THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 276, No. 1, Issue of January 5, pp. 844–849, 2001
Printed in U.S.A.

Dependence of Soluble Guanylyl Cyclase Activity on Calcium Signaling in Pituitary Cells*

Received for publication, May 22, 2000, and in revised form, October 11, 2000
Published, JBC Papers in Press, October 12, 2000, DOI 10.1074/jbc.M004406200

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The role of nitric oxide (NO) in the stimulation of soluble guanylyl cyclase (sGC) is well established, but the mechanism by which the enzyme is inactivated during the prolonged NO stimulation has not been characterized. In this paper we studied the interactions between NO and intracellular Ca^{2+} in the control of sGC in rat anterior pituitary cells. Experiments were done in cultured cells, which expressed neuronal and endothelial NO synthases, and in cells with elevated NO levels induced by the expression of inducible NO synthase and by the addition of several NO donors. Basal sGC-dependent cGMP production was stimulated by the increase in NO levels in a time-dependent manner. In contrast, depolarization of cells by high K^+ and Bay K 8644, an L-type Ca^{2+} channel agonist, inhibited sGC activity. Depolarization-induced down-regulation of sGC activity was also observed in cells with inhibited cGMP-dependent phosphodiesterases but not in cells bathed in Ca^{2+}-deficient medium. This inhibition was independent from the pattern of Ca^{2+} signaling (oscillatory *versus* nonoscillatory) and NO levels, and was determined by averaged concentration of intracellular Ca^{2+}. These results indicate that inactivation of sGC by intracellular Ca^{2+} serves as a negative feedback to break the stimulatory action of NO on enzyme activity in intact pituitary cells.

Soluble guanylyl cyclases (sGC)[1] are heterodimeric cytoplasmic proteins composed of α and β subunits that function biologically as intracellular nitric oxide (NO) receptors and effectors. Coexpression of both subunits is required to obtain enzyme activity and NO regulation. The binding of NO to the sGC heme activates the enzyme, resulting in the conversion of GTP to cGMP (1). Together with particulate guanylyl cyclase and adenylyl cyclase (AC), these enzymes compose a family of proteins that is involved in a broad array of cellular functions in cardiovascular, neuronal, neuroendocrine, and other cell types (2). In this respect, sGC-generated cGMP production allows this enzyme to transmit the NO signal to the downstream elements of the signaling cascade, such as cGMP-dependent protein kinase (3), cyclic nucleotide-gated channels (CNGs) (4), and cGMP-regulated phosphodiesterases (5).

In contrast to the well established role of NO in the activation of sGC, the mechanisms of inactivation for this enzyme have been incompletely characterized. This is especially important for cells in which the highly diffusible NO is intimately involved in the control of Ca^{2+} signaling by facilitating Ca^{2+} release and/or Ca^{2+} influx pathways, as in hepatocytes (6, 7), glial cells (8), and neuroendocrine cells (9). Because the elevated intracellular calcium concentration ($[Ca^{2+}]_i$) stimulates the activity of two NO-producing enzymes, neuronal NO synthase (nNOS) and endothelial NOS (eNOS), such up-regulation would lead to overloading the cells with Ca^{2+}. Thus, it is reasonable to propose that the negative feedback effect of Ca^{2+} on sGC would provide the necessary mechanism to coordinate the regulation of intracellular cGMP and Ca^{2+} concentrations.

To test this hypothesis, we chose rat anterior pituitary cells, the majority of which exhibit spontaneous $[Ca^{2+}]_i$ transients of high amplitude that are sufficient to trigger hormone secretion (10). These cells also express the messages for rod, cone, and olfactory CNGs, which may participate in the generation of spontaneous $[Ca^{2+}]_i$ transients (11). Although not fully characterized, the NO-signaling pathway is also expressed in these cells (12–14) and is activated by several G protein-coupled receptors (15–17). In our study, we initially characterized the NOS subtypes expressed in pituitary cells and their participation in the delivery of NO and sGC-controlled cGMP. We further characterized the role of calcium in the control of sGC activity in intact 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 Farms (Germantown, NY). Pituitary cells were dispersed as described previously (18) and cultured in medium 199 containing Earle's salts, sodium bicarbonate, 10% horse serum, and antibiotics. Cell purification was done as described previously (18), and further identification of gonadotrophs and lactotrophs was done by the addition of specific Ca^{2+}-mobilizing agonists for these cells, gonadotropin-releasing hormone and thyrotropin-releasing hormone (Peninsula, San Carlos, CA), respectively.

To express iNOS, cells (10^6/well) were treated for 16 h with 30 μg/ml lipopolysaccharide + 1000 IU/ml interferon-γ (LPS+IFN-γ), both from Sigma. To elevate NO levels, cells were treated with three NO donors: sodium nitroprusside (SNP) from Research Biochemicals (Natick, MA) and *N*-ethylethanamine:1,1-diethyl-2-hydroxy-2-nitrosohydrazine (DEA) and 3,3′-(hydroxynitrosohydrazino)bis-1-propanamine (DPTA), both from Alexis Biochemicals (San Diego, CA). Basal and stimulated NOS activity

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[1] The abbreviations used are: sGC, soluble guanylyl cyclase(s); NO, nitric oxide; NOS, nitric acid synthase; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; AC, adenylyl cyclase; LPS+IFN-γ, 30 μg/ml lipopolysaccharide + 1000 IU/ml interferon-γ; SNP, sodium nitroprusside; DEA, *N*-ethylethanamine:1,1-diethyl-2-hydroxy-2-nitrosohydrazine; DPTA, 3,3′-(hydroxynitrosohydrazino)bis-1-propanamine; NS 2028, 4*H*-8-bromo-1,2,4-oxadiazolo(3,4-*d*)benz(b)(1,4)oxazin-1-one; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; Bay K 8644, 1,4,-dihydro-2,6-dimethyl-5-nitro-4; and CNG, cyclic nucleotide-gated channel.

