EXPERIMENTAL STUDY

Role of phosphodiesterase and protein kinase G on nitric oxide-induced inhibition of prolactin release from the rat anterior pituitary

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Abstract

Objective: In order to determine the mechanism by which nitric oxide (NO) inhibits prolactin release, we investigated the participation of cGMP-dependent cAMP-phosphodiesterases (PDEs) and protein kinase G (PKG) in this effect of NO.

Methods: Anterior pituitary glands of male rats were incubated with inhibitors of PDE and PKG with or without sodium nitroprusside (NP). Prolactin release, and cAMP and cGMP concentrations were determined by RIA.

Results and conclusions: The inhibitory effect of NP (0.5 mmol/l) on prolactin release and cAMP concentration was blocked by EHNA (10^−4 mol/l) and HL-725 (10^−4 mol/l), inhibitors of cGMP-stimulated cAMP-PDE (PDE2). 8-Br-cGMP (10^−4 and 10^−3 mol/l), which mimics cGMP as a mediator of NP effects on prolactin release, also decreased cAMP concentration. Zaprinast (10^−4 mol/l), a selective inhibitor of specific cGMP-PDE (PDE5), potentiated the NP effect on cAMP concentration. Rp-8-[(4-chlorophenyl)thio]-cGMP triethylamine (Rp-8-cGMP, 10^−7 – 10^−6 mol/l), an inhibitor of PKG, reversed the effect of NP on prolactin release. The present study suggests that several mechanisms are involved in the inhibitory effect of NO on prolactin release. The activation of PDE2 by cGMP may mediate the inhibitory effect of NO on cAMP concentration and therefore on prolactin release. NO-activated PKG may also be participating in the inhibitory effect of NO on prolactin release.

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Introduction

Nitric oxide (NO) is a diffusible gas with an important role in neuroendocrine functions (1, 2). In the anterior pituitary, NO is synthesized in gonadotrophs and follicle-stellate cells (3, 4), and exerts direct effects on pituitary hormone secretion (5–10). We recently demonstrated that NO, by a paracrine mechanism, inhibits prolactin release from rat anterior pituitaries in vitro (9). We also found that NO increases pituitary cGMP concentration and decreases cAMP concentration (11). In addition, NO induced a transient decrease in free intracellular calcium levels in anterior pituitary cells (12). cAMP and calcium are intracellular messengers that play important roles in the signal transduction mechanisms regulating prolactin secretion (13, 14).

cGMP regulates cellular responses through several different downstream mechanisms, including calcium influx (15, 16), protein phosphorylation by cGMP-dependent protein kinase G (PKG) (16, 17) and stimulation or inhibition of phosphodiesterase (PDE) activity (18, 19). Several PDEs able to hydrolyze cAMP are regulated by cyclic nucleotides and hormones (19, 20). At least two cAMP-PDE families are controlled by cGMP (18): the cGMP-stimulated PDE (cGS-PDE or PDE2), cGMP-phosphodiesterase (PDE2), 8-Br-cGMP (10^−4 and 10^−3 mol/l), which mimics cGMP as a mediator of NP effects on prolactin release, also decreased cAMP concentration. Zaprinast (10^−4 mol/l), a selective inhibitor of specific cGMP-PDE (PDE5), potentiated the NP effect on cAMP concentration. Rp-8-[(4-chlorophenyl)thio]-cGMP triethylamine (Rp-8-cGMP, 10^−7 – 10^−6 mol/l), an inhibitor of PKG, reversed the effect of NP on prolactin release. The present study suggests that several mechanisms are involved in the inhibitory effect of NO on prolactin release. The activation of PDE2 by cGMP may mediate the inhibitory effect of NO on cAMP concentration and therefore on prolactin release. NO-activated PKG may also be participating in the inhibitory effect of NO on prolactin release.

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indirect demonstrations of their presence are reported by different authors (24, 25).

The relative contribution of PDE and PKG in the inhibitory effect of NO on prolactin release remains unknown. The aim of this study was to investigate some of the mechanisms by which NO decreases prolactin release. Since NO increased pituitary cGMP and decreased cAMP concentrations (11), we studied whether cGMP, through cGMP-stimulated PDE (PDE2), could mediate the NO effect on cAMP concentration and prolactin release. We also studied the participation of PKG in the effect of NO on prolactin release.

The results of this study show that NO through cGMP stimulates PDE2 activity, thus decreasing pituitary cAMP concentration and prolactin release. The stimulation of PKG activity by the NO/cGMP system is also involved in the effect of NO on prolactin release.

Materials and methods

Rats

Male Wistar rats (200–250 g) were kept in controlled conditions of light (12 h light:12 h darkness cycle) and temperature (21–24 °C) with food and water freely available. Rats were kept according to the US National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The animals were killed by decapitation and the anterior pituitary lobes removed, cut longitudinally into halves, and placed in Krebs–Ringer bicarbonate buffer, pH 7.4 containing 10 mmol/l glucose, 25 mmol/l Hapes, 0.1 mmol/l bacitracin and 0.1% BSA (KRB buffer).

