Spontaneous and Receptor-Controlled Soluble Guanylyl Cyclase Activity in Anterior Pituitary Cells

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Nitric oxide (NO)-dependent soluble guanylyl cyclase (sGC) is operative in mammalian cells, but its presence and the role in cGMP production in pituitary cells have been incompletely characterized. Here we show that sGC is expressed in pituitary tissue and dispersed cells, enriched lactotrophs and somatotrophs, and GH_3 immortalized cells, and that this enzyme is exclusively responsible for cGMP production in unstimulated cells. Basal sGC activity was partially dependent on voltage-gated calcium influx, and both calcium-sensitive NO synthases (NOS), neuronal and endothelial, were expressed in pituitary tissue and mixed cells, enriched lactotrophs and somatotrophs, and GH_3 cells. Calcium-independent inducible NOS was transiently expressed in cultured lactotrophs and somatotrophs after the dispersion of cells, but not in GH_3 cells and pituitary tissue. This enzyme participated in the control of basal sGC activity in cultured pituitary cells. The overexpression of inducible NOS by lipopolysaccharide + interferon-γ further increased NO and cGMP levels, and the majority of *de novo* produced cGMP was rapidly released. Addition of an NO donor to perifused pituitary cells also led to a rapid cGMP release. Calcium-mobilizing agonists TRH and GnRH slightly increased basal cGMP production, but only when added in high concentrations. In contrast, adenylyl cyclase agonists GHRH and CRF induced a robust increase in cGMP production, with EC_{50}s in the physiological concentration range. As in cells overexpressing inducible NOS, the stimulatory action of GHRH and CRF was preserved in cells bathed in calcium-deficient medium, but was not associated with a measurable increase in NO production. These results indicate that sGC is present in secretory anterior pituitary cells and is regulated in an NO-dependent manner through constitutively expressed neuronal and endothelial NOS and transiently expressed inducible NOS, as well as independently of NO by adenylyl cyclase coupled-receptors. (Molecular Endocrinology 15: 1010–1022, 2001)

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INTRODUCTION

Guanylyl cyclases (GCs) catalyze the formation of cGMP, an intracellular messenger molecule that is involved in regulation of various cellular functions by activating specific cGMP-receptor proteins. These include cGMP-dependent protein kinases I and II (1), cyclic nucleotide-gated (CNG) channels (2), and cyclic nucleotide-regulated phosphodiesterases (PDEs) (3). Whereas in mammalian cells, adenylyl cyclases (ACs) exist only in membrane-bound forms, GCs occur in both particulate (pGC) and soluble (sGC) forms. Receptor-linked pGCs are stimulated by peptide hormones (4), and sGC functions biologically as intracellular nitric oxide (NO) receptor and effector (5). sGC is a heterodimeric cytoplasmic protein composed of α- and β-subunits, and coexpression of both subunits is required to obtain enzyme activity. In the absence of NO, sGC-derived cGMP production is negligible, and three members of NO synthases (NOS), neuronal (nNOS), endothelial (eNOS), and inducible (iNOS), supply cells with NO by catalyzing the oxidation of L-arginine to L-citrulline and NO (6). The first two enzymes are constitutively expressed and their activation is dependent on a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), whereas iNOS does not require elevation in $[Ca^{2+}]_i$ to generate NO. The released NO stimulates sGC by binding to the heme part of the enzyme, causing an elevation in cGMP production (6–8).

Earlier studies have suggested that pituitary cells express pGC, and that this enzyme's activity is regulated by natriuretic peptides (9, 10). A lot of indirect evidence has also suggested the operation of sGC in these cells. Two NOS subtypes, nNOS and iNOS, were

detected in normal and immortalized pituitary cells (11–17) and were confirmed to be active by measurements of NO, NO_2, and NO_3 under different experimental paradigms (18, 19). Consistent with the Ca^{2+}-calmodulin sensitivity of nNOS (6), agonists that increase $[Ca^{2+}]_i$, including the Ca^{2+}-mobilizing TRH and Ca^{2+}-influx-dependent GHRH (20), were found to modulate NO levels and hormone secretion (19, 21). However, the effects of NO donors and NOS inhibitors on basal and agonist-induced hormone secretion (22–27) are not necessarily good indicators of the status of sGC in these cells. For example, in the absence of cGMP measurements, it is difficult to separate the direct messenger roles of NO (28) from those mediated by cGMP.

Here we studied the expression and regulation of sGC in rat anterior pituitary cells. Specifically, we addressed the coupling of sGC activity with spontaneous voltage-gated Ca^{2+} influx. We also studied the role of AC-coupled and Ca^{2+}-mobilizing receptors and inflammatory factors on sGC activity. The results of these investigations indicate that sGC is operative in unstimulated cells and is partially controlled by NOS. Calcium-insensitive iNOS also participates in the control of basal sGC activity. sGC activity is further stimulated by elevation of iNOS expression, which leads to an increase in NO production, and by activation of adenylyl cyclase-coupled receptors, which act in an NO-independent manner.

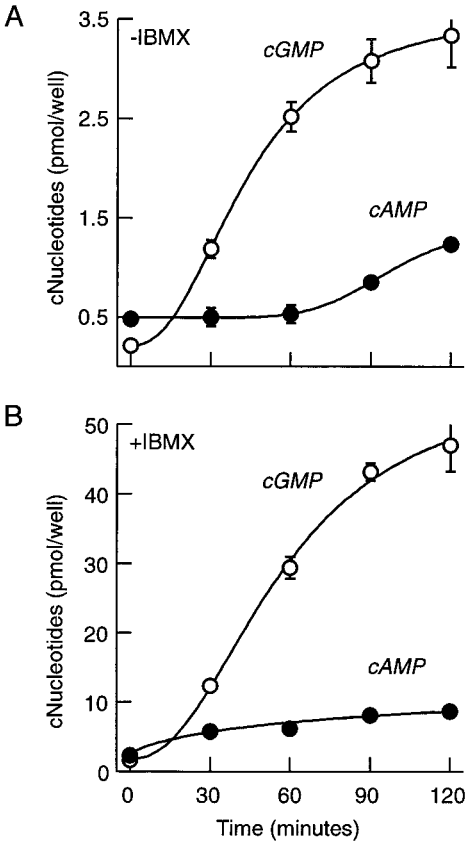

Fig. 1. Characterization of Basal Cyclic Nucleotide Production in Pituitary Cells

A and B, Time course of total (cell content and released) cGMP and cAMP production in the absence (A) and presence (B) of 1 mM IBMX, a nonselective PDE inhibitor. In this and the following figures, data points are means ± SEM from six replicates in one of at least three similar experiments.

RESULTS

Dependence of Basal cGMP Production on sGC

Total cyclic nucleotide levels (cell content + released in medium) were estimated in anterior pituitary cells 16 h after dispersion. Cells were washed and incubated in serum-free medium in the absence or presence of 1 mM 3-isobutyl-1-methylxanthine (IBMX), a nonselective PDE inhibitor. At the end of the incubation period, cells were dialyzed in an IBMX-containing medium, and cyclic nucleotide concentrations were determined by RIA. As shown in Fig. 1A, in the absence of IBMX during incubation in serum-free medium pituitary cells produce cGMP in a time-dependent manner, with the calculated half-time of 45 min. cAMP levels also increased, but more gradually, with the calculated half-time of 80 min. In five experiments done under similar experimental conditions, the steady-state levels of cGMP, estimated 2 h after incubation, were 3- to 8-fold higher than cAMP.

