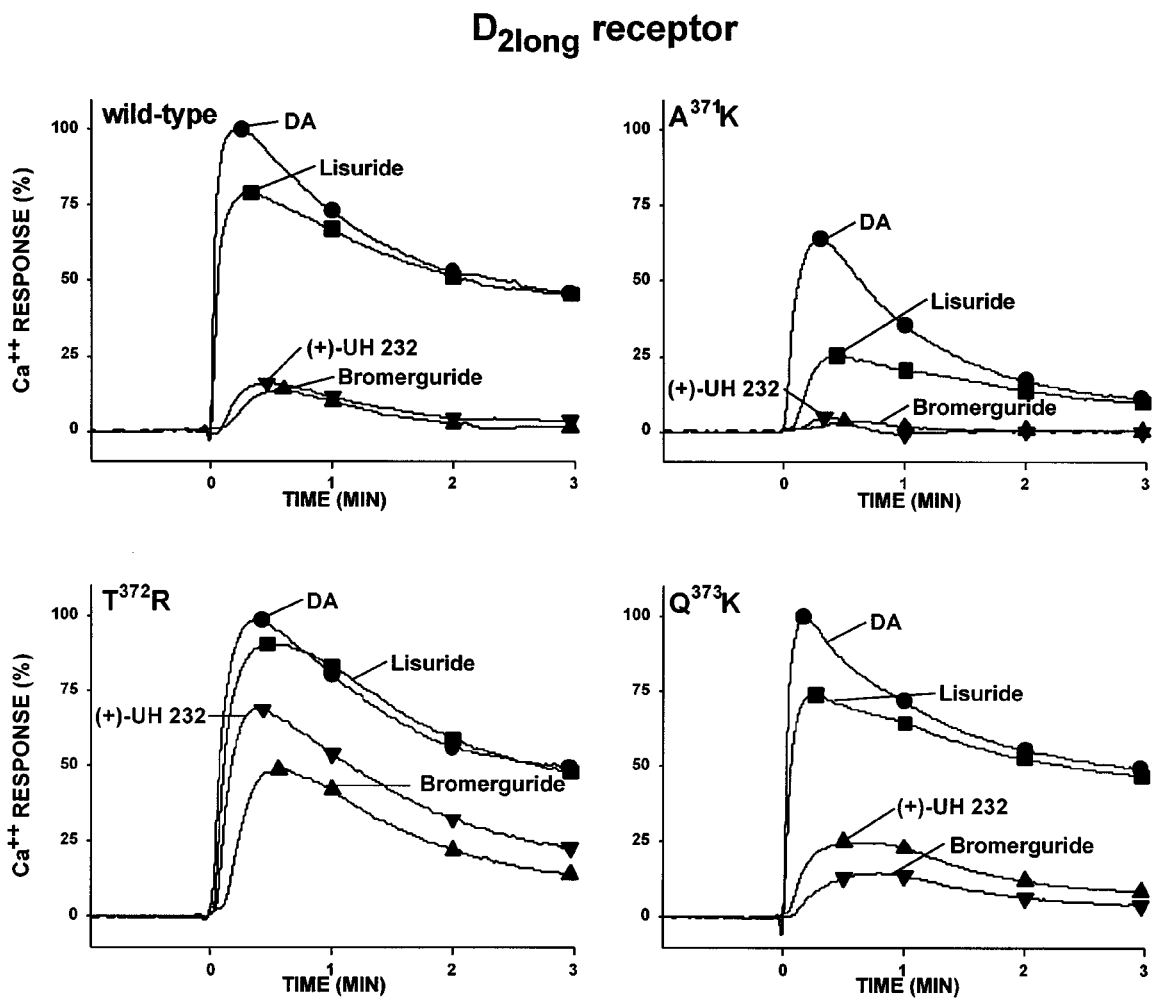

Figure 3 Dopaminergic ligand-mediated Ca^{2+} responses by wild-type and mutant D_{2long} receptors in the co-presence of a $G_{\alpha q/o}$ protein in CHO-K1 cells. Ca^{2+} responses were measured with either 0.1 μM dopamine, 1 μM lisuride, 1 μM (+)-UH 232 or 1 μM bromerguride as described in Methods and expressed in percentage *versus* the Ca^{2+} response as mediated by 10 μM dopamine. Curves illustrate a representative experiment. Mean onset-times of maximal activation (T_{max}), pEC_{50} and E_{max} values$\pm$s.e.mean are summarized in Tables 3 and 4.

