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ORIGINAL ARTICLE

Non-competitive interaction between raclopride and spiperone on human D_{2L}-receptors in intact Chinese hamster ovary cells

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ABSTRACT

We recently investigated the binding properties of the antagonists [^{3}H]-raclopride and [^{3}H]-spiperone to intact Chinese hamster ovary cells expressing recombinant human D_{2long}-dopamine receptors (CHO-D_{2L} cells). Compared with saturation binding with [^{3}H]-raclopride, raclopride reduced [^{3}H]-spiperone binding with to low potency in competition binding experiments. The present findings illustrate the ability of spiperone to inhibit [^{3}H]-raclopride binding non-competitively. While raclopride only decreases the apparent K_D of [^{3}H]-raclopride in saturation binding experiments, spiperone only decreases the number of sites to which [^{3}H]-raclopride binds with high affinity. Also, while the IC_{50} of raclopride depends on the concentration of [^{3}H]-raclopride in competition experiments, this is not the case for spiperone. Kinetic studies reveal that the binding of raclopride at its high affinity sites does not affect the association of subsequently added [^{3}H]-spiperone nor the rebinding of freshly dissociated [^{3}H]-spiperone to the same or surrounding receptors. Yet, spiperone does not affect the dissociation rate of [^{3}H]-raclopride and raclopride does not affect the (genuine) dissociation rate of [^{3}H]-spiperone. The easiest way to interpret the present findings in molecular terms is to assume that D_{2L}-receptors or their dimeric complexes possess two distinct binding sites: one with high affinity/accessibility for [^{3}H]-raclopride and the other one with high affinity/accessibility for [^{3}H]-spiperone. The ability of bound spiperone to inhibit high affinity raclopride binding while the reverse is not the case suggests for the occurrence of non-reciprocal allosteric interactions. These new findings could point at the occurrence of allosteric interactions between different classes of D_2-receptor antagonists.

INTRODUCTION

Dopamine receptors belong to the large family of G protein-coupled receptors (GPCRs) [1]. They are divided into five subclasses, termed D_1 to D_5. D_1 and D_5-receptors stimulate cAMP production while D_2-, D_3- and D_4-receptors produce the opposite effect by exerting negative control on the adenylyl cyclase activity [2,3]. D_2-receptors exist as two variants with comparable pharmacological properties, D_{2short} (D_{2S}) and D_{2long} (D_{2L}) with an extra 29 amino acid insert in the third intracellular loop [4].

D_2-receptors mediate some of the most important physiological actions of dopamine, such as control of movement and prolactin secretion [5]. Their blockade is of particular interest in clinical therapy as it contributes to attenuating the psychotic phases in patients suffering from schizophrenia [6,7]. To this end, various neuroleptic drugs have been developed, ranging from those with high D_2-recepor affinity and slow dissociation kinetics

(i.e. classical neuroleptics/antipsychotics) [8] to those with low affinity and fast dissociation (i.e. atypical neuroleptics/antipsychotics) [9]. The high potency and long-lasting D_2-receptor blockade of the classical antipsychotics have been held responsible for their predisposition for producing extrapyramidal side effects, as a result of the refractoriness of the occupied D_2-receptors in the striatum in responding to fast fluctuations in local dopamine concentration. The clinical findings with antipsychotics clearly illustrate that receptor binding kinetics may influence the utility of a therapeutic drug [10,11].

A vast majority of previously published D_2-receptor binding studies have been carried out on plasma membranes and/or cell homogenates and were often performed with the tritiated antagonists [^{3}H]-raclopride (benzamide and hydrophilic) and [^{3}H]-spiperone (butyrophenone and highly hydrophobic). Some of these studies have shed light on marked differences in the binding properties of these ligands. Among them, distinct spiperone/raclopride potency ratios show up when comparing competition experiments with [^{3}H]-spiperone and [^{3}H]- raclopride (513- and 16-fold, respectively in [12,13]).

However, GPCRs (and membrane-associated proteins in general) lose part of their natural environment when cells have been disrupted [14,15] and it is thus quite conceivable that their observed characteristics in membrane preparations differ from those that pertain to intact cells. Because of this concern, we recently compared the [^{3}H]-raclopride and [^{3}H]-spiperone binding characteristics in intact recombinant Chinese hamster ovary cells stably expressing human D_{2L}-receptors (i.e. CHO-D_{2L} cells) [16]. In these intact cell experiments, specific binding of both radioligands occurred to a comparable number of sites. However, 'competition binding' experiments with [^{3}H]-spiperone and [^{3}H]-raclopride showed the same marked differences in the spiperone/raclopride potency ratios for the intact CHO-D_{2L} cells (692- and 17-fold respectively) as in earlier studies on membranes [12,13]. Simulations with the classical equations according to the law of mass-action, with the kinetic parameters of both ligands and with the assumption that spiperone and raclopride are competitive with one another, failed to reproduce this behaviour.

