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Nitric Oxide Action on Growth Factor-elicited Signals

PHOSPHOINOSITIDE HYDROLYSIS AND $[Ca^{2+}]_i$ RESPONSES ARE NEGATIVELY MODULATED VIA A cGMP-DEPENDENT PROTEIN KINASE I PATHWAY*

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The role of nitric oxide (NO) in the phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis and intracellular Ca^{2+} release responses induced by epidermal, platelet-derived, and fibroblast growth factors was investigated in three cell lines, a clone of NIH-3T3 fibroblasts overexpressing epidermal growth factor receptors and the tumoral epithelial cells A431 and KB. In all three cell types, pretreatment with NO donors decreased growth factor-induced PIP_2 and Ca^{2+} responses, whereas pretreatment with NO synthase inhibitors increased them. The Ca^{2+}-dependent PIP_2 hydroysis induced by micromolar concentrations of the Ca^{2+} ionophore, ionomycin, was also modulated negatively and positively by NO donors and synthase inhibitors, respectively. In contrast, the Ca^{2+} content of the intracellular stores was unaffected by the various pretreatments employed. NO donors and synthase inhibitors induced an increase and decrease, respectively, of the intracellular cGMP formation in all three cell lines investigated. All of the effects of the NO donors were mimicked by 8-bromo-cGMP administration and abolished by pretreatment with the specific blocker of the cGMP-dependent protein kinase I, KT5823, which by itself mimicked the effects of the synthase inhibitors. Together with previous observations on G protein-coupled receptors, the present results demonstrate that PIP_2 hydrolysis and Ca^{2+} release occur under the feedback control of NO, independently of the phospholipase C (β, γ, or δ type) involved and of the mechanism of activation. Such a control, which appears to be effected by the cGMP-dependent protein kinase I acting at the level of the phospholipases C themselves, might ultimately contribute to the inhibitory role of NO on growth previously observed with various cell types.

Individual molecules of the signal transduction cascades turned on by receptor agonist binding can play important roles not only in the intracellular activation process but also in the fine feedback regulation of signaling itself. In this respect particular attention has been devoted to nitric oxide (NO).[1] In the cells competent for the Ca^{2+}-dependent, constitutive forms of NO synthases (NOSs), this highly reactive radical gas, generated in response to appropriate increases of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), works as the controller of a number of enzymes including guanylyl cyclase (1). The ensuing increase of cGMP formation, with activation of cGMP-dependent protein kinase I (G kinase), yields responses that may be variable from cell to cell (2, 3). In the case of receptors coupled to phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis via the activation of heterotrimeric G proteins of the Gq family (4), negative modulations by NO have been described, with decreased generation of the two second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol, and ensuing blunting of Ca^{2+} release from intracellular stores (3). With these receptors the modulation was shown to depend upon G kinase activation, and the site of action was proposed at the G protein/phospholipase C (PLC) interface (5).

PIP_2 hydrolysis is induced not only by G protein-coupled receptors but also by growth factor receptors, working, however, on different PLCs and by a different activation process, *i.e.* by direct tyrosine phosphorylation of the PLCs of the γ family rather than by G protein activation of those of the β family (4, 6). Because of these distinct molecular and functional differences, we thought it worth investigating whether NO and cGMP had any effect on the growth factor-induced PIP_2 hydrolysis and $[Ca^{2+}]_i$ responses.

The results reported here indicate in three types of cells that the above responses induced by growth factors are indeed inhibited by NO working through the cGMP/G kinase I pathway. Similar inhibition by NO was observed also when the same responses were induced by persistent $[Ca^{2+}]_i$ increases triggered by the Ca^{2+} ionophore, ionomycin. Under the latter condition activation is not restricted to a single family of PLCs but affects them all: β, γ, and δ families altogether (7–10). We conclude therefore that the G kinase-sustained negative modulation is a $[Ca^{2+}]_i$ and NO-induced feedback regulation process occurring most probably at the level of PLC.

EXPERIMENTAL PROCEDURES

Materials—Culture sera and media were purchased from Life Technologies, Inc.; epidermal growth factor (EGF), platelet-derived growth factor AB heterodimer (PDGF), basic fibroblast growth factor (FGF),

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[1] The abbreviations use are: NO, nitric oxide; NOS, nitric oxide synthase; G kinase, cGMP-dependent protein kinase; PIP_2, phosphatidylinositol 4,5,-bisphosphate; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; L-NIO, L-*N*-(1-iminoethyl)-ornithine; SNP, sodium nitroprusside; KRH, Krebs-Ringer-Hepes medium; IP, inositol phosphate; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPases; Br, bromo.