Table 3 Onset-time of maximal activation by dopaminergic ligands at wild-type and mutant dopamine D_{2long} receptors as determined for the high-magnitude Ca^{2+} responses in the co-presence of a $G_{\alpha q/o}$ protein in CHO-K1 cells

		Onset-time of maximal activation (T_{max}, s)			
D_{2long} receptor		*wt*	*A371K*	*T372R*	*Q373K*
Dopamine	0.1 μM	12$\pm$2	14$\pm$1	19$\pm$2	12$\pm$2
	10 μM	11$\pm$2	16$\pm$3	20$\pm$3	11$\pm$2
Lisuride	1 μM	19$\pm$3	24$\pm$2	21$\pm$3	16$\pm$3
(+)-UH 232	1 μM	28$\pm$1		22$\pm$1	26$\pm$2
Bromerguride	1 μM	35$\pm$1		32$\pm$2	34$\pm$2

Onset-time of maximal activation was derived from ligand-mediated high-magnitude Ca^{2+} responses as illustrated in Figure 3. Data represent mean values$\pm$s.e.mean of four independent transfection experiments.

Antagonism of dopamine-mediated Ca^{2+} responses by wild-type and mutant dopamine D_{2long} receptors

Antagonism of dopamine-mediated Ca^{2+} responses was assayed for haloperidol, bromerguride, (+)-UH 232, (+)- and (−)-butaclamol over a period of 10 min (Figure 4 and Table 5). Haloperidol (1 μM) completely blocked the dopamine-mediated high- and low-magnitude Ca^{2+} responses at both wild-type and each of the mutant D_{2long} receptors on condition it was pre-incubated 10 min prior to 1 μM dopamine. A similar result was observed with 1 μM of bromerguride and 1 μM (+)-butaclamol, though bromerguride displayed a large positive Ca^{2+} response (53% *versus* dopamine) at the T372R receptor mutant when assayed in the absence of dopamine. Comparison of the antagonist properties upon simultaneous incubation of the putative antagonist and dopamine at time zero indicated the following features: the rank order of antagonist capacity of the dopamine-mediated Ca^{2+} response was for each of these ligands:

Table 4 Intrinsic activities of dopaminergic ligands at wild-type and mutant dopamine D_{2long} receptors as determined by Ca^{2+} responses in the co-presence of a $G_{\alpha q/o}$ protein in CHO-K1 cells

D_{2long} receptor		*Wild-type*	n	*A371K*	n	*T372R*	n	*Q373K*	n
Dopamine	pEC_{50}	8.10±0.10	12	7.20±0.04	7	8.38±0.07	8	8.19±0.08	7
Lisuride	E_{max} (%)	75±5	4	29±4	3	91±4	3	75±2	3
	pEC_{50}	8.54±0.13	4	7.83±0.37	3	8.21±0.10	3	8.68±0.37	3
(+)-UH 232	E_{max} (%)	19±3	10	2±0	10	67±4	3	24±3	10
	pEC_{50}	7.70±0.04	2			8.01±0.22	3	7.92±0.00	2
Bromerguride	E_{max} (%)	14±2	3	2±0	12	53±12	4	19±4	3
	pEC_{50}					7.10±0.04	4	6.94±0.28	2
(+)-Butaclamol	E_{max} (%)	0±0	9	5±2	3	−1±0	3	2±1	3
Haloperidol	E_{max} (%)	0±0	13	1±0	9	0±0	8	0±0	8
Clozapine	E_{max} (%)	0±0	3	2±0	3	2±0	3	0±0	3

Ca^{2+} responses were measured in the co-presence of a $G_{\alpha q/o}$ protein as described in Methods. E_{max} values are presented in percentage of respective maximal Ca^{2+} response induced by 10 μM dopamine. *n*: number of independent transfection experiments, each one performed in quadruplicate.