The major incongruence with the simulations was the low potency of raclopride in the competition binding studies with [^{3}H]-spiperone. This discordant behaviour prompted us to examine the potential occurrence of non-competitive interactions among raclopride and spiperone at their receptor in intact CHO-D_{2L} cells by a variety of approaches. Contrary to unlabelled raclopride, spiperone was found to inhibit the binding of [^{3}H]-raclopride in a non-competitive fashion. On the other hand, at concentrations sufficient to saturate its high affinity sites, raclopride failed to affect the rate of [^{3}H]-spiperone association as well as [^{3}H]-spiperone 'rebinding'. These findings hint at allosteric interactions between both ligands.

MATERIALS AND METHODS

Materials

[^{3}H]-raclopride (60–63 Ci/mmol) and [^{3}H]-spiperone (79–113 Ci/mmol) were obtained from PerkinElmer (Boston, MA, USA) and Amersham (Buckinghamshire, UK), respectively. Clozapine, haloperidol, raclopride and spiperone were purchased from Tocris (Avonmouth, UK) and (+)-butaclamol and bovine serum albumin (BSA) from Sigma-Aldrich (St. Louis, MO, USA). Chinese hamster ovary cells (CHO-K1) stably transfected with the cDNA for apo-aquorin of *Aquoria victoria* and the GTP-binding protein $G_{\alpha 16}$ (CHO-AEQ cells) were kindly donated by Dr M. Detheux (Euroscreen s.a., Gosselies, Belgium).

Cell culture and stable transfection

Chinese hamster ovary cells stably transfected with the cDNA for apo-aquorin of *Aquoria victoria* cells were cultured in 5% CO_2 at 37 °C in 75 cm^2 flasks in supplemented Dulbecco's Modified Essential Medium (DMEM), i.e. with 2 mM L-glutamine, 2% of a stock solution containing 5000 IU/mL penicillin and 5000 μg/mL streptomycin (Life Technologies, Merelbeke, Belgium), and 10% foetal bovine serum (Life Technologies). The pcDNA3.1 expression vector containing the entire coding region of the human D2DR (transcript variant 1) was obtained from UMR cDNA Resource Center (Rolla, MO, USA) and was transfected into the CHO-AEQ cells using lipofectamine (Life Technologies) according to the manufacturer's instructions. Seventy-two hours after transfection the supplemented DMEM was replaced with the same medium containing 1 mg/mL geneticin (Life Technologies). After approximately five passages, the resulting stably transfected cells (denoted as CHO-D_{2L} cells) were further grown in supplemented DMEM and used in the present studies.

Binding experiments on intact cells

Chinese hamster ovary cells-AEQ stably transfected with the cDNA of the human D2DR (transcript variant 1) cells

were plated in polystyrene Costar® Corning® Cellbind® Surface 24-well plates (Elscolab, Kruibeke, Belgium) and cultured until confluence. Before the experiment, cells were washed three times with 500 μL/well of HEPES/DMEM (Life Technologies) at room temperature. Incubations were carried out at 37 °C in a final volume of 500 μL/well of HEPES/DMEM buffer (pH 7.4) either alone (for [^{3}H]-raclopride) or supplied with 0.2% (w/v) BSA (for [^{3}H]-spiperone). Non-specific binding was determined by performing the incubation with radioligand in the presence of 1 μM spiperone (for [^{3}H]-raclopride) or 1 μM (+)-butaclamol (for [^{3}H]-spiperone). Intermediate wash steps involved replacement of the supernatant with 1 mL HEPES/DMEM buffer at 37 °C and prompt removal of this buffer.

For [^{3}H]-raclopride competition binding (*Figure 1*), cells were incubated for 30 min with radioligand (0.5 or 8 nM) either alone (control) or in the presence of simultaneously added increasing concentrations of unlabelled ligands. For [^{3}H]-raclopride saturation binding (*Figure 2*), cells were incubated for 30 min with increasing concentrations (0.25–8 nM) of radioligand either alone (control) or in presence of simultaneously added (i.e. co-incubation) spiperone (0.25 nM) or raclopride (4 nM). For [^{3}H]-raclopride dissociation assays (*Figure 3*), cells were incubated for 30 min with 2 nM [^{3}H]-raclopride and its dissociation was initiated by addition of 1 μM raclopride, 1 μM spiperone or both together.