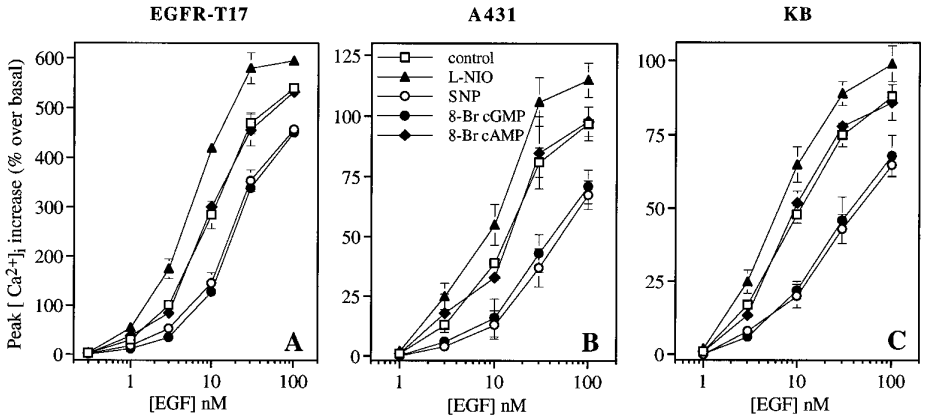

FIG. 1. **Effects of L-NIO, SNP, 8-Br-cGMP, and 8-Br-cAMP on EGF-evoked Ca^{2+} release.** Fura-2-loaded EGFR-T17 (*A*), A431 (*B*), and KB (*C*) cell suspensions were incubated for 15 min at 37 °C in KRH medium, alone (control) or supplemented with L-NIO (200 μM), SNP (30 μM), 8-Br-cGMP (200 μM), or 8-Br-cAMP (200 μM). Cell aliquots were then challenged in Ca^{2+}-free KRH medium with increasing concentrations of EGF. Values are expressed as percent peak increase over basal, resting $[Ca^{2+}]_i$ levels. Basal $[Ca^{2+}]_i$ values were, on average, 141 ± 15, 112 ± 28, and 126 ± 16 nM for EGFR-T17, A431, and KB cell lines, respectively, and were not modified by the various pretreatments. The graphs show the results of 8–10 experiments (mean values ± S.D.).

KT5823, fura-2 AM, thapsigargin, ionomycin, and L-*N*-(1-iminoethyl)-ornithine (L-NIO) from Calbiochem, San Diego, CA; *S*-nitroso-*N*-acetylpenicillamine was obtained from Alexis, Laufelfingen, Switzerland. $^{45}Ca^{2+}$ and *myo*-[2-^{3}H]inositol were purchased from Amersham Corp. Sodium nitroprusside (SNP), N^{ω}-nitro-D-arginine methylester, N^{ω}-nitro-L-arginine methylester, UTP, 8-Br-cGMP, 8-Br-cAMP, and the remaining chemicals were from Sigma. The EGFR-T17 cell clone was a kind gift of L. Beguinot, Milano, Italy.

Cell Culture and Preparation—The NIH 3T3 cell clone EGFR-T17, overexpressing the human EGF receptor, A431, and KB carcinoma cell lines were routinely grown as previously described (11) and used before the 10th week from thawing. The day of the experiment, cell monolayers were detached from Petri dish by gentle trypsinization and resuspended in Krebs-Ringer-Hepes (KRH) medium containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose, 0.8 mM L-arginine, 25 mM Hepes-NaOH (pH 7.4). Cells were then washed three times by centrifugation and resuspended as required by the various experimental procedures. Cell viability in the presence or absence of the various drugs employed was more than 95%, as assessed by the trypan blue exclusion test.

$[Ca^{2+}]_i$ Measurements—Cell suspensions were loaded with the Ca^{2+}-sensitive dye fura-2/AM (3 μM final concentration) for 30 min at 25 °C in KRH medium and kept at 37 °C until use. Cell aliquots (4×10^6 cells) were then transferred to a thermostatted cuvette in a Perkin-Elmer LS-5B fluorimeter and maintained at 37 °C under continuous stirring. Preincubations with the various drugs interfering with the L-arginine/NO pathway were carried out for 15 min at 37 °C, a condition selected in preliminary experiments to be long enough for maximal drug effects in the time range effective in other cell types (12–15). One min before addition of the Ca^{2+}-releasing agents (EGF, PDGF, FGF, UTP, or thapsigargin), the samples were supplemented with excess EGTA (2 mM; estimated extracellular $[Ca^{2+}]$, $<10^{-8}$ M). Under this condition only Ca^{2+} release from intracellular stores, rather than Ca^{2+} influx, can be detected. Traces were recorded and analyzed as described elsewhere (16). Results shown are averages of 8–10 separate experiments ± S.D.

$^{45}Ca^{2+}$ Measurements—During the last 72 h of cell growth the incubation medium was supplemented with $^{45}Ca^{2+}$ (4 μCi/ml). Labeled cells were extensively washed and resuspended in KRH medium. A pellet obtained from the suspension was used for the measurement of total cell $^{45}Ca^{2+}$ content. The rest was incubated at 37 °C in EGTA-containing Ca^{2+}-free KRH medium. At different times, aliquots of 4×10^6 cells were centrifuged, and the $^{45}Ca^{2+}$ recovered into the medium was assayed in a Beckman β-counter (for further details, see Fasolato *et al.* (17)). Results shown are means of four separate experiments ± S.D.

Inositol Phosphate Measurements—Cells labeled for 24 h with 3 μCi/ml *myo*-[2-^{3}H]inositol in basal Eagle's diploid modified medium containing 8% inositol-free fetal calf serum were detached, washed, and resuspended in KRH medium. After preincubation (15 min at 37 °C) with the various drugs interfering with the L-arginine/NO pathway, in the presence (total inositol phosphates (IPs) measurements) or absence (IP_3 measurements) of 20 mM LiCl, aliquots of 4×10^6 cells were challenged with either growth factors or ionomycin and the reaction stopped by the addition of ice-cold formic acid (20 mM final concentration). Samples were kept on ice for 30 min, then centrifuged, and the supernantants were loaded onto anion-exchange columns. Radioactive IPs or IP_3 were separated by stepwise elution as previously described (18), and radioactivity was counted in a Beckman β-counter. Results shown are means of four to six independent experiments ± S.D. Control experiments in which production of IPs and IP_3 was stimulated in Ca^{2+}-free KRH medium gave similar results.