A371K > wild-type = Q373K > T372R; the transient high-magnitude Ca^{2+} peak response was rarely antagonized (i.e., 55±3% by haloperidol at A371K D_{2long} receptor); and the onset-time ($T^{1/2}$ E_{asym}, s) of full blockade of the low-magnitude Ca^{2+} phase showed the following rank order: haloperidol (29±5) < bromerguride (58±6) < (+)-butaclamol (99±21) [i.e., wild-type D_{2long} receptor, $P<0.05$, Newman-Keuls multiple-comparison test after significant randomized block ANOVA (F (2,5) = 21.10, $P<0.004$]. Preincubation of (+)-UH 232 (1 μM) for 10 min partially (74−84 %) blocked the Ca^{2+} response as mediated by the wild-type and mutant D_{2long} receptors under conditions of an approximately equivalent response by dopamine; no significant difference was observed between each of the receptor mutants. Note that the magnitude of the Ca^{2+} response by dopamine upon pre-incubation with (+)-UH 232 was smaller than its intrinsic agonist effect at the empty, dopamine-free T372R receptor; this was also observed with bromerguride. Simultaneous incubation of (+)-UH 232 with dopamine displayed partial antagonism of the dopamine-mediated Ca^{2+} response (A371K > wild-type = Q373K) in contrast to weak antagonism at the T372R D_{2long} receptor. The inactive (−)-enantiomer of butaclamol produced no antagonism of the dopamine-mediated Ca^{2+} response, irrespective of the selected D_{2long} receptor or incubation condition (Table 5).

Discussion

The present study demonstrates modulation of the activation state of the D_{2long} receptor in a G_{α} protein-dependent manner by mutation of the distal $Lys^{369}LysAlaThrGln^{373}$ motif in its third intracellular loop. Both the A371K and T372R D_{2long} receptor mutants lost 80% of their capacity to respond to dopamine in the co-presence of a $G_{\alpha o}Cys^{351}Ile$ protein. These receptor mutants could be activated in a ligand-dependent manner *via* either a $G_{\alpha q/o}$ or a $G_{\alpha 15}$ protein; the magnitude of the dopamine-mediated Ca^{2+} responses being inferior to that of the wild-type D_{2long} receptor. The dopamine-mediated Ca^{2+} responses in the co-presence of a $G_{\alpha q/o}$ protein consisted of a rapid transient increase with a high-magnitude phase followed by a low-magnitude phase which continued for the recorded period (10 min). The partial agonists lisuride, (+)-UH 232 and bromerguride displayed dissimilar kinetic properties; their onset-times of maximal activation varied and could differ by a factor of three *versus* dopamine, as was the case for bromerguride at the wild-type D_{2long} receptor. The T372R receptor mutant displayed distinct kinetic properties; the onset-time of maximal activation by dopamine was delayed in contrast to that mediated by the A371K receptor mutant. Lisuride and (+)-UH 232 behaved with a similar onset-time of maximal activation as dopamine, whereas bromerguride was unaffected at the T372R receptor mutant. Hence, the kinetic data as provided by the Ca^{2+} responses may be an alternative way to discern differences in receptor activation as a consequence of a point-mutation, coupling to a distinct G_{α} protein or interaction with a different ligand.

The investigated mutations in the D_{2long} receptor did not (under the above described experimental conditions) demonstrate constitutive activity. The agonist-independent (basal) [^{35}S]-GTPγS binding response was not significantly enhanced by the BBXXB mutations and could not detectably be attenuated by putative dopamine antagonists such as haloperidol. This observation contrasts with several mutations (Kjelsberg *et al*., 1992; Ren *et al*., 1993; Egan *et al*., 1998; Herrick-Davis *et al*., 1997; Pauwels *et al*., 1999; 2000a; Rossier *et al*., 1999) in the BBXXB region of α_{1B}-adrenergic, α_{2A}-adrenergic, $5-HT_{1B}$, $5-HT_{2A}$ and $5-HT_{2C}$ receptors that have shown to yield constitutive activation with a differentiation between ligands, classically defined as antagonists, as either weak partial agonists, neutral antagonists, and partial to inverse agonists. Most of the dopamine antagonists have nonetheless been suggested to act as inverse agonists at the dopamine D_2 receptor (Hall & Strange, 1997; Strange, 1999), 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 (Malmberg *et al*., 1996; Backlund Höök *et al*., 1999; Choi *et al*., 2000) as compared to other G protein-coupled receptor systems (Daeffler & Landry, 2000) is perhaps one possible explanation for the apparent lack of differentiation between the intrinsic activity of these putative inverse agonists. We found clear differences in the activation profiles of both A371K and T372R D_{2long} receptor mutants by the partial agonists lisuride, (+)-UH 232 and bromerguride. A371K and T372R receptor mutants displayed an attenuated and enhanced response to these partial agonists, respectively. Therefore, the substitution of the Ala^{371} or Thr^{372} receptor position by a basic amino acid determines an opposite role in the agonist-dependent D_{2long} receptor activation. Mutation of

