EXPERIMENTAL STUDY

Nitric oxide decreases the production of inositol phosphates stimulated by angiotensin II and thyrotropin-releasing hormone in anterior pituitary cells

Miguel O Velardez, Ariel H Benitez, Jimena P Cabilla, Cristian C A Bodo and Beatriz H Duvilanski

Centro de Investigaciones en Reproducción, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso 10, Buenos Aires C1121ABG, Argentina

(Correspondence should be addressed to B H Duvilanski; Email: neuroend@fmed.uba.ar)

Abstract

Objective: Nitric oxide (NO) affects the synthesis of several second messengers, such as cyclic nucleotides, arachidonic acid metabolites and the intracellular calcium concentration, involved in the anterior pituitary hormone release. The present study was performed to investigate the effect of NO on phosphoinositide metabolism.

Methods: The synthesis of inositol phosphates (IPs) was studied in primary cultures of anterior pituitary cells from Wistar male rats. IPs (mono, bis and tris phosphates) were determined by ionic exchange chromatography.

Results: Sodium nitroprusside and DETA NONOate (DETA/NO) significantly decreased IP synthesis and prolactin release stimulated by angiotensin II (AngII) and thyrotropin-releasing hormone (TRH). These effects were not observed with decayed DETA NONOate (unable to release NO). LY-83583, a guanylyl cyclase inhibitor, completely reversed the inhibitory effect of DETA/NO on AngII-induced IP production. However, BAY 41-2272, a novel stimulator of the soluble guanylyl cyclase, did not mimic the effect of NO donors. Likewise, neither 8-Bromine-cyclic GMP (8-Br-cGMP), an analog of cGMP, nor Sp-8-pCPT-cGMPs triethylamine, a cGMP-dependent protein kinase (PKG) stimulator, decreased IP synthesis stimulated by AngII. In addition, Rp-8-pCPT-cGMPs triethylamine, a PKG inhibitor, did not block the effect of NO. The decrease of IPs induced by DETA/NO was fully reversed by guanosine 5'-O-(3-thiotriphosphate) tetralithium salt, a non-hydrolyzable analog of GTP.

Conclusions: The present work indicated that NO decreases IP synthesis stimulated by Ang II and TRH in anterior pituitary cells by a soluble guanylyl cyclase/cGMP/PKG-independent pathway, and suggested that NO affects some regulatory factor located between the plasma membrane receptor and G-protein.

European Journal of Endocrinology 148 89–97

Introduction

Nitric oxide (NO), generated from L-arginine by NO synthase (NOS), is a free radical involved in the regulation of many physiologic functions, such as endothelium-dependent vasodilation, apoptosis, neurotransmission and hormone secretion (1). Immunocytochemical studies and *in situ* hybridization have demonstrated the presence of all three types of NOS in the rat anterior pituitary gland (2–4).

NO participates in the regulation of prolactin, growth hormone (GH) and luteinizing hormone (LH) secretion and its effect can be directly exerted at the pituitary level (2, 5–7).

The secretion of anterior pituitary hormones is affected by a large variety of stimuli provided by the environment and the internal milieu. Such stimuli

are mostly transduced by the hypothalamus which elaborates a series of releasing and inhibiting factors (8, 9). Thyrotropin-releasing hormone (TRH) and angiotensin II (AngII) are two of the most important neuropeptides that stimulate hormone release in the rat anterior pituitary. Signaling events triggered by both TRH and AngII G-protein-coupled receptors involve an increase of phosphoinositide breakdown through phospholipase C (PLC) activation (10, 11).

It is well established that inositol 1,4,5-trisphosphate (IP₃) stimulates prolactin, adrenocorticotropin, follicle-stimulating hormone, GH, LH and thyrotropin release from anterior pituitary cells or their derived cell lines (12–16).