Measurements of cGMP Levels—Cell suspensions were incubated for 15 min at 37 °C in KRH medium supplemented with 0.6 mM 3-isobutyl-1-methylxanthine, with or without L-NIO (200 μM). NOS activity was stimulated by cell exposure to EGF, PDGF, or FGF for 5 min at 37 °C. As a control, cGMP formed upon stimulation for 5 min with SNP (30 μM) was also measured. Reactions were terminated by addition of ice-cold trichloroacetic acid (final concentration, 7.5%). After ether extraction, cGMP levels were measured by radioimmunoassay kit (DuPont) and normalized on cellular proteins, determined using the bicinchoninic acid assay (BCA protein assay reagent; Pierce). Results shown are means ± S.D. of three separate experiments.

RESULTS

All the results shown are from experiments in which L-NIO was used as a NOS inhibitor, and SNP as a NO donor. Qualitatively similar results were obtained using the NOS inhibitor, N^{ω}-nitro-L-arginine methylester, and the NO donor, *S*-nitroso-*N*-acetylpenicillamine. With the less active enantiomer of N^{ω}-nitro-L-arginine methylester, N^{ω}-nitro-D-arginine methylester, the results did not differ significantly from those obtained with untreated, control cells.

Effects of NO on Growth Factor-induced Ca^{2+} Release—Suspensions of fura-2 loaded EGFR-T17, A431, and KB cells were incubated for 15 min at 37 °C in KRH medium, with or without the NOS inhibitor, L-NIO (200 μM), the NO donor, SNP (30 μM), or the membrane permeant, stable analogues of cGMP or cAMP, 8-Br-cGMP (200 μM) and 8-Br-cAMP (200 μM). They were then supplemented with excess EGTA and challenged with increasing concentrations of EGF. Under these experimental conditions any $[Ca^{2+}]_i$ changes can be safely attributed to Ca^{2+} release from intracellular stores (4). In all three cell lines investigated, pretreatment with L-NIO potentiated Ca^{2+} release induced by EGF, while SNP exerted an inhibitory effect with respect to untreated, control cells (Fig. 1). With 8-Br-cGMP, the effect observed on Ca^{2+} release was analogous to that of SNP, while 8-Br-cAMP was without any appreciable effect (Fig. 1).

EGFR-T17 cells are known to exhibit $[Ca^{2+}]_i$ responses not only to EGF but to other growth factors, *i.e.* PDGF and FGF (19). The effects of L-NIO, SNP, 8-Br-cGMP, and 8-Br-cAMP on Ca^{2+} release induced by the latter agonists were therefore investigated, with results qualitatively similar to those induced by EGF. Ca^{2+} release elicited by PDGF and FGF was potentiated when cells were preincubated with L-NIO, inhibited after treatment with SNP or 8-Br-cGMP, and almost unaffected by 8-Br-cAMP (Fig. 2, *A* and *B*). The effects of NO-modulating drugs on Ca^{2+} release induced by growth factors were then compared to those exerted on the responses elicited by activation of a G protein-coupled receptor. Fig. 2*C* shows the results obtained with UTP, a receptor agonist specific for the puriner-

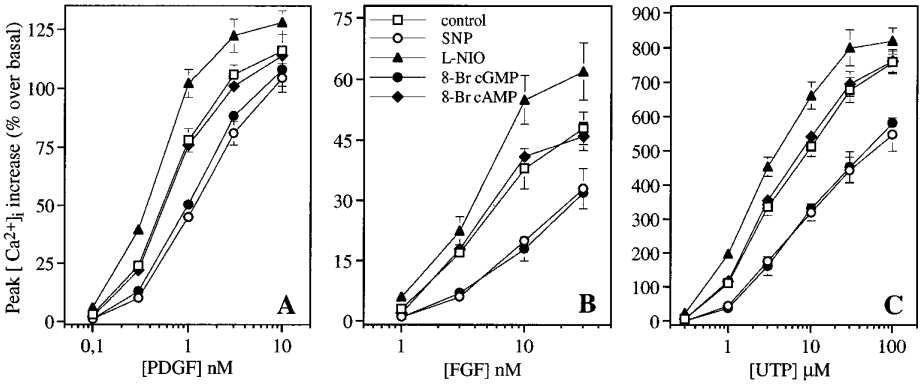

FIG. 2. **Effects of L-NIO, SNP, 8-Br-cGMP, and 8-Br-cAMP on agonist-evoked Ca^{2+} release.** Fura-2-loaded EGFR-T17 cell suspensions were incubated for 15 min at 37 °C in KRH medium, alone (control) or supplemented with L-NIO (200 μM), SNP (30 μM), 8-Br-cGMP (200 μM), or 8-Br-cAMP (200 μM). Cell aliquots were then challenged in Ca^{2+}-free KRH medium with increasing concentrations of either PDGF (*A*), FGF (*B*), or UTP (*C*). Values are expressed as percent peak increase over basal, resting [Ca^{2+}]$_i$ levels. The graphs show the results of 8–10 experiments (mean values ± S.D.).

gic P_{2U} receptor (20). As already demonstrated in PC12 cells (15), preincubation with L-NIO increased, that with SNP or 8-Br-cGMP decreased, the UTP-induced Ca^{2+} release to extents similar to those observed with growth factor-induced [Ca^{2+}]$_i$ responses.

