0026-895X/95/061068-10$3.00/0

MOLECULAR PHARMACOLOGY, 48:1068–1077 (1995).

Growth Factor-Induced Ca^{2+} Responses Are Differentially Modulated by Nitric Oxide via Activation of a Cyclic GMP-Dependent Pathway

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Received March 30, 1995; Accepted September 12, 1995

SUMMARY

Nitric oxide (NO) plays a modulatory role on cell growth and differentiation, biological processes that occur under the control of various signal transduction mechanisms, including those triggered by activation of membrane receptors for polypeptide growth factors. The increases in intracellular Ca^{2+} concentration elicited by the activation of these receptors are sustained by release of the cation from intracellular stores and by stimulation of its influx from the extracellular medium. Using NIH 3T3 cells overexpressing the human epidermal growth factor receptor, we investigated both of these processes stimulated by the administration of epidermal and platelet-derived growth factors as the receptor agonists. Pharmacological and functional analyses carried out on Fura-2-loaded cells showed that Ca^{2+} influx elicited by both growth factors is the summation of two distinct pathways, with the major pathway dependent on and the minor pathway independent of store depletion. Exposure of the cells to either NO donors or NO synthase inhibitors induced increase and inhibition, respectively, of the two components of Ca^{2+} influx. When Ca^{2+} release was investigated, the above drugs were also active but in the opposite direction. The effects of NO were mimicked by the cGMP analogue 8-Br-cGMP and abolished by two cGMP-dependent protein kinase I inhibitors, whereas the cAMP analogue 8-Br-cAMP and two protein kinase A inhibitors had no appreciable effects. In addition, growth factors induced an increase in cGMP formation, an effect that was prevented by NO synthase inhibitors. In conclusion, NO appears to exert a feedback modulatory control on Ca^{2+} responses to growth factor administration. Such a control might contribute to the inhibitory effect of NO on growth previously reported with various cell types.

NO is a highly reactive radical gas involved in the regulation of crucial events in intracellular signaling, including those leading to cell growth and differentiation (1, 2). The role of NO is well established in the inhibition of mitogenesis and growth of various cell systems, including vascular smooth muscle (2; also see Ref. 3), rat hepatocytes (4), and retinal pigmented epithelial cells (5), as well as in the inhibition of bone marrow cell proliferation and development (6). In addition, NO induces differentiation of neuroblastoma cells (7) and is a messenger molecule for myoblast fusion (8).

Cell growth and differentiation occur under the control of various agents, including polypeptide growth factors acting through their cognate tyrosine kinase receptors (9). The signals delivered by these receptors include variations in $[Ca^{2+}]_i$ sustained by both release from intracellular stores and influx across the plasmalemma (9, 10), which contribute to the overall effects of growth factors. Stable expression of truncated IP_3 receptors has been found to induce inhibition of cell growth with suppression of transformation in EGF-stimulated NIH 3T3 fibroblasts (11), whereas pharmacological blockade of Ca^{2+} influx decreased substantially the mitogenic effect of EGF (12). Moreover, virally transformed cells exhibit a modified regulation of Ca^{2+} homeostasis (13) and appear to be dependent on an intact Ca^{2+} influx system for their proliferation (14). A detailed characterization of the relationships among NO synthesis, growth factor receptors, and Ca^{2+} homeostasis could therefore provide important in-

This work was supported in part by grants from the Italian Association of Cancer Research and target project ACRO of the Italian Consiglio Nazionale delle Ricerche.

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ABBREVIATIONS: NO, nitric oxide; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IP_3, inositol-1,4,5-trisphosphate; EGF, epidermal growth factor; SMOC, second messenger-operated Ca^{2+} channel; SDC, store-dependent Ca^{2+} channel; PDGF, platelet-derived growth factor; Tg, thapsigargin; L-NIO, L-*N*-(1-iminoethyl)-ornithine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; Rp 8-Br-cGMP[S], (*R*)-*p*-8-bromo-cGMP monophosphorothioate; Rp 8-Br-cAMP[S], (*R*)*p*-8-bromo-cAMP monophosphorothioate; SNP, sodium nitroprusside; D-NAME, N^{ω}-nitro-D-arginine methylester; L-NAME, N^{ω}-nitro-L-arginine methylester; KRH, Krebs-Ringer-HEPES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NOS, nitric oxide synthase; G kinase, cGMP-dependent protein kinase.

formation regarding the intricate network of intracellular signals responsible for control of proliferation and transformation processes.