When cultured in the presence of IBMX, accumulation of cyclic nucleotides dramatically increased over time, with the calculated half-times of 55 and 75 min for cGMP and cAMP, respectively (Fig. 1B). In further parallelism to experiments without IBMX, cGMP production was 4–8 times higher than cAMP when cells were bathed in the presence of 1 mM IBMX. These results indicate that both enzymes, GC and AC, are operative in unstimulated cells, and *de novo* cGMP production dominates over cAMP production. Thus, cGMP, rather than cAMP, has a potential to act as a messenger in unstimulated pituitary cells.

In general, cGMP production in unstimulated pituitary cells could reflect basal pGC and/or basal sGC activity, the latter controlled by basal NOS activity. To dissociate between these two possibilities, cells were bathed in medium containing nonselective NOS inhibitors, N^w-monomethyl-L-arginine (L-NMMA) and N^w-nitro-L-arginine methyl ester (L-NAME) (7). As shown in Fig. 2, A and B, both compounds inhibited basal cGMP production in a concentration-dependent manner. Consistent with the specificity of their action, only a minor decrease in basal cAMP levels was observed in L-NMMA- and L-NAME-stimulated cells. The residual cGMP production observed in the presence of high NOS inhibitor concentrations was less than 10%, indicating that the majority of basal cGMP production is derived by NO-sensitive sGC. In line with this, RT-PCR and Western blot analysis revealed the presence of sGC in pituitary tissue and mixed cells, enriched lac-

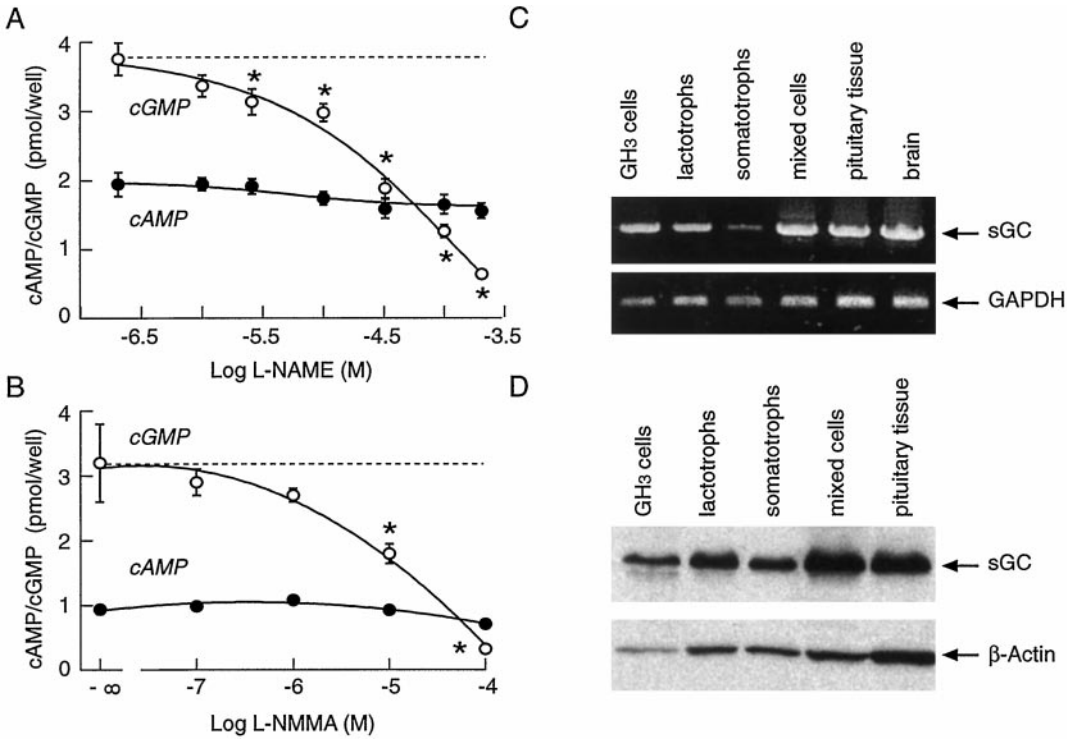

Fig. 2. Dependence of Basal cGMP Production on sGC Activity

A and B, Concentration-dependent effects of two nonselective NOS inhibitors, L-NAME (A) and L-NMMA (B), on cAMP and cGMP accumulation in pituitary cells during a 30-min incubation in the absence of PDE inhibitors. C, Expression of sGC transcripts in pituitary cells. Reverse transcriptional PCR analysis was performed using the sGC-β1 (*upper panel*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, *bottom panel*) specific primers. D, Western blot analysis of sGC expressed in pituitary cells.

totrophs and somatotrophs, and GH_3 immortalized cells (Fig. 2, C and D). These results indicate that in a mixed population of pituitary cells NOS are active and produce NO to levels that are sufficient to trigger cGMP production by sGC.

Expression of NOS in Pituitary Cells

To examine the expression of NOS, RT-PCR analysis was done in pituitary tissue, dispersed pituitary cells, enriched somatotrophs and lactotrophs, GH_3 immortalized cells, and control tissues. The transcripts for nNOS were present in both cultured pituitary cells (Fig. 3A, lane 1) and pituitary tissue (lane 3). Our study also revealed the presence of nNOS transcripts in GH_3 cells (Fig. 3B, lane 1) and enriched lactotrophs (lane 3) and somatotrophs (lane 5). The transcripts for eNOS were also detected in pituitary tissue (Fig. 4A, lane 3), mixed populations of pituitary cells (lane 1), as well as in lactotrophs (Fig. 4B, lane 3), but not somatotrophs (lane 5). In further agreement with the expression of eNOS in secretory cells, the transcripts for eNOS were also detected in GH_3 cells (Fig. 4B, lane 1).

mRNA for iNOS was also observed in mixed population of pituitary cells (Fig. 5A, lane 3) and enriched lactotrophs (Fig. 5B, lane 3) and somatotrophs (lane 5), all cultured for 16 h. However, no transcripts for iNOS were detected in pituitary tissue (Fig. 5A, lane 1) or GH_3 cells (Fig. 5B, lane 1). Time course study with mixed anterior pituitary cells revealed that iNOS expression began during the initial 2-h incubation at 37 C (Fig. 5C, lane 8) and progressed during the next 4 h (lanes 6 and 4). In 24-h-old cultures, the message was hardly detectable (line 2), indicating the transient nature of iNOS expression in pituitary cells.

Extracellular Calcium Dependence of Cyclic Nucleotide Production

Earlier studies have shown that the majority of anterior pituitary cells exhibit periods of spontaneous firing of action potentials, which are sufficient to generate global calcium signals (reviewed in Ref. 29). Because the activity of several ACs and two NOS is known to depend on $[Ca^{2+}]_i$ (30), we tested the dependence of pituitary AC and sGC activity on extracellular Ca^{2+}. Cells cultured for 16 h were bathed for 60 min in IBMX-free (Fig. 6A) and IBMX-containing media (Fig. 6B), both supplemented with variable Ca^{2+} concentrations. In cells bathed in Ca^{2+}-deficient medium (free Ca^{2+} about 200 nM), cGMP levels were 2- to 3-fold higher than cAMP, and an increase in Ca^{2+} concentration in medium led to a progressive increase in cGMP production. This pattern was observed in both

