Abstract

Prolactin secretion from the anterior pituitary gland is regulated by multiple factors including prolactin-release inhibiting factors (PIFs) and prolactin releasing factors. PIFs, however, usually dominate to exert a tonic inhibition in the biological system, and the physiological PIF is believed to be dopamine. However, there is accumulating evidence that dopamine can not only inhibit but also stimulate prolactin release. Many investigators believe that this is achieved by activating inhibitory and stimulatory subtypes of dopamine receptors. We tried to demonstrate that one subtype of dopamine receptors is capable of both inhibiting or stimulating prolactin release using GH4ZR7 cells. GH4ZR7 cells express only a short form of dopamine D2 receptors (D2s). Low concentrations of three well-established D2 receptor agonists (dopamine, apomorphine and bromocriptine) stimulated prolactin release from GH4ZR7 cells while high concentrations inhibited the release. Haloperidol, a D2 receptor antagonist, blocked the inhibitory action, but was unable to block the dopamine-induced stimulatory action. Pretreatment of cells with phenoxybenzamine, a receptor alkylating agent, abolished both the dopamine-induced stimulatory and inhibitory actions. Our results support the thesis that the stimulation of prolactin release induced by dopamine is mediated through dopamine D2s receptors since the GH4ZR7 cells have only D2s receptors among dopamine receptors. We have concluded that the D2s receptor is capable of both stimulating and inhibiting prolactin release, probably via the activation of a Gs protein by low concentrations and a Gi protein by high concentrations of dopaminergic agents.

Introduction

The neuroendocrine control of prolactin secretion from the anterior pituitary gland involves multiple factors including prolactin releasing factors (PRFs) and prolactin-release inhibiting factors (PIFs); however, the latter usually predominate to exert a tonic inhibitory control (1–4). The concept of inhibitory regulation is supported by evidence that hypothalamic lesions (5) and transplantation of the anterior pituitary to extracranial sites such as the kidney capsule (5) resulted in a marked increase in prolactin secretion. Convincing evidence has been given that dopamine (DA), secreted by the tuberfundibular dopaminergic (TIDA) neurons of the hypothalamus, is the main physiological PIF (4, 6–9), but DA alone cannot satisfy all the criteria to be the PIF and we have recently concluded that the physiological PIF is DA plus ascorbate (10). PRFs, on the other hand, are important in mediating the rise in prolactin release under a variety of physiological and experimental conditions. A number of hypothalamic stimulating factors such as thyrotropin-releasing hormone (TRH), cholecystokinin, vasoactive intestinal polypeptide (VIP), and neurotensin have been identified, but their role in the physiological regulation of prolactin secretion is not yet certain (11). Under several of the stimulatory conditions studied, for example during suckling, the level of DA in portal blood decreases, and there has been a suggestion that the fall of DA in portal blood, although very transient, is a pre-requisite for a full prolactin response to PRF (2, 12).

Although DA is established as the PIF, there is substantial evidence showing that DA, at much lower concentrations than those required for inhibition of prolactin secretion, stimulates prolactin release both in vitro (13–20) and in vivo (21). DA exerts its action through the binding and activation of specific receptors which belong to the G protein-coupled receptor superfamily. Five distinct DA receptor subtypes (D1–D5), which are encoded by separate genes, have been identified (22–25), yet it is widely believed that only the D2 receptors are responsible for the inhibitory actions of DA in the anterior pituitary (26). The genomic sequence of the dopamine D2 receptor is alternatively spliced to...
generate two isoforms (a short isoform (D2s) and a long isoform (D2l)) which differ by a 29 amino acid insert in the third cytoplasmic domain (27–30). D2s and D2l receptors are structurally very similar, and they are also pharmacologically indistinguishable.

We have previously shown that DA at very low concentrations, stimulates, and at high concentrations, inhibits prolactin secretion from cloned GH4ZR7 cells (20). The GH4ZR7 cells express only the short isoform of the rat dopamine D2 receptor by transfecting the D2s receptors into GH4C1 cells (31). The GH4C1 cells do not express any catecholamine receptors (32). In this study, we further explored the nature of the stimulatory action of DA on prolactin release using different DA agonists and antagonists. We selected three well-established D2 receptor agonists (dopamine, apomorphine and bromocriptine) to investigate whether the DA-induced stimulatory effect on prolactin secretion in GH4ZR7 cells is unique to DA, or if it is a general phenomenon of other DA agonists. In addition, we used a dopamine D2 receptor antagonist (haloperidol) and an alkylation agent (phenoxybenzamine) of adrenergic and DA receptors (33) to confirm that all the observed dopaminergic actions were indeed mediated via activation of dopamine D2 receptors on the GH4ZR7 cells.

