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Carmoxirole is able to reduce amisulpride-induced hyperprolactinemia without affecting its central effect

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Abstract

Prolactin blood level and apomorphine-induced yawning were studied in rats treated with the substituted benzamide amisulpride in association with bromocriptine or carmoxirole; two dopamine D_2 receptor agonists with high or low propensity to cross the brain–blood barrier, respectively. Administration of amisulpride produced a maximum increase in rat serum prolactin level ($315 \pm 18\%$) vs. vehicle-treated animals ($ED_{50} = 0.25 \pm 0.017$ mg/kg, s.c.). The concurrent administration of carmoxirole or bromocriptine completely reversed the hyperprolactinemia induced by amisulpride (0.5 mg/kg, s.c.) ($ID_{50} = 14.9 \pm 0.8$ mg/kg and 0.81 ± 0.03 mg/kg, respectively). Carmoxirole (15 mg/kg, i.p.) did not affect yawning induced by apomorphine (0.08 mg/kg, s.c.) nor amisulpride (0.5 mg/kg, s.c.) blockade of apomorphine-induced yawning. Conversely, a significant increase in the number of yawns was observed when bromocriptine (0.8 mg/kg, i.p.) was associated with apomorphine in the absence or presence of amisulpride. These results suggested that a peripheral dopamine D_2 receptor agonists could be a useful tool in alleviating amisulpride-induced hyperprolactinemia without possibly affecting its central effect.

Keywords: Antipsychotic; Benzamide; Depression; Prolactin

1. Introduction

The finding that low doses of amisulpride, an atypical antipsychotic of the substituted benzamides class, improved negative symptoms of schizophrenia (Boyer et al., 1995) led to the hypothesis that, at small doses, this drug could be useful in depressive symptoms (Puech et al., 1981). On such basis, amisulpride has been largely and successfully employed in dysthymia and major depression (Lecrubier et al., 1997; Smeraldi, 1998; Ravizza, 1999). This therapeutic property of amisulpride has been associated to its selective antagonism for dopamine D_2 and D_3 autoreceptors (Perrault et al., 1997). Indeed, as it has been amply demonstrated, the dopamine D_2-like autoreceptors are involved in the inhibition of dopamine release, neuronal firing and tyrosine hydroxylase activation (Lejeune and Millan, 1995; Saud Chagny et al., 1991; Walters and Roth, 1976). The selective antagonism of these autoreceptors by low doses of amisulpride should then produce an increase in the dopaminergic neurons firing (Di Giovanni et al., 1998) and release (Shoemaker et al., 1997) in the limbic system, which are accounted for the antidepressant properties of the drug. In line with this hypothesis, different studies conducted on laboratory animals indicated that low doses of amisulpride selectively block dopamine D_2 autoreceptor in vivo. For instance, very low doses of amisulpride (0.2–0.3 mg/kg) were able to antagonize rat yawning and hypomotility induced by pre-synaptic doses of apomorphine, while only very high doses of amisulpride (30–100 mg/kg) are needed to reduce rat hypermotility and stereotypies induced by post-synaptic doses of apomorphine (Perrault et al., 1997; Shoemaker et al., 1997).

Although these studies showed that amisulpride displays a pre-synaptic selectivity, clinical studies on healthy volun-

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teers demonstrated that, even at small doses, amisulpride increases prolactin blood level (Wetzel et al., 1994), indicating that the same doses of amisulpride which antagonize mesolimbic dopamine autoreceptors are able, outside the brain–blood barrier, to block pituitary dopamine D_2 postsynaptic receptors producing hyperprolactinemia as a side effect.

The presence of dopamine D_2 receptor in the anterior and intermediate lobes of the pituitary gland has been extensively demonstrated with biochemical and pharmacological techniques (Enjalbert and Bockaert, 1983; Memo et al., 1986) and the gene encoding for both the dopamine D_{2S} and D_{2L} receptor isoforms has been later found in pituitary lactotroph cells (Dal Toso et al., 1989; Meador-Woodruff et al., 1989; Bunzow et al., 1988). The stimulation of pituitary dopamine D_2 receptor by dopamine released from tuberoinfundibular neurons was proven to reduce prolactin secretion from the pituitary gland, as confirmed by cell culture studies on lactotroph cells and by in vivo evidence (Burris et al., 1991; Ben-Jonathan, 1985). The block of pituitary dopamine D_2 receptors by amisulpride should then reduce the dopaminergic inhibition of the prolactin release, leading to hyperprolactinemia in patients (Wetzel et al., 1994).