Drugs

HL-725, [2,3,6,7-tetrahydro-9,10-dimethoxy-3-methyl-2-[2,4,6-trimethylphenyl]limino]-4H pirimido[6,1-a]isoquinolin-4-one hydrochloride], and zaprinast, 1,4-dihydro-5-[2-propoxyphenyl]-7H-1,2,3-triazolo[4,5-d]-a[isoquinolin-4-one hydrochloride], and zaprinast, 1,4-dihydro-5-[2-propoxyphenyl]-7H-1,2,3-triazolo[4,5-d]-pyrimidine-7-one, were purchased from ICN Pharm. Inc., CA, USA; EHNA, erythro-9-[2-hydroxy-3-nonyl]-adenine, and Rp-8-cGMP, Rp-8-[4-chlorophenyl]thio]-cGMP triethylamine, from RBI, Natick, MA, USA. All other drugs were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sodium nitroprusside (NP) was prepared 1 min before addition to the assay buffer.

Incubation of anterior pituitary glands

One hemipituitary per tube was preincubated for 60 min in 0.5 ml KRB buffer at 37 °C to stabilize the tissue, in an atmosphere of 95% O2:5% CO2 with constant shaking at 60 cycles/min. Afterwards, the tissue was incubated in 0.45 ml fresh KRB buffer containing the different inhibitors being studied (to give the inhibitors time to take effect before addition of NP). Fifteen minutes after starting the incubation, 0.05 ml 5 mmol/l NP (to final concentration of 0.5 mmol/l) or KRB buffer was added and incubations continued for 15 or 45 min more. Controls were incubated in medium alone or with the corresponding vehicles. At the end of the incubation period, the media were aspirated and samples stored at −20 °C pending prolactin determination. Tissues were immediately frozen on dry ice and then processed for cyclic nucleotide and protein determination.

Cyclic nucleotide determination

Tissues were heated at 100 °C in 50 mmol/l sodium acetate buffer, pH 6.2, for 5 min. Tissues were sonicated for 30 s and centrifuged. Supernatants were stored at −70 °C pending cyclic nucleotide measurement by specific RIAs, with rabbit cAMP antibody for RIA pool CV-27 provided by National Institute of Diabetes, Digestive and Kidney Diseases of the NIH (Bethesda, MD, USA). The intra- and interassay coefficients of variation were less than 9%.

Prolactin determination

Prolactin was measured in the incubation medium by a double-antibody RIA, with kits provided by NIDDK, NIH. The results were expressed in terms of rat prolactin (RP-3) standard. The intra- and interassay coefficients of variation were less than 9%.

Protein concentration in tissue was determined by the Lowry method after sonication.

Statistical analysis

The results are expressed as means ± s.e.m. and evaluated by one- or two-way ANOVA, followed by the Student–Newman–Keuls multiple comparison test for unequal replicates or Dunnett’s test. The differences between groups were considered significant at P < 0.05. Results were confirmed by at least three independent experiments.

Results

Role of PDE2 in the NP effect on prolactin release and on cAMP and cGMP content

In order to examine the effect of NP on prolactin release and cAMP concentration in the presence of PDE2 inhibitors, EHNA (IC50: 10−6 mol/l) and HL-725 (IC50: 10−10 mol/l for the purified enzyme) (18, 26–28), we first tested the effect of these inhibitors alone on prolactin release. After 30 min incubation, neither EHNA nor HL-725 alone, at either concentration tested, affected prolactin release (control, 1.07 ± 0.08 µg/mg protein; EHNA, 10−3 mol/l, 1.07 ± 0.05, 10−5 mol/l, 1.08 ± 0.07, n = 6, Dunnett’s test: control,
1.01 ± 0.06 µg/mg protein; HL-725, 10−6 mol/l, 0.86 ± 0.05, 10−4 mol/l, 0.90 ± 0.04, n = 6, Dunnett’s test). Similar results were observed when pituitaries were incubated for 60 min with EHNA and HL-725 (data not shown). Both EHNA and HL-725, at a concentration that did not modify per se prolactin release (10−4 mol/l), abolished the inhibitory effect of NP on this release (Fig. 1).

Both PDE2 inhibitors did not modify per se basal cAMP concentration. The inhibitory effect of NP on cAMP concentration was not only blocked but even significantly increased in the presence of both EHNA and HL-725 (Fig. 2). We also evaluated the effect of NP on intracellular cGMP concentration in the presence of PDE2 inhibitors. Neither EHNA nor HL-725 (10−4 mol/l) had any effect per se on cGMP concentration, but significantly increased the effect of NP on this nucleotide (Fig. 2).

**Effect of exogenous and endogenous cGMP on intracellular cAMP concentration**

cGMP, which mediates the NO effect on prolactin release (11), also seems to mediate its effect on cAMP concentration. 8-Br-cGMP, a slowly hydrolyzable, membrane-permeable analogue of cGMP, significantly reduced cAMP concentration (control, 2.40 ± 0.22 pmol/mg protein; 10−4 mol/l 8-Br-cGMP, 1.25 ± 0.11, 10−3 mol/l 8-Br-cGMP, 1.42 ± 0.23, n = 5, both P < 0.01 vs control, ANOVA followed by Dunnett’s test).