Val^{385} to Ala in the distal portion of the third intracellular loop of the muscarinic m_2 receptor (equivalent to A371K position in the D_{2long} receptor) has also been shown to display a 16-fold attenuated potency for carbachol (Kostenis

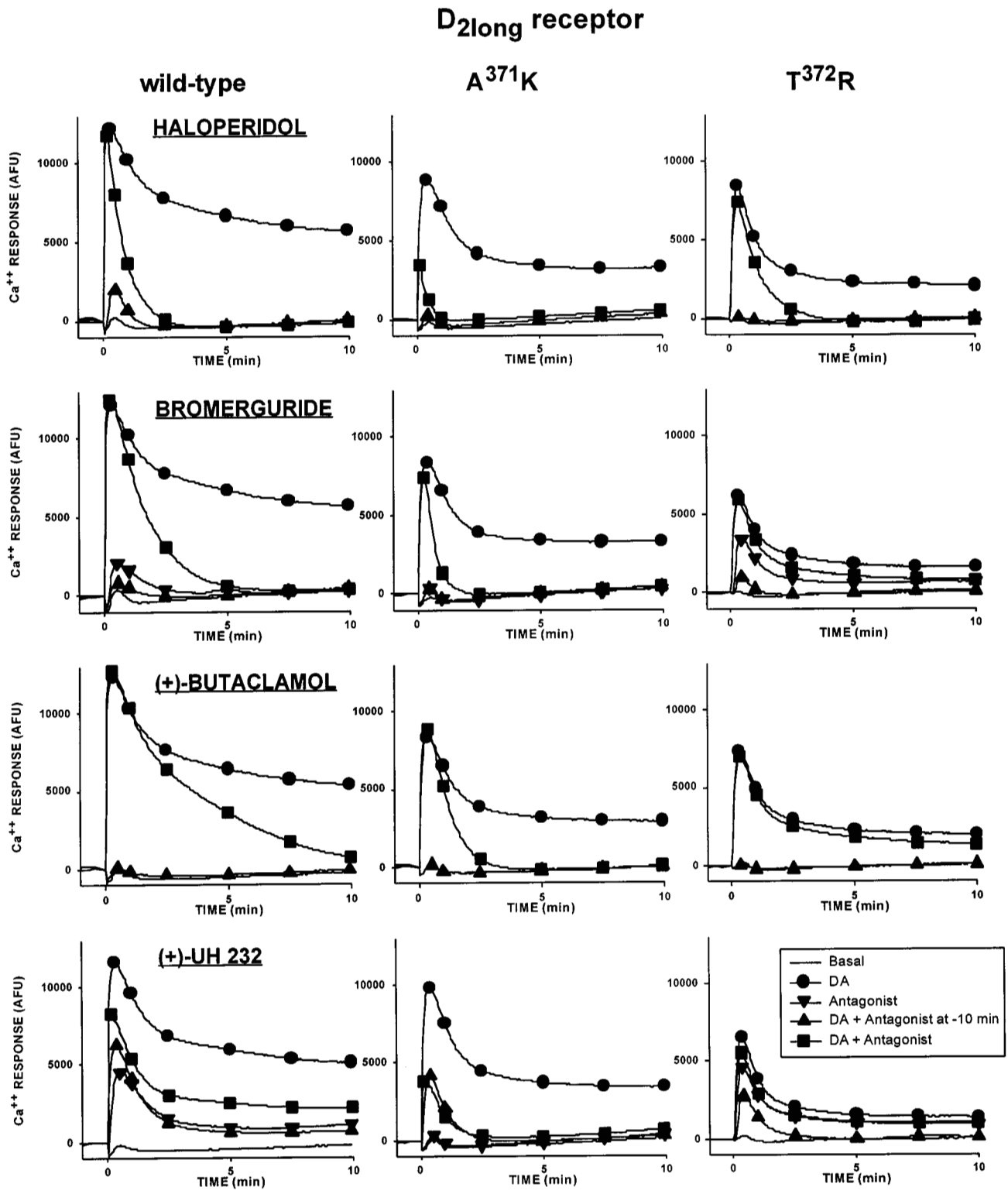

Figure 4 Antagonism of dopamine-mediated Ca^{2+} responses in the co-presence of a $G_{\alpha q/o}$ protein by wild-type, mutant A371K and T372R D_{2long} receptors in CHO-K1 cells. Antagonist (1 μM) was either preincubated for 10 min (DA + Antagonist at −10 min) or co-administrated with dopamine (1 μM except 0.1 μM for (+)-UH 232 at wild-type and T372R D_{2long} receptors) at time zero (DA + Antagonist). For comparison, the Ca^{2+} response of 1 μM bromerguride and 1 μM (+)-UH 232 at the dopamine-free receptor (Antagonist) was also displayed; this was not shown for haloperidol, (+)- and (−)-butaclamol as they were virtually free of intrinsic activity. Curves illustrate a representative experiment. Mean antagonist capacity values ± s.e.mean are summarized in Table 5.

Table 5 Antagonist capacities of putative dopamine antagonists at wild-type and mutant dopamine D_{2long} receptors as determined by Ca^{2+} responses in the co-presence of a $G_{\alpha q/o}$ protein in CHO-K1 cells

D_{2long} receptor		*Antagonist capacity (%)* *Wild-type*	*A371K*	*T372R*	*Q373K*
Haloperidol	T_{-10}	98±1	98±1	101±2	98±0
	T_0	85±2	92±3	67±4	86±3
Bromerguride	T_{-10}	98±1	100±1	97±2	100±1
	T_0	63±3	88±1	21±5	64±5
(+)-butaclamol	T_{-10}	98±2	99±1	101±1	99±1
	T_0	39±5	74±3	12±6	40±3
(+)-UH 232	T_{-10}	74±2	84±2	83±2	74±3
	T_0	55±2	81±2	20±6	56±3
(−)-butaclamol	T_{-10}	2±1	0±3	6±4	7±3
	T_0	−5±2	2±4	5±7	0±3