For [^{3}H]-spiperone association kinetics (*Figure 4a*), cells were pre-incubated for 30 min with buffer alone (control curve) or with 100 nM raclopride, washed two times and further incubated for the indicated periods of time with 1 nM [^{3}H]-spiperone. For preventing the 'rebinding' of [^{3}H]-spiperone (*Figure 4b*), cells were incubated for 30 min with 1 nM [^{3}H]-spiperone, washed two times and further incubated for 120 min (wash out-time) with buffer either alone (control) or including increasing concentrations of unlabelled ligands.

At the end of all experiments, the plates were placed on ice, washed three times with ice-cold PBS and cells were solubilized by addition of 300 μL 1 M NaOH and

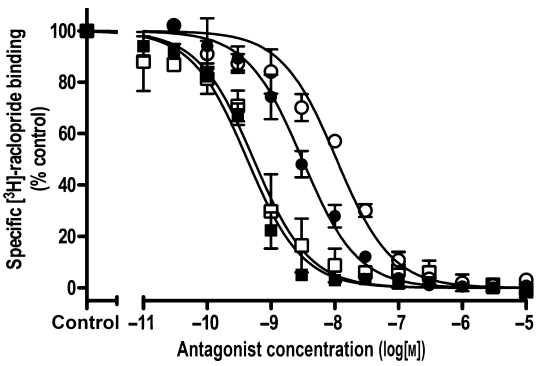

Figure 1 [^{3}H]-Raclopride 'competition binding' experiments with spiperone and raclopride. Intact CHO-D_{2L} cells were co-incubated for 30 min at 37 °C with 0.5 nM (■, ●) or 8 nM (□, ○) [^{3}H]-raclopride in the presence of increasing concentrations of spiperone (■, □) or raclopride (●, ○). Specific binding is expressed as percentage of control binding (i.e. binding in buffer only), n = 3.

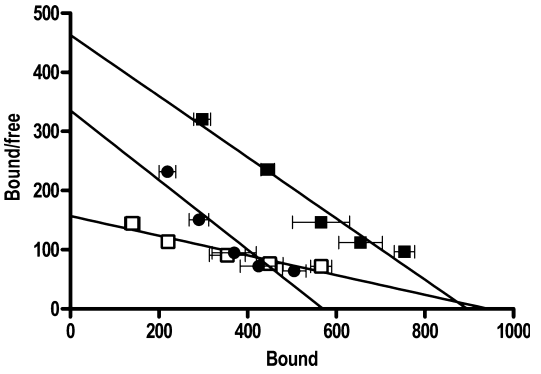

Figure 2 Effect of spiperone and raclopride on [^{3}H]-raclopride saturation binding. Intact CHO-D_{2L}-cells were co-incubated for 30 min at 37 °C with increasing concentrations of [^{3}H]- raclopride either in the absence (■) or presence of 0.25 nM spiperone (●) or 4 nM raclopride (□). Saturation binding data (specific binding) is presented under the form of Scatchard plots [34], n = 3.

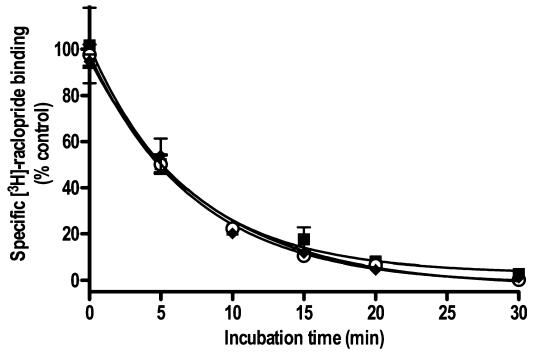

Figure 3 Dissociation of [^{3}H]-raclopride from intact CHO-D_{2L} cells. Cells were incubated for 30 min at 37 °C with 2 nM [^{3}H]-raclopride and its dissociation was initiated by addition of 1 μM raclopride (■), 1 μM spiperone (○) or a combination of both (◆). Remaining specific binding was measured after the indicated periods (abscissa) and expressed as percentage of control binding (i.e. [^{3}H]-raclopride binding immediately after removal of the free radioligand), n = 3.

