ELSEVIER

Inverse Agonism at Dopamine D_2 Receptors

Haloperidol-Induced Prolactin Release from GH_4C_1 Cells Transfected with the Human D_2 Receptor Is Antagonized by R(-)-n-propylnorapomorphine, Raclopride, and Phenoxybenzamine

Christer L. Nilsson, M.D., Agneta Ekman, B.Sc., Monika Hellstrand, B.Sc., and Elias Eriksson, Ph.D.

Our earlier observation that the antipsychotic drug haloperidol in the absence of dopamine increases cAMP formation and prolactin release in two prolactin-producing cell lines expressing rat dopamine D_2 receptors (GH_3, GH_4ZR_7), but not in similar cells devoid of D_2 receptors (GH_4C_1), prompted us to suggest that haloperidol may act as an inverse (or negative) agonist, rather than as a neutral antagonist, at the D_2 receptor (Nilsson and Eriksson 1993). In the present study it is shown that haloperidol elicits a dose-dependent increase in prolactin release also in prolactin-producing GH_4C_1 cells transfected with the human dopamine D_2 receptor (short isoform) (GH_4C_1-hD_2s); in addition, it is shown that another antipsychotic drug, flupenthixol, also causes prolactin release per se in this cell line. The effect of haloperidol on prolactin release in GH_4C_1-hD_2s is calcium dependent and counteracted by pretreatment either with the D_2 receptor agonist R(-)-n-propylnorapomorphine or with a D_2 receptor antagonist that does not affect prolactin release per se (raclopride). In addition, pretreatment with the alkylating compound phenoxybenzamine at a concentration causing a marked reduction of D_2 receptor density in GH_4C_1-hD_2s cells significantly counteracted haloperidol-induced prolactin release. **[*Neuropsychopharmacology 15:53–61, 1996*]**

KEY WORDS: *Inverse agonism; Reverse agonism; Negative antagonism; GH_4C_1 cells; Dopamine D_2 receptor; Prolactin; Haloperidol; Flupenthixol; R(-)-n-Propylnorapomorphine; Raclopride; Phenoxybenzamine*

We have earlier reported that the dopamine D_2 receptor antagonist haloperidol, also in the absence of agonist, increases prolactin release and cAMP formation in cultured clonal prolactin-producing pituitary cells expressing rat dopamine D_2 receptors spontaneously (GH_3) or by means of transfection (GH_4ZR_7) (Nilsson and Eriksson 1993). Because no prolactin-releasing effect was observed in cells devoid of D_2 receptors (GH_4C_1), it was suggested that this effect of haloperidol in GH_3 and GH_4ZR_7 cells is mediated by D_2 receptors; thus, we proposed that haloperidol in this experimental setting may act as an inverse (negative) agonist rather than as a neutral antagonist vis-à-vis the dopamine D_2 receptor. In contrast, the D_2 receptor antagonist raclopride, while inhibiting agonist-induced prolactin suppression from GH_4ZR_7 cells, does not cause prolactin release per se (Nilsson and Eriksson 1995).

The purpose of the present study was to explore further whether the observed effect of haloperidol on prolactin release in vitro is indeed mediated by D_2 recep-

From the Institute of Physiology and Pharmacology, Department of Pharmacology, Göteborg University, Sweden.

Address correspondence to Christer L. Nilsson, M.D., Institute of Physiology and Pharmacology, Department of Pharmacology, Göteborg University, Medicinaregatan 7, S-413 90 Göteborg, Sweden.

Received April 27, 1995; revised July 20, 1995; accepted August 4, 1995.

Published by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0893-133X/96/$15.00
SSDI 0893-133X(95)00156-5

tors. To this end we show that the effect of haloperidol on prolactin release previously observed in cell lines expressing the rat D_2 receptor (GH_4ZR_7, GH_3) is observed also in cells transfected with the human D_2 receptor (short isoform) (GH_4C_1-hD_2s). The effect is shown to be calcium dependent and to be counteracted by pretreatment with the D_2 receptor agonist R(-)-n-propylnorapomorphine, the neutral D_2 receptor antagonist raclopride, or the receptor alkylating compound phenoxybenzamine.

MATERIALS AND METHODS

Drugs and Chemicals

Dopamine hydrochloride (Sigma, St. Louis, MO, USA), raclopride tartrate (Astra AB, Södertälje, Sweden), and vasoactive intestinal peptide (VIP, Sigma) were all dissolved in Earle's Balanced Salt Solution (EBSS, Biochrom) supplemented with 0.2% bovine serum albumin (BSA, Sigma). R(-)-n-propylnorapomorphine hydrochloride (NPA, RBI, Natick, MA, USA), and *cis*-flupenthixol hydrochloride (Lundbeck AB, Helsingborg, Sweden) were dissolved in water. Phenoxybenzamine (RBI) was solubilized using 50% ethanol and diluted in EBSS, yielding a final concentration of ethanol <0.5%. Haloperidol (Leo AB, Helsingborg, Sweden) was solubilized in tartaric acid and ethanol and diluted in EBSS; the final concentrations of tartaric acid and ethanol were <0.5 nM and <0.5%, respectively.

