

THE EFFECT OF CLOZAPINE ON PROLACTIN SECRETION AT THE LEVEL OF THE LACTOTROPH

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Summary

Clozapine is an antipsychotic drug which is unusual in that it has no dopamine receptor-blocking activity. Previous studies gave conflicting results whether administration of clozapine induces hyperprolactinemia.

In the present study it was shown that a wide concentration range of clozapine does not interfere with dopamine-mediated inhibition of prolactin (PRL) secretion by normal cultured rat pituitary cells. This in contrast to other neuroleptics, like haloperidol and trifluoperazine. Clozapine does also not antagonize norepinephrine-mediated inhibition of PRL secretion. Clozapine exerts at micromolar concentrations a direct inhibitory action on PRL release by cultured normal rat pituitary cells. In cultured rat pituitary tumor cells, these high concentrations of clozapine directly inhibit PRL release as well as the DNA content of the cells, suggesting a direct antimitotic action. In this model clozapine was about 5-10 times less potent than trifluoperazine. Clozapine and trifluoperazine exert an additive inhibitory action both on PRL release and on the DNA content of the pituitary tumor cells.

It is concluded that clozapine does not interfere at the pituitary level with dopamine-mediated inhibition of PRL release. At micromolar concentrations clozapine may act on lactotrophs as a calmodulin-inhibitor. These observations suggest that the transient PRL-releasing effects which have been observed in both animal and human studies after clozapine administration are mediated via supra-pituitary actions of the drug.

Clozapine, a dibenzazine derivative, is an antipsychotic drug which is unusual in that it has no typical antidopaminergic profile (1-4). Its strong antipsychotic and sedative actions are accompanied with only mild extrapyramidal side effects. The drug was recently found to be especially successful in treatment-resistant schizophrenics (5). This last observation probably means that clozapine in the near future will be part of the therapeutic armament of psychiatric institutions throughout the world, despite other potential severe side-effects, like an increased risk of agranulocytosis (6).

One of the commonly occurring side-effects of the chronic use of neuroleptic drugs is hyperprolactinemia. This often causes galactorrhea, amenorrhea and/or loss of libido (7). However, previous reports with regard to the effects of clozapine on prolactin (PRL) release were inconclusive in that the drug causes

hyperprolactinemia in rats (8-10), but not, or only to a minimal extent in man (11-15). The mechanism of action of the clozapine-mediated increase in circulating PRL levels in rats is unclear (8). The compound was shown to displace  $^3\text{H}$ -dihydroergocryptine binding from bovine anterior pituitary membranes (16), and  $^3\text{H}$ -spiroperidol binding from rat pituitary membranes (17), but in both studies micromolar concentrations of clozapine were needed. In the present study we evaluated whether clozapine exerts a direct action on PRL release at the pituitary level. We found that the drug does not affect dopamine-mediated inhibition of PRL secretion, but acts at high concentration probably as a calmodulin inhibitor.

#### Materials and Methods

Male Rp rats or female Rp rats (in any stage of the estrous cycle), obtained from T.N.O., Rijswijk, The Netherlands, weighing 180-200 g were used for the preparation of cultured normal pituitary cells. The isolation methods have been described in detail by Hofland et al (18). The culture medium used in all experiments was phenol red-free Eagle's Minimal Essential Medium with Earle's salts (MEM) supplemented with 1% MEM non-essential amino acids, sodium pyruvate (1 mmol/l), penicillin ( $10^3$  U/l), streptomycin (100 mg/l), fungizone (0.5 mg/l), L-glutamin (2 mmol/l), sodium bicarbonate (2.2 g/l), and 10% fetal calf serum which had been treated with dextran-coated charcoal. The cells were cultured at a density of  $10^5$  cells per well in 1 ml of culture medium in 48-well plates. On day 4 of culture the medium was changed and after an additional medium change on day 7 of culture 4 hr incubations were carried out.

Female Buffalo rats (R.B.I., Rijswijk, The Netherlands), weighing 150-170 g were inoculated subcutaneously between the scapula with a suspension of the transplantable PRL-secreting rat pituitary tumor 7315b, as described in detail elsewhere (19). The 7315b tumor is a pure PRL secreting pituitary tumor, which was derived from the mixed ACTH/PRL secreting pituitary tumor 7315a. The 7315b tumor has dopamine receptors, but is resistant to dopaminergic inhibition, presumably because of an intracellular defect. Four weeks after inoculation of the tumor cell suspension a tumor of approximately 20 cm<sup>3</sup> had grown dorsally on the animals. 7315b Pituitary tumor cells were isolated by mechanical dispersion. In order to separate vital cells from non-vital cells the suspension was layered on Ficoll-Isopaque (density 1.077 g/ml; prepared by the Dijkzigt Hospital Pharmacy, Rotterdam, The Netherlands) and centrifuged at 1000 x g for 20 min. The interphase containing vital cells was collected and washed twice. Finally the cells were resuspended and cultured at a density of 25.000 cells per well in 1 ml of culture medium, consisting of MEM with 10% untreated fetal calf serum. On day 6 of culture the media plus cells were collected and stored at -20°C until analysis.