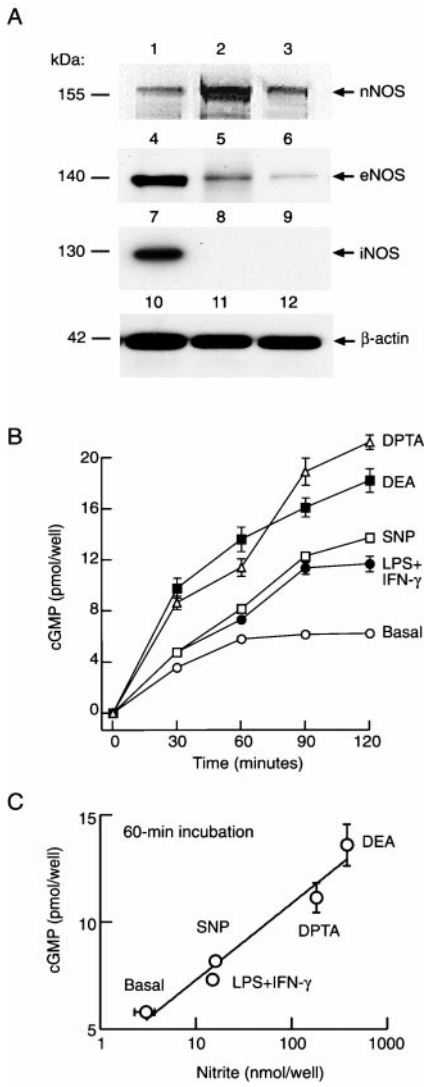

FIG. 1. **Characterization of the NO signaling pathway in anterior pituitary cells.** *A,* Western blot analysis of NOS expressed in pituitary tissue (*2*, *5*, and *8*), cultured pituitary cells (*3*, *6*, and *9*), pituitary cells stimulated with LPS+IFN-γ (*7*), cerebellum (*1*), and aorta (*4*), and β-actin expression in aorta (*10*), pituitary tissue (*11*), and cultured pituitary cells (*12*). *B,* time dependence of cGMP production in controls (basal) and in LPS+IFN-γ-, SNP-, DEA-, and DPTA-stimulated cells. *C*, correlation between cGMP and nitrite levels in samples collected 60 min after stimulation. In this and the following figures, cells were cultured in medium not containing phosphodiesterase inhibitors, if not otherwise specified. To express iNOS, pituitary cells were stimulated with LPS+IFN-γ for 16 h prior to experiment. SNP (1 mM), DEA, and DPTA (both 0.1 mM) were added immediately after replacing medium. The results shown are mean ± S.E. from sextuplicates in one of at least three similar experiments. All experiments were done in cultures 16 h after dispersion of cells.

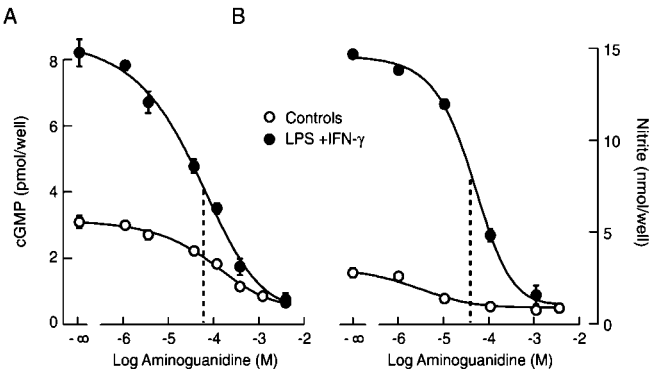

FIG. 2. **Dose-dependent effects of aminoguanidine, an NOS inhibitor, on cGMP (*A*) and nitrite (*B*) production in pituitary cells.** Aminoguanidine was ineffective in inhibiting cGMP production induced by 1 mM SNP (not shown). *Dotted lines*, calculated IC_{50} values. For other details see the legend of Fig. 1.

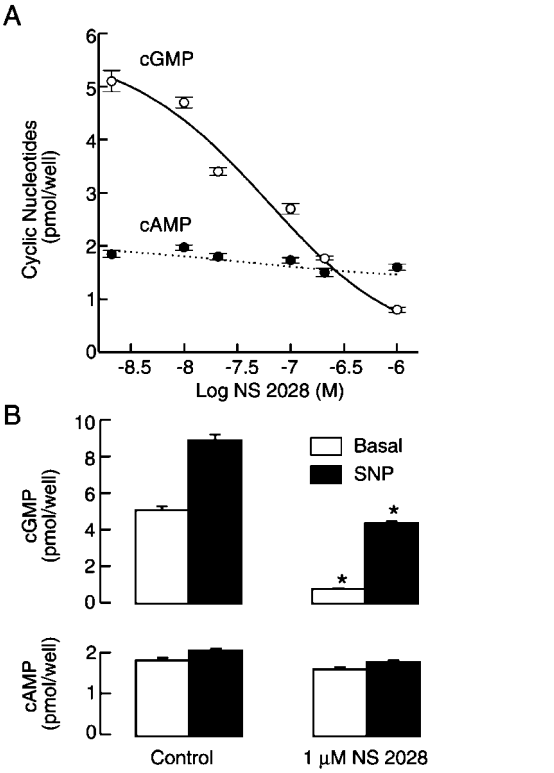

FIG. 3. **Dependence of cAMP and cGMP production on sGC activity (I).** *A,* dose-dependent effects of NS 2028, a specific inhibitor of sGC, on cGMP (*open circles*) and cAMP (*closed circles*) production. *B,* comparison of the effects of NS 2028 on basal and SNP-stimulated cAMP and cGMP production. In this and the following figures, measurements were done in cultures 60 min after stimulation, if not otherwise specified. *, $p < 0.01$ *versus* controls.

was inhibited by aminoguanidine (RBI), an NOS inhibitor. sGC activity was inhibited by 4*H*-8-bromo-1,2,4-oxadiazolo(3,4-*d*)benz(b)(1,4)oxazin-1-one (NS 2028) and 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), and cGMP-specific phosphodiesterases were inhibited by vinpocetine and dipyridamole (all from Calbiochem).