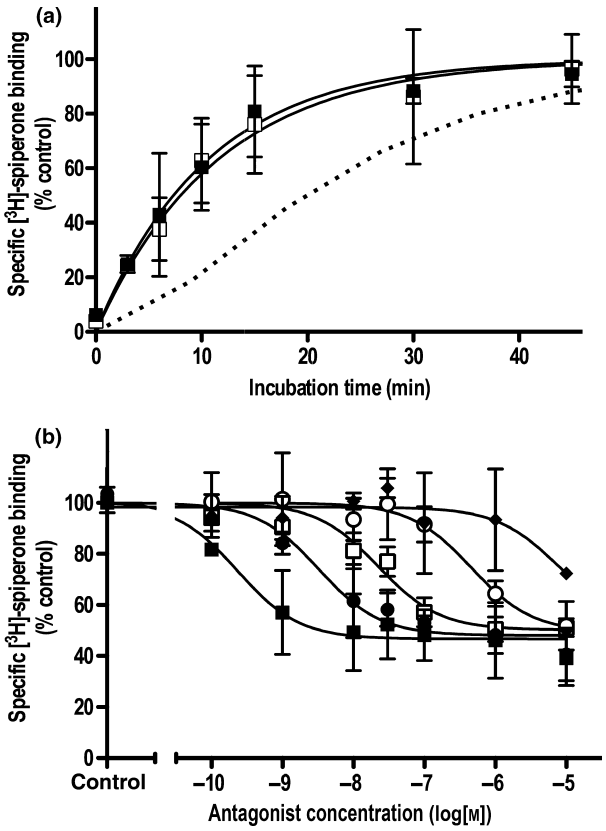

Figure 4 (a) Effect of raclopride on [^{3}H]-spiperone association binding kinetics. Intact CHO-D_{2L} cells were pre-incubated for 30 min at 37 °C with buffer alone (■) or with 100 nM raclopride (□), washed twice and further incubated for different periods with 1 nM [^{3}H]-spiperone. Specific binding was measured after the indicated periods (abscissa) and expressed as percentage of control binding (i.e. binding after 90 min in buffer only), $n = 3$. Dotted line: Simulated [^{3}H]-spiperone association binding curve (for more information, please see Materials and methods) for raclopride-pretreated cells under the same experimental conditions based on the assumption that both ligand–receptor interactions follow the law of mass-action and are mutually exclusive. (b) Effect of increasing concentrations of unlabelled D_2-receptor antagonists on [^{3}H]-spiperone dissociation. Intact CHO-D_{2L} cells were pre-incubated for 30 min at °C with 1 nM [^{3}H]-spiperone, washed twice and further incubated for 120 min in buffer without (control binding) or with increasing concentrations of spiperone (■), haloperidol (□), (+)-butaclamol (●), raclopride (○) and clozapine (♦). Remaining specific binding is expressed as percentage of control binding, $n = 4$.

200 µL H_2O per well. After 60 min treatment at room temperature, solutes were transferred in scintillation vials. The radioactivity was counted for 3 min in a liquid scintillation counter after addition of 3 mL of scintillation liquid (Optiphase Hisafe, PerkinElmer; Boston, MA, USA).

Simulations

Computer-assisted simulations (dotted line in *Figure 4a*) were performed as previously described [17] to generate a [^{3}H]-spiperone/D_{2L}-receptor association binding curve in raclopride-pretreated cells when assuming that both ligand–receptor interactions follow the law of mass-action and are mutually exclusive. The association (k_{+1}: 5.50×10^7 and 6.30×10^7 M/min for raclopride and spiperone, respectively) and dissociation (k_{-1}: 0.12 and 0.007 per min for raclopride and spiperone, respectively) rate constants of both antagonists were calculated from the kinetic studies shown in Packeu et al. [16]. These kinetic constants are regarded to be the same for the unlabelled and labelled form of the same antagonist. Simulated data rely on integrating the outcomes of the differential equations (yielding changes in the percentage of free and ligand bound receptors after very small time periods) for a total time period of 30 min for the pre-incubation with 100 nM raclopride and for increasing time periods up to 45 min for the ensuing incubation with 1 nM [^{3}H]-spiperone.