Antagonist capacity for antagonists (1 μM) was measured against 1 μM dopamine (0.1 μM for (+)-UH 232 at wild-type, T372R and Q373K D_{2long} receptors), as illustrated in Figure 4. The antagonist capacity (%) was calculated as the surface area between the dopamine and ligand condition for a period of 10 min upon addition of dopamine. Data represent mean values±s.e.mean of four independent transfection experiments. T_{-10}: antagonist preincubated for 10 min prior to dopamine; T_0: antagonist added simultaneously with dopamine.

et al., 1997). This amino acid portion has recently been demonstrated to be involved in cell type-specific internalization of the muscarinic m_2 receptor (Schlador *et al*., 2000). A double mutation in the $5-HT_{1A}$ receptor ($Thr^{343}Lys+Val^{344}$-Glu equivalent to Ala^{371} and Thr^{372} in the D_{2long} receptor) has been reported (Malmberg & Strange, 2000) to alter receptor: G protein coupling, allowing ligand-dependent coupling of receptor to G_s in addition to $G_{i/o}$ proteins. We extend the implication of these non-basic amino acid positions of the BBXXB motif as being critical for dopamine D_{2long} receptor: G_α protein interactions.

Bromerguride and (+)-UH 232 showed weak but significant positive intrinsic activity at the wild-type dopamine D_{2long} receptor; this activity could be enhanced at the facilitating T372R D_{2long} receptor mutant. Both enantiomers of UH 232 have also been reported as partial agonists by measuring the extracellular acidification rate at the D_{2long} receptor stably transfected in CHO-K1 cells (Coldwell *et al*., 1999). Monitoring forskolin-stimulated cyclic AMP accumulation, Hall & Strange (1997) suggested (+)-UH 232 to be a weak partial inverse agonist rather than a truly neutral antagonist at the stably transfected D_{2short} receptor. It cannot be excluded that these minor differences in intrinsic activities for UH 232 reflect effector-dependent features.