Dopamine, norepinephrine and trifluoperazine were purchased from Sigma chemical company (St Louis, Missouri, USA). Clozapine was a gift from Sandoz (Basel, Switzerland).

Rat PRL concentrations in the culture media were measured by a double antibody RIA using materials and protocols supplied by the distribution officer of the NIADDK. All results are expressed in rat PRL reference preparation (RP-1). The DNA content of the tumor cells was determined as described in detail elsewhere (20). The method is based on a DNA dependent fluorescence enhancement of a fluorochrome. Fluorescence of experimental samples was referenced to a standard curve of calf thymus DNA (type II, no-D-3636; Sigma). The data are expressed as means  $\pm$  SEM.

The statistical significance of the differences between mean values was determined using analysis of variance (ANOVA). When significant overall effects were obtained by ANOVA, multiple comparisons were made with the Newman-Keuls

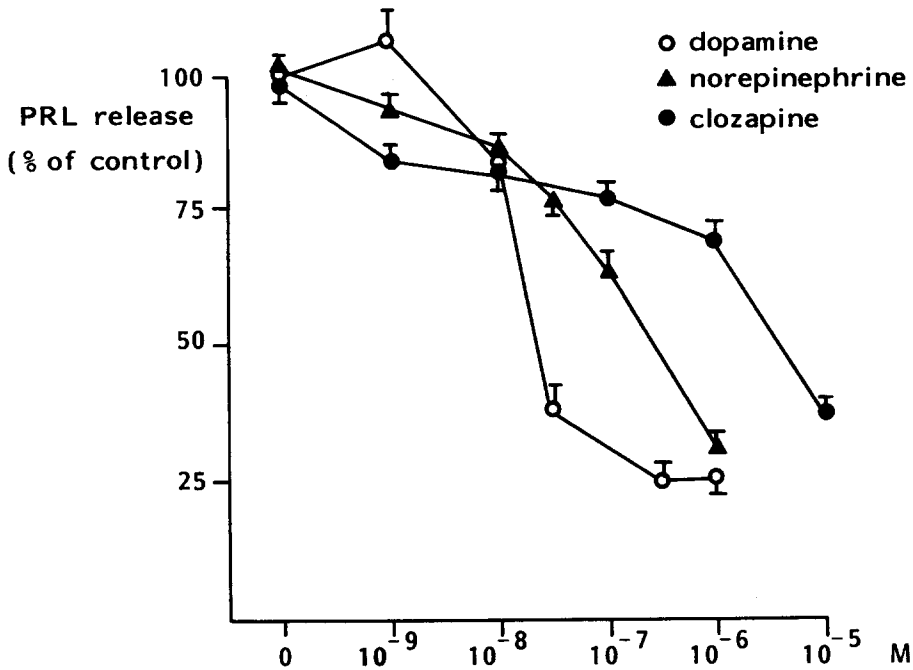


Fig. 1

The effects of dopamine, norepinephrine and clozapine on PRL release by normal cultured rat pituitary cells. The cells had been precultured for 3 days. Incubation time 4 h. Mean  $\pm$  SEM (n=4 wells per group).

test. All data shown represent the results of at least three experiments which gave similar results.

### Results

The effects of dopamine, norepinephrine and clozapine on PRL release by normal cultured rat pituitary cells are shown in fig. 1. All three compounds inhibited PRL release in a dose-dependent manner, the  $IC_{50}$  for dopamine, norepinephrine and clozapine being 30 nM, 400 nM and 6  $\mu$ M, respectively.

The inhibition of PRL secretion by dopamine (50-500 nM) was not antagonized by a wide dose-range of 1 nM - 1  $\mu$ M clozapine, while in the same experiments the dopamine-receptor antagonist haloperidol (5 nM) at least partially neutralized dopamine-mediated inhibition of PRL release (Table I).

The inhibition of PRL secretion by norepinephrine (100 nM- 1  $\mu$ M) was not affected by a wide dose-range of 1 nM - 1  $\mu$ M clozapine. The inhibitory effect of norepinephrine on PRL release was attenuated by haloperidol (5 nM), however, but not by fentolamine (100 nM - 10  $\mu$ M), prazosine (100 nM - 10  $\mu$ M) or propranolol (100 nM-10  $\mu$ M; data not shown).