Zaprinast (10−8 mol/l), a selective inhibitor of cGMP-PDE (scG-PDE, PDE5) (IC50: 10−6 mol/l) (18, 29, 30), did not affect cGMP or cAMP concentrations after 30 min incubation, but significantly increased the NP effect on cGMP and cAMP concentrations (Table 1). Zaprinast failed to modify the inhibitory effect of NP on prolactin release (Table 1), but after 60 min incubation, reduced basal prolactin release to levels similar to those reached by NP alone or with NP plus zaprinast (control, 1.54 ± 0.09 µg/mg protein; 0.5 mmol/l NP,
Role of PKG in the NP effect on prolactin release

Since NO through cGMP can stimulate PKG, we next considered the role of these enzymes in the effect of NP on prolactin release. For this purpose, we used Rp-8-cGMP, an inhibitor of PKG (31, 32). Anterior pituitary glands were incubated with increasing concentrations of Rp-8-cGMP (10^{-8}–10^{-6} mol/l) for 30 and 60 min. At these concentrations, PKG inhibitor did not modify basal prolactin release per se at either incubation time (Table 2). However, when anterior pituitaries were incubated with NP, the PKG inhibitor reversed the inhibitory effect of NP on prolactin release (Fig. 3).

Discussion

The aim of this study was to characterize some of the intracellular mechanisms by which NO inhibits prolactin release. Our present data show that at least two mechanisms, the activation of the PDE2 and the PKG, may be involved in the inhibitory effect of the NO/cGMP system on prolactin release.

cGMP regulates several of the PDEs that hydrolyze cAMP (18), such as PDE2 and PDE3. Both PDEs hydrolyze cAMP and cGMP. However, PDE2 contains a non-catalytic binding site for cGMP that, in the presence of cGMP, increases its affinity for cAMP, with greater hydrolyzing capacity for cAMP than for cGAMP (18, 19). Although the cellular distribution of PDE isoenzymes within the anterior pituitary remains unknown, it has been observed that PDE2 prevails in tissues where the two cyclic nucleotides have opposite effects (18, 19). Our data show that NP and 8-Br-cGMP, which mimics cGMP, reduce pituitary cAMP concentration. Since the effect of NP on cAMP content is potentiated by inhibiting cGMP breakdown, we can assume that cGMP may be activating PDE2. Supporting our hypothesis, when PDE2 was inhibited by EHNA or HL-725, NP not only failed to decrease cAMP concentration but even increased it. This increase in cAMP caused by cGMP could result from two simultaneous effects, one being the inability of cGMP to stimulate PDE2 in the presence of EHNA and HL-725, and the other being exerted by cGMP on PDE3. Also, since PDE2 hydrolyzes cGMP as well, NP induced a higher cGMP concentration in the presence of the PDE2 inhibitors.

NO inhibits prolactin by a paracrine mechanism (9) and increases intrapituitary cGMP content by stimulating guanylyl cyclase activity. cGMP mediates NO effects on prolactin release (11). Although evidence for cGMP production in lactotrophs has not been obtained to date (33), our results indicate that this cyclic nucleotide participates in the regulation of prolactin release.

HL-725 is a potent inhibitor of purified PDE2 in vitro (27, 28), though it is not totally selective (34, 35). Because NP produced a higher increase in cAMP with HL-725 than with EHNA, it is possible that HL-725...
Table 2 Effect of increasing concentrations of Rp-8-cGMP, an inhibitor of PKG, on prolactin release. Anterior pituitary glands were incubated with Rp-8-cGMP for 30 or 60 min. Results are means ± S.E.M. (n = 6).

<table>
<thead>
<tr>
<th>Rp-8-cGMP (mol/l)</th>
<th>Prolactin (µg/mg protein)</th>
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<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>Control</td>
<td>0.83 ± 0.08</td>
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<tr>
<td>10⁻⁸</td>
<td>1.09 ± 0.10</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>1.12 ± 0.11</td>
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<tr>
<td>10⁻⁶</td>
<td>1.09 ± 0.09</td>
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No statistical significance, determined by one-way ANOVA followed by Dunnett’s test.

cAMP on prolactin release could be masked by opposite effects of cGMP through other mechanisms influenced by NO/cGMP. Supporting this hypothesis, the inhibition of PKG, the main protein receptor of cGMP, also reversed the inhibitory effect of NP on prolactin release. These findings suggest that PKG may be involved in the inhibitory effect of NO on prolactin release. The increase of cGMP induced by NO may activate both PDE2 and PKG enzymes. Through these mechanisms the NO/cGMP system decreases anterior pituitary cAMP content and may affect events associated with calcium mobilization. Thus, NO may control prolactin release through multiple and integrated actions.

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