Until now, the effects of NO on the Ca^{2+} responses elicited by stimulation of growth factor receptors had not been investigated, which is at variance with the responses induced by another class of membrane receptors: those coupled to phosphatidylinositol-4,5,-bisphosphate hydrolysis and IP_3 generation via heterotrimeric G proteins. Activation of these receptors triggers both Ca^{2+} release from intracellular, IP_3-sensitive stores and influx of the cation from the outside medium (10). The latter occurs through two concomitantly activated but independently regulated pathways, including SMOCs and SDCs, whose current is referred to as Ca^{2+} release-activated current (15–17). In various cell systems, the effect of NO on Ca^{2+} release was shown to consist of an inhibitory modulation mediated by increased cGMP formation (e.g., see Refs. 18–20), whereas in the case of SDCs, the effect of NO was a stimulation, possibly mediated not only by cGMP (21) but also by other, unidentified signaling pathway(s) (22). In contrast, the few existing data suggest SMOCs to be insensitive to NO action (19, 22), although an effect of the gaseous messenger cannot be entirely excluded (23).

In the present study, the investigation of the role of NO in the modulation of Ca^{2+} homeostasis was extended to the processes stimulated by the activation of growth factor receptors. Although the Ca^{2+} responses induced by these receptors resemble those presented above for G protein-coupled receptors, the mechanisms and molecules involved are known to be different, at least in part, and therefore their modulation by NO could not be predicted. As the experimental system, we focused on Ca^{2+} release and influx triggered by EGF and PDGF administration in a clone of NIH 3T3 cells overexpressing the human EGF receptor (24). The results obtained show that also in the case of the two growth factors, Ca^{2+} influx occurs through distinctly activated SMOCs and SDCs and that NO exerts a complex, modulatory effect on both pathways, as well as on EGF- and PDGF-stimulated Ca^{2+} release from intracellular stores.

Experimental Procedures

Materials. Culture sera and media were purchased from GIBCO (Basel, Switzerland); EGF, PDGF (AB heterodimer), KT5823, KT5720, Fura-2, Tg, and L-NIO from Calbiochem (San Diego, CA); SNAP from Alexis (Laufelfingen, Switzerland); $^{45}Ca^{2+}$ from Amersham Int. (Little Chalfont, UK); Rp 8-Br-cGMP[S] and Rp 8-Br-cAMP[S] from Biolog (Bremen, Germany); SNP, D-NAME, L-NAME, 8-Br-cGMP, and the remaining chemicals from Sigma and Aldrich (Milano, Italy). LU52396 was a kind gift from Knoll AG, Ludwigshafen, Germany.

Cell culture and preparation. EGFR-T17 cell line, a clone of NIH-3T3 cells overexpressing the human EGF receptor, was routinely grown as described (24) and used before the 10th week after thawing. On the day of the experiment, cell monolayers were detached from Petri dishes by gentle trypsinization and resuspended in 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, 1 mM L-arginine, and 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 used was >95%, as assessed by trypan blue exclusion test.

$[Ca^{2+}]_i$ measurements. Cell monolayers, detached and washed as described above, were resuspended, loaded in KRH medium with the Ca^{2+}-sensitive dye Fura-2 for 30 min at 25° and then maintained at 37° until use. The experiments were initiated by incubation of cell aliquots (4×10^6 cells) with various drugs interfering with the L-arginine/NO pathway for 15 min at 37°, a condition selected in preliminary experiments to be sufficient for maximal drug effects, in the time range previously demonstrated to be effective in other cell types (19–22). Cells were subsequently centrifuged and resuspended in KRH supplemented with 250 μM sulfinpyrazone to prevent dye leakage. The samples were then transferred to a thermostat-equipped cuvette (37°), maintained under continuous stirring, and analyzed in a Perkin Elmer LS-5B fluorimeter as described (25). In the experiments in which Ca^{2+} release and Ca^{2+} influx were analyzed separately, cell samples were resuspended in a KRH medium from which $CaCl_2$ had been omitted and 10 μM EGTA had been added (Ca^{2+}-free KRH medium (estimated extracellular calcium concentration $<10^{-8}$ M). After stimulation with Tg, EGF, or PDGF, the recording was continued until the end of the first $[Ca^{2+}]_i$ peak (intracellular Ca^{2+} release). Ca^{2+} (2 mM) was then introduced into the medium, and the ensuing second peak (Ca^{2+} influx) was recorded. Results are shown as traces and graphs. Traces are representative of results obtained in 8–10 experiments; graphs show the mean ± standard deviation values of 6–10 experiments.