Haloperidol, bromerguride and (+)-butaclamol completely antagonized the high- and low-magnitude Ca^{2+} responses mediated by the wild-type and mutant D_{2long} receptors when the cells were incubated with antagonist prior to 1 μM dopamine. Whereas this condition apparently optimizes the interaction of the antagonist with the D_{2long} receptor before exposure to dopamine, this situation is unlikely to occur in a physiological condition. Moreover, certain dopamine pathways have been postulated to be overactive in schizophrenia (Niedermier & Nasrallah, 1997), though a true hyperdopaminergic state has not been established in schizophrenia (Goldstein, 1999). The application of the antagonist simultaneously with dopamine may mimic a more physiological condition to investigate antagonist capacity. Under this condition, the capacity of the dopamine antagonists [haloperidol, bromerguride, and (+)-butaclamol] to block the transient high-magnitude Ca^{2+} phase by the wild-type D_{2long} receptor was absent; full blockade of the low-magnitude Ca^{2+} phase appeared later on, haloperidol being faster than bromerguride and (+)-butaclamol. This implies that dopamine has the capacity to induce Ca^{2+} mobilization for a transient period with a variable duration (i.e., 2.5 min for haloperidol) in the co-presence of an antagonist. The question thus arises as to what are the cellular consequences of such a transient Ca^{2+} phase as compared to the maximally effective dopamine-mediated Ca^{2+} response. The herein described Ca^{2+} data suggest that the duration of this transient Ca^{2+} phase may vary, dependent on the investigated dopamine antagonist. It would be interesting to investigate the properties of an antagonist which is capable to entirely block the transient high-magnitude Ca^{2+} phase.

A similar antagonist profile was also found at the A371K receptor mutant though the efficacy of the blockade was greater as could be expected with a less potent dopamine response at this receptor mutant. Conversely, the blockade of the low-magnitude Ca^{2+} phase was attenuated (haloperidol) or almost absent [(+)-butaclamol and bromerguride] at the T372R receptor mutant. Note that bromerguride, in contrast to (+)-butaclamol, displayed dopamine-like intrinsic activity at the presumably unoccupied, dopamine-free receptor while both compounds were virtually ineffective as antagonist. This observation exemplifies that a weak antagonism cannot be solely explained by the putative presence or, for that matter, absence of the ligand's intrinsic activity. Similar observations have been made for closely related compounds at the wild-type α_{2C}-adrenoceptor (Pauwels & Colpaert, 2000). Remarkably, bromerguride and also (+)-UH 232, when applied prior to dopamine, demonstrated a lower Ca^{2+} response as compared to their intrinsic Ca^{2+} response at the unoccupied D_{2long} receptor. Thus, these Ca^{2+} data again suggest that dopaminergic compounds act differently at the unoccupied, dopamine-free D_{2long} receptor and dopamine-bound receptor. The comparison between these two dopamine D_{2long} receptor states may reveal distinct antagonist properties that are not apparent from other analyses.

In conclusion, mutagenesis of the Ala^{371} and Thr^{372} positions in the BBXXB motif affects in an opposite way the ligand-dependent activation and blockade of the D_{2long} receptor. The Ca^{2+} data further suggest a different spectrum in the antagonist capacity of the Ca^{2+} response as observed by analyses of dopamine: antagonist interactions at the wild-

type and mutant D_{2long} receptors. The occurrence of a Ca^{2+} phase with different decay-times, dependent on the investigated dopamine antagonist, may reveal distinct properties of these ligands.

We sincerely thank Dr W. Koek for helpful analyses of the antagonist Ca^{2+} data. Stéphanie Brignatz is greatly acknowledged for secretarial assistance.

References

BACKLUND HÖÖK, B., BREGE, C., LINNAMEN, T., MALMBERG, A. & JOHANSSON, A.M. (1999). Derivatives of (R)-2-amino-5-methoxytetralin: antagonists and inverse agonists at the dopamine D_2 receptor. *Bioorg. Med. Chem. Lett.,* **9,** 2167–2172.

BOND, R.A., LEFF, P., JOHNSON, T.D., MILANO, C.A., ROCKMA, H.A., MCMINN, T.R., APPARSUNDARAM, S., HYEK, M.F., KENAKIN, T.P., ALLEN, L.F. & LEFKOWITZ, R.J. (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2-adrenoreceptor. *Nature,* **374,** 272–276.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254.