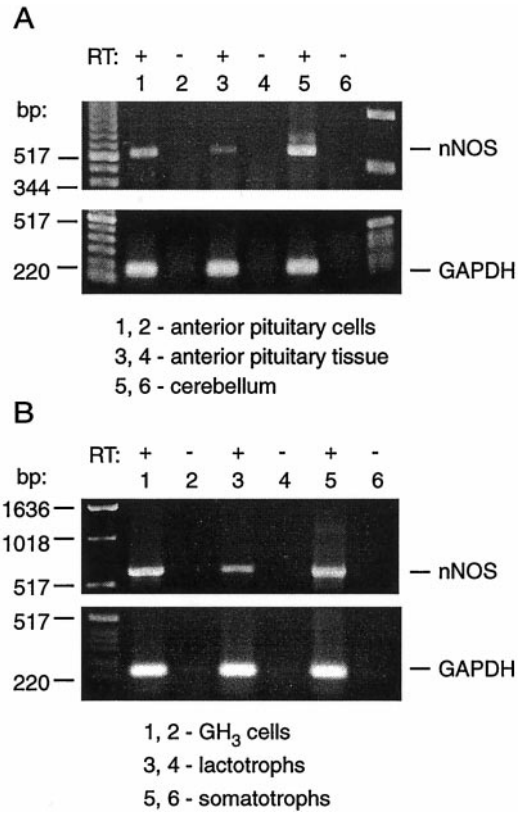

Fig. 3. Expression of nNOS Transcripts in Pituitary

A and B, Reverse transcriptional PCR analysis was performed using the nNOS (*upper panel*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, *bottom panel*) specific primers in pituitary tissue and cultured cells, as well as in control tissue (cerebellum). Negative controls were performed in the absence of enzyme in the RT reaction. The RT-PCR products were separated in 1% agarose gel and visualized with ethidium bromide.

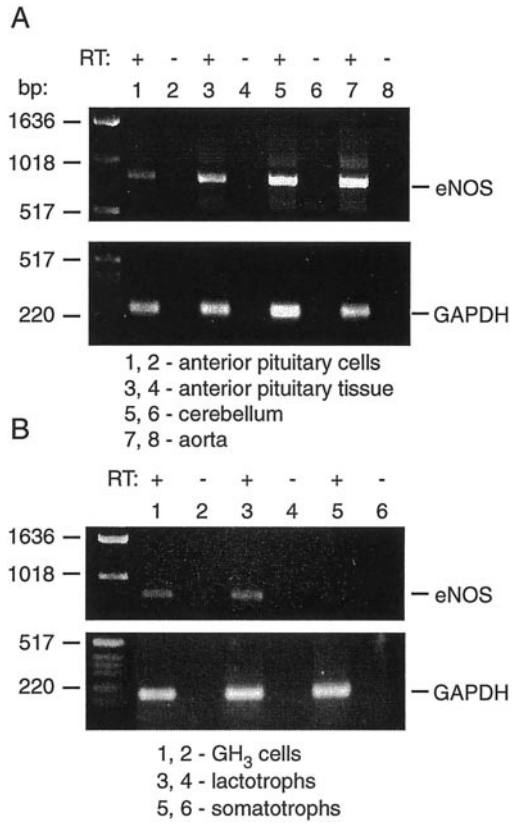

Fig. 4. Expression of eNOS Transcripts in Pituitary

A and B, RT-PCR analysis was performed using the eNOS (*upper panel*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, *bottom panel*) specific primers in pituitary tissue and cultured cells, as well as in cerebellum and aorta (control tissues). For details see Fig. 3 legend.

experimental conditions, with and without inhibited PDEs. In contrast, cAMP levels were not obviously affected by changing Ca^{2+} concentration in IBMX-free medium (Fig. 6A), but increased in cultures bathed in IBMX-containing medium (Fig. 6B).

In further experiments, cells were bathed in IBMX-free and Ca^{2+}-containing medium supplemented with 1 μM nifedipine, a specific blocker of L-type voltage-gated Ca^{2+} channels. Figure 6C shows that the blockade of voltage-gated Ca^{2+} influx was accompanied by a significant decrease in cGMP level, comparable to that observed in cells bathed in Ca^{2+}-deficient medium. On the other hand, cAMP levels were not significantly affected by addition of nifedipine. These results indicate that pituitary GC activity is partially dependent on spontaneous voltage-gated Ca^{2+} influx, and that a significant portion of cGMP production occurs independently of the status of extracellular Ca^{2+}. Adenylyl cyclase is also positively coupled to Ca^{2+} influx, but the basal PDE activity is sufficient to keep cAMP levels low.

Consistent with the expression of functional nNOS and its participation in control of basal sGC activity in unstimulated cells, addition of N^5-(1-imino-3-butenyl)-L-ornithine (vinyl-L-NIO), a relatively specific nNOS blocker (31), led to a dose-dependent decrease in basal cGMP production (Fig. 7A). The level of inhibition of cGMP production by high (10 μM) concentration of this blocker was similar to that observed in cells after removal of extracellular calcium, suggesting that nNOS mediates the coupling of voltage-gated Ca^{2+} influx with sGC signaling pathway. It is also likely that eNOS expressed in lactotrophs participates in the control of basal sGC activity. Since we do not know of a specific inhibitor of this enzyme, this hypothesis was not tested.

As described above, depletion of extracellular Ca^{2+} did not reduce cGMP production to the levels observed in cells bathed in the presence of high concentrations of nonselective NOS inhibitors. These results suggest that sGC in unstimulated pituitary cells used in our studies also produce cGMP independently of voltage-gated Ca^{2+} influx, *i.e.* through transiently expressed iNOS. Furthermore, extracellular Ca^{2+}-independent cGMP production was inhibited by amino-

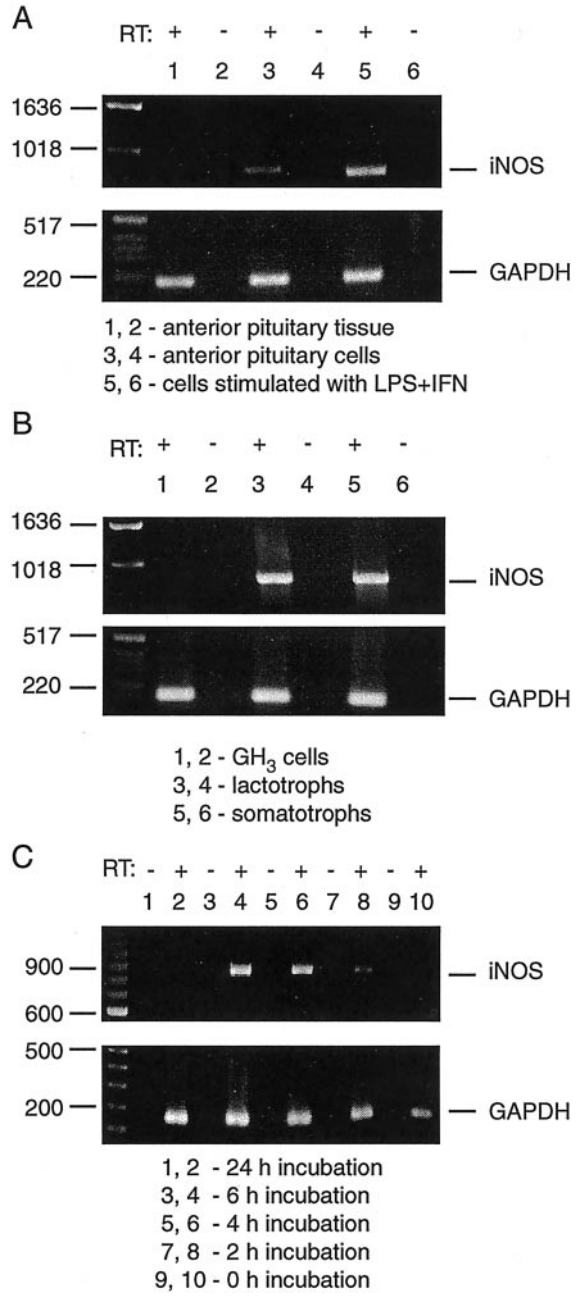

Fig. 5. Expression of iNOS Transcripts in Pituitary

A, Expression of iNOS transcripts in pituitary tissue, and mixed pituitary cells stimulated with and without LPS + IFN for 16 h. B, Expression of iNOS transcripts in GH_3 cells and enriched lactotrophs and somatotrophs. C, Time course of iNOS expression in mixed anterior pituitary cells. Tissue collection and dispersion of cells was done at 4 C, whereas trypsin digestion (15 min) and trypsin inhibitor incubations (15 min) were done at 37 C. After cell dispersion and their counting, a fraction of cells was immediately dialyzed (0 time point). The residual cells were plated and incubated at 37 C for indicated times.