In a previous work, we have demonstrated that NO decreases intracellular Ca²⁺ concentration in anterior pituitary cells (17). Several reports show that NO,

through cGMP-dependent protein kinase (PKG) activation, modulates phosphoinositide metabolism (18, 19) and IP₃ receptor activity (20, 21). PLC activation results in production of IP₃ which releases Ca²⁺ from non-mitochondrial intracellular stores (12, 22–24). However, the mechanisms of NO action on inositol phosphate (IP) production in anterior pituitary cells are not clear yet. The aim of this study was to investigate the effect of NO on the metabolism of phosphoinositides in rat anterior pituitary cells under basal and stimulated conditions of IP production.

Materials and methods

Animals

Male Wistar rats (200–250 g), kept under 12-h light: 12-h darkness cycles and controlled temperature (20–25 °C), were used. Food and water were supplied *ad libitum*. The animals were maintained in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*. The animals were killed by decapitation and the anterior pituitary glands were removed and placed in chambers containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 µl/ml MEM amino acids, 2 mmol/l glutamine, 5.6 µg/ml amphotericin B and 25 µg/ml gentamicin (DMEM-S) with 0.3% (w/v) bovine serum albumin (BSA).

Reagents

DETA NONOate (DETA/NO), guanosine 5'-O-(3-thiotriphosphate) tetralithium salt (GTPγS) and LY-83583 were purchased from Alexis, San Diego, CA, USA; [2-³H(N)]myo-inositol (22 Ci/mmol) from New England Nuclear, Buenos Aires, Argentina; Sp-8-[(4-Chlorophenyl)thio]-cGMPs triethylamine (Sp-8) and Rp-8-[(4-Chlorophenyl)thio]-cGMPs triethylamine (Rp-8) from RBI, Natick, MA, USA; and AG 1-X8 resin from Bio-Rad Laboratories, Buenos Aires, Argentina. All other drugs were obtained from Sigma Chemical Co., St Louis, MO, USA. All drugs were freshly prepared either in serum-free DMEM-S or HEPES-buffered salt solution B (see below) depending on the experimental approach. Decayed DETA/NO (DETA; unable to release NO) was obtained from a solution of 1 mmol/l DETA/NO that had been incubated for 48 h at 37 °C to allow the complete release of NO. LY-83583 (10 mmol/l) was solubilized in ethanol and successive dilutions were made in DMEM-S with 10 mmol/l LiCl reaching a final concentration of 0.01% (v/v) ethanol. In experiments with LY-83583, both control and experimental groups included 0.01% ethanol. In these conditions, no differences in both basal and stimulated IP production were observed between similar groups with or without ethanol. BAY 41-2272 (provided by Dr Andreas Knorr from BAYER AG Pharma Research, Wuppertol, Germany) was prepared in

transcutol (provided by Pharmacia, Buenos Aires, Argentina) and then diluted in the corresponding medium. Transcutol, at maximal concentration (0.1% v/v), did not modify IP values obtained in the control group without transcutol in experiments carried out in parallel. Likewise, 0.1% transcutol did not modify cell viability even when cells were incubated for 24 h with the drug (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay, data not shown).

Cell culture

The glands were washed twice in DMEM-S–BSA and cut into small fragments. Sliced fragments were dispersed enzymatically by successive incubations in the same medium containing 2.5 mg/ml trypsin (type I from bovine pancreas), 1 mg/ml DNase (deoxyribonuclease II, type V from bovine spleen) and 1 mg/ml trypsin inhibitor (type II-S from soybean). Finally, the cells were dispersed by gentle extrusion through a Pasteur pipet in Krebs–Ringer bicarbonate buffer without Ca²⁺ and Mg²⁺. Dispersed cells were washed twice with DMEM-S–BSA and resuspended in DMEM-S with 10% fetal bovine serum (FBS). Cell viability as assessed by trypan blue exclusion was always greater than 90%. Cells were seeded onto 6-well tissue culture plates (2.5 × 10⁶ cells/well) for IP quantification experiments and cultured for 3 days (37 °C, 5% CO₂ in air) in 4 ml DMEM-S–10% FBS. After this, the cells were washed twice and the media replaced with serum-free DMEM-S (1.4 ml/well) containing 2 µl [2-³H(N)]myo-inositol. The cells were incubated in this medium for 20–24 h prior to the experiments. A similar protocol was used for prolactin release experiments with cells seeded onto 96-well tissue culture plates (0.1 × 10⁶ cells/well). In these experiments, the addition of [³H]myo-inositol was omitted. Incubation of anterior pituitary cells for 24 h without FBS did not affect cell viability (determined by MTT assay, data not shown).