BRAESTRUP, C., SCHMIECHEN, R., NEEF, G., NIELSEN, M. & PETERSEN, E.N. (1982). Interactions of convulsive ligands with benzodiazepine receptors. *Science,* **216,** 1241–1243.

CHOI, D.-S., WANG, D., TOLBERG, L. & SADEE, W. (2000). Basal signaling activity of human dopamine D_2L receptor demonstrated with an ecdysone-inducible mammalian expression system. *J. Neurosci. Meth.,* **94,** 217–225.

COLDWELL, M.C., BOYFIELD, I., BROWN, A.M., STEMP, G. & MIDDLEMISS, D.N. (1999). Pharmacological characterization of extracellular acidification rate responses in human $D_{2(Long)}$, D_3 and $D_{4,4}$ receptors expressed in chinese hamster ovary cells. *Br. J. Pharmacol.,* **127,** 1135–1144.

CONKLIN, B.R., FARFEL, Z., LUSTIG, K.D., JULIUS, D. & BOURNE, H.R. (1993). Substitution of three amino acids switches receptor specificity of $G_{q\alpha}$ to that of $G_{i\alpha}$. *Nature,* **363,** 274–276.

COSTA, T. & HERTZ, A. (1989). Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 7321–7325.

DAEFFLER, L. & LANDRY, Y. (2000). Inverse agonism at heptahelical receptors: concept, experimental approach and therapeutic potential. *Fundam. Clin. Pharmacol.,* **14,** 73–87.

DE LIGT, R.A.F., KOUROUNAKIS, A.P. & IJZERMAN, A.D. (2000). Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *Br. J. Pharmacol.,* **130,** 1–12.

DUPUIS, D.S., TARDIF, S., WURCH, T., COLPAERT, F.C. & PAUWELS, P.J. (1999). Modulation of 5-HT_{1A} receptor signalling by point-mutation of cysteine^{351} in the rat $G_{\alpha o}$ protein. *Neuropharmacol.,* **38,** 1035–1041.

EGAN, C.T., HERRICK-DAVIS, K. & TEITLER, M. (1998). Creation of a constitutively activated state of the 5-Hydroxytryptamine$_{2A}$ receptor by site-directed mutagenesis: inverse agonist activity of antipsychotic drugs. *J. Pharmacol. Exp. Ther.,* **286,** 85–90.

GOLDSTEIN, J.M. (1999). Atypical antipsychotic drugs: beyond acute psychosis, new directions. *Emerging Drugs,* **4,** 127–151.

HALL, D.A. & STRANGE, P.G. (1997). Evidence that antipsychotic drugs are inverse agonists at D_2 dopamine receptors. *Br. J. Pharmacol.,* **121,** 731–736.

HAYES, G., BIDEN, T.J., SELBIE, L.A. & SHINE, J. (1992). Structural subtypes of the dopamine D_2 receptor are functionnally distinct: expression of the cloned D_{2A} and D_{2B} subtypes in a heterologous cell line. *Mol. Endocrinol.,* **6,** 920–926.

HERRICK-DAVIS, K., EGAN, C. & TEITLER, M. (1997). Activating mutations of the serotonin 5-HT_{2C} receptor. *J. Neurochem.,* **69,** 1138–1144.

KJELSBERG, M.A., COTECCHIA, S., OSTROWSKI, J., CARON, M.G. & LEFKOWITZ, R.J. (1992). Constitutive activation of the α_{1B}-adrenergic receptor by all amino acid substitutions at a single site: evidence for a region which constrains receptor activation. *J. Biol. Chem.,* **267,** 1430–1433.

KOSTENIS, E., CONKLIN, B.R. & WESS, J. (1997). Molecular basis of receptor/G protein coupling selectivity studied by coexpression of wild type and mutant m2 muscarinic receptors with mutant $G_{\alpha q}$ subunits. *Biochemistry,* **36,** 1487–1495.

LIU, Y.F., CIVELLI, O., GRANDY, D.K. & ALBERT, P.R. (1992). Differential sensitivity of the short form and long human dopamine D_2 receptor subtypes to protein kinases C. *J. Neurochem.,* **59,** 2311–2317.

MALMBERG, A., HÖÖK, B.B., JOHANSSON, A.M. & HACKSELL, U. (1996). Novel (R)-2-amino-5-fluorotetralins: dopaminergic antagonists and inverse agonists. *J. Med. Chem.,* **39,** 4421–4429.