Measurements of Intracellular Calcium Ion Concentration—For $[Ca^{2+}]_i$ measurements, cells were incubated in Krebs-Ringer buffer, supplemented with 2 μM fura-2/AM (Molecular Probes, Eugene OR) at 37 °C for 60 min. Coverslips with cells were washed with this buffer and mounted on the stage of an Axiovert 135 microscope (Carl Zeiss, Oberkochen, Germany) attached to the Attofluor digital fluorescence microscopy system (Atto Instruments, Rockville, MD). Cells were examined under a ×40 oil immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 520 nm was measured. The ratio of light intensities, F_{340}/F_{380}, which reflects changes in Ca^{2+} concentration, was followed in several single cells simultaneously. To calculate the area under the $[Ca^{2+}]_i(t)$ curve, SigmaPlot 2000 software by SPSS was used. Average $[Ca^{2+}]_i(t)$ curves, shown in Fig. 8, were calculated from traces obtained from 73 to 256 cells, base-line values were subtracted, and the area was calculated using the trapezoidal rule.

cGMP, cAMP, and Nitrite Measurements—Cells (1 million/well) were plated in 24-well plates and incubated overnight at 37 °C under 5% CO_2-air and saturated humidity. Prior to the experiments, the medium was removed and cells were washed with Ca^{2+}-containing medium 199 and stimulated at 37 °C under 5% CO_2-air and saturated humidity. cAMP and cGMP were measured in the medium and in dialyzed cells as described previously (18), using specific antisera provided by Albert Baukal (NICHD, Bethesda, MD). For measurement of total NO production (NO_2^- + NO_3^-), 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

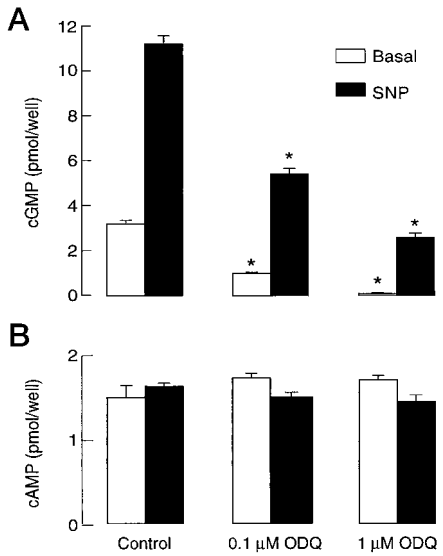

FIG. 4. **Dependence of cAMP and cGMP production on sGC activity (II).** *A,* effects of ODQ, a hemoprotein inhibitor, on cGMP production in controls and SNP-stimulated cells. *B,* lack of effects of ODQ on cAMP production in controls and SNP-stimulated cells. *, $p <$ 0.01 *versus* controls.

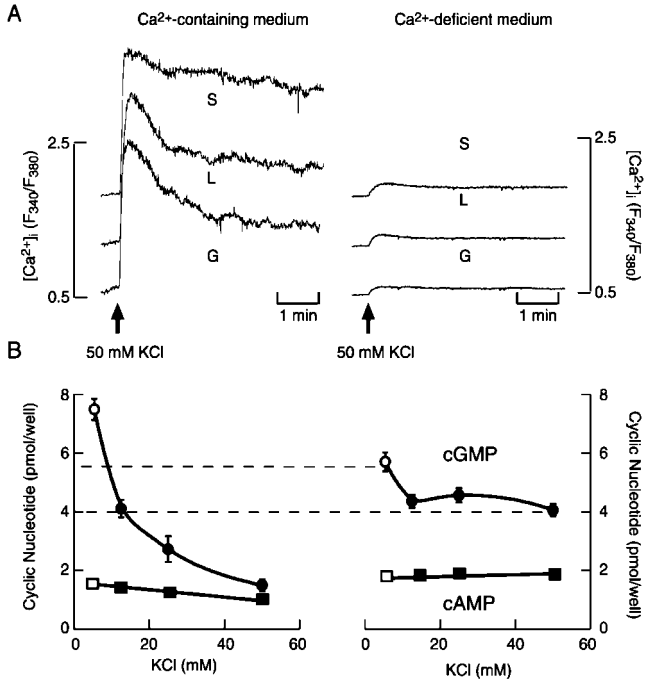

FIG. 5. **Effects of high potassium-induced depolarization of cells on Ca^{2+} signaling and cGMP production in pituitary cells.** *A,* extracellular Ca^{2+}-dependence of $[Ca^{2+}]_i$ signaling. *S*, somatotrophs, *L*, lactotrophs, *G*, gonadotrophs. *B,* effects of high potassium on cAMP (*squares*) and cGMP (*circles*) production in cells bathed in Ca^{2+}-containing and Ca^{2+}-deficient medium. *Open circles* and *squares*, controls bathed in 5 mM K^+-containing medium.

0.5% sulfanilamide and 0.05% naphthylethylenediamine in 2.5% phosphoric acid (all from Sigma); the mixture was incubated at room temperature for 10 min, and the absorbance was measured at 546 nm (19). In experiments with NO_2^- measurements, samples were not treated with nitrate reductase. In both measurements, nitrite concentrations were determined relative to a standard curve derived from increasing concentrations of sodium nitrite. Concentrations of cAMP, cGMP, and NO_2 are expressed as combined values in cell content and in medium.