Materials and methods

Cell culture

The GH4ZR7 cell line was kindly provided by Dr H Elsholtz, Clinical Biochemistry, University of Toronto, Canada. The cells were cultured in a mixture (3:4) of Ham’s Nutrient Mixture F-12 (Connaught Laboratories Ltd, Willowdale, Canada) and Dulbecco’s modified Eagle’s medium (DMEM) (Gibco Lab., Grand Island, NY, USA) supplemented with 2.5% fetal calf serum, 15% horse serum (Gibco Lab.) and penicillin 50 IU/ml (Sigma Chemical Co., St Louis, MO, USA) (culture medium). The cells were maintained in tissue culture flasks (75 cm² style, Becton Dickinson Labware, Lincoln Park, NJ, USA) which were incubated at 37°C under a humidified atmosphere of 5% CO2:95% air in a water-jacketed incubator (Forma Scientific, Marietta, OH, USA), and were passaged once per week into new flasks. For collecting cells from the monolayer in the flask, we used jet streams of medium (obtained by squeezing medium in and out of a Pasteur pipette) to lift up cells from the surface without any treatment with a protease and then we harvested the lifted cells by centrifugation (700 x g for 5 min). This method eliminates the possibility that receptors may be damaged by enzyme treatment.

Chemicals

All experimental solutions were prepared immediately prior to use. Dopamine (dopamine-HCl, Sigma Chemical Co.) was dissolved in appropriate volumes of DMEM containing bovine serum albumin (1 mg/ml) (DMEM-BSA) to make solutions of different concentrations. Bromocriptine and haloperidol (Sigma Chemical Co.) were first dissolved in absolute alcohol (10 mmol/l) and then diluted with DMEM-BSA to the appropriate concentrations.

Incubation experiments

The collected cells were resuspended in culture medium at a concentration of 10⁵ cells/ml and 0.5 ml cell suspension was distributed to each well of a 24-well microtiter plate (Corning Glass Work, Corning, NY, USA). Cultures were returned to the humidified incubator and maintained at 37°C in a 5% CO2:95% air atmosphere for 24 to 48 h. On the day of experiment, plated cells were rinsed twice with DMEM-BSA. All the experimental solutions were prepared in DMEM-BSA, and 1 ml experimental solution was applied to each well. After the cells were incubated with the experimental solutions in the incubator for 4 h, the supernatant from each well was gently pipetted from the wells and stored in disposable cups (24x14 mm, Sarstedt Canada Inc., V-St Laurent, Quebec, Canada) at −20°C until assay.

Experiments were run at least twice and they were highly reproducible. Quadruplicate tests were normally performed in each experiment using 24-well plates. When we repeated experiments with the wider ranges of doses, additional data were added to the original observations.

Radioimmunoassay

One hundred microliters of the medium were assayed in triplicate using a radioimmunoassay kit for rat prolactin kindly supplied by Drs A F Parlow and P F Smith through the Rat Pituitary Hormone Distribution Program. The quantity of prolactin was expressed in terms of National Institute of Diabetes and Digestive and Kidney Diseases (NIADDK) rat prolactin PR-3. Coefficients of variation for inter- and intra-assay variability were 14.5 and 7.2% respectively. The sensitivity was 0.03 ng/tube.

Data analysis

The prolactin level in a well was expressed as a percentage of the mean prolactin level of the control wells (receiving vehicle only) on the same plate because prolactin levels often showed some inter-plate variability. Differences between groups were evaluated by one-way ANOVA with Bonferroni post-tests in GraphPad Prism 2.0 (GraphPad, San Diego, CA, USA). A P value <0.05 was considered to be statistically significant. Data were expressed as the mean±standard error.
of the mean (S.E.M.) with the indicated number of replicates performed.

**Results**

**Effects of dopamine**

The dose–response relationships between the DA concentrations and the amounts of prolactin released into media were tested in a wide range of DA concentrations between $10^{-10}$ and $10^{-3}$ mol/l after 4 h incubation. Mean values of prolactin concentrations of DA ($10^{-10}$ and $10^{-9}$ mol/l)-treated groups were 104 ± 22% and 112 ± 26% respectively, relative to control values (100 ± 2%) but were not statistically significant. However, the prolactin concentration was gradually elevated by increasing concentrations of DA peaking at $10^{-8}$ mol/l DA (139 ± 14%) which was significantly higher than the control group (100 ± 2%) (Fig. 1). As expected, higher concentrations ($10^{-7}$ and $10^{-6}$ mol/l) of DA progressively lowered prolactin concentrations to 126 ± 10%, 114 ± 9% and 104 ± 5% respectively of the control level as compared with the peak level of 139 ± 14%. When even higher concentrations of DA ($10^{-5}$ and $10^{-4}$ mol/l) were used, prolactin concentrations dropped further to below the control level (87 ± 7% and 64 ± 7% respectively). However, only the highest concentration ($10^{-3}$ mol/l) of DA induced a statistically significant decrease in prolactin secretion from the cells (Fig. 1). The inhibitory action of DA on GH4ZR7 cells is much less sensitive than on primary cultured rat pituitary cells.