Effects of NO on Ca^{2+} Storage—In all cell types exchangeable Ca^{2+} is known to be distributed into at least three major pools: (i) the IP_3-sensitive Ca^{2+} store, a subcompartment of the endoplasmic reticulum endowed with SERCA ATPases; (ii) an additional endoplasmic reticulum-located Ca^{2+} pool also accumulated by SERCAs but insensitive to IP_3; (iii) a large, still poorly characterized pool, insensitive to IP_3 and devoid of SERCAs, that can be discharged by Ca^{2+}-specific ionophores (17, 21). In order to investigate the effects of NO on these pools, cell monolayers were loaded to equilibrium (72 h) with $^{45}Ca^{2+}$, pretreated with L-NIO or SNP for 15 min at 37 °C, and then challenged in sequence with EGF (to release Ca^{2+} stored in the IP_3-sensitive Ca^{2+} store) followed by thapsigargin (an irreversible SERCA blocker that induces within a few minutes the leakage and emptying of Ca^{2+} from the entire endoplasmic reticulum) (21), and finally by ionomycin (an electroneutral Ca^{2+} ionophore) (15, 17, 21). Fig. 3*A* summarizes the results obtained with EGFR-T17 cells; qualitatively similar observations were made with A431 and KB cells. Pretreatment with L-NIO increased, that with SNP decreased the amount of $^{45}Ca^{2+}$ released by EGF (100 nM) (Fig. 3*A*). In contrast, release by the subsequent administration first of thapsigargin (100 nM) and then of ionomycin (1 μM) was unchanged by both pretreatments. Moreover, neither L-NIO nor SNP appeared to affect the basal leak of Ca^{2+} from the resting cells. To further confirm these findings, thapsigargin-induced Ca^{2+} release was analyzed also by the fura-2 technique. Again, no significant differences in Ca^{2+} release induced by the SERCA blocker were revealed in cells pretreated with L-NIO or SNP in comparison with controls (Fig. 3*B*).

Effects of NO on PIP_2 Hydrolysis—The effects of L-NIO, SNP, and 8-Br-cGMP on growth factor-induced PIP_2 hydrolysis were next investigated. Fig. 4*A* shows the results obtained with EGF in EGFR-T17 cells. Pretreatment with L-NIO potentiated total IP accumulation stimulated by increasing concentrations of the growth factor, administered for 20 min in the presence of 20 mM LiCl. Under parallel conditions both SNP and 8-Br-cGMP exerted an inhibitory effect. Similar results were observed when the time-course of IP_3 production, generated after administration of 30 nM EGF, was assayed (Fig. 4*B*). The results obtained with PDGF and FGF were consistent with those of EGF (not shown). In additional experiments, total IP accumulation was investigated in EGFR-T17 cells in which PLC activity was stimulated by the Ca^{2+} ionophore, ionomycin, administered at concentrations (1 or 3 μM) that in preliminary experiments gave rise to persistently high values of [Ca^{2+}]$_i$ (10^{-6}–10^{-5} M). With both concentrations of ionomycin tested, preincubation