The hyperprolactinemia due to different pathological status is currently treated by stimulating pituitary dopamine D_2 receptor with dopamine D_2 receptor agonists, such as bromocriptine, that are among the most effective drugs able to normalize plasma prolactin levels (Pinzone et al., 2000).

An attempt to reduce the increase in prolactin secretion induced by benzamides class of compounds with the use of dopamine D_2 receptors agonists was carried out. For instance, bromocriptine was administered to rat treated with the benzamide derivative sulpiride (Hofmann et al., 1979). However, since bromocriptine readily crosses the brain–blood barrier, it lowered sulpiride-induced hyperprolactinemia, but it also suppressed the 3,4-dihydroxyphenylacetic acid (DOPAC) accumulation induced by sulpiride in the nucleus accumbens, thus interfering with the sulpiride-induced increase in dopamine synthesis which is thought to be at the basis of its action as an antidepressant (Tagliamonte et al., 1975).

Recently, a new dopamine D_2 receptor agonist carmoxirole has been synthesized (Haase et al., 1991). Carmoxirole was proven to reduce basal prolactin blood levels in naive rat and showed low propensity to cross the blood–brain barrier, since it exerted no effect on striatal L-DOPA accumulation up to the dose of 100 mg/kg and it was able to affect the central levels of biogenic amine only at high doses (Haase et al., 1991).

In the present paper, we evaluated the possibility that carmoxirole may reverse the amisulpride-induced hyperprolactinemia without affecting the central effect of this substituted benzamide. For this purpose, we compared the effect of carmoxirole and bromocriptine on prolactin blood level and on apomorphine-induced yawning in rats acutely treated with low doses of amisulpride.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley albino rats (Charles River, Como, Italy) weighting 100–125 g were kept on a 12/12 dark/light cycle (7:00 a.m./7:00 p.m.) with food and tap water available ad libitum. All experimental protocols were approved by the Ethical Committee at the University of Cagliari and performed in strict accordance with the E.C. regulation for care and use of experimental animals (EEC No. 86/609).

2.2. Drugs and chemicals

Carmoxirole 3-(4-(4-phenil-1,2,3,6-tetrahydro-1-pyridyl)-butyl) was a generous gift from E. Merck (Darmstadt, Germany). Amisulpride hydrochloride was supplied from Sanofi-Synthélabo (Bagneaux, France). Bromocriptine-methanesulfonate and apomorphine hydrochloride and dimethylsulfoxide sodium salt were purchased from Sigma (St. Louis, MO, USA) and [^{3}H]YM-09151-2 (specific activity 85 Ci/mmol) was from NEN Life Science Product (Boston, MA, USA). For animal treatment amisulpride was dissolved in NaCl 0.9%, carmoxirole, bromocriptine, or apomorphine in 25 μl glacial acetic acid and tamponated (pH 7.2) using a solution 0.1 M of sodium bicarbonate in distilled water.

2.3. [^{3}H]YM-09151-2 homogenate binding

Rats were killed by decapitation, the pituitaries were rapidly dissected and homogenized in 100 volumes of ice cold 50 mM Tris–HCl, pH 7.4, using a polytron apparatus. The homogenates were centrifuged at 48,000 × g for 20 min at 4 °C and the resultant pellets resuspended and centrifuged two more times at 48,000 × g for 20 min at 4 °C. The final pellets were stored at −70 °C until assayed. Pellets obtained from rat pituitary were rapidly thawed and homogenated in 100 volumes of ice cold Tris–HCl buffer containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM EDTA and 5.7 mM ascorbic acid, pH 7.4. After centrifugation at 48,000 × g for 20 min at 4 °C, the pituitary membranes were re-suspended in 70 volumes of the same buffer.

[^{3}H]YM-09151-2 binding was determined by the method developed by Niznik et al. (1985). Briefly, 200 μl (100–150 μg protein) of pituitary membrane homogenate was added to the incubation medium containing 25 pM [^{3}H]YM-09151-2 and different concentrations of the drugs tested (eight concentrations dissolved in dimethylsulfoxide). To avoid possible undesired effects on radioligand binding, dimethylsulfoxide concentration in the different assays never exceeds 0.1% (v/v). (−)-Sulpiride (10 μM) was used to define non-specific binding. Saturation binding assays were carried out using 10 concentrations of [^{3}H]YM-09151-2 ranging from 1