Thereafter we investigated the effects of clozapine on cultured PRL-secreting pituitary tumor cells prepared from the transplantable rat pituitary tumor 7315b. As a comparison the effects of trifluoperazine were also investigated. Both clozapine and trifluoperazine inhibited PRL release by the cultured tumor

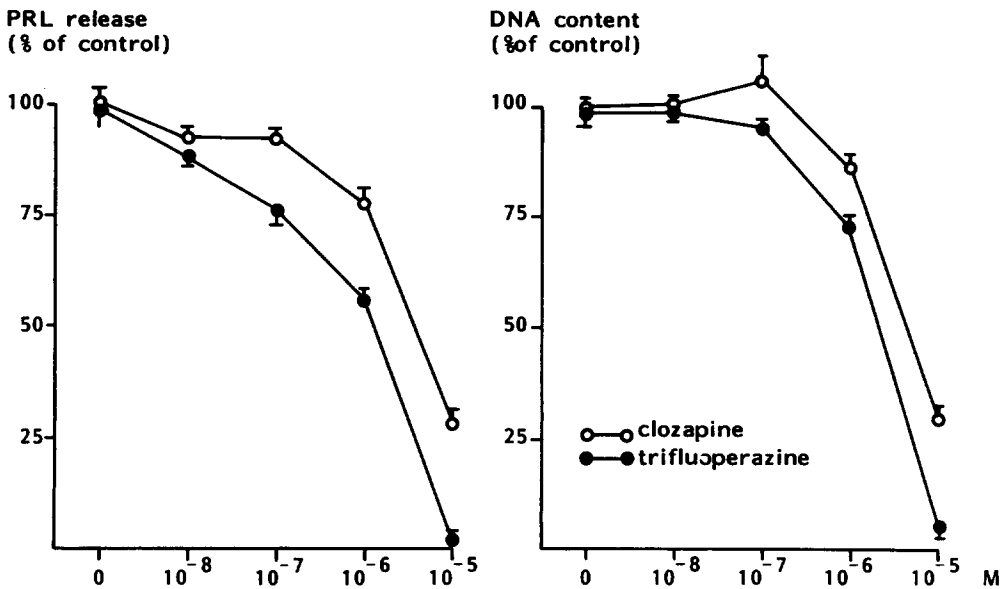


Fig. 2

The effects of clozapine and trifluoperazine on PRL release (left) and the DNA content (right) of cultured cells prepared from the transplantable 7315b rat pituitary tumor. Culture time was 6 days. Mean  $\pm$  SEM (n=4 dishes wells group).

cells and the DNA content of the cells after 6 days of culture in a dose-dependent manner (fig. 2). Clozapine and trifluoperazine exerted similar PRL release-inhibitory and antimetabolic effects, trifluoperazine being 5-10 times more potent than clozapine.

In a final experiment (table II) it was shown that both compounds exert similar actions in this tumor cell model, because the combination of 5  $\mu$ M clozapine and 1  $\mu$ M trifluoperazine inhibited PRL release and the DNA content of the cells in an additive fashion (table II).

#### Discussion

The antipsychotic efficacy of the neuroleptic class of drugs is closely correlated to antagonism of the D-2 dopamine receptor (7,21-23). The antipsychotic action of these drugs may be mediated primarily at D-1 dopaminergic receptors in the limbic area, whereas action at striatal D-2 receptors and/or haloperidol-sensitive sigma receptors may be responsible for extrapyramidal side-effects while their action at the pituitary D-2 receptors causes hyperprolactinemia which often results in galactorrhea, amenorrhea and/or loss of libido (7,23,24).

Clozapine is a piperazine derivative of the dibenzodiazepine group, with a strong antipsychotic and sedative action (1-5). It has a weak D-1 dopamine receptor blocking effect, with special affinity to mesolimbic areas, while it also has strong anti-noradrenergic, anti-histaminergic, and anti-cholinergic

TABLE I

The effects of clozapine and haloperidol on dopamine-mediated inhibition of PRL release by normal cultured female rat pituitary cells.

	PRL release as a percentage of control
dopamine (500 nM)	52.9 ± 3.1 <sup>a</sup>
dopamine + clozapine (1 nM)	45.3 ± 3.1 <sup>a</sup>
dopamine + clozapine (10 nM)	50.0 ± 1.6 <sup>a</sup>
dopamine + clozapine (100 nM)	49.1 ± 2.1 <sup>a</sup>
dopamine + clozapine (1 μM)	46.8 ± 3.4 <sup>a</sup>
dopamine + haloperidol (5 nM)	74.8 ± 1.4 <sup>a, b</sup>

The cells had been precultured for 3 days. Incubation time 4 h.  
Mean ± SEM (n= 4 wells per group)  
<sup>a</sup>p<0.01 vs control  
<sup>b</sup>p<0.01 vs dopamine (500 nM)

TABLE II

The effect of clozapine, trifluoperazine and their combination on PRL release and the DNA content of cultured 7315b rat pituitary tumor cells.