Mn^{2+} quenching of Fura-2 fluorescence. The Mn^{2+} permeability of the cell plasmalemma was measured as previously described (16). Cells loaded with Fura-2 were suspended in an incubation medium from which $CaCl_2$ was omitted, KH_2PO_4 was reduced to 0.6 mM, and $MgCl_2$ was substituted for $MgSO_4$. Fluorescence was excited at 360 nm, the Ca^{2+}/Fura-2 isosbestic wavelength at which changes are caused by Mn^{2+} quenching (26). Emission was recorded at 500 nm. Mn^{2+} was added to the medium 4 min after Tg at a final concentration of 25 μM, a condition in which rate of quenching of fluorescence is almost constant, to allow careful quantitative analysis of the data. At the end of the recordings, maximal Mn^{2+} quenching values were estimated in each preparation by cell permeabilization with Triton X-100 (0.1%). Traces shown are representative of results obtained in 8–12 experiments; graphs show the mean ± standard deviation values of six to nine experiments.

$^{45}Ca^{2+}$ measurements. EGFR-T17 cells were grown as described above, except that during the last 72 hr, their incubation medium was supplemented with $^{45}Ca^{2+}$ (4 μCi/ml). Labeled cells were washed extensively and resuspended in KRH medium. An aliquot (4×10^6 cells) of the suspension was immediately centrifuged, and the ensuing pellet was used for measurement of total cell $^{45}Ca^{2+}$ content. The other aliquots were incubated with or without L-NIO (100 μM) or SNP (30 μM) for 15 min at 37° and then, for an additional 5 min, with or without Tg (30 nM) in EGTA-containing, Ca^{2+}-free KRH medium. Finally, the samples were centrifuged, and the $^{45}Ca^{2+}$ recovered in the medium was measured in a Beckman β-counter (for further details, see Ref. 27). Results described are mean ± standard deviation values of five separate experiments.

Measurements of cGMP levels. Cell monolayers, detached and washed as described above, were resuspended and incubated for 15 min at 37° in KRH medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, with or without L-NIO (100 μM). NOS activity was stimulated by exposing cell suspensions to EGF or PDGF for 0.5–15 min at 37°. The reaction was terminated by the addition of ice-cold trichloroacetic acid (final concentration, 7.5%). After ether extraction, cGMP levels were measured using a radioimmunoassay kit (DuPont, Milano, Italy) and normalized on cellular proteins, determined using the bicinchoninic acid assay (BCA protein assay reagent; Pierce, Milano, Italy). Results shown are mean ± standard deviation values of three separate experiments.

Results

Effects of L-NIO and SNP on $[Ca^{2+}]_i$ responses induced by EGF and PDGF. In agreement with previously reported data (24), exposure of Fura-2-loaded EGFR-T17 cells to EGF (30 nM) or PDGF (3 nM), administered in Ca^{2+}-containing KRH medium, gave rise to a biphasic $[Ca^{2+}]_i$ response consisting of an initial peak followed by a sustained plateau phase (Fig. 1, A and E): the first originates primarily from intracellular Ca^{2+} release, and the second originates from Ca^{2+} influx (10). Under these experimental conditions, preexposure (15 min at 37°) of EGFR-T17 cell suspensions to the NOS inhibitor L-NIO (100 μM) induced a slight, yet appreciable increase with respect to untreated, control cells of both of the growth factor-induced $[Ca^{2+}]_i$ responses: the transient, peak phase and the following sustained plateau (Fig. 1, B and F). Conversely, preincubation with the NO donor SNP (30 μM) resulted in an inhibition of both peak and plateau responses (Fig. 1, C and G). The action of SNP was presumably mediated by NO because the drug was ineffective when solubilized 24 hr before use (data not shown). When the cells were incubated (15 min at 37°) with the cGMP analogue 8-Br-cGMP (200 μM), the effect on both the peak and the plateau phases of the $[Ca^{2+}]_i$ response elicited by EGF and PDGF was similar to that observed with SNP (Fig. 1, D and H). In control experiments, addition to the cell suspensions of SNP, L-NIO, or 8-Br-cGMP alone (30, 100, and 200 μM, respectively) did not induce any changes in the basal $[Ca^{2+}]_i$ levels (Fig. 1, I and L, and data not shown).