Data analysis

All experimental values are shown as the mean ± SD of at least three independent experiments (each performed in duplicate or triplicate). The half maximal inhibitory concentration values (IC_{50}) from competition binding experiments, kinetic constants from time curves and B_{max} and K_D from the saturation binding experiments were calculated by non-regression analysis by GraphPad Prism™ (version 4.0 for Mac OSX, GraphPad Software, San Diego, CA, USA) based on a one-site bimolecular reaction obeying the Law of Mass Action. For curve fitting in the competition binding experiments, a one-site model was used with free-floating 100% and 0% equal to non-specific binding (i.e., in presence of 1 µM spiperone or 1 µM (+)-butaclamol for the [^{3}H]-raclopride or [^{3}H]-spiperone binding experiments, respectively).

RESULTS

Using intact recombinant CHO cells (CHO-D_{2L} cells) [16], we here explore for the potential occurrence of non-competitive interactions among raclopride and spiperone by radioligand binding approaches. Only specific (D_{2L}-dopamine receptor) binding is considered below.

[^{3}H]-raclopride binding experiments

In competition binding experiments, intact CHO-D_{2L} cells were co-incubated for 30 min with 0.5 or 8 nM [^{3}H]-

raclopride along with increasing concentrations of the unlabelled ligands. Care was hereby taken to add unlabelled ligand and radioligand simultaneously to prevent the potential interference of insurmountable interactions [18]. As shown in *Figure 1*, all the competition binding curves were steep. In agreement with competitive bimolecular ligand–receptor interactions, the potency of raclopride was inversely related to the [^{3}H]-raclopride concentration, i.e. pIC_{50} = 8.51 ± 0.03 and 7.97 ± 0.04 at 0.5 and 8 nM radioligand, respectively. By contrast, the potency of spiperone was independent of the [^{3}H]-raclopride concentration, i.e. pIC_{50} = 9.37 ± 0.03 and 9.27 ± 0.05 at 0.5 and 8 nM radioligand, respectively.

In [^{3}H]-raclopride saturation binding experiments (*Figure 2*), co-incubation with a D_{2L}-dopamine receptor sub-saturating concentration of raclopride (4 nM), produced an apparent decrease in the equilibrium dissociation constant of the radioligand (i.e. K_D = 6.0 nM vs. 1.9 nM in control experiments in medium only) whereas a sub-saturating concentration of spiperone (0.25 nM) did not (i.e. K_D = 1.7 nM). Conversely, spiperone produced a net decrease in the number of sites to which [^{3}H]-raclopride binds with high affinity (i.e. B_{max} = 569 fmol/mg protein vs. 896 fmol/mg protein in the control experiments) while raclopride did not show this effect (i.e. B_{max} = 943 fmol/mg protein). Of note is that the Scatchard plots in *Figure 2* only serve as visual clue and that the above K_D and B_{max} values were obtained by non-linear regression analysis of the saturation binding curves. Here again, only the result with the raclopride/[^{3}H]-raclopride combination is compatible with competitive bimolecular ligand–receptor interactions.

For the dissociation experiments with [^{3}H]-raclopride (*Figure 3*), cells were incubated with 2 nM of the radioligand for 30 min at 37 °C and the dissociation was initiated by addition of 1 μM raclopride, 1 μM spiperone or a mixture of both. The time-wise decline in [^{3}H]-raclopride binding was mono-exponential and nearly complete after 30 min under the three conditions. The corresponding dissociation rate constants (k_{-1}) were very similar: i.e. 0.15 ± 0.03/min ($t_{1/2}$ = 4.60 min) with raclopride, 0.14 ± 0.01/min ($t_{1/2}$ = 4.93 min) with spiperone and 0.12 ± 0.01/min ($t_{1/2}$ = 5.75 min) with raclopride and spiperone. These results indicate that spiperone was not able to affect the dissociation rate of [^{3}H]-raclopride to a different extent as raclopride itself.

[^{3}H]-spiperone binding experiments

Competition and saturation binding assays as in *Figures 1 and 2* but with [^{3}H]-spiperone did not yield sufficiently precise results because of the high degree of non-specific binding at high concentrations of this radioligand. However, kinetic experiments were feasible as they could be carried out with a relatively modest [^{3}H]-spiperone concentration.

Pre-incubation of the CHO-D_{2L} cells for 30 min with an otherwise receptor-saturating concentration of raclopride (100 nM), after brief washing of the cells, affected neither the association rate nor the binding capacity of 1 nM [^{3}H]-spiperone (*Figure 4a*). For comparison, the dotted line in *Figure 4a* refers to a simulated [^{3}H]-spiperone association curve in the case that both ligand–receptor interactions follow the law of mass-action and are mutually exclusive.