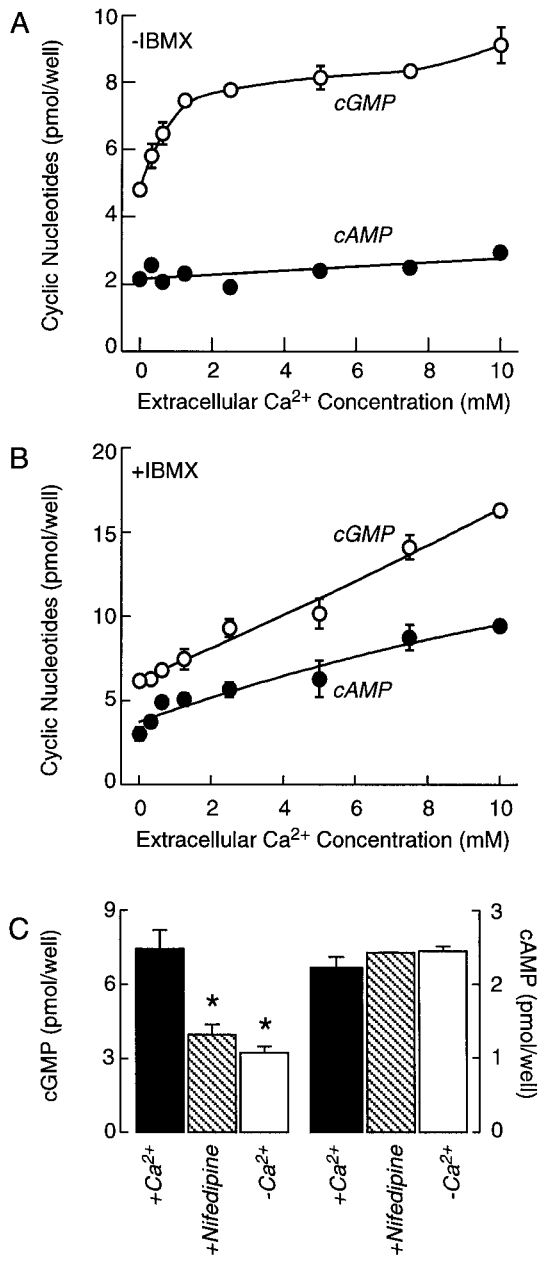

Fig. 6. Dependence of AC and GC Activity on Extracellular Ca^{2+} Influx

A and B, Concentration-dependent effects of extracellular Ca^{2+} on basal cAMP and cGMP production in cells bathed for 60 min in the absence (A) and presence (B) of 1 mM IBMX. C, Comparison of the effects of nifedipine, a specific blocker of L-type voltage-gated Ca^{2+} channels, with depletion of extracellular Ca^{2+} on cAMP and cGMP accumulation during 60-min incubation. *Asterisks* indicate $P < 0.05$ *vs.* control ($+Ca^{2+}$).

guanidine, a specific blocker of iNOS (32), in a concentration-dependent manner, with an EC_{50} of about 70 μM (Fig. 7A), comparable to that observed in other tissues expressing iNOS (33). Basal NO production was also inhibited by aminoguanidine in a concentration-dependent manner and with an EC_{50} comparable to that observed in cGMP measurements (Fig. 7B, *inset*).

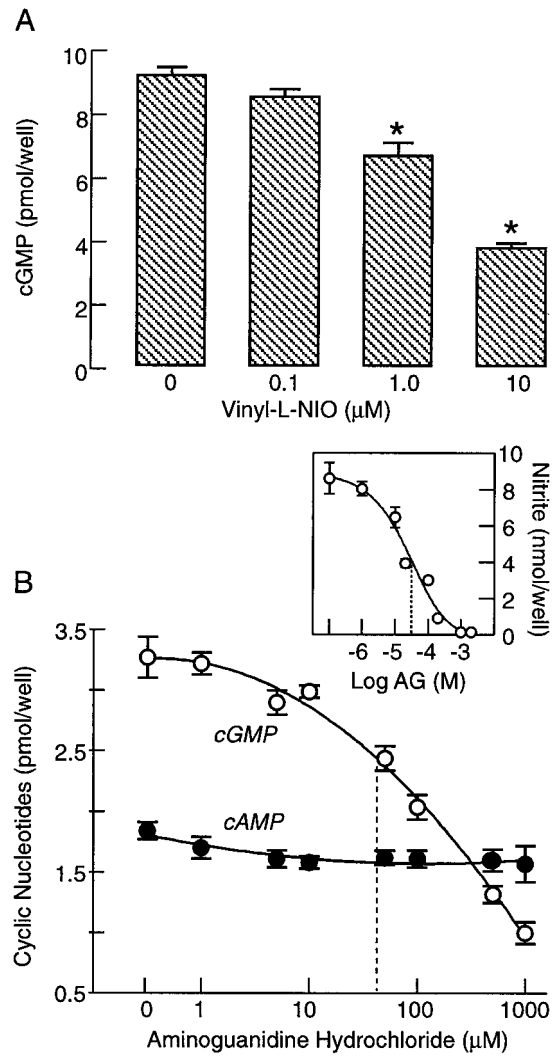

Fig. 7. Effects of NOS-Specific Inhibitors on Basal cGMP and NO Production

A, Concentration-dependent effects of vinyl-L-NIO, a relatively specific nNOS inhibitor, on basal cGMP production in pituitary cells incubated for 60 min in the absence of PDE inhibitors. *Asterisks* indicate $P < 0.05$ *vs.* control. B, Concentration-dependent effects of aminoguanidine, a relatively specific iNOS inhibitor, on basal cAMP and cGMP production in pituitary cells incubated for 60 min in the absence of PDE inhibitors. To exclude extracellular Ca^{2+}-dependent cGMP production, cells were bathed in medium containing about 200 nM Ca^{2+}. *Inset*, Effects of aminoguanidine (AG) on nitrite production in pituitary cells cultured overnight. *Dotted lines* illustrate the calculated EC_{50} values.

Dependence of sGC Activity on NO Levels

To increase NO levels above the basal, pituitary cells were treated with lipopolysaccharide + interferon-γ (LPS + IFN) for 16 h. As expected, these cells exhibited an elevated expression of mRNA for iNOS compared with untreated cells (Fig. 5A, line 5 *vs.* line 3, respectively). Consistent with the functional expression of this enzyme, nitrite accumulation in cells overexpressing iNOS was significantly elevated compared with control cells in all time points examined (Fig. 8A). cGMP production was also significantly elevated in LPS + IFN-treated cells compared with untreated cells (Fig. 8B). Under these experimental conditions, there was a linear relationship between NO_2, a stable product of NO, and cGMP levels (Fig. 8C). Aminoguanidine inhibited NO production in cells overexpressing iNOS in a concentration-dependent manner (Fig. 8D). Finally, removal of extracellular Ca^{2+} only slightly (~10%) reduced NO and cGMP production in cells overexpressing iNOS. Thus, the increase in NO production in pituitary cells is accompanied with proportional elevation in cGMP production. Furthermore, pituitary cells have a potential to respond to inflammation by expressing iNOS, an action that leads to increase of two messengers, NO and cGMP.