Measurement of IPs in intact cells

At the end of the labeling period, the cells were washed twice with 2 ml DMEM and preincubated in serum-free DMEM-S with 10 mmol/l LiCl (to inhibit IP degradation) for 30 min (37 °C, 5% CO₂ in air). After this preincubation period, the medium was changed for 2 ml of the same medium containing the different drugs being studied and incubated for another 30 min. When Rp-8 or LY-83583 were used, they were present during both preincubation and incubation periods. DETA/NO was prepared 1 h before the beginning of experiments to allow NO concentration to reach a plateau. After the incubation period, the plates were placed on ice, the medium was quickly aspirated and 0.5 ml/well cold 0.5 mol/l HClO₄ was added. The cells were scraped with a rubber spatula and transferred to 5 ml tubes. The wells were washed with 0.7 ml cold HClO₄ and

this was combined with the previous extract. The extracts were neutralized by the addition of 0.6 ml 0.72 mol/l KOH/0.6 mol/l KHCO_3 , stirred, and placed on ice for 30 min. The precipitated KClO_4 with cellular particulated remains was removed by centrifugation and the supernatant saved for analysis of the IP content. The supernatant was mixed with 0.5 ml 100 mmol/l myo-inositol, adjusted to 5 ml with distilled H_2O and chromatographed on 0.5×4.0 cm columns of Dowex (AG 1-X8 200–400 mesh, formate form) according to the method described by Ascoli *et al.* (25). The columns were prewashed with 15 ml 10 mmol/l myo-inositol. After sample application, the columns were sequentially washed with 10 ml 10 mmol/l myo-inositol (to wash residual [^3H]-myo-inositol), 5 ml 5 mmol/l sodium borate/60 mmol/l sodium formate (to elute glycerophosphoinositols), 5 ml 0.1 mol/l formic acid/0.2 mol/l ammonium formate (to elute inositol monophosphate; IP_1), 5 ml 0.1 mol/l formic acid/0.4 mol/l ammonium formate (to elute inositol bisphosphates; IP_2), and 5 ml 0.1 mol/l formic acid/1.0 mol/l ammonium formate (to elute IP_3). Aliquots (2 ml) of each wash were mixed with 5 ml scintillation liquid. Radioactivity was measured by liquid scintillation counting and the results are expressed as a percentage of incorporated radioactivity.

Measurement of IPs in permeabilized cells

Permeabilized cells were obtained as described by Koshiyama & Tashjian (26) with minor modifications. Briefly, at the end of the labeling period, the cells were washed twice with 2 ml HEPES-buffered salt solution A containing 118 mmol/l NaCl, 4.6 mmol/l KCl, 1 mmol/l CaCl_2 , 10 mmol/l glucose and 20 mmol/l HEPES. Then, the cells were washed twice with 2 ml HEPES-buffered salt solution B containing 125 mmol/l KCl, 2 mmol/l KH_2PO_4 , 0.25 mmol/l CaCl_2 , 2.5 mmol/l MgCl_2 , 10 mM LiCl, 10 mmol/l glucose, 1 mmol/l EGTA, 25 mmol/l HEPES and 1 mg/ml BSA. Afterwards, the cells were incubated for 7 min at room temperature with 5 $\mu\text{mol/l}$ digitonin, quickly washed twice with salt solution B and preincubated in this solution for 15 min (37°C, 5% CO_2 in air). After this, 1.8 ml salt solution B containing the different drugs being studied was added and the cells were incubated for 30 min (37°C, 5% CO_2 in air). At the end of the incubation period, the plates were placed on ice, the medium was quickly put in tubes containing 0.2 ml cold 5 mol/l HClO_4 , the cells were then scraped and transferred to the same tubes. Subsequent steps were performed as described above in *Measurement of IPs in intact cells*.