The previous wash out experiments with intact CHO-D_{2L} cells revealed that freshly dissociated [^{3}H]-spiperone is prone to bind again to the same or surrounding receptor molecules. This 'rebinding' phenomenon is perceived as an apparent decrease in dissociation rate of [^{3}H]-spiperone. However, this phenomenon can be effectively prevented when unlabelled ligands are present in the wash out medium at high concentration [16]. The present experiments are focused on the concentration-dependency of this preventing effect by raclopride, spiperone and other unlabelled ligands. To this end, [^{3}H]-spiperone pretreated cells were washed and further incubated for a fixed time (120 min) in fresh buffer without or with increasing concentrations of unlabelled ligands. As shown in *Figure 4b*, all the ligands produced a concentration-dependent decline in the amount of specific [^{3}H]-spiperone binding after 120 min wash out, indicating that they prevented [^{3}H]-spiperone rebinding concentration-dependently. All curves were steep and the order of potency of the ligands was the same as in earlier competition binding experiments [16]; i.e. spiperone > (+)-butaclamol > haloperidol > raclopride > clozapine. When the ligands had sufficiently high potency (i.e. for all except clozapine), it could be shown that they produce the same maximal decline in [^{3}H]-spiperone binding (*Figure 4b* and *Table 1*). This is compatible with earlier experiments showing that [^{3}H]-spiperone dissociates with the same rate from the D_{2L}-receptors when its rebinding is prevented by various unlabelled ligands at high concentration [16].

DISCUSSION

The previous work with intact recombinant CHO cells stably expressing the D_{2L}-receptor [16] shed light on marked differences in the spiperone/raclopride potency

Table I Antagonist pIC_{50}-values for the D_{2L}-receptors in intact CHO-D_{2L} cells.

	pIC_{50}	
Antagonist	I	II
Spiperone	9.16 ± 0.07*	9.63 ± 0.30
(+)-Butaclamol	8.77 ± 0.08	8.50 ± 0.19
Haloperidol	7.89 ± 0.10*	7.65 ± 0.12
Raclopride	6.20 ± 0.08*	6.34 ± 0.20
Clozapine	5.10 ± 0.10*	5.13 ± 1.19

Antagonist pIC_{50}-values from (I) competition binding experiments with [^{3}H]-spiperone and (II) for preventing [^{3}H]-spiperone rebinding.

(I) Intact CHO-D_{2L} cells were pre-incubated for 30 min at 37 °C in the presence of increasing concentrations of unlabelled antagonists, followed by a 30 min incubation in their presence along with 1 nM [^{3}H]-spiperone. *Values are calculated from curves shown in fig. 10 in Packeu et al. [16], n = 3–5.

(II) Intact CHO-D_{2L} cells were pre-incubated for 30 min at 37 °C with 1 nM [^{3}H]-spiperone, washed twice and further incubated for 120 min in buffer with increasing concentrations of unlabelled antagonists. Values are calculated from curves shown in *Figure 4*, n = 4.

ratios in 'competition binding' experiments with [^{3}H]-spiperone and [^{3}H]-raclopride (i.e. 692- and 17-fold, respectively). Simulations with the classical equations according to the law of mass-action, with the kinetic parameters of both [^{3}H]-spiperone and [^{3}H]-raclopride and with the assumption that spiperone and raclopride are competitive with one another failed to reproduce this behaviour. These simulations clearly pointed out that, even though raclopride was added to the cells first, it impeded [^{3}H]-spiperone binding with much lower potency than expected from the K_D of [^{3}H]-raclopride in saturation binding experiments. In line with this non-conventional picture, we show here that raclopride and spiperone bind to the D_{2L}-receptor in a non-competitive fashion.

Non-competitive interactions can manifest themselves in a variety of ways, including functional antagonism (i.e. by blocking one of the biochemical processes downstream to receptor activation) and allosteric interactions at a single receptor molecule or molecular complex. For radioligand binding studies, a number of approaches have proven to be particularly useful for the detection of such allosteric interactions. They include saturation and competition binding experiments as well as kinetic approaches with special attention to radioligand dissociation [19]. In this respect, any interpretation of such binding data is only pertinent if the investigated ligands bind to the same receptor population. This concern is especially relevant in the present situation because of the contention that [^{3}H]-raclopride only binds to cell surface receptors while [^{3}H]-spiperone is also potentially able to bind to intracellular receptors [20–22]. However, the similar D_{2L}-receptor binding capacity of [^{3}H]-spiperone and [^{3}H]-raclopride [16] suggests that the same population of cell surface receptors is labelled by both radioligands in our experimental paradigm. This is also in line with studies showing that D_2-receptor internalization is only minimal under basal conditions and that, while it can be readily triggered by dopamine, antagonists are ineffective to this end [22,23].