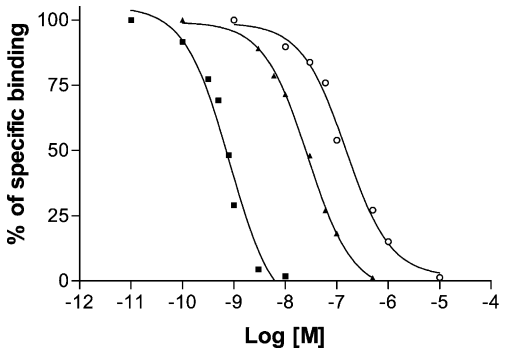

Fig. 1. Competition curves of bromocriptine (■), amisulpride (▲) and carmoxirole (○) on [^{3}H]YM-09151-2 binding. Results are representative of one of three independent experiments.

to 100 pM. Experimental conditions were set up to avoid a ligand depletion higher than 10%. After 60 min of incubation at 25 °C in the dark, samples were filtered through Whatmann GF-B filter using a Brandell 96-sample harvester apparatus (Brandell, Gaithersburg, MD, USA), filters were rinsed four times with 4 ml of ice cold Tris–HCl buffer, pH 7.4. Radioactivity was measured in a liquid scintillation counter (Tricarb 2100, Packard, Meridien, USA) using 3 ml of scintillation fluid (Ultima Gold MV, Packard, Meridien, USA). Saturation and competition curves were analyzed using a computer program (Kell 6.0, Biosoft, UK). All the experiments were performed in triplicate and each result was expressed as mean ± S.E.M of three independent experiments. Protein content was determined using the Bio-Rad Dc Kit (Bio-Rad Laboratories, Munich, Germany) and following manufacturer's instructions.

2.4. Serum prolactin determination

Rats (10 rats/group for each determination), previously habituated to the guillotine frame, were decapitated and ~ 800 μl of blood was collected. Blood was stored at 4 °C and the serum was separated after centrifugation at 3000 × *g* and 4 °C for 8 min.

Serum prolactin levels were measured using a Rat Prolactin Radioimmunoassay Kit (Biocode, Liege, Belgium) and following manufacture's instructions. Results were expressed as ng/ml. The experimental procedure was repeated three times for ED_{50} (mean ± S.E.M.) determination and statistical analyses. In order to estimate the effect of different drugs on serum prolactin level, amisulpride was administered (s.c.) 30 min before bleeding, while carmoxirole and bromocriptine 45 min before bleeding.

2.5. Locomotor activity

Locomotor activity was measured in Plexiglas cages (area: 25 × 40 cm^2; height: 14 cm) equipped with a grid of eight horizontal infra-red beams (two in the frontal side and six in the lateral side of the cage) positioned 4 cm above the floor to measure horizontally. Beam breaks were recorded automatically and analyzed by the computerized monitoring apparatus, a TSE ActiMot/Motil (TSE Systems, Bad Homburg, Germany). The locomotor activity of each individual rat was measured by means of meters covered by the rat as recorded by the infra-red sensors during 20 min.

During dark time (8:00 to 10:00 p.m.), the rats (10 rats/group) were injected i.p. with different doses of carmoxirole (20–60 mg/kg). Forty-five minutes or 3 h after injection, the rats were individually placed in the activity cage and the locomotion was monitored.

2.6. Apomorphine-induced yawning

The number of yawns was counted for 20 min, as described by Mogilnicka and Klimek (1977), starting 10 min after injection of apomorphine (0.08 mg/kg s.c.) or vehicle in rat (10 rats/group) placed in individual Plexiglas cages. Amisulpride (0.5 mg/kg s.c.), carmoxirole (15 mg/kg i.p.), bromocriptine (0.8 mg/kg i.p.) or their respective vehicles were administered 30 or 45 min before observation, respectively. The observer was blind to the treatment.

2.7. Statistical analysis

The statistical significance of the effect of any compound was evaluated by *t*-test, one- or two-way analysis of variance (ANOVA). When a significant ($P<0.05$) interaction was demonstrated, the Newman–Keuls post hoc test was used to compare the effect of the different drugs.