**Effects of apomorphine**

Apomorphine effects on prolactin release were tested in a wide range of concentrations between $10^{-12}$ and $10^{-4}$ mol/l after 4 h incubation, and a biphasic effect was observed. Apomorphine at a very low concentration ($10^{-10}$ mol/l) significantly elevated prolactin concentrations in the medium to 138 ± 13% of the control level (100 ± 3%). On the other hand, higher concentrations of apomorphine progressively decreased prolactin concentrations, and the reduction reached a statistically significant level at concentrations above $10^{-7}$ mol/l.

---

**Figure 1** Dose–response relationship between DA concentrations and prolactin release. Plated cells were incubated with DMEM containing 0.1% BSA plus various concentrations of DA for 4 h at 37°C. Results are expressed as a percentage of control prolactin (PRL) concentration (100 ± 2% when DA=0 mol/l). The numbers in parentheses represent the number of repetitions of the particular experiment. Vertical bars= S.E.M. *P<0.05.
significant level only at $10^{-4}$ mol/l apomorphine which lowered the level of prolactin secretion to only $51 \pm 9\%$ of the control level (Fig. 2). In our experimental conditions, the stimulatory action of apomorphine was more potent (peak concentration $10^{-11}$ mol/l) than that of DA (peak concentration $10^{-8}$ mol/l) while the inhibitory actions of DA and apomorphine were approximately equal.

**Effects of bromocriptine**

Figure 3 shows the dose-dependent effects of bromocriptine on prolactin release from the cultured cells. After a 4-h incubation period, low concentrations of bromocriptine (between $10^{-12}$ and $10^{-7}$ mol/l) did not significantly change prolactin concentrations in the medium when compared with the control group, but the prolactin concentration was progressively elevated with concentrations of bromocriptine from $10^{-9}$ mol/l and peaked at $155 \pm 14\%$ of control level ($100 \pm 2\%$) with $10^{-6}$ mol/l bromocriptine (Fig. 3). In contrast, a higher concentration of bromocriptine ($10^{-4}$ mol/l) significantly inhibited prolactin release to only $41 \pm 5\%$ of the control level (Fig. 3). All three DA agonists showed biphasic responses.

**Effects of haloperidol on dopamine-induced prolactin release**

Effects of different concentrations of DA were tested in medium containing haloperidol ($10^{-4}$ mol/l). The haloperidol was unable to block the stimulatory action of $10^{-8}$-$10^{-6}$ mol/l DA on prolactin release (Fig. 4). The level of prolactin release was elevated to $143 \pm 18\%$ of the control value when cells were incubated with $10^{-8}$ mol/l DA plus haloperidol ($10^{-4}$ mol/l), and this increase was comparable to that seen when cells were incubated with $10^{-8}$ mol/l DA alone (Fig. 1). On the other hand, the inhibitory effect of $10^{-4}$-$10^{-3}$ mol/l DA was abolished in the presence of this DA antagonist. There was no difference in the prolactin concentrations between the control and the treated group with either $10^{-4}$ mol/l ($98 \pm 9\%$) or $10^{-3}$ mol/l ($105 \pm 8\%$) DA plus haloperidol.
Effects of pretreatment of cells with phenoxybenzamine on dopamine-induced prolactin release

Pre-incubation of cells with phenoxybenzamine (10⁻⁶ mol/l) for 1 h markedly reduced both the stimulatory and inhibitory effects of DA on prolactin release. Prolactin concentrations remained at about the basal level when the cells were incubated with DA at concentrations between 10⁻⁸ and 10⁻⁶ mol/l (Fig. 5). When cells were incubated with 10⁻⁵ and 10⁻⁴ mol/l DA prolactin concentrations were 115 ± 14% and 116 ± 14% respectively, but neither increase was statistically significant.