Under strict co-incubation conditions the IC_{50} of raclopride depended on the concentration of [^{3}H]-raclopride in competition binding experiments (*Figure 1*) and raclopride only produced an apparent decrease in the K_D of [^{3}H]-raclopride in the saturation binding experiments (*Figure 2*). This behaviour is to be expected in the case of competitive interactions. On the other hand, spiperone behaved in a non-competitive fashion under the same conditions. Indeed, the IC_{50} of spiperone did not depend on the concentration of [^{3}H]-raclopride in competition experiments (*Figure 1*). Moreover, spiperone only decreased the apparent binding capacity (B_{max}) of [^{3}H]-raclopride in the saturation experiments (*Figure 2*). Of note is that the B_{max} values were calculated according to a one-site model so that a decrease thereof could result from a sizable drop in affinity of [^{3}H]-raclopride for part of its binding sites instead of a mere drop in its binding capacity.

Allosteric-type non-competitive interactions could, in principle, also allow one ligand to speed-up or to delay the dissociation of the other ligand [19]. However, when [^{3}H]-raclopride dissociation was monitored in the presence of an excess of raclopride or spiperone, no significant difference in dissociation rate was noticed (*Figure 3*). However, this does not rule out that spiperone is capable of interfering with raclopride binding by preventing or delaying its association to the D_{2L}-receptor.

A number of experiments show that, at concentrations sufficient for binding to most of its high affinity sites, raclopride does not seem to affect [^{3}H]-spiperone binding. In this respect, it was already shown that raclopride decreases the binding of [^{3}H]-spiperone in competition binding experiments with much lower potency than expected from [^{3}H]-raclopride saturation binding (fig. 10 in [16]). Comparable results were presently also obtained by kinetic experiments. The first one is a 'delayed radioligand association' experiment in which receptors are pre-incubated with a saturating concentration of unlabelled ligand and, after a brief wash step to remove

the free unlabelled ligand molecules, further incubated with radioligand for increasing time periods. Provided that the ligand and radioligand interact in a competitive fashion with the receptor, radioligand binding can only take place after the receptors have been liberated from the previously bound unlabelled ligand molecules. Based on this principle, the dissociation rate of the unlabelled ligands can be calculated based on their ability to delay the association of a subsequently added radioligand [24]. In compliance to this, pre-incubation of the CHO-D_{2L} cells with a receptor-saturating concentration of raclopride (100 nM) delayed the association of subsequently added [^{3}H]-raclopride (fig. 5B in [16]) and the extent of this delay was in good agreement with the dissociation rate of [^{3}H]-raclopride in wash out experiments. However, when similar experiments were performed with [^{3}H]-spiperone as radioligand, 100 nM raclopride (occupying >97% of its high affinity sites at the D_{2L}-receptor) failed to delay the association of this radioligand (*Figure 4a*). This behaviour is quite distinct from the expected delay in [^{3}H]-spiperone asociation (dotted line in *Figure 4a*, generated by computer-assisted simulations) when assuming that both ligand–receptor interactions follow the law of mass-action and are mutually exclusive. The second kinetic experiment is focused on the propensity of freshly dissociated [^{3}H]-spiperone molecules to undergo rebinding to the same or surrounding receptor molecules (a phenomenon which produces an apparent delay in the dissociation of this radioligand [16] and deals with the ability of unlabelled ligands to prevent this phenomenon. To this end, receptors are pre-incubated with a saturating concentration of radioligand and, after a brief wash-step to remove the free radioligand molecules, further incubated with or without unlabelled ligand for increasing time periods in the final wash out step [25]. Provided that the ligand and radioligand interact in a competitive fashion with the receptor, the unlabelled ligands should decrease the rebinding of dissociated radioligand molecules in a concentration-dependent fashion and with a potency that is compatible with their affinity for the receptors in question. A convenient way to estimate this potency is to monitor the decline in radioligand binding after a fixed-time wash out with increasing concentrations of unlabelled ligand [26]. Using the same method, it is shown in *Figure 4b* that unlabelled D_2-receptor antagonists lessen the residual [^{3}H]-spiperone binding in a concentration-dependent fashion and with the same potency order as in competition binding studies (*Figure 4b* and *Table I*). While this is in agreement with the alleged role of the unlabelled antagonists to prevent rebinding of [^{3}H]-spiperone by occupying the receptors, raclopride can once again only effectively prevent the rebinding of [^{3}H]-spiperone when its concentration is well beyond what is necessary to saturate the D_{2L}-receptors. Spiperone, butaclamol, haloperidol and raclopride were sufficiently potent to completely prevent [^{3}H]-spiperone rebinding at the highest concentrations tested. Under those conditions, [^{3}H]-spiperone dissociated with the same rate in the presence of all the unlabelled antagonists (*Figure 4b* and [16]). Similar observations were also made with AT_1-angiotensin II receptor antagonists [26].