cGMP determination

Cells were heated at 100°C in 50 mmol/l sodium acetate buffer, pH 6.2, for 5 min. Then cells were sonicated

for 30 s and samples stored at -70°C pending cGMP determination by specific radioimmunoassay (RIA). A rabbit polyclonal antibody (final dilution 1:300) was used (Chemicon, Temecula, CA, USA). Samples (100 μl) were acetylated with 10 μl of a triethylamine: acetic anhydride (2:1) solution, mixed with 25 μl antibody and 25 μl [^{125}I]-acetylated cGMP (20 000 c.p.m./tube) and stored overnight at 4°C. The reaction was stopped by the addition of 100 μl BSA (10% w/v) and 2 ml cold ethanol. Samples were centrifuged at 2000 r.p.m. for 20 min at 4°C and supernatants were discarded. Radioactivity was measured in a gamma-counter. The intra- and interassay coefficients of variation were lower than 9%.

Prolactin determination

Prolactin was measured by a double-antibody RIA with reagents provided by Dr A F Parlow (National Hormone and Pituitary Program, Torrance, CA, USA). RP-3 was used as the reference preparation and NIDDK-anti-rPRL-S-9 as the antiserum. The intra- and interassay coefficients of variation were lower than 10%.

Statistics

The results are expressed as means $\pm$ S.E.M. and evaluated by Student's *t*-test or one- or two-way analysis of variance (ANOVA) followed either by Student–Newman–Keuls multiple comparison test for unequal replicates or Dunnett's test, depending on the experimental design. Differences between groups were considered significant if $P < 0.05$. Results were confirmed by at least three independent experiments.

Results

Effect of NO donors on basal and stimulated IP synthesis

In order to investigate the effect of NO on PLC activity in primary cultures of anterior pituitary cells, we examined the effect of DETA/NO and sodium nitropruside (NP), two NO donors, on basal and stimulated IP production. Neither DETA/NO (Fig. 1) nor NP (0.1–2.0 mM, data not shown) had any effect on basal IP production. To stimulate IP synthesis, we used either 10^{-8} mol/l AngII or 10^{-7} mol/l TRH because of their abilities to increase PLC activity in anterior pituitary cells (10, 11). AngII significantly stimulated IP synthesis, and DETA/NO (1 and 2 mmol/l) partially decreased this stimulation (Fig. 1). NP also decreased AngII-stimulated IP synthesis (IP_3 data, as % of incorporated radioactivity: 10^{-8} mol/l AngII, 3.07 ± 0.07 ; AngII+1 mmol/l NP, 2.32 ± 0.04 ; $n = 6$; $P < 0.001$, Student's test). Similar results with both DETA/NO and NP were observed when TRH was used as stimulator of PLC activity (data not shown).

In order to test the specificity of NO action and to discard any artificial effect of NO donors, we used decayed DETA (unable to release NO). DETA (1 mmol/l) did not modify AngII-stimulated IP synthesis (Fig. 2). Similar results were obtained on TRH-stimulated IP production (IP₃ data, as % of incorporated radioactivity: control, 0.90 ± 0.09 ; 10^{-7} mol/l TRH, $4.46 \pm 0.46^*$; TRH+1 mmol/l DETA/NO, $2.89 \pm 0.27^\Delta$; TRH+1 mmol/l DETA, $3.98 \pm 0.29^\#$; $n = 6$; $*P < 0.05$ vs respective control without TRH, $^\Delta P < 0.05$ vs respective control without DETA/NO,

$^\#P < 0.05$ vs TRH+DETA/NO; ANOVA followed by Student–Newman–Keuls test).