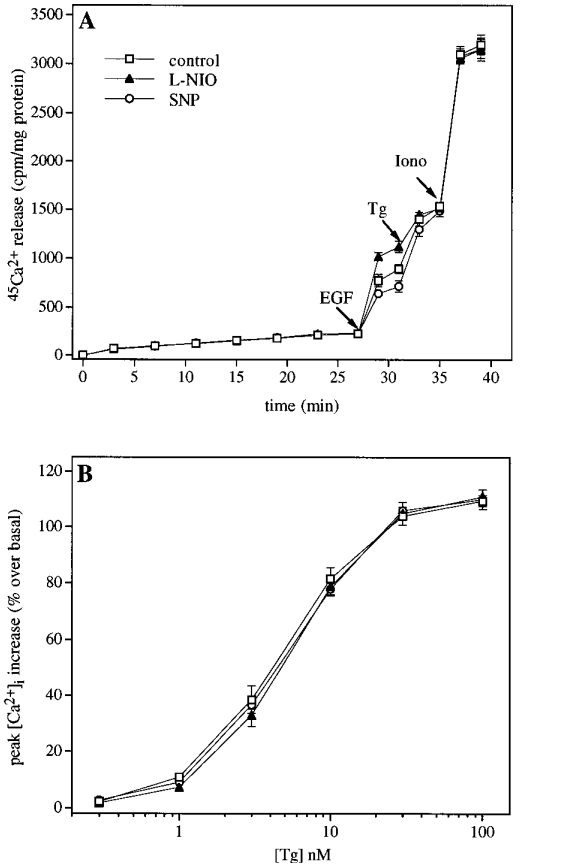

FIG. 3. **Effects of L-NIO and SNP on the release of Ca^{2+} from the various intracellular Ca^{2+} pools.** *A*, EGFR-T17 cells were loaded to equilibrium with $^{45}Ca^{2+}$, then incubated for 15 min at 37 °C in KRH medium alone (control) or supplemented with L-NIO (200 μM) or SNP (30 μM). After addition of excess EGTA (Ca^{2+}-free medium), basal $^{45}Ca^{2+}$ leak was analyzed before challenging the cells by sequential addition of EGF (100 nM), thapsigargin (*Tg*) (100 nM), and ionomycin (*Iono*) (1 μM) where indicated. Results illustrated are the averages of four experiments ± S.D. *B*, fura-2-loaded EGFR-T17 cells, preincubated for 15 min with KRH medium, alone or supplemented with L-NIO or SNP as in Fig. 1, were challenged in Ca^{2+}-free KRH medium with increasing concentrations of thapsigargin. The graph shows the mean percent [Ca^{2+}]$_i$ increases over basal (± S.D.) in eight experiments.

with L-NIO yielded higher levels, and those with SNP and 8-Br-cGMP lower levels of total IP accumulation (Fig. 4*C*).

Role of G Kinase I as NO Effector—Many effects of NO are known to be indirect, mediated by increases in cytosolic cGMP levels and activation of G kinase I (3, 12). The involvement of such a mechanism in our experiments was already suggested by the parallel effects of SNP and 8-Br-cGMP on both [Ca^{2+}]$_i$ and total IP accumulation. The lack of effect of 8-Br-cAMP excludes that the effects of 8-Br-cGMP are mediated via cross

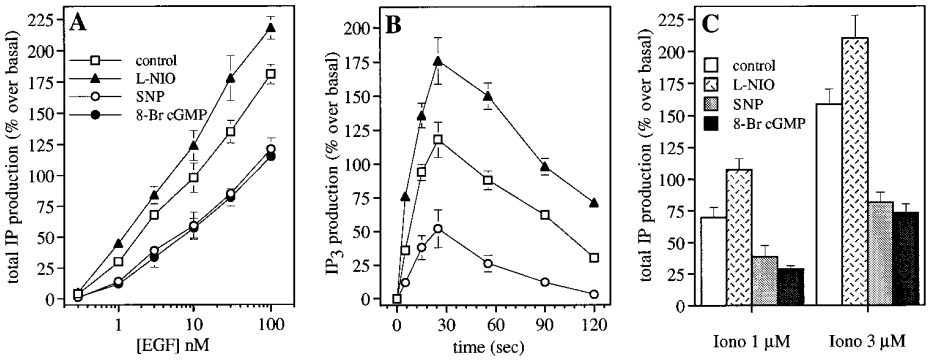

FIG. 4. **Effects of L-NIO, SNP, and 8-Br-cGMP on total IP accumulation and IP_3 generation.** EGFR-T17 cells, labeled with 3 μCi/ml *myo*-[2-^{3}H]inositol, were incubated for 15 min at 37 °C in KRH medium, alone (control) or supplemented with L-NIO (200 μM), SNP (30 μM), or 8-Br-cGMP (200 μM) as described under "Experimental Procedures." *A*, total IP accumulation measured in cells challenged with increasing concentrations of EGF for 15 min in the presence of 20 mM LiCl. *B*, IP_3 generation induced by 30 nM EGF, followed up to 120 s after EGF addition. *C*, total IP accumulation measured in cells challenged with the indicated concentrations of ionomycin (*Iono*). The average basal radioactivity of total IPs and IP_3, here and in the experiments of Figs. 5 and 6, were $6.1 \pm 2.3 \times 10^3$ and $1.3 \pm 0.9 \times 10^3$ cpm/mg of protein, respectively. No appreciable difference in basal radioactivity was observed between cell preparations treated with L-NIO, SNP, or 8-Br-cGMP. Results are expressed as percent increase of radioactivity over basal. Graphs show the results obtained in six independent experiments (averages ± S.D.).

activation of protein kinase A, a mechanism known to take place in other cell systems (22, 23). The drugs employed in the present study did indeed induce considerable changes of the endogenous cGMP production in all the cell lines investigated: decrease (−30–40%) with L-NIO and increases (10–16 fold; 3.5–5 fold) with SNP and EGF, the latter almost completely prevented by prior L-NIO treatment (Table I).

In further experiments SNP preincubation of EGFR-T17 cell suspensions was carried out with or without KT5823, a widely employed inhibitor of the G kinase I activity (15, 24, 25). Fig. 5*A* shows that in the presence of KT5823 (10 μM) the inhibitory effect of SNP on EGF-induced Ca^{2+} release was almost completely abolished. Similarly, SNP inhibition of total IP accumulation triggered by either EGF or ionomycin was largely prevented by the kinase blocker (Fig. 5, *B* and *C*). KT5823 was also administered alone or in combination with L-NIO. Fig. 6 shows the results obtained in EGFR-T17 cells challenged with EGF (*A* and *B*) or ionomycin (*C*). The effects of the kinase blocker on $[Ca^{2+}]_i$ variations and total IP accumulation induced by either stimulant resembled those induced by L-NIO; when KT5823 and L-NIO were administered together, no additive effect was measured. Similar results were found when the effects of KT5823 were investigated in A431 and KB cells (not shown).