3. Results

3.1. Homogenate binding

The binding of [^{3}H]YM-09151-2 to pituitary synaptosomal membranes was clearly saturable and the specific

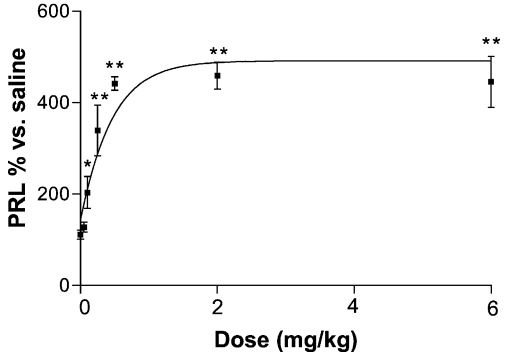

Fig. 2. Effect of different doses of amisulpride on blood prolactin level. Results are representative of one of three independent experiments. Each point represents the mean ± S.E.M. of 10 rats. Statistical significance has been analyzed using one-way ANOVA test ($F(6,63)=41.1$, $P<0.01$) followed by Newman–Keuls post hoc test (*$P<0.05$; **$P<0.01$ vs. vehicle-treated rats).

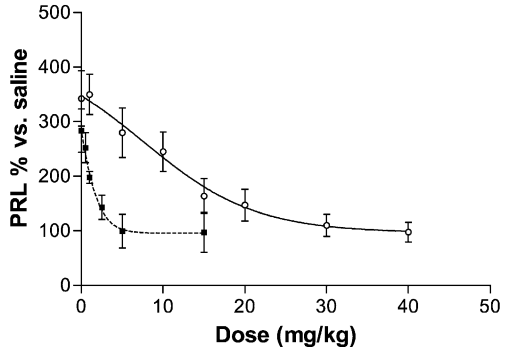

Fig. 3. Effect of different doses of bromocriptine (■) or carmoxirole (○) on prolactin serum level after administration of amisulpride (0.5 mg/kg, s.c.). Results are representative of one of three independent experiments. Each point represents the mean ± S.E.M. of 10 rats.

binding represented the 95% of total binding at half the maximum specific binding while the ligand depletion was lower than 10%. Scatchard plots of the data from the saturation experiments could be fitted to a single site model with a K_d of 23.5 ± 0.3 pM and a B_{max} of 38 ± 5 fmol/mg protein (Hill coefficient 0.997). The studies of competition curves (Fig. 1) for the [^{3}H]YM-09151-2 of the ligands examined exhibited the statistically different affinities (one-way ANOVA $F(2,6) = 80.7$): bromocriptine ($K_i = 0.36 \pm 0.01$ nM; $P < 0.01$ vs. carmoxirole), amisulpride ($K_i = 12.2 \pm 0.4$ nM; $P < 0.01$ vs. carmoxirole), carmoxirole ($K_i = 23.3 \pm 0.15$ nM).

3.2. Serum prolactin level

The effects of incremental doses of amisulpride on serum prolactin levels are depicted in Fig. 2. Amisulpride increased the serum prolactin level in rats with an ED_{50} of 0.25 ± 0.017 mg/kg and the maximal stimulation was observed from the dose of 0.5 mg/kg (315 ± 18%). Carmoxirole (10 mg/kg) reduced prolactin level in untreated animals (47.9 ± 2.9% with respect to vehicle-treated rats; *t*-test $P < 0.01$, vs. vehicle) (data not shown). Moreover, carmoxirole inhibited the increase in prolactin level due to the administration of a maximal dose (0.5 mg/kg) of amisulpride in a dose-dependent manner with an ID_{50} of 14.9 ± 0.8 mg/kg (Fig. 3). The dose of 30 mg/kg completely reversed the increase in prolactin secretion produced by amisulpride. Bromocriptine strongly inhibited the hyperprolactinemia mediated by amisulpride with an ID_{50} of 0.81 ± 0.03 mg/kg (Fig. 3).

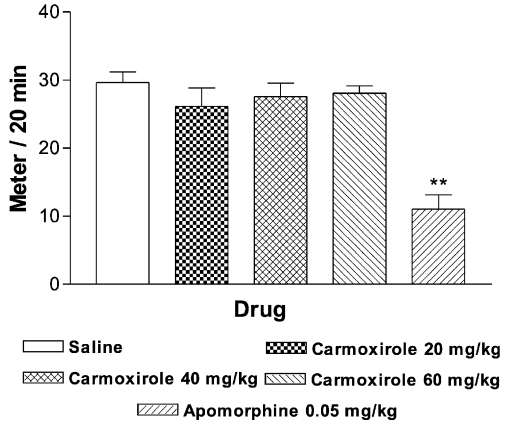

Fig. 4. Effect of different doses of carmoxirole and apomorphine (0.05 mg/kg) on rat spontaneous locomotor activity. Each bar represents the mean ± S.E.M. of 10 rats. Statistical significance has been analyzed using one-way ANOVA test followed by Newman–Keuls post hoc test (** $P < 0.01$ vs. vehicle-treated rats).