It was amply demonstrated that both AngII and TRH increased anterior pituitary hormone release through

Figure 1 Effect of DETA/NO on IP production. The anterior pituitary cells were incubated with (solid line) or without (broken line) 10^{-8} mol/l AngII in the presence of increasing concentrations of DETA/NO for 30 min. Points represent the mean (% of incorporated radioactivity) $\pm$ S.E.M. ($n = 6$). ** $P < 0.01$ vs AngII without DETA/NO (Dunnett's test).

Figure 2 Decayed DETA (unable to release NO) did not affect AngII-stimulated IP synthesis. The anterior pituitary cells were incubated with 10^{-8} mol/l AngII in the presence of DETA/NO (1 mmol/l) or decayed DETA (1 mmol/l) for 30 min. Bars represent the mean (% of incorporated radioactivity) $\pm$ S.E.M. ($n = 6$). ** $P < 0.01$ vs respective control without AngII. # $P < 0.05$, $^\Delta P < 0.01$ vs AngII+DETA/NO (Student–Newman–Keuls test).

an up-regulation of IP metabolism. In order to investigate the physiological relevance of NO-induced IP decrease on anterior pituitary hormone secretion, we used prolactin release as an indicator of changes in IP metabolism under conditions stimulated by AngII or TRH. In accordance with the results obtained in a previous work (27), in the present study both DETA/NO (1.0–2.0 mmol/l) and NP (0.5 and 1.0 mmol/l) also inhibited basal prolactin release (data not shown). In stimulated conditions, both DETA/NO (0.5–2.0 mmol/l) and NP (0.5 and 1.0 mmol/l) partially reversed the stimulation of prolactin release induced by AngII (10^{-8} mol/l) (Table 1). Neither NO donor at 0.1 mmol/l concentration had any effect on basal or AngII-stimulated prolactin release.

Involvement of the guanylyl cyclase/cGMP/PKG pathway in the effect of NO on AngII-stimulated IP synthesis

Soluble guanylyl cyclase is one of the most important heme proteins stimulated by NO. A consequence of the activation of this enzyme is the increase of cGMP levels with a potential turning on of PKG activity and other cGMP-regulated proteins. We therefore investigated the role of the guanylyl cyclase/PKG pathway in the effect of NO on IP synthesis. LY-83583, an inhibitor of soluble guanylyl cyclase, completely reversed the inhibitory effect of NO on AngII-stimulated IP synthesis (Fig. 3), suggesting an essential role of guanylyl cyclase activation in the effect of NO on IP production in stimulated conditions. On the basis of this result, at least cGMP and probably PKG could be involved in the effect of NO. Therefore, the treatment of the cells with cGMP or with a PKG stimulator should result in a similar decrease of IP synthesis as observed with NO donors. However, when we used 8-Bromide-cGMP (8-Br-cGMP), a cGMP analog more resistant to phosphodiesterases than cGMP which can

mimic the cellular effects of cGMP, or Sp-8, a PKG stimulator, no effect was observed on AngII-stimulated IP synthesis (Fig. 4). In addition, Rp-8 (10^{-7} mol/l), a PKG inhibitor, did not reverse the inhibitory effect of DETA/NO on AngII-stimulated IP production (IP₃ data, as a percentage of incorporated radioactivity: control, 1.03 ± 0.05 ; 10^{-8} mol/l AngII, $3.07 \pm 0.68^*$;

Figure 3 Participation of guanylyl cyclase in the inhibitory effect of NO on AngII-stimulated IP synthesis. The anterior pituitary cells were preincubated with or without LY-83583 for 30 min, then incubated with 10^{-8} mol/l AngII and/or 1 mmol/l DETA/NO for 30 min. Bars represent the mean (% of incorporated radioactivity) $\pm$ S.E.M. ($n = 6$). * $P < 0.05$, ** $P < 0.01$ vs control without AngII. Δ $P < 0.05$, $\Delta\Delta$ $P < 0.01$ vs AngII. ## $P < 0.01$ vs respective control without LY-83583 (Student–Newman–Keuls test).