Western Blot Analysis—Postmitochondrial fractions of anterior pituitary tissue and dispersed pituitary cells, cerebellum, and aortic rings were obtained from adult female Sprague-Dawley rats. Concentration

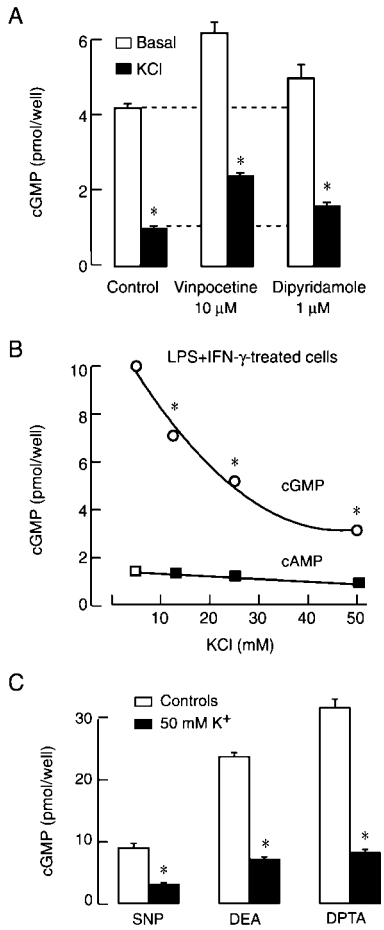

FIG. 6. **Characterization of depolarization-induced inhibition of cGMP production.** *A,* effects of high K^+ on cGMP production in controls and cells with inhibited cGMP-dependent phosphodiesterases with 10 μM vinpocetine and 1 μM dipyridamole. *B,* dose-dependent effects of high K^+ on cGMP and cAMP production in cells expressing iNOS. *C,* effects of high K^+ on cGMP production in cultures stimulated with NO donors. *, $p < 0.01$ *versus* controls.

of proteins was estimated by the Bradford method using bovine serum albumin as a standard (20). Equal amounts of protein (22 μg) from each postmitochondrial fraction were run on one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a discontinuous buffer system (NOVEX, San Diego, CA). The immunodetections on nNOS and iNOS were done with primary antibodies from Affinity BioReagents, Inc. (Golden, CO); eNOS antibody was from Transduction Laboratories (Lexington, KY); and β-actin antibody was from Oncogene Research Products (Boston, MA). The secondary antibody for all assays was an anti-mouse IgG (rabbit) from Life Technologies, Inc. (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.

RESULTS

The Characterization of NO-derived Enzymes in Pituitary Cells—Western blot analysis confirmed the presence of detectable levels of nNOS in pituitary tissue (Fig. 1*A*, *line 2*), cultured cells (*line 3*), and control tissue (cerebellum; *line 1*). eNOS was also detectable in pituitary tissue (*line 5*), dispersed pituitary cells (*line 6*), and control tissue (aorta; *line 4*). iNOS was not detectable in pituitary tissue (*line 8*) and dispersed cells (*line 9*), but was expressed in pituitary cells stimulated with LPS+IFN-γ for 16 h (*line 7*). In these experiments, β-actin expression was used as an internal standard (*lines 10, 11*, and *12*). These results indicate that nNOS and eNOS are constitutively expressed in mixed populations of anterior pituitary cells and that the expression of iNOS can be induced in dispersed pituitary cells.

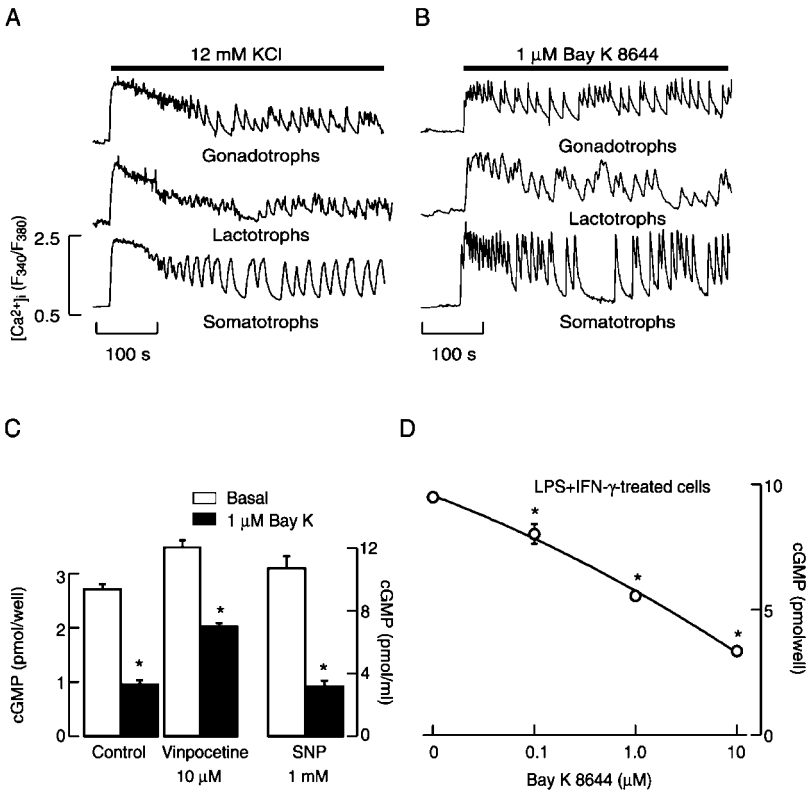

FIG. 7. **Effects of Bay K 8644, an L-type Ca^{2+} channel agonist, on Ca^{2+} signaling and cGMP production in pituitary cells.** A and *B*, initiation of Ca^{2+} spiking in quiescent somatotrophs, lactotrophs, and gonadotrophs by 12 mM K^+ and 1 μM Bay K 8644. *C,* inhibitory effects of Bay K 8644 on cGMP production in control and in vinpocetine- and SNP-treated cells. *D,* dose-dependent effects of Bay K 8644 on cGMP production in pituitary cells expressing iNOS. *, $p < 0.01$ *versus* controls.