Discussion

It is well established that DA inhibits prolactin release; however, we have observed that a low concentration of DA stimulates prolactin release while a high concentration inhibits prolactin release from primary cultured rat pituitary cells (13) and, more recently, in the cultured pituitary tumor cell line (GH₃ZR₇) (20). Several other groups of workers (14–19) have also reported a stimulatory effect of DA in primary cultured lactotrophs, and their observations, along with ours, strongly support the notion that DA has dual actions – stimulatory and inhibitory – on prolactin release. However, it is not established whether the stimulatory action originates from activation of a stimulatory subtype of DA receptor, or whether a dopamine D₂ receptor subtype is responsible for both the stimulatory and inhibitory actions.

Multiple types and subtypes of DA receptors are expressed in pituitary cells. While the D₂ receptor has been widely accepted as the inhibitory receptor, the stimulatory receptor, which mediates the stimulatory signals of dopaminergic agents, has not been clearly defined as yet (34). Dopamine D₅ receptors are present in primary cultured pituitary cells, and stimulate prolactin release in vitro (17) suggesting that the...
stimulatory action of DA on prolactin release could be mediated by the stimulatory DA receptor subtype. Nevertheless, the existence of a stimulatory subtype in the primary cultured cells does not exclude the possibility that one DA receptor subtype could mediate the two different signals of DA via different signal pathways. Therefore, we chose GH4ZR7 cells in this study. GH4ZR7 cells are derived from GH4C1 cells by transfecting D2s receptors (31), and the GH4C1 cells have many different receptors such as TRH and VIP receptors, but not DA receptors (32). Dopamine inhibits prolactin release via the inhibition of cyclic AMP production. When GH4C1 and GH4ZR7 cells were treated with dopamine, cyclic AMP production was not inhibited in GH4C1 cells, but it was effectively inhibited in GH4ZR7 cells (35) indicating that GH4C1 cells do not have dopamine receptors. The use of GH4ZR7 cells is ideal to clarify whether one subtype of D2 receptors (D2s) can stimulate both a stimulatory and an inhibitory action on prolactin release. In fact, we have recently reported that a low concentration of DA (10^{-7} mol/l) stimulates while a high concentration of DA (10^{-4} mol/l) inhibits prolactin release in GH4ZR7 cells using a perifusion system (20). In the present study, we continued to clarify the nature of the dopaminergic effects, focusing on the stimulatory action on prolactin release. Since the studied cells express only one subtype of DA receptor, the responses induced by dopaminergic agonists should be the result of activating dopamine D2s receptors, supporting the concept that the D2s receptors alone can mediate both stimulatory and inhibitory signals of DA in lactotrophs.

Three well-established dopaminergic agonists (dopamine, apomorphine and bromocriptine) were tested at various concentrations under specified conditions. These dopaminergic agonists inhibit prolactin release but their inhibitory characteristics are very different (36). All three dopaminergic agonists have stimulatory as well as inhibitory actions on prolactin release in GH4ZR7 cells, but their potencies regarding the stimulatory action are different. We concluded that the stimulatory effects on prolactin release are not limited only to DA but are common to other dopaminergic agents.
Prolactin release is usually inhibited by approximately $10^{-8}$ mol/l DA in primary cultured rat pituitary cells (36), but it is inhibited by about $10^{-4}$ mol/l DA in GH4ZR7 cells, indicating that GH4ZR7 cells are much less sensitive to DA than primary cultured lactotrophs. The difference in the potencies is probably due to different numbers of active D2 receptors (10). In contrast to our finding that DA ($10^{-6}$ mol/l) stimulates prolactin release from GH4ZR7 cells, Burris and Freeman (18), and Porter and coworkers (17) observed that $10^{-6}$ mol/l DA inhibited prolactin release and no stimulatory action was shown. The DA concentration ($10^{-6}$ mol/l) which inhibited prolactin release in the other studies is much lower than that which we observed ($10^{-8}$-$10^{-3}$ mol/l) to be the inhibitory concentration. Differences in experimental conditions, such as different number of passages of cloned cells, the presence or the absence of a DA stabilizer (ascorbic acid and sodium metabisulfite), and different incubation media, can produce different results. Under our experimental conditions, the stimulatory actions were shown in both static monolayer culture (Figs 1–3) and perifusion systems (20). The significance of our study is not the different potencies of DA agonists but the demonstration that one subtype of D2 receptors can both stimulate and inhibit prolactin release depending on the concentrations of agonists.