Table 1 Effect of NO donors on AngII-stimulated prolactin release. Values are means $\pm$ S.E.M. ($n = 6$). The anterior pituitary cells were incubated in the presence of increasing concentrations of DETA/NO with or without 10^{-8} M AngII for 30 min.

	Prolactin (μ g/well)
Control	0.316 ± 0.051
10^{-8} mol/l AngII	$1.235 \pm 0.147^{\Delta\Delta}$
10^{-8} mol/l AngII + 0.1 mmol/l DETA/NO	1.270 ± 0.095
10^{-8} mol/l AngII + 0.5 mmol/l DETA/NO	$0.576 \pm 0.093^{**}$
10^{-8} mol/l AngII + 1.0 mmol/l DETA/NO	$0.655 \pm 0.196^{**}$
10^{-8} mol/l AngII + 2.0 mmol/l DETA/NO	$0.690 \pm 0.057^{**}$
10^{-8} mol/l AngII + 0.1 mmol/l NP	0.874 ± 0.240
10^{-8} mol/l AngII + 0.5 mmol/l NP	$0.533 \pm 0.132^*$
10^{-8} mol/l AngII + 1.0 mmol/l NP	$0.539 \pm 0.186^*$

$\Delta\Delta$ $P < 0.01$ vs respective control without AngII; * $P < 0.05$, ** $P < 0.01$ vs respective control without DETA/NO or NP (Student–Newman–Keuls test).

AngII+1 mM DETA/NO, $1.72 \pm 0.25^{\Delta}$; 10^{-7} M Rp-8, 1.05 ± 0.08 ; Rp-8+AngII, $2.58 \pm 0.20^{*}$; Rp-8+AngII+DETA/NO, $1.84 \pm 0.13^{\Delta}$; $n = 6$; $^{*}P < 0.05$ vs control without AngII, $^{\Delta}P < 0.05$ vs respective control

Figure 4 Role of cGMP and PKG in the inhibitory effect of NO on AngII-stimulated IP synthesis. The anterior pituitary cells were incubated with or without 10^{-8} mol/l AngII and 8-Br-cGMP (10^{-4} mol/l) or Sp-8 (10^{-7} mol/l) for 30 min. Bars represent the mean (% of incorporated radioactivity) $\pm$ S.E.M. ($n = 6$). $^{**}P < 0.01$ vs respective control without AngII (Student–Newman–Keuls test).

without DETA/NO; ANOVA followed by Student–Newman–Keuls test).

The results obtained with LY-83583, 8-Br-cGMP and PKG stimulator and inhibitor are in contradiction, leaving the role for soluble guanylyl cyclase in the effect of NO unclear. In order to test whether the action of NO is dependent on soluble guanylyl cyclase activation or not, we used BAY 41-2272, a novel, NO-independent activator of this enzyme (28). First, we evaluated the capability of BAY 41-2272 to stimulate cGMP synthesis in anterior pituitary cells. BAY 41-2272 (10^{-5} mol/l) was 3.5–4 times more potent than the NO donors being used to stimulate cGMP production (cGMP as pmol/well: control, 2.10 ± 0.06 ; 1 mmol/l NP, $3.15 \pm 0.42^{*}$; 1 mmol/l DETA/NO, $3.51 \pm 0.14^{*}$; 10^{-6} mol/l BAY 41-2272, $10.03 \pm 1.38^{**}$; 10^{-5} mol/l BAY 41-2272, $12.24 \pm 2.66^{**}$; $^{*}P < 0.05$, $^{**}P < 0.01$ vs control; ANOVA followed by Student–Newman–Keuls test). In spite of the fact that BAY 41-2272 was able to stimulate cGMP production, it failed to modify IP₃ production, both in basal (data not shown) or in stimulated conditions (Fig. 5).