We also examined the kinetics of cGMP release in controls and cells overexpressing iNOS. The time course study indicated that the majority of *de novo* produced cGMP in control cells was released in medium (Fig. 9A, *left panel*). A similar ratio of released *vs.* intracellular cGMP levels was observed in cells overexpressing iNOS (Fig. 9A, *right panel*). Nitrite accumulation in and outside of cells was also analyzed. As shown in Fig. 9B, cell content of nitrite in unstimulated and LPS + IFN-treated cells was high and further increased during incubation. On the other hand, no nitrites were detected in medium immediately after washing the cells, and there was a progressive increase during incubation. Because NO easily diffuses between intracellular and extracellular medium, these results indicate that the majority of NO was transformed to NO_2 in medium. Thus, in both unstimulated and stimulated cells medium nitrite concentration reflects the activity of NOS better than its cellular concentration.

These experiments in static cultures raised the possibility that the kinetics of liberation of cGMP could be examined in perifusion system in a manner comparable to that commonly used for analysis of kinetics of pituitary hormone secretion. To do this, pituitary cells attached to beads (15×10^6/column) were perifused with medium containing 0.1 mM *N*-ethylethanamine: 1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEA), a rapidly releasable NO donor. Figure 10A (*open circles*) illustrates nitrite levels measured in medium after addition of DEA, and Fig. 10B (*open circles*) illustrates cGMP levels measured in the same samples. The profiles of DEA-derived nitrite were similar in columns with and without cells (not shown), confirming that transformation of NO into NO_2 does not require cells and occurs rapidly in water solution. Thus, the measured NO_2 levels reflect the kinetics of its accumulation in medium.

To fit experimental data (*open circles*, Fig. 10A) and calculate the rate of nitrite production, a sigmoidal curve (*full line*, Fig. 10A) was employed. The first derivative of this function (shown in Fig. 10B as *full line*) reflects the rate of NO_2 production after addi-

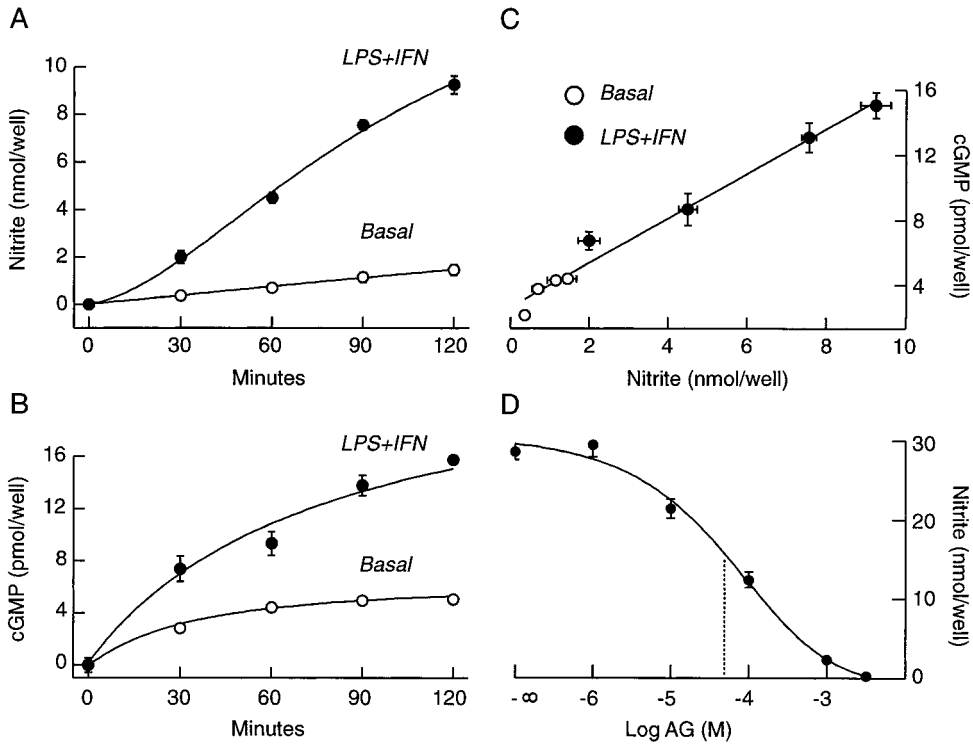

Fig. 8. Overexpression of iNOS in Pituitary Cells

A and B, Time course of nitrite (A) and cGMP (B) accumulation in controls (*open circles*) and cells overexpressing iNOS (*closed circles*). C, Relationship between nitrite levels and cGMP accumulation. D, Concentration-dependent effects of aminoguanidine (AG) on nitrite production in pituitary cells overexpressing iNOS. Data points are derived from panels A and B; $r = 0.97$. Basal cGMP levels present in cells at the beginning of stimulation (0 time point) were subtracted. Experiments were done in pituitary cells incubated in medium without PDE inhibitors.

tion of DEA in medium. There was an 8-min delay between the initial increase in NO_2 accumulation and initial cGMP release, and a 22-min delay in peak NO_2 and cGMP production (Fig. 10B). In the same samples, cAMP was not detectable. These results indicate that the kinetics of cGMP production by sGC reflect the kinetics of NO release and that rapid release of this nucleotide into medium protects against the action of PDEs.

Receptor-Regulation of sGC Activity

To examine the potential role of sGC in receptor-mediated signaling, cells were stimulated with several Ca^{2+}-mobilizing and AC-coupled receptors. The Ca^{2+}-mobilizing TRH receptors are expressed in thyrotrophs and lactotrophs, whereas GnRH receptors are expressed only in gonadotrophs. Their activation by TRH and GnRH also led to a small, but significant elevation in cGMP production when added in a submicromolar to micromolar concentration range (Fig. 11, A and B, *right panels*). Consistent with the cross-coupling of these receptors to AC, cAMP production was also elevated (*left panels*).

AC-coupled GHRH and CRF receptors are expressed in somatotrophs and corticotrophs, respectively. As expected, their activation led to an increase in cAMP production in a concentration-dependent manner (Fig. 12, A and B, *left panels*). GHRH and CRF also induced a concentration-dependent increase in cGMP production (*right panels*), indicating the cross-coupling of these receptors to sGC signaling pathway. In further accord with this, addition of NS 2028, a specific blocker of sGC, abolished GHRH and CRF-induced cGMP production, without affecting cAMP production (Fig. 12C). In parallel to LPS + IFN-stimulated cells, GHRH-induced stimulation of cGMP production was also observed in cells bathed in Ca^{2+}-deficient medium (not shown). In contrast to LPS + IFN-stimulated cells, no increase in NO production was detected in GHRH-stimulated cells (Table 1). These results indicate that AC-coupled receptors also stimulate sGC at basal NO production, *i.e.* independently of their action on Ca^{2+} signaling and NOS activation.