TABLE I
cGMP formation in EGF-treated KB, EGFR-T17, and A431 cells

Cell suspensions in KRH at 37°C were first preincubated with 600 μM 3-isobutyl-1-methylxanthine with or without L-NIO (200 μM). Subsequent treatments with SNP (30 μM) or EGF (100 nM) were for 5 min. Results shown are means ± S.D. of three separate experiments.

Cell treatments	cGMP formation		
	KB	EGFR-T17	A431
	pmol/mg min^{-1}		
Unstimulated, controls	1.24 ± 0.26	2.10 ± 0.18	1.31 ± 0.26
L-NIO preincubated	0.83 ± 0.35	1.29 ± 0.26	0.92 ± 0.25
EGF stimulated	4.58 ± 0.23	10.36 ± 0.24	7.50 ± 0.26
L-NIO preincubated, EGF stimulated	1.30 ± 0.42	2.85 ± 0.26	1.61 ± 0.21
SNP	11.91 ± 1.33	33.85 ± 2.31	19.76 ± 3.41

DISCUSSION

The results reported here demonstrate that NO has a role in the chain of intracellular events elicited by activation of growth factor receptors. While the Ca^{2+} storage machinery is unaffected by cell treatment with NO, the gaseous messenger is shown to modulate negatively PIP_2 hydrolysis and the ensuing generation of IP_3. An important consequence is the reduction of the growth factor-induced release of Ca^{2+} from the intracellular stores. Although obtained not by direct application of NO but by a pharmacological approach, our results appear unambiguous because of the contrast between the effects of NO donors, which induce release of the gas within the cells, with those of NOS blockers, which preclude the synthesis of endogenous NO. The fact that NO-induced negative signal modulations were observed in all three cell types investigated, NIH-3T3, A431, and KB lines, strongly suggests that they have a general significance. Moreover, their appearance with all the growth factors we have employed, *i.e.* EGF, PDGF, and FGF, suggests these effects to be generated at the level of the common signal cascade activated after receptor binding rather than at a level of receptors themselves. Indeed, it has been recently reported that EGF binding to its receptor is unaffected by NO (26).

Until now, negative effects of NO on PIP_2 hydrolysis and Ca^{2+} release had been reported only with receptors coupled to PLC via heterotrimeric G proteins. In the latter case, the site of NO action was proposed to occur at the G protein/PLC interface (5). The PLCs activated by G proteins, however, are a family of isoenzymes (defined as PLCβ) molecularly and functionally different from those activated by growth factors, the PLCγ, which are activated at the receptor level by direct tyrosine phosphorylation (4, 6). Taken together, the present and previous results indicate therefore that inhibition by NO is a widespread regulatory process that involves many (possibly all) types of transductive PLCs. In fact, also PIP_2 hydrolysis induced by administration of a Ca^{2+} ionophore, ionomycin, a mechanism effective with all types of the enzyme known so far (see Refs. 7, 8, 9, and 10, for PLC$\beta_{1,\,2,\,\text{and}\,3}$, PLC$\gamma_1$, PLC$\gamma_2$, and PLC$\delta_1$, respectively), was inhibited by NO. The inhibition by NO of PLC activity appears to be mediated by accumulation of cGMP and activation of G kinase I. Whether this kinase phosphorylates PLC(s) directly, or whether its effect is mediated through the phosphorylation of regulatory, yet unidentified protein(s), remains to be established. Also to be elucidated is the mechanism of inhibition of the PLCγ activity we have now observed. Various possibilities are open: decreased complex formation of the enzyme with growth factor receptors, of its degree of tyrosine phosphorylation, or of its activation level. Until now the results of preliminary experiments failed to reveal clear evidence supporting either one of the first two mechanisms (not shown).

Under unstimulated conditions the role of cGMP in the control of PIP_2 hydrolysis and $[Ca^{2+}]_i$ does not appear important,

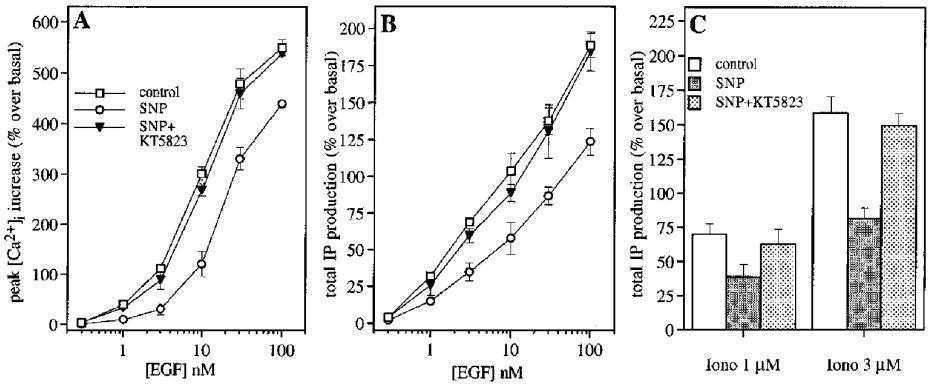

FIG. 5. **Effects of the G kinase inhibitor KT5823 on SNP-induced variations in $[Ca^{2+}]_i$ and total IP production elicited by EGF and ionomycin (*Iono*).** *A*, fura-2-loaded EGFR-T17 cells, preincubated for 15 min at 37 °C with KRH medium, alone or supplemented with SNP (30 μM) or SNP plus KT5823 (10 μm), were challenged in Ca^{2+}-free KRH medium with increasing concentrations of EGF. The graph shows the mean percent increases in peak $[Ca^{2+}]_i$ over basal ± S.D. in nine experiments. *B* and *C*, EGFR-T17 cells, labeled with 3 μCi/ml *myo*-[2-^{3}H]inositol, were incubated for 15 min at 37 °C in KRH medium, alone or supplemented with SNP or SNP plus KT5823 as above, then challenged with increasing concentrations of EGF or the indicated concentrations of ionomycin. Results shown are the mean percent increases of radioactivity over basal ± S.D. in six experiments. Experimental details as in Fig. 4.