It is interesting to point out that the three dopamine D2 receptor agonists seem to have very different potencies regarding their stimulatory actions in GH4ZR7 cells. Dopamine, apomorphine and bromocriptine produced their peak stimulatory effects at $10^{-8}$, $10^{-10}$ and $10^{-6}$ mol/l respectively. Regarding the inhibitory actions of the three DA agonists, on the other hand, our laboratory previously demonstrated that bromocriptine inhibited prolactin synthesis and release more potently than DA in primary cultured rat pituitary cells (36, 37), and Denef et al. (14) demonstrated earlier that apomorphine is more potent than DA in suppressing prolactin release in primary rat pituitary cells.
pituatory cultures. However, in this study we observed that their peak inhibitory actions were at similar concentrations, between $10^{-4}$ and $10^{-3} \text{ mol/l}$.

In the latter part of this study, we pre-treated GH4ZR7 cells with phenoxybenzamine before we introduced them to a dopaminergic agent. Exposure of D2 receptor-expressing cells (e.g., GH4C1-hD2) to the receptor alkylating compound, phenoxybenzamine, for 1 h could abolish D2 receptors (38), and we showed that the treatment markedly reduced the DA-mediated inhibition of prolactin release from primary cultured rat pituitary cells while phenoxybenzamine pretreatment was ineffective on somatostatin-, TRH-, and angiotensin-II-induced prolactin release (33). Our observation that phenoxybenzamine inactivates DA-induced prolactin release, is consistent with our previous studies (33). Taken together, our observations further support the concept that DA-induced prolactin release in the GH4ZR7 cells is mediated by dopamine D2a receptors.

D2 receptor agonists and antagonists act on the D2 receptor, but they have different potencies and affinities to the receptor. In addition, the patterns of prolactin release induced by these agonists and antagonists can also be different. For example, both DA and bromocriptine inhibit prolactin release via activating dopamine D2 receptors, yet only withdrawal of DA infusion, but not withdrawal of bromocriptine infusion, causes rebounding of prolactin release over the basal rate in a perfusion system of primary cultured rat pituitary cells (36). Both pimozide and d-butaclamol are well-established D2 receptor antagonists, but temporal patterns of prolactin release in male rats after injection of pimozide or d-butaclamol are substantially different (39). Moreover, we observed that haloperidol was unable to block the stimulatory action on prolactin release. These observations infer that these agonists and antagonists act on D2 receptors but that there are subtle differences in their modes of action.

It is possible that a broken-down product of dopamine may show stimulatory action by stimulating other receptors, since dopamine can be easily oxidized. However, no qualitative difference between freshly made dopamine and 1-h old dopamine was observed (10). Bromocriptine, a stable compound, also showed stimulatory action suggesting that no degradation product of dopamine generated the stimulatory action.

Dopamine should act on D2 receptors to inhibit and stimulate prolactin release, and haloperidol should also act on the same D2 receptors to block the dopamine actions. However, the haloperidol was unable to block the stimulatory action. We have tentatively proposed the following hypothesis to explain the inability of an antagonistic action of haloperidol on the stimulatory action. Dopamine binding to D2 receptors is largely blocked by haloperidol but haloperidol cannot completely block the receptor binding of dopamine since dopamine and haloperidol are in an equilibrium state on dopamine D2a receptors. High concentrations of dopamine–receptor complex are required to inhibit prolactin release and thus the inhibitory action was blocked by haloperidol. However, the reduced portion of dopamine–receptor complex may be sufficient to stimulate prolactin release.

Finally, the chemical nature of the physiological PRF is not yet established; several biologically active chemicals are considered to be PRFs (40). DA is the major PIF, but it may also play a role as a PRF since low concentrations of DA stimulate prolactin release.

Acknowledgements

This work was supported by the Medical Research Council of Canada. Appreciation is expressed to Dr A F Parlow and Dr S Raiti for supplying the rat prolactin radioimmunoassay kit through the NIADDK Rat Pituitary Hormone Distribution Program.

References


9 Lambergs SWJ & MacLeod RM. Regulation of prolactin secretion at the level of the lactotroph. Physiological Review 1990 70 279–318.


The $D_2$ receptor stimulates prolactin release


20 Chang A, Shin SH & Pang SC. Dopamine D2 receptor mediates both inhibitory and stimulatory actions on prolactin release. *Endocrine* 1997 7 177–182.


38 Nilsson CL, Hellström M, Ekman A & Eriksson E. Inverse agonism at dopamine $D_2$ receptors: haloperidol-induced prolactin release from GH4C1 cells transfected with the human $D_2$ receptor is antagonized by R(-)-n-propylnorapomorphine, raclopride, and phenoxybenzamine. *Neuropsychopharmacology* 1996 15 53–61.


Received 4 February 1999
Accepted 3 June 1999