3.3. Locomotor activity

As shown in Fig. 4, different doses of carmoxirole, ranging from 20 to 60 mg/kg, were not able to affect the spontaneous locomotor activity in rats 45 min after injection, while a reduced spontaneous motor activity was observed in rats treated with apomorphine 0.05 mg/kg

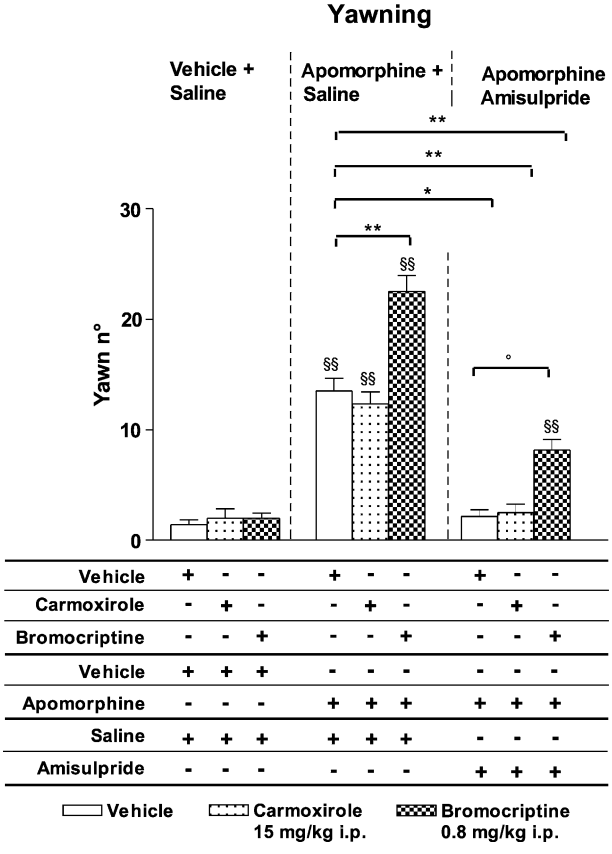

Fig. 5. Comparison between carmoxirole (15 mg/kg, i.p.) and bromocriptine (0.8 mg/kg, i.p.) on rat yawning. Rats were co-treated respectively with vehicle, apomorphine (0.08 mg/kg, s.c.), apomorphine (0.08 mg/kg, s.c.) plus amisulpride (0.5 mg/kg, s.c.). When administered, drugs were substituted with the respective vehicles in order to inject each rat three times (see treatment scheme below the graph). Statistical significance vs. respective control (vs. §vehicle; * apomorphine + vehicle; °apomorphine + amisulpride-treated rats) has been analyzed using two-way ANOVA test followed by Newman–Keuls post hoc test ($n = 10$; $^{+}P < 0.05$; $^{++}P < 0.01$).

(one-way ANOVA, $F(4,45)=6.73$, $P<0.01$). Similar results were obtained 3 h after carmoxirole administration (data not shown).

3.4. Apomorphine-induced yawning

As shown in Fig. 5, different associations of amisulpride, carmoxirole, bromocriptine or vehicle (drug group A) in the presence/absence of low doses of apomorphine (0.08 mg/kg, s.c.) and amisulpride (0.5 mg/kg s.c.) (drug group B) induced a statistically different yawning activity (two-way ANOVA: $F_{(\text{group A})}(2,63)=253.9$, $P<0.01$; $F_{(\text{group B})}(2,63)=48.6$, $P<0.01$; $F_{(\text{interaction A-B})}(4,63)=5.3$, $P<0.01$). Apomorphine induced a significant increase in yawn number vs. vehicle-treated rats ($P<0.01$). Amisulpride (0.5 mg/kg, i.p.) significantly reduced apomorphine-induced yawning ($P<0.01$), and the association of amisulpride plus apomorphine did not significantly differ vs. saline-treated rats ($P>0.05$). Carmoxirole (15 mg/kg, i.p.) administration did not show a significant effect on rat yawning when associated with vehicle, apomorphine or apomorphine plus amisulpride ($P>0.05$ vs. respective controls). Conversely, rat treated with bromocriptine (0.8 mg/kg, i.p.) and apomorphine or apomorphine plus amisulpride showed a significant increase in yawns when compared with the respective controls ($P<0.01$ and $P<0.05$, respectively). However, bromocriptine (0.8 mg/kg) by itself did not alter yawning in rats.