MALMBERG, A. & STRANGE, P.G. (2000). Site-Directed mutations in the third intracellular loop of the serotonin 5-HT_{1A} receptor alter G protein coupling from G_i to G_s in a ligand-dependent manner. *J. Neurochem.,* **75,** 1283–1293.

MILANO, C.A., ALLEN, L.F., ROCKMAN, H.A., DOLBER, P.C., MCMINN, T.R., CHIEN, K.R., JOHNSON, T.D., BOND, R.A. & LEFKOWITZ, R.J. (1994). Enhanced myocardial function in transgenic mice overexpression of the beta 2-adrenergic receptor. *Science,* **264,** 582–586.

NIEDERMIER, J.A. & NASRALLAH, H.A. (1997). New developments in schizophrenia. *J. Prac. Psych. Behav. Hlth.,* **3,** 191–201.

PARMA, J., DUPREZ, L., VAN SANDE J., PASCHKE, R., TONACCHERA, M., DUMONT, J., & VASSART, G. (1994). Constitutively active receptors as a disease-casuing mechanism. *Mol. Cell. Endocrinol.,* **100,** 159–162.

PAUWELS, P.J. & COLPAERT, F.C. (2000). Partial to complete antagonism by putative antagonists at the wild-type α_{2C}-adrenoceptor based on kinetic analyses of agonist: antagonist interactions. *Br. J. Pharmacol.,* **131,** 1385–1390.

PAUWELS, P.J., GOUBLE, A. & WURCH, T. (1999). Activation of constitutive 5-hydroxytryptamine$_{1B}$ receptor by a series of mutations in the BBXXB motif: positioning of the third intracellular loop distal junction and its $G_{o\alpha}$ protein interactions. *Biochem. J.,* **343,** 435–442.

PAUWELS, P.J., TARDIF, S., FINANA, F., WURCH, T. & COLPAERT, F.C. (2000a). Ligand-receptor interactions as controlled by wild-type and mutant Thr^{370}Lys α_{2B}-adrenoceptor: $G_{\alpha 15}$ fusion proteins. *J. Neurochem.,* **74,** 375–384.

PAUWELS, P.J., TARDIF, S., WURCH, T. & COLPAERT, F.C. (2000b). Facilitation of constitutive α_{2A}-adrenoceptor activity by both single amino acid mutation (Thr^{373}Lys) and $G_{\alpha o}$ protein coexpression: evidence for inverse agonism. *J. Pharmacol. Exp. Ther.,* **292,** 654–663.

REN, Q.H., KUROSE, H., LEFKOWITZ, R.J. & COTECCHIA, S. (1993). Constitutively active mutants of the α_2-adrenergic receptors. *J. Biol. Chem.,* **268,** 16483–16487.

ROSSIER, O., ABUIN, L., FANELLI, F., LEONARDI, A. & COTECCHIA, S. (1999). Inverse agonism and neutral antagonism at α_{1A}- and α_{1B}-adrenergic receptor subtypes. *Mol. Pharmacol.,* **56,** 858–866.

SCHLADOR, M.L., GRUBBS, R.D. & NATHANSON, N.M. (2000). Multiple topological domains mediate subtype-specific internalization of the M_2 muscarinic acetylcholine receptor. *J. Biol. Chem.,* **275,** 23295–23302.

STRANGE, P.G. (1999). Agonism and inverse agonism at dopamine $D_{2\text{-Like}}$ receptors. *Clin. Exp. Pharmacol. Physiol.,* **26,** S3–S9.

WILKIE, T.M., SCHERLE, P.A., STRATHMANN, M.P., SLEPAK, V.Z. & SIMON, M.I. (1991). Characterization of G-protein alpha subunits in the G_q class: expression in murine tissues and in stromal and hematopoietic cell lines. *Proc. Natl. Acad. Sci. U.S.A.,* **15,** 10049–10053.

ZHOU, Y.Y., SONG, L.S., LAKATTA, E.G., XIAO, R.P. & CHENG, H. (1999). Constitutive beta2-adrenergic signalling enhances sarcoplasmic reticulum Ca^{2+} cycling to augment contraction in mouse heart. *J. Physiol.,* **521,** 351–361.

(Received March 6, 2001
Revised June 4, 2001
Accepted June 21, 2001)