Cell Culture

Transfected GH_4C_1 cells expressing the short isoform of the human dopamine D_2 receptor (GH_4C_1-hD_2s) (batch zem 3#3) were kindly provided by Dr. Olivier Civelli, Hoffman-La Roche AG, Basel, Switzerland, and kept frozen in liquid nitrogen before being thawed, propagated, and passaged by means of trypsin-EDTA dissociation. Stock cultures (monolayers) of GH_4C_1-hD_2s cells were maintained in Nunclon T-25 flasks (Nunc A/S, Roskilde, Denmark) with approximately 10 ml of Ham's F-10 medium (Biochrom KG, Berlin, Germany) supplemented with 3.7 g/l sodium bicarbonate (Sigma), 15% horse serum (Biochrom), 2.5% fetal calf serum (Biochrom), 100 U/ml penicillin G + 100 μg/ml streptomycin, 0.6 μg/ml amphotericin B, and 1 mM l-glutamine (Sigma) and incubated at 37°C in a water-saturated atmosphere of 5% CO_2 in air. Passages were performed weekly; in addition, the total medium was changed once between each passage.

Radioligand Binding

GH_4C_1-hD_2s cells were grown in 100-mm tissue culture dishes in 10 ml of the same medium as in the stock culture. On the day of the experiment, cells were incubated for 60 minutes with either 15 μM phenoxybenzamine dissolved in EBSS + BSA or EBSS + BSA only. Each dish was rinsed with Dulbecco's phosphate-buffered saline (PBS); then the cells were scraped into cold PBS, pelleted by centrifugation (500 × g, 7 minutes), resuspended in cold buffer (50 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, pH 7.5 at 25°C), and homogenized using a dounce glass-Teflon homogenizer. The membrane suspension was centrifuged (250 × g, 7 minutes) in order to remove unlysed cells, the supernatant was retained, and the pellet was rehomogenized and centrifuged again (250 × g, 7 minutes); then the combined supernatants were centrifuged at 30,000 × g for 18 minutes. The final membrane pellet was resuspended in radioligand binding buffer (50 mM Tris-HCl, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM KCl, 1 mM EDTA, 120 mM NaCl, pH 7.5 at 25°C), and aliquots were stored at −70°C until used in radioligand binding experiments.

For determination of dopamine D_2 receptor density and affinity, triplicates of cell membrane suspension (1 ml corresponding to 18–26 μg protein) were incubated for 20 minutes at 37°C in radioligand binding buffer (as described) with five to six concentrations (30–1,000 pM) of [^{3}H]spiperone (97–107 Ci/mmol, Amersham) dissolved in radioligand binding buffer with 0.1% ascorbic acid and 0.01% BSA. For each concentration of [^{3}H]spiperone, nonspecific binding was assessed by adding 10 μM of the D_2 receptor agonist 6,7-ADTN (RBI) to triplicate samples. The reaction was terminated by rapid filtration through Whatman GF/B filters coated with 0.1% polyethyleneimine using a cell harvester (Brandel, Gaithersburg, MD, USA) followed by washing with cold buffer (4 ml 50 mM Tris-HCl, three times). The radioactivity trapped on the filters was assessed using a liquid scintillation counter (efficiency approximately 30%). Protein concentration was determined according to a modified Lowry assay using BSA as standard (Markwell et al. 1978). Data were analyzed using the nonlinear least-squares curve-fitting computer program LIGAND (Munson and Rodbard 1980).

Prolactin Experiments

One week before an experiment, GH_4C_1-hD_2s cells were seeded in Nunclon 16 mm 24-multiwell plates in an amount of 200.000 cells per well in 500 μl of the same medium as in stock culture but without amphotericin B. In order to avoid "edge effects" that could affect cell growth, in most experiments only the central eight wells on each plate were used, whereas the 16 peripheral wells were filled with 500 μl of sterile water.

Immediately before the experiments, the cells were washed twice (15 minutes each time) in warmed, CO_2-equilibrated EBSS supplemented with 0.2% BSA (our experimental medium).

To avoid systematic errors due to factors related to the position of the well on the plate, every second well was given the experimental drug (e.g., haloperidol), whereas every second well served as control. In the dose-response experiment, all different doses of haloperidol—and vehicle—were added in random order to the wells on each plate; in this experiment all 24 wells on each plate were used.

In all experiments the experimental incubation lasted for 30 minutes; then the supernatants were gently pipetted from the wells, centrifuged (300 × g, 5 minutes) in order to remove any remaining cells, and frozen for subsequent analysis of prolactin by means of radioimmunoassay (as described later).

Because prolactin levels often showed some interplate variability, the prolactin level in a well was always calculated as a percent of the mean prolactin level of the control wells (receiving vehicle only) on the same plate. Thus, all values are presented as percent of controls.

Generally, prolactin levels in wells situated on the same plate and exposed to the same treatment showed small variability. However, occasionally, the prolactin level in a sample from one well was highly aberrant compared to that from the other wells on the same plate receiving the same treatment. This phenomenon can probably be related to various experimental artifacts, such as the removal of a large number of poorly attached cells from the well during the washes preceding the incubation or remaining whole cells in the supernatant used for prolactin determination. As a rule, a prolactin value of a control well being 30% higher or lower than the mean value of the three other control wells from the same plate was excluded from further calculations. In wells given prolactin-releasing agents (such as haloperidol), the variability was generally somewhat higher than in vehicle-treated wells; for these wells the rule applied was that values being 50% higher or lower than the mean value of the three other wells given the same treatment were excluded.