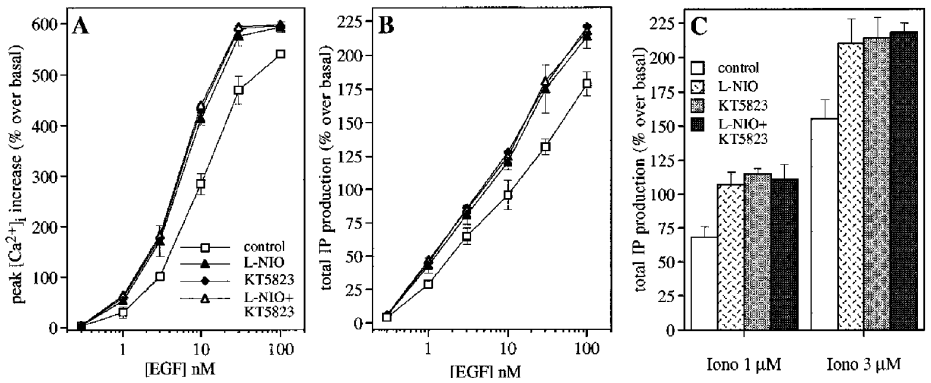

FIG. 6. **Effects of the G kinase inhibitor KT5823 on $[Ca^{2+}]_i$ variations and total IP production elicited by EGF and ionomycin (*Iono*) in the presence or absence of L-NIO.** *A*, fura-2-loaded EGFR-T17 cells, preincubated for 15 min at 37 °C with KRH medium, alone or supplemented with L-NIO (200 μM), KT5823 (10 μM), or a combination of the two, were challenged in Ca^{2+}-free KRH medium with increasing concentration of EGF. The graph shows the mean percent increases in peak $[Ca^{2+}]_i$ over basal ± S.D. in four experiments. *B* and *C*, EGFR-T17 cells, labeled with 3 μCi/ml *myo*-[2-^{3}H]inositol, were incubated for 15 min at 37 °C with KRH medium, alone or supplemented with L-NIO, KT5823, or both as above, then challenged with increasing concentrations of EGF or the indicated concentrations of ionomycin. Results shown are the mean percent increases of radioactivity over basal ± S.D. in four experiments. Experimental details as in Fig. 4.

inasmuch as the decrease of the nucleotide level following preincubation with a NOS blocker was not accompanied by any appreciable changes of basal IPs and $[Ca^{2+}]_i$ values. Only after stimulation with growth factors (and also with UTP and ionomycin) did the inhibition by NO become clear. NO appears therefore to work as a feedback controller. In NOS-competent cells, any increase in $[Ca^{2+}]_i$ is in fact expected to activate the enzyme. The NO thus produced would then modulate negatively all PLCs, via cGMP and the cognate kinase, thus contributing to the control of intracellular Ca^{2+} homeostasis.

NO- and cGMP-induced inhibitory modulation of growth factor receptor function may account for a number of cell growth effects that up to now had remained without an adequate explanation. Inhibition of mitogenesis by the gas and G kinase have been reported in various cell systems including vascular smooth muscle (3), rat hepatocytes (27), and retinal pigmented epithelial cells (28). Moreover, proliferation and development of bone marrow were reported to be inhibited (29), and neuronal and muscular differentiation to be stimulated by NO (30–32). In the array of intracellular signals elicited by growth factor receptor activation, PIP_2 hydrolysis and $[Ca^{2+}]_i$ responses are now recognized to promote mitogenesis and differentiation in various cell systems (33, 34), while impaired Ca^{2+} homeostasis or altered Ca^{2+} release exert an inhibitory effect on growth (see *e.g.* Refs. 20, 35, and 36). The possibility can therefore be considered that the effects of NO and cGMP on cell growth are mediated, at least in part, by their negative modulatory actions here described. Whether these actions are accompanied by others as yet still unknown, also induced by NO and cGMP, remains to be investigated.

In conclusion, our results expand the importance of the NO-cGMP-mediated modulation in transmembrane signaling, demonstrating that it covers the entire PIP_2 hydrolysis field, independent of the PLC isoform families involved and the mechanisms of their activation. In addition to the inhibition of cell growth discussed above, an important role of the NO modulation of PIP_2 hydrolysis could be in the control of Ca^{2+} homeostasis, with prevention of excess Ca^{2+} release. This possibility is supported by the recently recognized, NO-induced positive modulation of the surface Ca^{2+} channels of the type activated by intracellular Ca^{2+} release (13, 14), which are believed to be responsible for the replenishment of discharged Ca^{2+} stores.