4. Discussion

When used in small doses (50–100 mg) the benzamide derivative amisulpride possesses an antidepressant effect (Lecrubier et al., 1997; Ravizza, 1999) and this drug is successfully used, at these doses, in the treatment of negative symptoms of schizophrenia and of dysthymia. However, like other substituted benzamides, even low doses of amisulpride elevate plasma prolactin levels in patients, posing the risk of low compliance (Wetzel et al., 1994; Von Bahr et al., 1991).

In the present work, using the rat as an animal model, we confirmed the high propensity of very small doses of amisulpride to induce hyperprolactinemia. Furthermore, consistently with other studies (Scatton et al., 1997; Perrault et al., 1997), we found that amisulpride (0.5 mg/kg) was able to completely reverse apomorphine-induced rat yawning.

Although some pharmacological or anatomo-physiological controversies exist concerning the different brain region involved (Argiolas and Melis, 1998), the apomorphine-induced yawning is generally considered as a centrally mediated effect due to a direct stimulation of the dopamine D_2 receptor. In our condition the same doses of amisulpride that were able to antagonize this behavioral effect of apomorphine produced a maximal increase in prolactin blood level. Indeed, similarly to what has been reported in humans (Wetzel et al., 1994), in the rat we found a dose-related association between the hyperprolactinemia induced by amisulpride and the central effect mediated by the benzamide (i.e. the antidepressant effect in humans and the antagonism of apomorphine-mediated yawning in rats).

The present results indicate that low doses of bromocriptine decreased amisulpride-induced hyperprolactinemia, but they also reduced the effect of amisulpride on apomorphine-induced yawning. On the other hand, the peripheral dopamine D_2 agonist carmoxirole was able to reduce the amisulpride-induced hyperprolactinemia at doses much higher than bromocriptine, but without affecting the amisulpride ability to reduce apomorphine-induced yawning.

The different effect of the two dopamine D_2 receptor agonists on amisulpride-induced hyperprolactinemia reflects their different affinity for the pituitary dopamine D_2 receptors, as observed in our binding study. On the other side, the lower propensity of carmoxirole to cross the brain–blood barrier may explain the different effect, with respect to bromocriptine, on amisulpride-induced central effect. Bromocriptine at low doses is able to reduce cocaine-induced hypermotility and is known to alter dopamine synthesis and release in naive rats (Brannan et al., 1993; Jackson et al., 1995). On the other hand, carmoxirole (10–100 mg/kg, s.c.) exerts no effect on hypothalamic L-3,4-dihydroxyphenylalanine (L-DOPA) accumulation and produces only a small reduction of striatal DOPAC and homovanillic acid (HVA) levels, suggesting that carmoxirole is devoid of central effect at such doses (Haase et al., 1991). Consistent with this possibility, we found that carmoxirole did not affect spontaneous locomotor activity up to the dose of 60 mg/kg.

Interestingly, bromocriptine affected the apomorphine and apomorphine plus amisulpride effect on yawning at a dose devoid per se of any behavioral effects. A peculiarity that was also observed using apomorphine to antagonize cocaine behavioral arousal (Campbell et al., 1989). It is possible that the reported in vivo slow dissociation of bromocriptine from the dopamine receptor may account for an over-effect of the drug when in the presence of a dopamine receptor stimulation or antagonism (Bannon et al., 1980). In any case, this property of bromocriptine is not in favor of any possible use in reducing hyperprolactinemia induced by amisulpride in depressive patients, in spite of its widespread clinical use as an anti-hyperprolactinemia agent (Pinzone et al., 2000).

In conclusion, our result indicated that, even in very small doses, amisulpride is able to produce hyperprolactinemia and to reverse centrally mediated apomorphine-induced yawning in rats. The comparison of the effects of carmoxirole and bromocriptine on amisulpride-induced hyperprolactinemia and yawning indicated that only carmoxirole was able to restore normal prolactin blood level without affecting the central activity of amisulpride in rats. These results might suggest that, in humans, the combination of carmoxirole and amisulpride may leave intact the

central effect of this benzamide, which is relevant for its therapeutic response in affective and cognitive symptoms of dysthymia and negative symptoms of schizophrenia, while antagonizing the endocrine peripheral effect of amisulpride. However, further studies will be needed in order to evaluate the other actions of carmoxirole in the periphery (such as those on blood vessels, kidney, etc.) and particularly when given in combination with a substituted benzamide.

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