Experiment 1: For the assessment of the effect of dopamine on VIP- (300 nM) induced prolactin release in GH_4C_1-hD_2s cells, dopamine (50 nM) dissolved in 400 μl of experimental medium was given to half of the wells on each plate; 5 minutes later, 100 μl of experimental medium containing VIP was added to all wells. After VIP had been added, cells were incubated for 30 minutes.

Experiment 2: In the second prolactin experiment, the prolactin-releasing effect of various doses of haloperidol in otherwise untreated cells was studied. Haloperidol was dissolved in the entire experimental medium (500 μl); control wells received experimental medium only.

Experiment 3: In the third prolactin experiment the prolactin-releasing effect of flupenthixol (1 or 10 μM) in otherwise untreated cells was studied. Flupenthixol was dissolved in the entire experimental medium (500 μl) whereas the other wells on the same plate received experimental medium only.

Experiment 4: In the fourth experiment the effect of calcium on haloperidol- (1 μM) induced prolactin release was studied. On half of the plates all wells were given ordinary experimental medium (i.e., EBSS + BSA), whereas on the remaining plates all wells were given the same medium but without calcium. Half of the wells on each plate received haloperidol dissolved in the entire medium (500 μl); the other wells on the same plate received experimental medium only.

Experiment 5: In the fifth experiment the effect of NPA (10 μM) on haloperidol- (1 μM) induced prolactin release was studied. NPA dissolved in 400 μl of medium was given to all wells on half of the plates; 5 minutes later 100 μl of experimental medium containing haloperidol was added to half of the wells whereas the other half received 100 μl of medium only. On the control plates all wells received experimental medium only instead of medium containing NPA; again, haloperidol was added to half of the wells. For cells incubated with NPA, this compound was also present in the experimental medium during the second of the washes preceding the experimental incubation.

Experiment 6: In the sixth experiment the effect of raclopride (100 μM) on haloperidol- (1 μM) induced prolactin release was studied. The design of the experiment was the same as in fifth experiment, the only exception being that raclopride was given instead of NPA.

Experiment 7: In the seventh experiment the effect of preincubation of GH_4C_1-hD_2s with phenoxybenzamine (15 μM) on haloperidol-induced prolactin release was studied. Cells were exposed to phenoxybenzamine dissolved in experimental medium (500 μl) for 60 minutes; then the cells were washed twice and incubated with or without haloperidol (1 μM) for 30 minutes, as described. The cells were not exposed to phenoxybenzamine during the washes or during the experimental incubation.

Experiment 8: In the eighth experiment the effect of treatment with raclopride (100 μM) or pretreatment with phenoxybenzamine (15 μM) on VIP- (300 nM) induced prolactin release was studied. One-third of the plates were preincubated with phenoxybenzamine (as in Experiment 7), whereas one-third were treated with raclopride (as in Experiment 6); one-third of the plates served as controls. To wells containing 400 μl of experimental medium were added either 100 μl of VIP-containing medium or 100 μl of experimental medium only.

Prolactin Radioimmunoassay

Prolactin was analyzed by radioimmunoassay, for which materials were kindly provided by Dr. Philip F. Smith from the National Hormone and Pituitary Program, Ogden Bioservices Corporation, Rockville, MD, USA. NIDDKD rat prolactin RP-3 was used as reference standard. The chloramine-T (Fluka, Buchs, Switzerland) method was used for [^{125}I] iodination. All samples from one experiment were always analyzed in the same assay to avoid the influence of interassay variation. The intraassay variation was always lower than 10%.

RESULTS

Radioligand Binding Experiments

Saturation experiments on cell membrane preparations from GH_4C_1-hD_2s cells, using [^{3}H]spiperone as ligand, yielded a dissociation constant (K_D) of 59 ± 3 pM (mean ± SEM, n = 3) and a calculated B_{max} value of 750 ± 123 fmol/mg protein (mean ± SEM, n = 3).

After incubation of GH_4C_1-hD_2s cells for 60 minutes with 15 μM of phenoxybenzamine, specific binding of [^{3}H]spiperone was undetectable (n = 3).

Prolactin Experiments

Mean baseline prolactin levels in vehicle-treated wells varied considerably between experiments (lowest mean: 5 ng/ml, highest mean: 53 ng/ml). No correlation between baseline prolactin levels and percent prolactin response to haloperidol (or any of the other prolactin-releasing agents tested) was observed.

Experiment 1: Dopamine (50 nM) significantly reduced VIP- (300 nM) induced prolactin release in GH_4C_1-hD_2s cells (vehicle + VIP: 100 ± 3, n = 24 wells; dopamine + VIP: 52 ± 2, n = 24 wells; $p < .0001$; values given as % of vehicle/VIP controls).

Experiment 2: Haloperidol induced a dose-dependent increase in prolactin release from GH_4C_1-hD_2s cells (Figure 1).

Experiment 3: Flupenthixol, in a concentration of 10 μM, significantly increased prolactin release from GH_4C_1-hD_2s cells; in contrast, 1 μM of flupenthixol had no significant effect (Table 1).