The Characterization of NO-controlled cGMP Production—Basal cGMP production was analyzed in mixed populations of anterior pituitary cells cultured in the absence of phosphodiesterase inhibitors. Under these conditions, cGMP was produced in a time-dependent manner, reaching the steady-state plateau response 60–90 min after replacing medium in cultured cells (Fig. 1*B*, *open circles*). Consistent with the role of the NO signaling pathway in the control of cGMP production, the addition of NO donors, SNP, DEA, and DPTA (21), was accompanied by a significant increase in cGMP accumulation. In cells stimulated with LPS+IFN-γ for 16 h, cGMP production was also significantly elevated compared with controls and was comparable with levels observed in cells stimulated with SNP. Nitrite levels, which are commonly used as indicators of NO production (22), were significantly elevated in treated cells. As expected, the profiles of nitrite levels expressed as the sum of both NO_2^- and NO_3^- *versus* NO_2^- only (without nitrate reductase treatment) were highly comparable. The levels of total nitrite measured 1 h after stimulation were similar in SNP- and LPS+IFN-γ-stimulated cells and significantly higher in cells treated with DPTA and DEA. Finally, the levels of cGMP progressively increased with the elevation in nitrite levels (Fig. 1*C*).

Both basal and LPS+IFN-γ-induced cGMP productions were completely inhibited by aminoguanidine, an NOS inhibitor (23), in the micromolar to millimolar concentration range (Fig. 2*A*). In parallel to cGMP production, nitrite accumulation in cultured medium was inhibited by aminoguanidine (Fig. 2*B*). The blockers of sGC, NS 2028 (24), and ODQ (25) also inhibited cGMP productions in a dose-dependent manner. Fig. 3*A* illustrates the effect of NS 2028 on cGMP accumulation in pituitary cells during the first 60 min of incubation. In the same samples, NS 2028 treatment did not significantly affect cAMP production, indicating that AC is not sensitive to this compound. NS 2028 also inhibited the SNP-induced cGMP production (Fig. 3*B*) without affecting cAMP production. Likewise, basal and SNP-induced cGMP production, but not cAMP production, was significantly inhibited by ODQ (Fig. 4, *A* and *B*).

The Dependence of sGC Activity on $[Ca^{2+}]_i$—To study the effects of cytosolic Ca^{2+} on sGC activity, the $[Ca^{2+}]_i$ was elevated by depolarizing cells with high K^+. Fig. 5*A* illustrates that depolarization of cells with 50 mM K^+ led to similar patterns of $[Ca^{2+}]_i$ response in somatotrophs, lactotrophs, and gonadotrophs (shown on *left panels*), as well as all other unidentified cells (not shown). A high K^+-induced increase in $[Ca^{2+}]_i$ was accompanied by a significant and dose-dependent inhibition of cGMP production to about 20% of that observed in controls (Fig. 5*B*, *left panel*). Both depolarization-induced elevation in $[Ca^{2+}]_i$ and inhibition of cGMP production were almost abolished in cells bathed in Ca^{2+}-deficient medium (Fig. 5, *A* and *B*, *right panels*), indicating that depolarization *per se* is not responsible for the observed effects. Consistent with the role of $[Ca^{2+}]_i$ in the control of nNOS and eNOS, depletion of extracellular Ca^{2+} was accompanied with a significant decrease in cGMP production (illustrated by the *upper dashed line* in Fig. 5*B*). Manipulation of extracellular Ca^{2+} concentration did not obviously affect cAMP production (Fig. 5*B*).

The addition of two inhibitors of cGMP-specific phosphodiesterases, vinpocetine and dipyridamole (26, 27), elevated basal cGMP levels (Fig. 6*A*). In cells with inhibited phosphodiesterases, however, depolarization of cells inhibited cGMP production with a comparable efficiency. High K^+-induced inhibition of cGMP production, but not cAMP production, was also observed in cells expressing iNOS (Fig. 6*B*). Furthermore, high K^+ inhibited cGMP production in control cells treated with SNP, DEA, and DPTA (Fig. 6*C*). These results indicate that elevated $[Ca^{2+}]_i$ inhibits sGC activity and that this inhibition occurs in the presence of elevated NO levels.

High K^+-induced elevation in $[Ca^{2+}]_i$ (50, 75, and 100 mM) occurred in a nonoscillatory manner. In contrast, depolarization of cells with 10–20 mM K^+ frequently led to the generation of an oscillatory $[Ca^{2+}]_i$ response after an initial spike response. Fig. 7*A* illustrates the effects of 12 mM K^+ on the pattern of $[Ca^{2+}]_i$ signaling in identified somatotrophs, lactotrophs, and gonadotrophs. At that particular concentration of K^+, a significant inhibition of cGMP production was observed

in control cells (Fig. 5*B*), as well as in cells expressing iNOS (Fig. 6*B*). In further studies, the effects of low K^+ were compared with those induced by Bay K 8644, an L-type Ca^{2+} channel agonist. Addition of Bay K 8644 initiated $[Ca^{2+}]_i$ oscillations in quiescent somatotrophs, lactotrophs, gonadotrophs (Fig. 7*B*), and other pituitary cells and increased the amplitude of transients in oscillating cells (not shown). As in K^+-stimulated cells, the rise in $[Ca^{2+}]_i$ induced by Bay K 8644 was accompanied with the inhibition of cGMP production in controls and vinpocetine-treated cells (Fig. 7*C*, *left panels*). SNP-induced cGMP production was also inhibited by Bay K 8644 (Fig. 7*C*, *right panel*). Finally, Bay K 8644 inhibited cGMP production by LPS+IFN-γ-treated cells in a dose-dependent manner (Fig. 7*D*).