NO action on PLC pathway

In order to investigate the possibility that NO may be acting on PLC, we used GTP γ S, a stable analog of GTP that preferentially stimulates G-proteins. NO action could take place either up- or downstream of G-protein. The GTP analog allows us to focus on NO action in the receptor/G-protein/PLC system. GTP γ S increased basal IP production in a concentration-dependent manner, reaching a maximum of stimulation at 10^{-6} mol/l GTP γ S (data not shown).

Figure 5 Effect of BAY 41-2272, a novel soluble guanylyl cyclase stimulator, on AngII-stimulated IP₃ synthesis. The anterior pituitary cells were incubated with or without 10^{-8} mol/l AngII in the presence of 1 mmol/l DETA/NO or increasing concentrations of BAY 41-2272 (BAY) for 30 min. Bars represent the mean (% of incorporated radioactivity) $\pm$ S.E.M. ($n = 6$). $^{**}P < 0.01$ vs respective control without AngII. $^{\Delta\Delta}P < 0.01$ vs AngII (Student–Newman–Keuls test).

Figure 6 Role of GTP in the inhibitory effect of NO on AngII-stimulated IP synthesis. Permeabilized anterior pituitary cells were incubated in the presence of 10^{-6} mol/l GTP γ S with or without 10^{-8} mol/l AngII and/or 1 mmol/l DETA/NO for 30 min. Bars represent the mean (% of incorporated radioactivity) $\pm$ S.E.M. ($n = 6$). ** $P < 0.01$ vs respective control without AngII (Student–Newman–Keuls test).

In the presence of 10^{-6} mol/l GTP γ S, 10^{-8} mol/l AngII weakly increased IP synthesis and 1 mmol/l DETA/NO was not able to modify the effect of AngII (Fig. 6).

Discussion

The present study shows that NO inhibits IP production under stimulated conditions in rat anterior pituitary cells by a cGMP/PKG-independent mechanism.

IP₃ is one of the most important second messengers that stimulates hormone release in response to several hypothalamic secretagogues such as AngII, TRH and neurotensin (12, 22–24, 29). In our study, both DETA/NO and NP, two NO donors with different molecular structures that only share their ability to release NO, decreased both AngII- and TRH-stimulated IP synthesis and prolactin release. Since decayed DETA (unable to release NO) did not modify the production of IPs induced by AngII or TRH, it can be surmised that these effects were specifically exerted by NO. On the other hand, NO seems not to play any role on basal IP synthesis because the addition of NO donors did not modify basal IP production. However, they were able to decrease basal prolactin release. Since different mechanisms are involved in the inhibitory effect of NO on basal prolactin release (27, 30, 31), the present results indicate that NO may be acting on different biomolecules, and these biomolecules may be able to respond to NO depending on the physiologic status of the target cell.

In many biological systems, the effects of NO are mediated by cGMP. This signaling cascade involves acti-

vation of soluble guanylyl cyclase, up-regulation of cellular cGMP and activation of PKG (32, 33). It has been suggested that NO may modulate PLC activity through a cGMP/PKG pathway (18, 19). Since LY-83583, an inhibitor of soluble guanylyl cyclase, blocked the inhibitory effect of NO on AngII-stimulated IP production, it can be suggested that guanylyl cyclase activation is necessary for NO action. Nevertheless, neither the NO-independent, soluble guanylyl cyclase stimulator BAY 41-2272, nor the cGMP analog, nor the PKG stimulator mimicked NO action. Moreover, the PKG inhibitor did not block the inhibitory effect of NO on IP production. Collectively, these results suggest that the effect of NO on IP production under stimulated conditions is independent of the cGMP/PKG pathway.