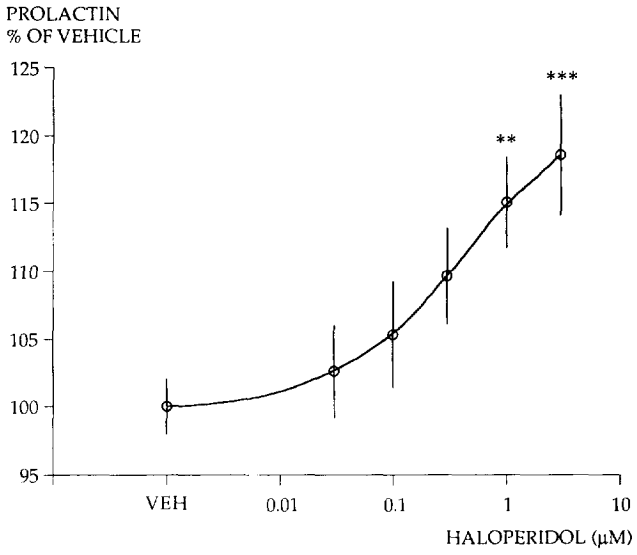

Figure 1. Effect of various concentrations of haloperidol (30 nM, 100 nM, 300 nM, 1 μM, 3 μM) on prolactin release from GH_4C_1-hD_2s cells. The curve was analyzed using UltraFit (BioSoft, Ferguson, MO, USA); goodness of fit (logistic sigmoid) was 0.97; EC_{50} was 425 nM, n = 28. Stars indicate level of significance versus vehicle: $^{**}p < .01$, $^{***}p < .001$ (ANOVA followed by Fisher's PLSD test).

Experiment 4: When GH_4C_1-hD_2s cells were incubated in a calcium-free medium, no effect of haloperidol (1 μM) on prolactin release was observed (Table 2). Prolactin levels in control wells on plates given the calcium-containing experimental medium appeared higher than the corresponding levels on plates given calcium-free medium.

Experiment 5: Pretreatment with NPA in a concentration of 10 μM significantly antagonized haloperidol- (1 μM) induced prolactin release in GH_4C_1-hD_2s cells (Table 2). A comparison of absolute prolactin levels in vehicle-treated wells on plates administered NPA or not lent no support for an effect of NPA per se on spontaneous prolactin release.

Experiment 6: Pretreatment with raclopride in a concentration of 100 μM significantly antagonized haloperidol-

Table 1. Effect of Flupenthixol on Prolactin Release in GH_4C_1-hD_2s Cells.

	n of wells	PRL (% of controls, mean ± SEM)	Difference versus Vehicle (p value)
A Vehicle	24	100 ± 2	NS
B Flupenthixol (1 μM)	24	103 ± 2	
A Vehicle	24	100 ± 1	0.0003
B Flupenthixol (10 μM)	24	108 ± 2	

Differences between groups were statistically evaluated by t-test.

Table 2. Effect of Calcium-Free Medium [Ca(−)] or Pretreatment with NPA (10 μM), Raclopride (100 μM), or Phenoxybenzamine (15 μM) on Haloperidol- (1 μM) Induced Prolactin Release in GH_4C_1-hD_2s Cells

		n of wells	PRL (% of controls, mean ± SEM)	Halo versus Vehicle: (*p* value)	B versus D (*p* value)
A	Ca(+)/veh	45	100 ± 2		
B	Ca(+)/halo	47	107 ± 3	0.02	
C	Ca(−)/veh	43	100 ± 2		
D	Ca(−)/halo	47	98 ± 2	NS	0.01
A	Veh/veh	24	100 ± 2		
B	Veh/halo	23	112 ± 3	0.001	
C	NPA/veh	24	100 ± 1		
D	NPA/halo	24	104 ± 2	NS	0.02
A	Veh/veh	33	100 ± 2		
B	Veh/halo	35	109 ± 4	0.04	
C	Raclo/veh	36	100 ± 2		
D	Raclo/halo	36	98 ± 2	NS	0.008
A	Veh/veh	67	100 ± 1		
B	Veh/halo	68	112 ± 2	0.0001	
C	PBZ/veh	66	100 ± 2		
D	PBZ/halo	68	105 ± 2	NS	0.01

Abbreviations: Veh = vehicle, halo = haloperidol, raclo = raclopride, PBZ = phenoxybenzamine. Second row from the right, *p* values when the effect of haloperidol was compared to that of vehicle in the different groups (*t* test); Row furthest to the right, *p* values for the comparisons of the magnitude of the responses to haloperidol in wells with or without calcium, or with or without pretreatment (*t* test).

(1 μM) induced prolactin release in GH_4C_1-hD_2s cells (Table 2); in contrast, raclopride concentrations of 30 μM or lower did not significantly alter the response to haloperidol (data not shown). A comparison of absolute prolactin levels in vehicle-treated wells on plates administered raclopride or not lent no support for an effect of raclopride per se on spontaneous prolactin release (Nilsson and Eriksson 1995).

Experiment 7: In GH_4C_1-hD_2s cells preexposed to phenoxybenzamine (15 μM) for 60 minutes, haloperidol- (1 μM) induced prolactin release was significantly blunted. A comparison of absolute prolactin levels in vehicle-treated wells on plates administered phenoxybenzamine or not lent no support for an effect of phenoxybenzamine per se on spontaneous prolactin release.