DISCUSSION

The hypotheses addressed in this study emerged from investigations on cyclic nucleotides and calcium signaling in pituitary and other neuroendocrine cells. Two recent reports suggest the expression of CNG-like channels in pituitary and hypothalamic cells and their

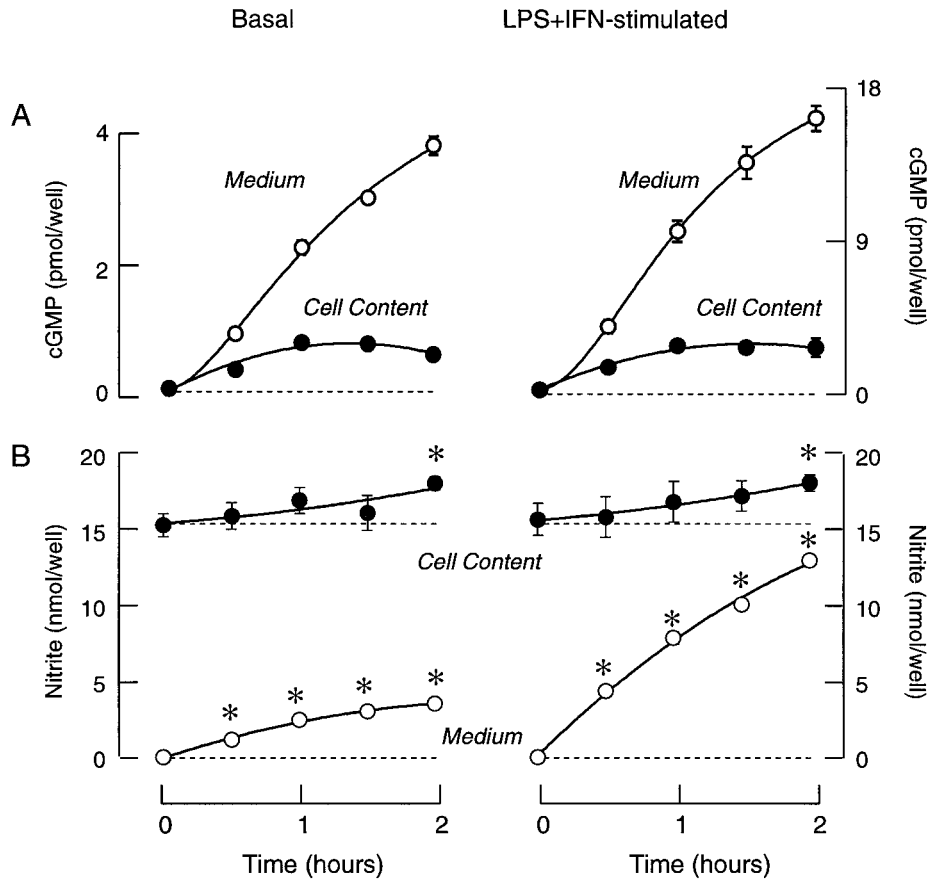

Fig. 9. Relationship between Cell Content and Released cGMP in Unstimulated Cells

A and B, Time course of cGMP (A) and NO (B) accumulation in cells and medium in unstimulated cells (*left panel*) and cells overexpressing iNOS (*right panel*), cultured in medium without PDE inhibitors. *Asterisks* indicate $P < 0.05$ *vs.* control (0 time point).

potential roles in the control of Ca^{2+} signaling and secretion (34, 35). There are three types of CNG channels: rod, cone, and olfactory; cGMP and cAMP are equipotent in the activation of cone and olfactory CNG channels, whereas cGMP is more potent in regulating rod CNG channels (2). Pituitary cells also express NOS (14, 15, 36), and the majority of dispersed and *in situ* pituitary cells exhibit periods of spontaneous electrical activity associated with voltage-gated Ca^{2+} influx (29, 37). Thus, it is possible that the relationship between cGMP and Ca^{2+} signaling pathways in pituitary cells is bidirectional, *i.e.* the rise in $[Ca^{2+}]_i$ may represent an effective signal for the activation of NOS, and the NO-controlled sCG may supply the cells with sufficient cGMP to activate CNG channels.

Once elevated, $[Ca^{2+}]_i$ can also modulate the activity of several other proteins involved in cyclic nucleotide signaling pathway. Two AC isozymes, ACI and ACVIII, are stimulated by Ca^{2+}, whereas ACV and ACVI are inhibited by this cation (30), suggesting that cAMP has a potential to act as a messenger in control of Ca^{2+} signaling in unstimulated cells, as it does in GHRH- and CRF-stimulated cells (29). Calcium also inhibits sGC directly (38, 39) and enhances the activity of PDEs (3), leading to the termination of cyclic nucleotide intracellular signaling functions. Furthermore, Ca^{2+} inhibits CNG channels (2) and stimulates NO- and cGMP kinase-regulated K^+ channels (28, 40), and both actions should inhibit electrical activity and voltage-gated Ca^{2+} influx. These observations indicate that the interactions between Ca^{2+} and cyclic nucleotide signaling pathways are extremely complex and cell specific, *i.e.* they depend on the subtypes of enzymes and channels expressed in a particular cell type.

In general, Ca^{2+} sensitivity of ACI and ACVIII in excitable cells provides a potential coupling mechanism between voltage-gated Ca^{2+} influx and cAMP production in the absence of receptor stimulation. Here we show that pituitary AC activity is facilitated by spontaneous voltage-gated Ca^{2+} influx, but the impact of Ca^{2+} influx-dependent cAMP production is diminished by PDEs. Only during receptor activation does AC-derived cAMP production dominate over PDE activity, as documented in experiments with GHRH. Basal cGMP production in pituitary cells is also dependent on spontaneous voltage-gated Ca^{2+} influx, and steady-state cGMP levels are 3- to 8-fold higher than cAMP levels. This finding suggests that cGMP, rather than cAMP, plays a messenger role in unstimu-

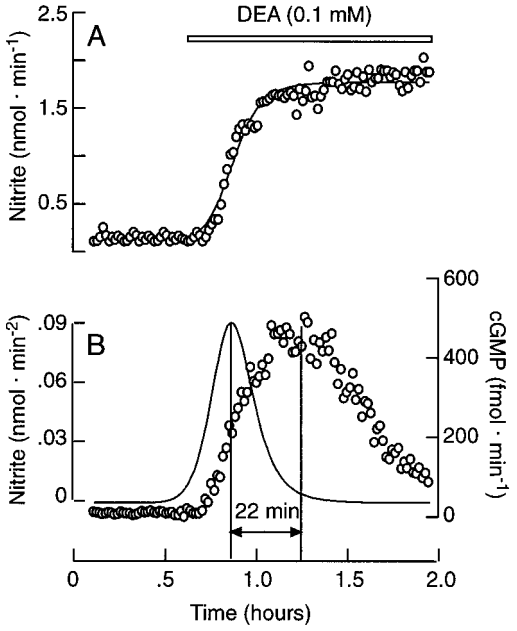

Fig. 10. Release of cGMP by Perifused Pituitary Cells

A, Time course of nitrite accumulation in medium from perifused cells (*open circles*). To calculate the rate of nitrite production, a sigmoidal curve (*solid line*) was employed to fit experimental data. B, Comparison of the rate of nitrite production and cGMP release. The first derivative of sigmoidal function (*solid line*) reflects the calculated rate of NO_2 production after addition of DEA in medium. *Open circles* indicate the measured cGMP in medium from perifused pituitary cells. Samples were collected every minute during a 2-h perifusion.

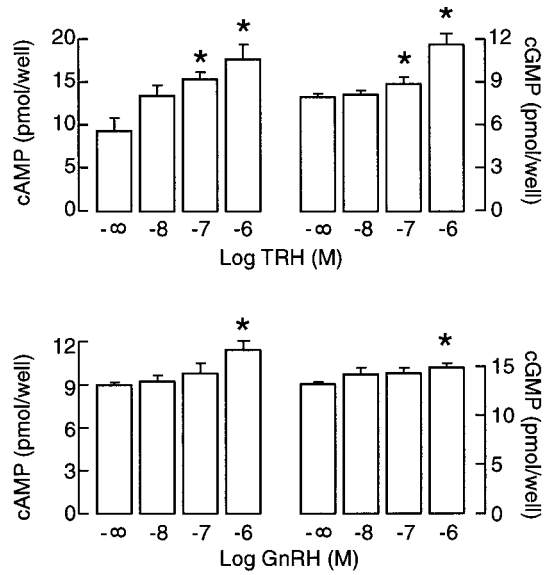

Fig. 11. Concentration-Dependent Effect of TRH and GnRH on Cyclic Nucleotide Accumulation

Cells were bathed in medium with IBMX for 60 min. *Asterisks* indicate $P < 0.05$ *vs.* control.

lated cells, including control of voltage-gated Ca^{2+} influx.