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REFERENCES

1. Knowles, R. G., and Moncada, S. (1994) *Biochem. J.* **298,** 249–258
2. Bredt, D. S., and Snyder, S. H. (1994) *Annu. Rev. Biochem.* **63,** 175–195
3. Lincoln, T. M., Komalavilas, P., and Cornwell, T. L. (1994) *Hypertension* **23,**

1141–1147
4. Berridge, M. J. (1993) *Nature* **361,** 315–325
5. Nguyen, B. L., Saitoh, M., and Ware, J. A. (1991) *Am. J. Physiol.* **261,** H1043–H1052
6. Schlessinger, J., and Ullrich, A. (1992) *Neuron* **9,** 383–391
7. Jhon, D.-Y., Lee, H.-H., Park, D., Lee, C.-W., Lee, K.-H., Yoo, O. J., and Rhee, S. G. (1993) *J. Biol. Chem.* **268,** 6654–6661
8. Wahl, M. I., Jones, G. A., Nishibe, S., Rhee, S. G., and Carpenter, G. (1992) *J. Biol. Chem.* **267,** 10447–10456
9. Homma, Y., Emori, Y., Shibasaki, F., Suzuki, K., and Takenawa, T. (1990) *Biochem. J.* **269,** 13–18
10. Banno, Y., Okano, Y., and Nozawa, Y. (1994) *J. Biol. Chem.* **269,** 15846–15852
11. Pandiella, A., Magni, M., Lovisolo, D., and Meldolesi, J. (1989) *J. Biol. Chem.* **264,** 12914–12921
12. Geiger, J., Nolte, C., Butt, E., Sage, S. O., and Walter, U. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 1031–1035
13. Xu, X., Star, R. A., Tortorici, G., and Muallem, S. (1994) *J. Biol. Chem.* **269,** 12645–12653
14. Clementi, E., Vecchio, I., Corasaniti, M. T., and Nisticò, G. (1995) *Eur. J. Pharmacol.* **289,** 113–123
15. Clementi, E., Vecchio, I., Sciorati, C., and Nisticò, G. (1995) *Mol. Pharmacol.* **47,** 517–524
16. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–4350
17. Fasolato, C., Zottini, M., Clementi, E., Zacchetti, D., Meldolesi, J., and Pozzan, T. (1991) *J. Biol. Chem.* **266,** 20159–20167
18. Seuwen, K., Lagarde, A., and Pouyssegur, J. (1988) *EMBO J.* **7,** 161–168
19. Pandiella, A., Magni, M., and Meldolesi, J. (1989) *Biochem. Biophys. Res. Commun.* **163,** 1325–1331
20. Fischer, G. A., Clementi, E., Raichman, M., Südhof, T., Ullrich, A., and Meldolesi, J. (1994) *J. Biol. Chem.* **269,** 19216–19224
21. Pozzan, T., Rizzuto, R., Volpe, P., and Meldolesi, J. (1994) *Physiol. Rev.* **74,** 595–636
22. Forte, L. R., Thorne, P. K., Eber, S. L., Krause, W. J., Freeman, R. H., Francis, S. H., and Corbin, J. D. (1992) *Am. J. Physiol.* **263,** C607–C615
23. Cornwell, T. L., Arnold, E., Boerth, N. J., and Lincoln, T. M. (1994) *Am. J. Physiol.* **267,** C1405–C1413
24. Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Mitsuri, T., Murakata, C., Sato, A., and Kaneko, M. (1987) *Biochem. Biophys. Res. Commun.* **142,** 436–440
25. Gadbois, D. M., Crissman, H. A., Tobey, R. A., and Bradbury, E. M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8626–8630
26. Peranovich, T. M. S., da Silva, A. M., Fries, D. M., Stern, A., and Monteiro, H. P. (1995) *Biochem. J.* **305,** 613–619
27. Miyazaki, M., Wahid, S., Bai, L., and Namba, M. (1992) *Exp. Cell Res.* **200,** 404–409
28. Goureau, O., Lepoivre, M., Becquet, F., and Curtois, Y. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4276–4280
29. Punjabi, C. J., Laskin, D. L., Heck, D. E., and Laskin, J. D. (1992) *J. Immunol.* **149,** 2179–2184
30. Lee, K. H., Baek, M. Y., Moon, K. Y., Song, W. K., Chung, C. H., Ha, D. B., and Kang, M. S. (1994) *J. Biol. Chem.* **269,** 14371–14374
31. Muñoz-Fernàndez, M. A., Cano, E., O'Donnell, C. A., Doyle, J., Liew, F. Y., and Fresno, M. (1994) *J. Neurochem.* **62,** 1330–1336
32. Peunova, N., and Enikolopov, G. (1995) *Nature* **375,** 68–73
33. Smith, M. R., Liu, Y. L., Kim, H., Rhee, S. G., and Fung, H. F. (1990) *Science* **247,** 1074–1077
34. Valius, M., and Kazlauskas, A. (1993) *Cell* **73,** 321–334
35. Rasmussen, C. D., and Means, A. R. (1989) *Trends Neurosci.* **12,** 433–438
36. Short, A. D., Bian, J., Ghosh, T. K., Waldron, R. T., Rybak, S. L., and Gill, D. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 4986–4990

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