Experiment 8: VIP-induced prolactin release in GH_4C_1-hD_2s cells was not reduced by pretreatment with raclopride (100 μM); neither was it significantly affected by preexposure of the cells to phenoxybenzamine (15 μM) (Table 3).

DISCUSSION

For the present experiments prolactin-producing clonal GH_4C_1 cells stably transfected with the cDNA for the human dopamine D_2 receptor (short isoform) (GH_4C_1-hD_2s) were used. That these cells indeed express dopamine D_2 receptors was confirmed by means of radioligand binding; thus, whereas in wild-type GH_4C_1

Table 3. Effect of Pretreatment with Raclopride (100 μM) or Phenoxybenzamine (15 μM), respectively, on VIP- (300 nM) induced prolactin release in GH_4C_1-hD_2s cells

		n of wells	PRL (% of controls, mean ± SEM)	Controls versus B, C, D (*p* value)	B versus C, D (*p* value)
A	Veh/veh	13	100 ± 2	—	—
B	Veh/VIP	16	150 ± 5	< .0001	—
C	Raclo/VIP	15	145 ± 5	< .0001	NS
D	PBZ/VIP	16	137 ± 5	< .0001	NS

Abbreviations: Veh = vehicle, raclo = raclopride, PBZ = phenoxybenzamine.

Second row from the right, the difference with respect to prolactin release between wells receiving VIP and wells receiving vehicle on plates exposed to the same treatment. Furthest row to the right, differences between the groups exposed to different pretreatments with respect to the magnitude of VIP-induced prolactin release. Data statistically evaluated using ANOVA followed by Fisher's PLSD test.

cells no or very few D_2 receptors can be detected (Albert et al. 1990; Allard et al. 1993; unpublished data), in the transfected cell line the B_{max} for the D_2 receptor, as assessed using [^{3}H]-spiperone as ligand, was 750 fmol/mg protein. Also, by means of Northern Blot, human D_2 mRNA could be detected in GH_4C_1-hD_2s cells but not in wild-type GH_4C_1 cells (unpublished data). That the D_2 receptors expressed in GH_4C_1-hD_2s cells are functionally coupled to intracellular transduction mechanisms is supported by the finding that dopamine effectively antagonized VIP-induced prolactin release in GH_4C_1-hD_2s cells; in contrast, in wild-type GH_4C_1 cells, VIP-induced prolactin release is unaffected by dopamine D_2 agonists (unpublished data).

We have previously shown (Nilsson and Eriksson 1993) that haloperidol, also in the absence of agonist, causes cAMP formation and prolactin release in prolactin-producing cells spontaneously expressing rat D_2 receptors (GH_3, mainly long isoform) (Johnston et al. 1991; Missale et al. 1991; Zhang et al. 1993) and from GH_4C_1 cells transfected with the rat D_2 receptor (GH_4ZR_7, short isoform) (Albert et al. 1990), but not from wild-type GH_4C_1 cells lacking both D_2 receptor mRNA and D_2 receptor binding sites (Albert et al. 1990; Allard et al. 1990; Burris and Freeman 1994). The present finding that haloperidol, in the absence of agonist, causes a dose-dependent prolactin release also from cells transfected with the human D_2 receptor (short isoform) lends further support for the assumption that haloperidol-induced prolactin release in vitro is indeed D_2 mediated; thus, we have now been able to demonstrate a haloperidol-induced prolactin release from three out of three cell lines expressing D_2 receptors, but not from related cells lacking D_2 receptors. Moreover, we have previously reported that haloperidol, in the absence of agonist, causes an increase in cAMP formation (i.e., the effect on cAMP opposite to that induced by dopamine) in GH_3 and GH_4ZR_7 cells (Nilsson and Eriksson 1993). Similarly, preliminary experiments indicate that haloperidol increases the accumulation of cAMP after pretreatment with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) also in GH_4C_1-hD_2s cells (unpublished data).

Calcium is important for spontaneous as well as secretagogue-induced prolactin release (Bjoro et al. 1984). The present observation that the effect of haloperidol was calcium dependent supports the notion that it is not due to a toxic influence on the cells but to an interaction with the transduction mechanisms involved in the physiological control of prolactin secretion.

The magnitude of the effect of haloperidol on prolactin release from GH_4ZR_7, GH_3, and GH_4C_1-hD_2s usually varies from one experiment to another. For example, the response is often weak or even absent when the cells have reached a high density in the wells in which they are grown the week before the experiment; the response also usually declines in cell populations that have been the subject of many passages; both observations are probably related to a reduction in D_2 receptor expression and/or receptor/G-protein coupling. We have previously reported that in GH_4ZR_7 and GH_3 cells haloperidol may cause prolactin release at concentrations as low as 30 nM (Nilsson and Eriksson 1993); in the present study the IC_{50} of haloperidol was 400 nM. However, because of the large interexperimental variability of the prolactin response to nanomolar concentrations of haloperidol, in most experiments presented in this article, haloperidol was administered at a concentration of 1 μM.