To quantify the dependence of sGC activity on $[Ca^{2+}]_i$, pituitary cells were stimulated with increasing concentrations of K^+ (10, 18, 30, 50, 75, and 100 mM) and Bay K 8644 (0.1 and 1 μM), and the averaged area $[Ca^{2+}]_i(t)$ curves during 15 min of stimulation were calculated and compared with cGMP production. Fig. 8, *A* and *B*, illustrates the dependence of cGMP production and the elevation in $[Ca^{2+}]_i$ on the level of cell depolarization. The relationship between $[Ca^{2+}]_i$ and cGMP in lower potassium (*closed squares*), high potassium (*open circles*), and Bay K 8644-treated cells (*closed circles*) is shown in Fig. 8*B*. A linear relationship between the $[Ca^{2+}]_i$ and cGMP production indicates that Ca^{2+}-mediated inhibition of sGC was not dependent on the pattern of signaling (nonoscillatory *versus* oscillatory) but was determined by the averaged $[Ca^{2+}]_i$.

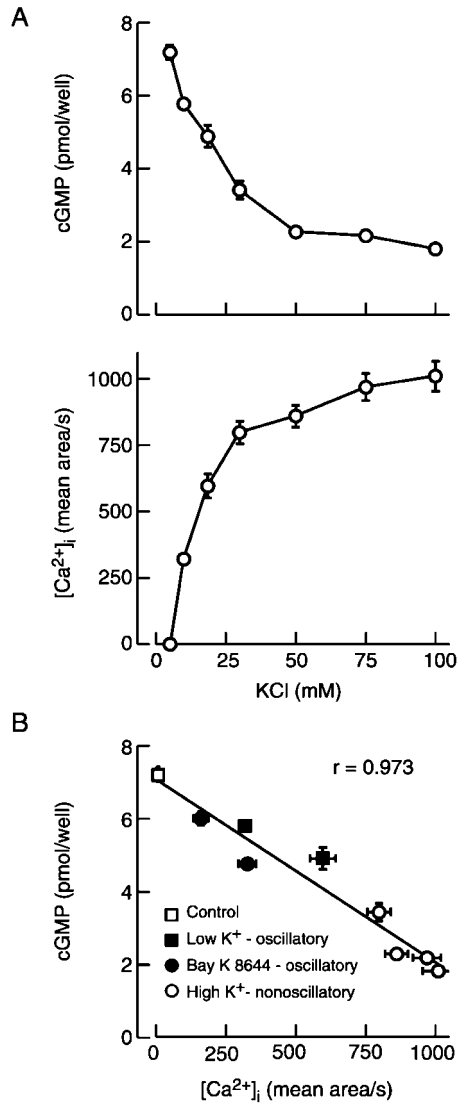

FIG. 8. **Dependence of cGMP production on $[Ca^{2+}]_i$.** *A,* dose-dependent effects of potassium on cGMP production (*upper panel*) and $[Ca^{2+}]_i$ response (*bottom panel*). *B,* relationship between $[Ca^{2+}]_i$ and cGMP production in pituitary cells. In *A* and *B*, $[Ca^{2+}]_i$ was expressed as a mean area per second under the curve subtracted for basal $[Ca^{2+}]_i$ during 15 min of stimulation with high K^+ and Bay K 8644. cGMP was measured in cells cultured for 60 min. Cells were stimulated with 5 (controls), 10, 18, 30, 50, 75, and 100 mM KCl and with 0.1 and 1 μM Bay K 8644.

DISCUSSION

The NO-cGMP signaling pathway exhibits a complex action on Ca^{2+} signaling in a number of cells. A cross-talk between NO and Ca^{2+} is critical in cells with intra- and intercellular Ca^{2+} oscillations, which are dependent on Ca^{2+} release mechanisms, such as those reported recently in hepatocytes (6). NO-cGMP also facilitates the Ca^{2+} influx pathway by stimulating CNGs, channels that depolarize cells to the level needed for activation of voltage-gated Ca^{2+} channels (28). The same pathway may also inhibit Ca^{2+} influx by activating Ca^{2+}-controlled K^+ channels in a cGMP-dependent protein kinase-dependent manner (8, 29). Because the rise in $[Ca^{2+}]_i$ is required to activate nNOS and eNOS, the latter pathway would lead to the coordinated regulation of $[Ca^{2+}]_i$ and cGMP. That is not the case with cells expressing CNGs, where the positive feedback effects of Ca^{2+} on NOS would elevate $[Ca^{2+}]_i$ in an uncontrolled manner.

Two recent reports have indicated the expression of CNGs in pituitary cells (11) and hypothalamic immortalized neurons (30); pituitary somatotrophs express the message for rod CNGs, whereas gonadotropin-releasing hormone-secreting neurons express the olfactory subtype of these channels. In general, activation of these channels by cAMP and cGMP leads to depolarization of cells and the subsequent facilitation of voltage-gated Ca^{2+} influx (28). These channels may provide a rationale for the dual control of Ca^{2+} signaling and secretion in neuroendocrine cells by the NO signaling pathway and by AC-coupled receptors. The NO-cGMP pathway is operative in pituitary and hypothalamic cells (12–17) and provides a rationale for the control of spontaneous $[Ca^{2+}]_i$ fluctuations, which were observed in both normal and immortalized pituitary cell types (31–36). Mixed populations of pituitary cells express two enzymes, nNOS and eNOS (Fig. 1) (17, 37). These cells also express iNOS in response to LPS+IFN-γ stimulation (Fig. 1) (38). AC-coupled receptors are also operative in these cells and stimulate Ca^{2+} influx (39–42).