In 1999, Prasad and co-workers (34) suggested that LY-83583 stimulates the PLC pathway in the hepatocyte-derived clone 9 cell line (34). In our system, BAY 41-2272 strongly stimulated cGMP synthesis (more than NO donors). The fact that BAY 41-2272 did not decrease AngII-stimulated IP production reinforces the idea that the reversion of the NO effect on IP production observed with LY-83583 may be due to an action of the guanylyl cyclase inhibitor on the PLC pathway. Thus, NO seems to be affecting the PLC pathway in a cGMP-independent manner.

It is well established that AT_{1A}, AT_{1B}, TRH-R1 and TRH-R2, AngII and TRH receptor subtypes, respectively, trigger PLC activation via the mediation of heterotrimeric Gq/G₁₁ subfamily members of G-proteins (24, 35). G-proteins participate in signal transduction and stimulus-secretion coupling in cells capable of exocytotic secretion, including anterior pituitary cells (12, 22–24). NO only inhibited IP production under AngII- or TRH-stimulated but not under basal conditions. Therefore, NO might be modifying the functionality of the signal transduction system probably by acting at different levels, such as plasma membrane receptors, G-proteins, regulatory proteins of signal transducers or PLC. However, there are two observations which support the idea that NO is not acting directly on PLC protein. In the first place, NO donors did not decrease IP synthesis in basal conditions when PLC activity was independent of G-protein activation. Secondly, GTP γ S was able to prevent the inhibitory effect of DETA/NO on AngII-induced IP production. Taken together, these observations suggest that NO might be disrupting some step in the chain of events between G-protein-coupled receptors and their linked effectors. It has been demonstrated that NO can modulate signals initiated via receptors by acting on different factors of the signal transduction pathways. NO can affect the function of agonist-gated receptor (36–38), ion channels (39, 40) and tyrosine kinase receptor (41) by a mechanism involving events of nitrosylation. Likewise, heterotrimeric and small G-proteins are targets of NO action by both cGMP-dependent (42–44) and -independent mechanisms (45–47). Several G-protein-coupled

receptors (48), including AT1 receptor (49), as well as the phosphatidylinositol 4,5 diphosphate (PIP₂)/PLC system (50, 51) are mainly localized in caveolae. Considering that NO has been shown to interfere with the integrity of the caveolin-1 scaffolding function (52, 53), this interference can be proposed as another hypothetical mechanism of NO action on IP metabolism in anterior pituitary cells. Besides, since AngII stimulated IP synthesis in the presence of GTPγS, an additional, GTP-independent stimulatory pathway of IP synthesis by AngII may be suggested, and this pathway, in turn, seems not to be modified by NO either.

In previous works we demonstrated that NO inhibits basal prolactin release by a mechanism that involves soluble guanylyl cyclase activation, up-regulation of cGMP and cGMP-dependent phosphodiesterase stimulation with a consequent cAMP decrease (30, 31). NO also inhibits 5-lipo-oxygenase decreasing 5-hydroxyeicosatetraenoic acid content which participates in the regulation of calcium homeostasis and basal prolactin release (27). In the present work, we have found another intracellular pathway affected by NO. NO only inhibited IP production under AngII- or TRH-stimulated but not under basal conditions by a mechanism that seems to be independent of the soluble guanylyl cyclase/cGMP/PKG pathway. NO is disrupting the signal transduction system, probably by acting on some factor located between the plasma membrane receptor and G-protein. Together, these results indicate that NO is capable of acting on different second-messenger pathways depending on the secretory activity status of anterior pituitary cells.

Acknowledgements

We would like to thank Dr Andreas M Knorr and BAYER AG Pharma Research for providing BAY 41-2272. We also thank Mrs Nilda Ramirez for her excellent technical assistance. Financial support from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Universidad de Buenos Aires is gratefully acknowledged.

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Received 18 January 2002

Accepted 3 September 2002