In most displacement experiments using rat or human D_2 receptors, haloperidol has been attributed a K_i value of approximately 0.5 to 5 nM (Castro and Strange 1993); however, in some reports, the K_i of haloperidol has been considerably higher (30–100 nM) (Sander et al. 1994). When comparing these K_i values with EC_{50} of haloperidol observed in the present study, it should be taken into consideration that higher concentrations may be required in order to induce uncoupling of precoupled receptors than to displace an antagonist in radioligand binding experiments (Vogel et al. 1995). As yet, the experience of the doses required to achieve "functional" inverse agonism vis-à-vis G-protein-coupled receptors in intact cells is sparse; thus, whether the affinity to ligands of spontaneously precoupled receptors differs from that of uncoupled receptors remains to be established.

The stimulating effect of haloperidol on prolactin release from GH_4C_1-hD_2s cells could be counteracted by pretreatment with high concentrations of the D_2 receptor agonist NPA; similarly, we have previously found that pretreatment with NPA effectively antagonizes the effects of haloperidol on both cAMP formation and prolactin release in GH_4ZR_7 cells (unpublished data). The effect of NPA is well in line with the concept that haloperidol-induced prolactin release is D_2 mediated; however, because in GH_4C_1-hD_2s and GH_4ZR_7 cells dopamine receptor agonists inhibit prolactin release induced also by nondopaminergic receptors partly interacting with the same second messenger systems, such as those for VIP and TRH, the finding that NPA reduces haloperidol-induced prolactin release constitutes no conclusive evidence for an involvement of D_2 receptors in the effect of the latter drug.

In a previous study inverse agonism at beta-adrenoceptors could be counteracted by pretreatment with an antagonist displaying less inverse efficacy (Chidiac et al. 1994). We have earlier shown that the selective D_2 antagonist raclopride is devoid of prolactin-releasing effects per se at GH cells (Nilsson and Eriksson 1995); the present finding that pretreatment with high concentrations of raclopride significantly reduced haloperidol-induced prolactin release, while not affecting the prolac-

tin response to VIP, strongly supports the assumption that the prolactin release induced by haloperidol is indeed D_2 receptor mediated. It should be noted that high concentrations of raclopride were required to antagonize the effect of haloperidol; similarly, in the study by Chidiac and coworkers (1994), a concentration of 100 μM of labetalol was used to antagonize the inverse effects of timolol (100 nM) at beta-adrenoceptors.

It has earlier been shown (Shin et al. 1992) that preincubation of a primary culture of rat adenohypophyseal cells for 1 hour with the receptor alkylating compound phenoxybenzamine markedly reduces dopamine-mediated inhibition of prolactin release at a concentration not influencing changes in prolactin release induced by other receptors (somatostatin, TRH, angiotensin II). In the present study exposure of GH_4C_1-hD_2s cells to phenoxybenzamine was shown to abolish D_2 receptors as measured using [^{3}H]-spiperone binding; the finding that haloperidol-induced prolactin release was significantly blunted in phenoxybenzamine exposed cells whereas the response to VIP was less affected thus lends further support for the concept that haloperidol-induced prolactin release is indeed a D_2-mediated phenomenon.

Additional support for the involvement of D_2 receptors in haloperidol-induced prolactin release was obtained from the observation that another antipsychotic drug with affinity for D_2 receptors, the thioxanthene derivative flupenthixol, was also found to induce a significant increase in prolactin release from GH_4C_1-hD_2s cells.

All experiments in this study were undertaken in a serum-free, balanced salt solution and preceded by repeated washing of the cells; however, albeit unlikely, the possibility that the effects of haloperidol and flupenthixol are due to antagonism of small amounts of dopamine present in the serum-containing medium in which the cells were grown before the experiment should not be ignored. However, the fact that raclopride effectively antagonizes both dopamine-induced prolactin suppression (Nilsson and Eriksson 1995) and haloperidol-induced prolactin release (this report) without mimicking the prolactin releasing effect of haloperidol in the absence of agonists (Nilsson and Eriksson 1993) refutes the possibility that the effect of haloperidol is merely due to displacement of small amounts of dopamine from its receptors.

The concept of inverse (or negative) agonism originally emanates from studies on benzodiazepine receptors associated with the $GABA_A$/chloride channel complex; however, recently, several authors have suggested that G-protein coupled receptors, such as opioid (Costa and Herz 1989), muscarinic (Hilf and Jakobs 1992), dopamine D_{1B} (Tiberi and Caron 1994), $5HT_2$ (Barker et al. 1994; Labreque et al. 1994; Westphal and Sanders-Bush 1994), and β_2 (Adie and Milligan 1994; Chidiac et al. 1994; Mewes et al. 1994) receptors may also be the subjects of inverse agonism. The earliest of these reports were based on experiments using membrane preparations; however, recently several research groups have demonstrated inverse agonism in intact cells, too. The concepts of constitutive activity and of inverse agonism of G-protein coupled receptors have been thoroughly discussed in several recent papers (Costa et al. 1992; Schütz and Freissmuth 1992; Lefkowitz et al. 1993; Onaran et al. 1993; Tian and Deth 1993; Leeb-Lundberg et al. 1994; Milligan et al. 1995).