Several lines of evidence demonstrate that basal cGMP production in pituitary cells is mediated by sGC. First, basal cGMP production was dramatically reduced by addition of several NOS inhibitors, indicating its dependence on NO signaling. Second, the addition of NO donors led to an increase in cGMP production, with a delay in cGMP release of about 8 min. Third, inhibition of sGC by two specific inhibitors, 1*H*-(1, 2, 4)oxadiazolo(4, 3,–1)quinoxalin-1-one and 4*H*-8-bromo-1,2,4-oxadiazolo(3, 4-d)benz(b)(1, 4)oxazin-1-one, decreased basal cGMP (38). In accord with these findings, RT-PCR and Western blot analyses revealed the presence of sGC in pituitary tissue, mixed pituitary cells, enriched lactotrophs and somatotrophs, and GH_3 cells.

Our results indicate that the coupling of voltage-gated Ca^{2+} influx to sGC occurs indirectly, through activation of Ca^{2+}-sensitive NOS. The present data further indicate that nNOS is expressed in a mixed population of pituitary cells. The enzyme is also expressed in intact pituitary tissue, suggesting the potential *in vivo* relevance of the nNOS-sGC signaling pathway in normal physiological conditions. Several previously published studies have shown the expression of nNOS in folliculo-stellate and secretory pituitary cells, as well as in immortalized pituitary cells (14–17, 36). Here we show that enriched somatotrophs and lactotrophs also express nNOS. This enzyme probably mediates the coupling of voltage-gated Ca^{2+} influx with sGC, since extracellular Ca^{2+}-dependent cGMP production was inhibited by vinyl-L-NIO, an nNOS-selective inhibitor (31). In line with this idea, *in situ* measurements of electrical activity and $[Ca^{2+}]_i$ indicate that these cells are spontaneously active and can generate intra- and intercellular Ca^{2+} signals (29, 37). Furthermore, Western blot analysis indicates that nNOS expression in pituitary cells varies during the estrous cycle and that the proestrus rise in nNOS expression is accompanied by elevation of basal cGMP levels (12). Also, estrogens were found to down-regulate nNOS in anterior pituitary cells and GH_3 tumors (41).

The finding that eNOS is expressed in pituitary tissue and mixed pituitary cells is novel. It was believed that eNOS expression is restricted exclusively to vascular endothelium and that the presence of this enzyme in most tissues is generally attributable to the vascular endothelium contained in those tissues (6). However, here we show that eNOS transcripts are also present in enriched lactotrophs, as well as in GH_3 cells, demonstrating that secretory cells can also express this enzyme. In the absence of a specific blocker for this subtype of the enzyme, we were unable to estimate its participation in the control of basal sGC activity in secretory cells.

In addition to eNOS and nNOS, cultured pituitary cells also express transcripts for Ca^{2+}-insensitive iNOS. The expression of functional enzyme in cultured cells is indicated by the incomplete inhibition of cGMP

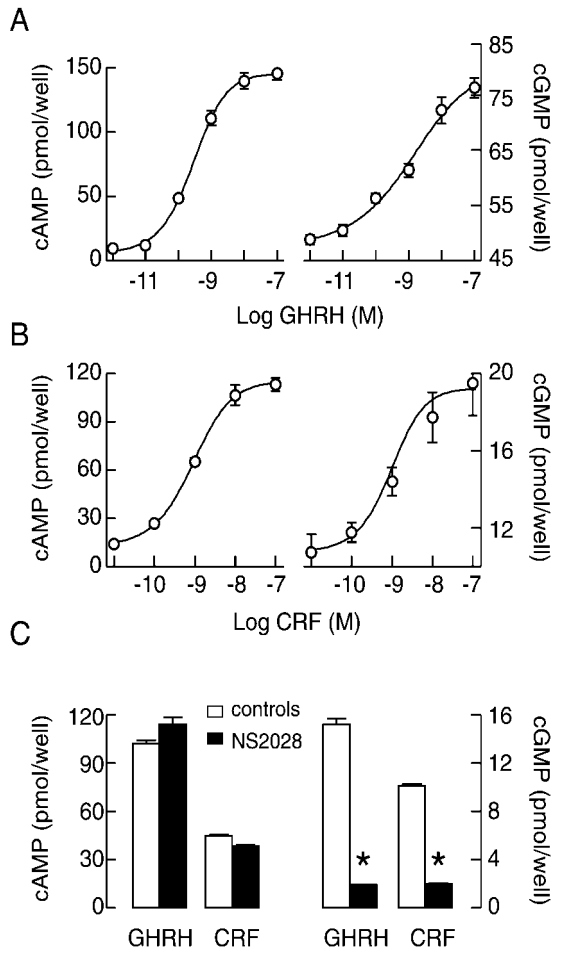

Fig. 12. Activation of AC and sGC by GHRH and CRF

A and B, Concentration-dependent effect of GHRH and CRF on cyclic nucleotide accumulation. C, Effects of NS2028, a specific inhibitor of sGC, on GHRH and CRF-induced cGMP production. Cells were bathed in medium with IBMX for 60 min. *Asterisks* indicate $P < 0.05$ *vs.* control.

Table 1. Dependence of sGC activity in mixed anterior pituitary cells on NO production

Treatment	cGMP (pmol/well)	Nitrite (nmol/well)
Basal	24.52 ± 1.37	1.27 ± 0.046
LPS + IFN	61.51 ± 5.87[a]	5.11 ± 0.11[a]
GHRH	41.02 ± 2.56[a]	1.22 ± 0.13

Overexpression of iNOS was induced by LPS + IFN as described in *Materials and Methods.*

[a]Significant difference ($P < 0.05$) *vs.* basal.

productions in cells bathed in Ca^{2+}-deficient medium and in cells with inhibited nNOS. Extracellular Ca^{2+}-independent NO and cGMP production was inhibited by aminoguanidine, in concentrations that are specific to iNOS (32). The expression of iNOS in pituitary cells, but not in pituitary tissue, reflects a reaction of cells to dispersion and/or culturing conditions and may affect spontaneous Ca^{2+} signaling in these cells. Transcripts for iNOS were first observed after incubation for 2 h after the cell dispersion and reached peak in expression after incubation for 6 h. During longer incubation times, a gradual decrease and disappearance of the transcripts for iNOS were observed. When cultured in serum-containing medium, immortalized P11 cells also express iNOS and produce a proinflammatory-like factor (11). The ability of mixed anterior pituitary cells to overexpress functional iNOS in response to LPS + IFN stimulation is also in accord with published data (11, 13, 18), suggesting that this enzyme may mediate the interactions between immune and endocrine systems.