	PRL release (ng/10 <sup>5</sup> cells/6 days)	DNA content (ng/well)
control	177.4 ± 2.5	747.5 ± 39.3
clozapine (5 μM)	75.0 ± 3.9 <sup>b</sup>	442.8 ± 103.1 <sup>a</sup>
trifluoperazine (1 μM)	101.3 ± 2.2 <sup>b</sup>	511.8 ± 30.3 <sup>a</sup>
clozapine+trifluoperazine	40.1 ± 2.6 <sup>a</sup>	155.3 ± 14.3 <sup>b</sup>

incubation time 6 days; mean ± SEM (n=4 wells/group)  
<sup>a</sup>p<0.05 vs control    <sup>b</sup>p<0.01 vs control  
<sup>c</sup>p<0.01 vs clozapine and trifluoperazine separately

effects (1-5). Clozapine does not produce extrapyramidal syndromes and was recently shown to be very effective in treatment-resistant schizophrenics (5).

Previous studies on the effects of clozapine on PRL release have been conflicting. The drug markedly increased serum PRL levels in rats, when injected intraperitoneally in doses of 5-100 mg/kg (8-10). The PRL-releasing effect of clozapine was only 20-50% of that of chlorpromazine, however. In patients treated with 200-600 mg clozapine per day PRL levels were reported to be moderately increased, especially during the first 4 h after drug administration (11,12), but not thereafter (11,15,22). Chronic therapy of psychotic patients for 2-4 weeks with 600 mg clozapine per day did not increase the PRL levels of the cerebrospinal fluid (12). So, clozapine is distinctly less potent in its *in vivo* effects on PRL secretion in both rat and man, but its mechanism of action is unexplained. Meltzer et al (8) suggested that the increase in serum PRL levels are attributed to clozapine's ability to produce dopamine blockade at the pituitary level or to inhibit nerve impulse-dopamine release or both. Indeed clozapine was found to displace <sup>3</sup>H-dihydroergocryptine binding from bovine anterior pituitary membranes (16) and <sup>3</sup>H-spiroperidol from rat pituitary membranes (17), but the IC<sub>50</sub>'s of clozapine were about 1000 nM in the former and 1320 ± 86 nM in the latter study.

Our present studies might help to understand the seemingly discrepant previous observations. Clozapine does not interfere with the dopamine-mediated inhibition of PRL release by cultured rat pituitary cells. This in contrast with the action of neuroleptics like haloperidol and trifluoperazine which block the dopamine effect at concentrations in the nanomolar range (25). Norepinephrine inhibits PRL release by lactotrophs via its direct effects at the dopamine receptor (16). This assumption is underlined by our observation that the norepinephrine-mediated inhibition of PRL release was antagonized by haloperidol, but not by prazosine, fentolamine and propranolol. Clozapine did not reverse norepinephrine-induced inhibition of PRL release, further excluding the possibility that it causes hyperprolactinemia via a blockade of  $\alpha$ -receptors at the pituitary level.

Finally it was shown that at micromolar concentrations, clozapine directly inhibited PRL release by normal and tumorous pituitary cells, as well as mitosis of the tumor cells. In this, its actions were quite similar (and additive) to those of high concentrations of trifluoperazine. It has been previously shown that phenothiazines, butyrophenones and tricyclic antidepressants bind to and inactivate calmodulin in the presence of calcium in several model systems (26,27). The inactivation of calmodulin by these compounds suppresses not only anterior pituitary hormone secretion which is dependent on calcium ions (27), but in malignant cells which have been shown often to contain elevated calmodulin levels (28), chronic blockade of its action results in direct antimitotic effects. In these studies trifluoperazine has been most often used: micromolar concentrations of the drug inhibited the growth of human breast and ovarian cancer cells (29,30). A similar mechanism of action may explain the effects of high concentrations of clozapin on normal and tumorous PRL secretion.

In conclusion, we showed that clozapine does not interfere at the pituitary level with dopamine-mediated inhibition of PRL release. Its binding to rat and bovine pituitary dopamine receptors is probably of no physiological significance, because the concentrations of clozapine needed for a 50% displacement in pituitary membrane preparations are in the micromolar range and we showed that at these concentrations the drug exerts in intact cells only inhibition of PRL release, probably via an inhibition of calmodulin action. Our observations suggest that the transient PRL releasing effect which has been observed after intraperitoneal injection of clozapine in rats and only to a minimal extent after oral administration in man, is caused via supra-pituitary actions which might involve brain serotonin, norepinephrine and/or acetylcholine metabolism, or an indirect change in the dopaminergic activity of the tubero-infundibular neurons.

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