Apart from our reports on haloperidol-induced cAMP formation and prolactin release in GH_3, GH_4ZR_7, and GH_4C_1-hD_2s cells (Nilsson and Eriksson 1993; this paper), reports on the negative efficacy of dopamine D_2 antagonists in the absence of agonists are as yet sparse. However, more than 15 years ago, chlorpromazine was in fact reported to stimulate adenylate cyclase activity in broken cell preparations from GH_3 cells (Clement-Cormier et al. 1977; Heindel and Clement-Cormier 1981); similar effects were also obtained with fluphenazine in fragments from human prolactin adenomas (De Camilli et al. 1979). More recently the D_2 antagonist (-)-sulpiride was reported to act as an inverse agonist at D_2 receptors with respect to protein kinase C activity in reconstituted synaptoneurosomes (Giambalvo and Wagner 1994); in addition, radioligand binding data supporting the concept that human D_2 receptors transfected into GH_4 cells are also partly activated in the absence of agonist were recently reported (Kramer and Deth 1995; Tian and Deth 1993). Finally, it should be mentioned that 10 years ago Wreggett and De Léan (1984), on the basis of computer modeling of experimental radioligand binding data obtained from membrane homogenates of bovine anterior pituitary glands, suggested that D_2 receptors could appear in a coupled state also in the absence of agonist and that this spontaneous precoupling could be counteracted by an antagonist.

Whether the concept of inverse agonism at G-protein-coupled receptors is of significance also in vivo remains to be established. Interestingly, recent data from our laboratory indicate that in male rats pretreatment with raclopride in a dose causing prolactin release per se counteracts the elevation of serum levels of prolactin induced by haloperidol (unpublished). Inverse agonism at D_2 autoreceptors regulating dopamine release was also recently suggested on the basis of in vivo microdialysis experiments (Carlsson et al. 1991).

In spite of the fact that raclopride displays high affinity for dopamine D_2 receptors, with respect to both extrapyramidal side effects and to antipsychotic efficacy, this compound appears somewhat less powerful than haloperidol (The British Isles Raclopride Study Group 1992). Given the present data, it is tempting to speculate that this difference between haloperidol and raclopride could at least be related partly to a difference in inverse efficacy at D_2 receptors in the striatum and the nucleus accumbens.

ACKNOWLEDGMENTS

GH_4C_1 cells transfected with human D_2 receptor was kindly donated by Dr. Olivier Civelli, Hoffman-La Roche AG, Basel, Switzerland. Radioimmunoassay materials were provided by Dr. Philip F. Smith from the National Hormone and Pituitary Program, Rockville, MD, USA. Excellent technical assistance was provided by Gunilla Bourghardt, Lucia Gaete, and Inger Oscarsson. The study was supported by the Swedish Medical Research Council (grants No. 08668 and 09299).

REFERENCES

Adie EJ, Milligan G (1994): Regulation of basal adenylate cyclase activity in neuroblastoma X glioma hybrid, NG108-15, cells transfected to express the human β_2 adrenoceptor: Evidence for empty receptor stimulation of the adenylate cyclase cascade. Biochem J 303:803–808

Albert PR, Neve KA, Bunzow J, Civelli O (1990): Coupling of a cloned rat dopamine-D_2 receptor to inhibition of adenylyl cyclase and prolactin secretion. J Biol Chem 265:2098–2104

Allard S, Lapointe S, Falardeau P (1993): Specific induction of a functional endogenous D_2 short dopamine receptor in GH_4C_1 cells. Biochem Biophys Res Comm 193:801–807

Barker, EL, Westphal RS, Schmidt D, Sanders-Bush E (1994): Constitutively active 5-hydroxytryptamine$_{2C}$ receptors reveal novel inverse agonist activity of receptor ligands. J Biol Chem 269:11687–11690

Bjøro T, Haug E, Sand O, Gautvik KM (1984): Vasoactive intestinal peptide causes a calcium-dependent stimulation of prolactin secretion in GH_4C_1 cells. Mol Cell Endocrinol 37:41–50

The British Isles Raclopride Study Group (1992): A double-blind comparison of raclopride and haloperidol in the acute phase of schizophrenia. Acta Psychiatr Scand 86:391–398

Burris TP, Freeman ME (1994): Comparison of the forms of the dopamine D_2 receptor expressed in GH_4C_1 cells. Proc Soc Exp Biol Med 205:226–235

Carlsson A, Waters N, Svensson K, Löfberg L (1991): New trends in the molecular biology and pharmacology of dopaminergic transmission. In Racagni G, Brunello N, Fukuda T (eds), Biological Psychiatry, vol 2, Amsterdam, Elsevier, pp 687–689

Castro SW, Strange PC (1993): Differences in the ligand binding properties of the short and long versions of the D_2 dopamine receptor. J Neurochem 60:372–375

Chidiac P, Hebert TE, Valiquette M, Dennis M, Bouvier M (1994): Inverse agonist activity of β-adrenergic antagonists. Mol Pharmacol 45:490–499

Clement-Cormier YC, Heindel JJ, Robison GA (1977): Adenylyl cyclase from a prolactin producing tumor cell: The effects of phenotiazines. Life Sci 21:1357–1364

Costa T, Herz A (1989): Antagonists with negative intrinsic activity at δ opioid receptors coupled to GTP-binding proteins. Proc Natl Acad Sci USA 86:7321–7325