It was obvious that in cells where the NO-cGMP pathway is positively coupled to Ca^{2+} influx, the inactivation of sGC is needed to break the stimulatory action of NO on Ca^{2+} signaling and Ca^{2+}-controlled cellular functions. However, it was not clear how sGC activity is controlled. Here we show that sCG activity in anterior pituitary cells is down-regulated by elevation in intracellular Ca^{2+} and that the inhibition of the enzyme activity is dependent on the $[Ca^{2+}]_i$. Our results further indicate that Ca^{2+}-dependent inhibition of sGC activity occurs in the presence of elevated NO, *i.e.* independently of the status of NOS, as well as when cGMP-specific phosphodiesterases were inhibited.

Ca^{2+}-dependent inhibition of sGC was observed in cells exhibiting oscillatory and nonoscillatory signaling. The effects of the nonoscillatory and high amplitude $[Ca^{2+}]_i$ signals, induced in our experiments with depolarization of cells with high potassium, are unlikely to occur under physiological situations and are here used to pharmacologically establish the relationship between $[Ca^{2+}]_i$ and sGC activity. On the other hand, the effects of oscillatory $[Ca^{2+}]_i$ signals observed in low potassium and Bay K 8644-stimulated cells are of relevance for the control

of sGC activity. Unstimulated pituitary cells frequently exhibit periods of spontaneous and extracellular Ca^{2+}-dependent fluctuations in $[Ca^{2+}]_i$ (11), which control basal hormone secretion (10). The activation of several AC-coupled receptors also leads to the initiation of extracellular Ca^{2+}-dependent $[Ca^{2+}]_i$ fluctuations in quiescent cells and to an increase in the frequency of fluctuations in spontaneously active cells (39–42). Finally, when activated, Ca^{2+}-mobilizing receptors generate extracellular Ca^{2+}-independent oscillatory Ca^{2+} signals in pituitary cells (10).

In accord with our observations concerning intact cells, it has also been shown recently that Ca^{2+} inhibits sGC in crude cell extract and immunopurified preparations (43). Such a role of Ca^{2+} signaling is not unique for sGC; intracellular Ca^{2+} also inhibits two other members of this family of enzymes, particulate guanylyl cyclase (44, 45) and adenylyl cyclase, particularly types V and VI (46). The common point in $[Ca^{2+}]_i$-dependent inhibition of these enzymes is in the reciprocal regulation of intracellular concentrations of cyclic nucleotides and Ca^{2+}. The unique characteristic of the action of elevated $[Ca^{2+}]_i$ on sGC signaling pathway is its dual action; it stimulates cGMP production by activating eNOS and nNOS but inhibits sGC even in the presence of elevated NO. This inhibition is not only required to balance the nNOS- and eNOS-generated NO production but also to protect the cells from overloading with Ca^{2+} when NO production occurs in a $[Ca^{2+}]_i$-independent manner, *i.e.* by iNOS (22) and phosphorylated eNOS (47).

In summary, here we show that sGC and AC are operative in unstimulated pituitary cells and differently regulated by $[Ca^{2+}]_i$. The basal levels of cGMP are 2–4-fold higher than cAMP when estimated in the absence of phosphodiesterase inhibitors. Facilitation of voltage-gated Ca^{2+} influx and removal of Ca^{2+} from medium were practically ineffective in modulating cAMP production. This suggests that the participation of $[Ca^{2+}]_i$-sensitive AC in unstimulated pituitary cells is minor. In contrast, both treatments affected sGC activity. Consistent with the role of $[Ca^{2+}]_i$ in facilitating nNOS and eNOS, cGMP production was inhibited but not abolished by culturing cells in Ca^{2+}-deficient medium. Inhibition was not observed in cells with elevated NO. An increase in $[Ca^{2+}]_i$ also inhibited cGMP production but independently of NO levels and the phosphodiesterase activity. The low cAMP production and its independence of $[Ca^{2+}]_i$ and the high level of cGMP and its dependence on $[Ca^{2+}]_i$ are consistent with the coupling of the NO signaling pathway, but not the AC signaling pathway, to spontaneous Ca^{2+} signaling in cultured pituitary cells.