The ability of AC-coupled GHRH receptors to stimulate sGC is in line with previously published findings that GHRH stimulates the synthesis of NO. This synthesis occurs at least partially through cAMP (21), suggesting that phosphorylation of NOS may account for elevation in NO production. GHRH also stimulates electrical activity and voltage-gated Ca^{2+} influx in pituitary somatotrophs (34, 42–44), suggesting that Ca^{2+} may mediate the activation of NOS by this agonist. Purified somatotrophs express the mRNA message for rod CNG channels (34), further indicating that both cAMP and cGMP may participate in GHRH-stimulated electrical activity in these cells. However, the present data clearly indicate that GHRH, as well as CRF, the other agonist acting through an AC signaling pathway, can induce a robust increase in cGMP production in extracellular Ca^{2+} and NO-independent manner. Ca^{2+}-mobilizing TRH and GnRH receptors were less potent in activating sGC, further indicating that signals other than Ca^{2+}/NO can elevate sGC activity. In accord with this, protein kinase A and protein kinase C were found to stimulate sGC activity *in vitro* (45, 46). Further studies are required to demonstrate the role of cAMP-protein kinase A in activation of sGC.

In summary, we show here that sGC is expressed in secretory pituitary cells and controls basal cGMP production. Our results indicate that the basal enzyme activity is high and partially dependent on voltage-gated Ca^{2+} influx and Ca^{2+}-dependent NOS, the rest being controlled by Ca^{2+}-independent iNOS. In contrast, basal cAMP levels are very low, and thus it is unlikely that cAMP acts as an intracellular messenger in unstimulated cells. nNOS was identified in enriched somatotrophs and lactotrophs, as well as in GH_3 cells, whereas eNOS was found in lactotrophs and GH_3 cells, but not in somatotrophs. Cell dispersion and/or culturing conditions lead to transient expression of iNOS in mixed pituitary cells, as well as in lactotrophs and somatotrophs. This enzyme participates in control of sGC activity *in vitro* during the initial 16-h incubation. Basal sGC activity is elevated by overexpression of iNOS, indicating the relevance of this enzymatic pathway in pathophysiological conditions. Activation of AC-coupled receptors also leads to a robust increase in sGC activity, the majority of which occurs in an extracellular Ca^{2+} and NO-independent manner. Finally, the presence of NOS in somatotrophs and

lactotrophs and the ability of GHRH, TRH, GHRH, and CRF to elevate sGC activity indicate that the sGC signaling pathway is common for all secretory anterior pituitary cells.

MATERIALS AND METHODS

Cell Cultures and Treatments

Experiments were performed on anterior pituitary cells from normal female Sprague Dawley rats obtained from Taconic Farm (Germantown, NY). Pituitary cells were dispersed as described previously (38), and cultured as mixed cells or enriched lactotrophs and somatotrophs in medium 199 containing Earle's salts, sodium bicarbonate, 10% heat-inactivated horse serum, and antibiotics. Purification of lactotrophs and somatotrophs was done by a two-stage Percoll discontinuous density gradient centrifugation as described in Refs. 34 and 42. The PRL-secreting GH_3 cells (obtained from ATCC, Manassas, VA) were cultured in Ham's F12K medium supplemented with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 15% heat-inactivated horse serum, and 2.5% FBS.

To express iNOS, cells (10^6/well) were treated for 16 h with 30 μg/well lipopolysaccharide + 1,000 IU/well interferon-γ (LPS + IFN), both from Sigma (St. Luis, MO). To elevate NO levels, cells were treated with NO donors DEA and DPTA, both from Alexis Biochemicals (San Diego, CA). Basal NOS activity was inhibited by L-NMMA), L-NAME, and vinyl-L-NIO) (RBI, Natick, MA). Phosphodiesterases were inhibited by IBMX (from Calbiochem, La Jolla, CA).

cGMP, cAMP, and Nitrite Measurements

Cells (1 million per well) were plated in 24-well plates in serum-containing M199 and incubated overnight at 37 C under 5% CO_2-air and saturated humidity. Before experiments, medium was removed and cells were washed with serum-free M199 and stimulated at 37 C under 5% CO_2-air and saturated humidity. cAMP/cGMP was measured in medium and in dialyzed cells as previously described (38), using specific antisera provided by Albert Baukal (NICHD, Bethesda, MD). The antisera used in our RIAs are highly specific for cAMP and cGMP, *i.e.* there was not any cross-reactivity at 100 pM and lower concentrations. All samples were diluted 2- to 5-fold and cyclic nucleotide concentrations were estimated using standard curves ranging from 5 fmol to 1 pmol.

For measurement of total NO production, samples were initially treated with nitrate reductase (Alexis Biochemicals) to convert nitrate to nitrite. Sample aliquots were then mixed with an equal volume of Greiss reagent containing 0.5% sulfanilamide and 0.05% naphthylethylenediamine in 2.5% phosphoric acid (all from Sigma), and the mixture was incubated at room temperature for 10 min and the absorbance measured at 546 nm (38). Nitrite concentrations were determined relative to a standard curve derived from increasing concentrations of sodium nitrite.

RNA Isolation and RT-PCR

Total RNA was extracted from a mixed population of anterior pituitary cells before and after purification using TRIZOL reagent (Life Technologies, Inc., Gaithersburg, MD), and its purity was determined spectrophotometrically. RNA samples were subjected to RT-PCR to determine whether these cells contain nNOS, iNOS and/or eNOS mRNA, as well as sGC-β1. To eliminate residual genomic DNA, RNA samples were treated with DNase I. Two micrograms of total RNA from each sample with DNase I treatment, were reverse transcribed into cDNA in a 20 μl reaction mixture containing oligo (dT)18 primer and Superscript II reverse transcriptase (Life Technologies, Inc.) according to the supplier's instructions. An aliquot of 5 μl of the RT reaction was amplified with PCR reagent system (Life Technologies, Inc.) in a final volume of 50 μl containing 1.5 mM $MgCl_2$, 0.4 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate, and 1.25 U of TaqDNA polymerase. Sequences for sense and antisense primers, respectively, were: iNOS, 5′-ATGGCTTGCCCCTGGAAGTTTCTC-3′ and 5′-CCTCTGATGGTGCCATCGGGCATCTG-3′; eNOS, 5′-TACGGAGCAGCAAATCCAC-3′ and 5′-CAGGCTGCAGTCCTTTGAT-3′; nNOS, 5′-CCGTCCTTTGAATACCAGCCTGATCCATG-3′ and 5′-CAGTTCCTCCAGGAGGGTGTCCACCGCAT-3′; and sGC-β1, 5″-GATCCGCAATTACGGCG-3′ and 5′-TGGAGAGGGATGTCACTACTCAG-3′. The PCR products were analyzed by agarose gel (1%) electrophoresis and visualized with ethidium bromide. The same volumes of samples used for NOS analysis were also subjected to PCR reaction using GAPDH-specific primers. Sequences for sense and antisense primers were: 5′-GGCATCCTGGGCTACACTG-3′ and 5′-TGAGGTCCACCACCCTGTT-3′, respectively. Samples of RNA isolated from rat cerebellum, aortic tissue, and a mixed population of anterior pituitary cells stimulated with LPS + IFN were used as positive controls for nNOS, eNOS, and iNOS, respectively. Reaction without RNA sample or RT served as negative controls.

Western Blot Analysis

Postmitochondrial fractions of anterior pituitary tissue and dispersed pituitary cells were obtained from adult female Sprague Dawley rats. Concentration of proteins was estimated by Bradford method using BSA as a standard (47). Equal amounts of protein (20 μg) from each postmitochondrial fraction were run on one-dimensional SDS-PAGE, using a discontinuous buffer system (Novex, San Diego, CA). The immunodetections on sGC were done with an antibody specific for β1-subunit (Cayman Chemical, Ann Arbor, MI). The secondary antibody for all assays was an antirabbit IgG (goat) from Kirkegaard & Perry Laboratories (Gaithersburg, MD) linked to horseradish peroxidase. The reactive bands were always determined with a luminol-based kit, and the reaction was detected by an enhanced chemiluminescence system, using x-ray film.

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