Costa T, Ogino Y, Munson PJ, Onaran HO, Rodbard D (1992): Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: Thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. Mol Pharmacol 41:549–560

De Camilli P, Macconi D, Spada A (1979): Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. Nature 278:252–254

Giambalvo CT, Wagner RL (1994): Activation of D_1 and D_2 dopamine receptors inhibits protein kinase C activity in striatal synaptoneurosomes. J Neurochem 63:169–176

Heindel JJ, Clement-Cormier YC (1981): Regulation of adenylate cyclase activity in GH_1 cells by chlorpromazine and a heat-stable factor. Endocrinology 108:310–317

Hilf G, Jakobs KH (1992): Agonist-independent inhibition of G protein activation by muscarinic acetylcholine receptor antagonists in cardiac membranes. Eur J Pharmacol [Mol Sect] 225:245–252

Johnston JM, Wood DF, Bolaji EA, Johnston DG (1991): The dopamine D_2 receptor is expressed in GH_3 cells. J Mol Endocrinol 7:131–136

Kramer ML, Deth RC (1995): Characterization of sodium-sensitive D_2 dopamine receptors via basal and ligand-regulated GTP binding studies. FASEB J 9: abstract 3879

Labrecque J, Dennis M, Fargin A (1994): Inverse efficacy of serotonergic antagonists at the 5HT-$_{2C}$ receptor expressed in Sf9 cells. Can J Physiol Pharmacol 72(Suppl 1): P18.9.35, 546

Leeb-Lundberg LMF, Mathis SA, Herzig MCS (1994): Antagonists of bradykinin that stabilize a G-protein-uncoupled state of the B_2 receptor act as inverse agonists in rat myometrial cells. J Biol Chem 269:25970–25973

Lefkowitz RJ, Cotecchia S, Samama P, Costa T (1993): Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. Trends Pharmacol Sci 14:303–307

Markwell MAK, Haas SM, Bieber LL, Tolbert NE (1978): A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem 87:206–210

Mewes T, Dutz S, Ravens U, Jakobs KH (1994): Evidence for G-protein involvement in spontaneous activity of cardiac empty β-adrenoceptors. Naunyn Schmiedeberg's Arch Pharmacol 349(suppl):R10

Milligan G, Bond RA, Lee M (1995): Inverse agonism: Pharmacological curiosity or potential therapeutic strategy? Trends Pharmacol Sci 16:10–13

Missale C, Boroni F, Castelletti L, dal Toso R, Gabelini N, Sigala S, Spano PF (1991): Lack of coupling of D_2 receptors to adenylate cyclase in GH_3 cells exposed to epidermal growth factor. Possible role of a differential expression of G_i protein subtypes. J Biol Chem 266: 23392–23398

Munson PJ, Rodbard D (1980): LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107:220–239

Nilsson CL, Eriksson E (1993): Haloperidol increases prolactin release and cyclic AMP formation in vitro: Inverse agonism at dopamine D_2 receptors? J Neural Transm [Gen Sect] 92:213–220

Nilsson CL, Eriksson E (1995): Effects of remoxipride and raclopride on prolactin release from clonal pituitary tumour cells. Pharmacol Toxicol 76:85–88

Onaran HO, Costa T, Rodbard D (1993): βγ-Subunits of guanine nucleotide-binding proteins and regulation of spontaneous receptor activity: Thermodynamic model for the interaction between receptors and guanine nucleotide-binding protein subunits. Mol Pharmacol 43:245–256

Sander P, Grünevald S, Bach M, Haase W, Reiländer H, Michel H (1994): Heterologous expression of the human D_{2S} dopamine receptor in protease-deficient *Saccharomyces cerevisiae* strains. Eur J Biochem 226:697–705

Schütz W, Freissmuth M (1992): Reverse intrinsic activity of antagonists on G protein-coupled receptors. Trends Pharmacol Sci 13:376–380

Shin SH, McAssey K, Heisler RL, Szabo MS (1992): Phenoxybenzamine selectively and irreversibly inactivates dopaminergic D_2 receptors on primary cultured rat lactotrophs. Neuroendocrinology 56:397–406

Tian W-T, Deth RC (1993): Precoupling of G_i/G_o-linked receptors and its allosteric regulation by monovalent cations. Life Sci 52:1899–1907

Tiberi M, Caron MG (1994): High agonist-independent activity is a distinguishing feature of the dopamine D_{1B} receptor subtype. J Biol Chem 269:27925–27931

Vogel WK, Mosser VA, Bulseco DA, Schimerlik MI (1995): Porcine m2 muscarinic acetylcholine receptor-effector coupling in Chinese hamster ovary cells. J Biol Chem 270:15485–15493

Westphal RS, Sanders-Bush E (1994): Reciprocal binding properties of 5-hydroxytryptamine type 2C receptor agonists and inverse agonists. Mol Pharmacol 46:937–942

Wreggett KA, De Léan A (1984): The ternary complex model: Its properties and application to ligand interactions with the D_2-dopamine receptor of the anterior pituitary gland. Mol Pharmacol 26:214–227

Zhang K, Kulig E, Jin L, Lloyd RV (1993): Effects of estrogen and epidermal growth factor on prolactin and Pit-1 mRNA in GH_3 cells. Proc Soc Exp Biol Med 202:193–200