Taken together, kinetic studies reveal that the binding of raclopride at its high affinity sites at the D_{2L}-receptor does not affect the association and (genuine) dissociation rate of [^{3}H]-spiperone (*Figure 4a, b*) while binding of spiperone prevents high affinity binding of [^{3}H]-raclopride (fig. 5B in [16]) without affecting its dissociation rate (*Figure 3*). This explains the ability of spiperone to decrease the apparent binding capacity of [^{3}H]-raclopride in saturation binding experiments (*Figure 2*) and the overlapping 'competition' binding curves of spiperone at different [^{3}H]-raclopride concentrations (*Figure 1*). The easiest way to interpret the present findings in molecular terms is to assume that D_{2L}-receptors or their dimeric complexes possess two distinct binding sites: one with high affinity/accessibility for [^{3}H]-raclopride and the other one with high affinity/accessibility for [^{3}H]-spiperone. To deal with the ability of high concentrations of raclopride to decrease binding and rebinding of [^{3}H]-spiperone (*Figure. 4a, b*, fig. 10 in [16]), these latter sites may also possess low affinity for raclopride. The 'general allosteric ternary complex' model is the simplest and most widely advocated model to describe the binding of ligands to two distinct sites at a receptor [19,27]. However, as this model predicts a strict reciprocity in the modulatory action of both ligands (i.e. 'whatever ligand A does to B, B does to A'), it does not apply to the present situation. A better candidate is the sequential 'KNF' model [28,29]. Indeed, as this model allows the end state/conformation of the receptor to depend on which ligand bound first, it allows non-reciprocal modulations to take place.

It should be mentioned that binding studies with benzamides and butyrophenones on brain membrane preparations [30] and on membrane preparations from D_{2S}-dopamine receptor expressing CHO cells [31] and insect Sf9 cells [32] also hinted at the occurrence of complex non-competitive interactions and the implication of sodium ions therein. However, no consistent

results were obtained, suggesting for a potentially important role of the test system in consideration. In this respect, this study constitutes the only one that has hitherto been performed on intact cells. The use of recombinant cells in this and preceding study [16] allows the use of the untransfected CHO-K1 cells for control experiments dealing with receptor-independent phenomena such as partitioning of hydrophobic ligands like spiperone into the membrane. Moreover, the stable expression of the D_{2L}-receptors in the CHO-D_{2L} cells allows consistent results to be obtained throughout the study. However, a potential drawback of such recombinant material is that the obtained results could be affected by the presence of human receptors in cells from a distinct species and by receptor overexpression. Although a previous study by our team showed that complex sartan-AT_1 angiotensin II receptor interactions are indistinguishable in studies performed on recombinant and endogenous AT_1 receptor expressing cell lines [33], it cannot be asserted that the same consistency also applies to D_{2L}-receptors. It should therefore be of great interest to repeat the present experiments on endogenous D_{2L}-receptor expressing cell lines. However, as a result of the low amount of specific binding of the tritiated antagonists and especially to the highly unfavourable specific- to non-specific binding ratio [^{3}H]-spiperone, preliminary data lacked the required precision.

We recently proposed a model in which the hydrophilic raclopride approaches the D_{2L}-receptor from the aqueous phase while the hydrophobic spiperone approaches the receptor by lateral diffusion within the membrane [16]. Still compatible with this model this findings hint at the occurrence of two antagonist binding sites with distinct pharmacological properties at the D_{2L}-receptor (or homodimeric complex). Clearly, further research is required to acquire a clearer insight into the molecular basis of these complex ligand–receptor interactions.

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