REFERENCES

1. Garbers, D. L. (1992) *Cell* **71,** 1–4
2. Denninger, J. W., and Marletta, M. A. (1999) *Biochim. Biophys. Acta* **1411,** 334–350
3. Smolenski, A., Burkhardt, A. M., Eigenthaler, M., Butt, E., Gambaryan, S., Lohmann, S. M., and Walter, U. (1988) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **358,** 134–139
4. Zagotta, W. N., and Siegelbaum, S. A. (1996) *Annu. Rev. Neurosci.* **19,** 235–263
5. Beavo, J. A. (1995) *Physiol. Rev.* **75,** 725–748
6. Patel, S., Robb-Gaspers, L. D., Stellato, K. A., Shon, M., and Thomas, A. P. (1999) *Nat. Cell Biol.* **1,** 467–471
7. Charles, A. (1999) *Nat. Cell Biol.* **1,** E193–195
8. Willmott, N. J., Wong, K., and Strong, A. J. (2000) *J. Neurosci.* **20,** 1767–1779
9. Yermolaieva, O., Brot., N., Weissbach, H., Heinemann, S. H., and Hoshi, T. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 448–453
10. Stojilkovic, S. S., and Catt, K. J. (1992) *Endocr. Rev.* **13,** 256–280
11. Tomic, M., Koshimizu, T., Yuan, D., Andric S. A., Zivadinovic, D., and Stojilkovic, S. S. (1999) *J. Biol. Chem.* **274,** 35693–35702
12. Hartt, D. J., Ogiwara, T., Ho, A. K., and Chik, C. L. (1995) *Biochem. Biophys. Res. Commun.* **214,** 918–926
13. Duvilanski, B. H., Zambruno, C., Seilicovich, A., Pisera, D., Lasaga, M., Diaz, M. C., Belova, N., Rettori, V., and McCann, S. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 170–174
14. Pinilla, L., Gonzalez, D., Tena-Sempre, M., and Aguilar, E. (1998) *Neuroendocrinology* **68,** 180–186
15. Kato, M. (1992) *Endocrinology* **131,** 2133–2138
16. Tsumori, M., Murakami, Y., Koshimura, K., and Kato, Y. (1999) *J. Neuroendocrinol.* **11,** 451–456
17. Lozach, A., Garrel, G., Lerrant, Y., Berault, A., and Counis, R. (1998) *Mol. Cell. Endocrinol.* **143,** 43–51
18. Tomic, M., Zivadinovic, D., Van Goor, F., Yuan, D., Koshimizu, T., and Stojilkovic, S. S. (1999) *J. Neurosci.* **19,** 7721–7731
19. Green, L. C., Wagner, A. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) *Anal. Biochem.* **126,** 131–138
20. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
21. Feelisch, M. (1998) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **358,** 113–122
22. Knowles, R. G., and Moncada, S. (1994) *Biochem. J.* **298,** 249–258
23. Mayer, B., and Andrew, P. (1998) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **358,** 127–133
24. Olesen, S.-P., Drejer, J., Axelsson, O., Moldt, P., Bang, L., Nielsen-Kudsk, J. E., Busse, R., and Mulsch, A. (1998) *Br. J. Pharmacol.* **123,** 299–309
25. Garthwaite, J., Southam, E., Boulton, C. L., Nielsen, E. B., Schmidt, K., and Mayer, B. (1995) *Mol. Pharmacol.* **48,** 184–188
26. Yamamoto, T., Yamamoto, S., Osborne, J. C., Manganiello, V. C., Vaughan, M., and Hidaka, H. (1983) *J. Biol. Chem.* **258,** 14173–14177
27. Cataldi, M., Secondo, A., D'Alessio, A. D., Sarnacchiaro, F., Colao, A. M., Amoroso, S., Di Renzo, G. F., and Annunziato, L. (1999) *Biochim. Biophys. Acta* **1449,** 186–193
28. Wei, J.-Y., Samantra, R., Leconte, L., and Barnstable, C. J. (1998) *Prog. Neurobiol. (Oxf.)* **56,** 37–64
29. Griffith, O. W., and Stuehr, D. J. (1995) *Annu. Rev. Physiol.* **57,** 707–736
30. Vitalis, E. A., Costantin, J. L., Tsai, P.-S., Sakakibara, H., Paruthiyil, S., Liri, T., Martini, J.-P., Taga, M., Choi, A. L. H., Charles, A. C., and Weiner, R. I. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1861–1866
31. Kwiecien, R., and Hammond, C. (1998) *Neuroendocrinology* **68,** 135–151
32. Schlegel, W., Winiger, B. P., Mollard, P., Vacher, P., Wuarin, F., Zahnd, G. R., Wollheim, C. B., and Dufy, B. (1987) *Nature* **329,** 719–721
33. Wagner, K. A., Yacono, P. W., Golan, D. E., and Tashjian, A. H., Jr. (1993) *Biochem. J.* **292,** 175–182
34. Charles, A. C., Piros, E. T., Evans, C. J., and Hales, T. G. (1999) *J. Biol. Chem.* **274,** 7508–7515
35. Holl, R. W., Thorner, M. O., Mandell, G. L., Sullivan, J. A., Sinha, Y. N., and Leong, D. A. (1988) *J. Biol. Chem.* **263,** 9682–9685
36. Fiekers, J. F., and Konpoka, L. M. (1996) *Cell Calcium* **19,** 327–336
37. Wolff, D., and Datto, G. A. (1992) *Biochem. J.* **128,** 201–206
38. Vancelecom, H., Matthys, P., and Denef, C. (1997) *J. Histochem. Cytochem.* **45,** 847–857
39. Kwiecien, R., Tseeb, V., Kurchikov, A., Kordon, C., and Hammond, C. (1997) *J. Physiol. (Lond.)* **499,** 613–623
40. Herrington, J., and Hille, B. (1994) *Endocrinology* **135,** 1100–1108
41. Lussier, B. T., French, M. B., Moor, B. C., and Kraicer, J. (1991) *Endocrinology* **128,** 592–603
42. Kuryshev, Y. A., Childs, G. V., and Ritchie, A. K. (1995) *Endocrinology* **136,** 3925–3935
43. Parkinson, S. J., Jovanovic, A., Jovanovic, S., Wagner, F., Terzic, A., and Waldman, S. A. (1999) *Biochemistry* **38,** 6441–6448
44. Wolbring, G., and Schnetkamp, P. P. M. (1996) *Biochemistry* **35,** 11013–11018
45. Dizhoor, A. M., Lowe, D. G., Olshevskaya, E. V., Laura, R. P., and Hurley, J. B. (1994) *Neuron* **12,** 1345–1352
46. Cooper, D. M. F., Mons, N., and Karpen, J. W. (1995) *Nature* **374,** 421–424
47. Butt, E., Bernhardt, M., Smolenski, A., Kotsonis, P., Frohlich, L. G., Sickmann, A., Meyer, H. E., Lohmann, S. M., and Schmidt, H. H. H. W. (2000) *J. Biol. Chem.* **275,** 5179–5187