Effects of L-NIO and SNP on Ca^{2+} release and influx induced by EGF and PDGF. The effects induced by the NO-modulating drugs on intracellular Ca^{2+} release and influx were analyzed separately, according to the Ca^{2+}-free/Ca^{2+}-introduction protocol (see $[Ca^{2+}]_i$ measurements). Figs. 2 and 3 show the results obtained with this protocol in parallel batches of EGFR-T17 cell suspensions, challenged

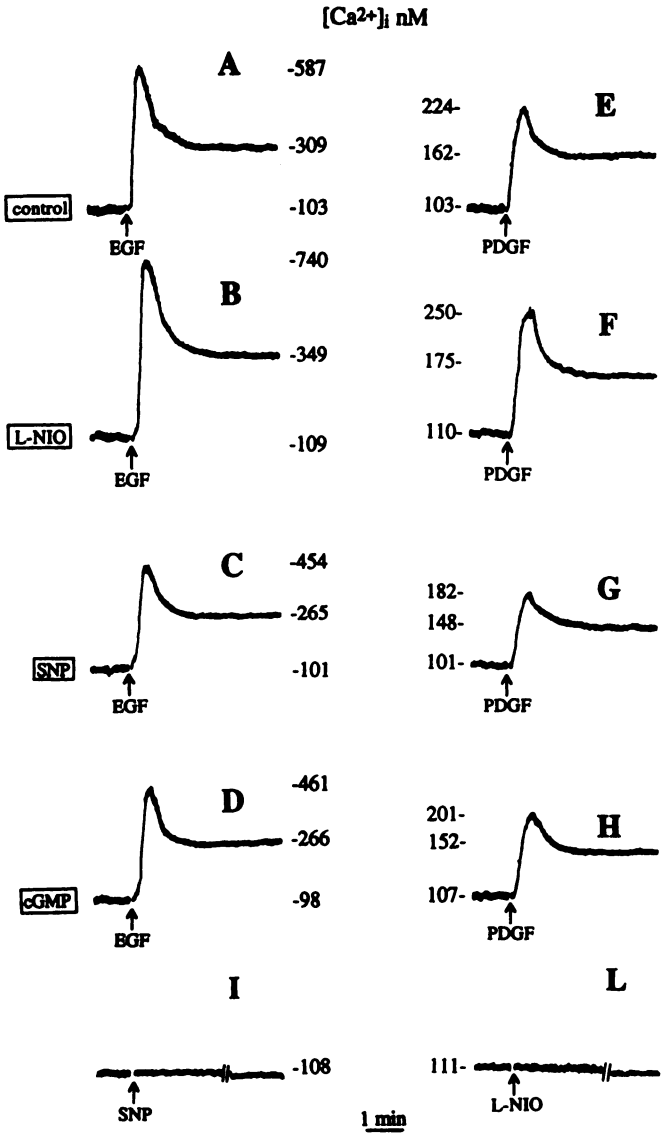

Fig. 1. Effects of pretreatment with L-NIO, SNP, and 8-Br-cGMP on EGF- and PDGF-induced $[Ca^{2+}]_i$ variations in EGFR-T17 cells. Fura-2-loaded cells, suspended in Ca^{2+}-containing KRH medium, were challenged with EGF (30 nM) or PDGF (3 nM) where indicated. *Traces A and E*, control cells. *Traces B and F, C and G, and D and H*, effects of 15-min pretreatment with L-NIO (100 μM), SNP (30 μM), or 8-Br-cGMP (200 μM), respectively. *Traces I and L*, effects of SNP and L-NIO administration on basal $[Ca^{2+}]_i$, followed up to 15 min (shown are the first 2.5 and the last 1.5 min). *Numbers in the middle*, $[Ca^{2+}]_i$ values.

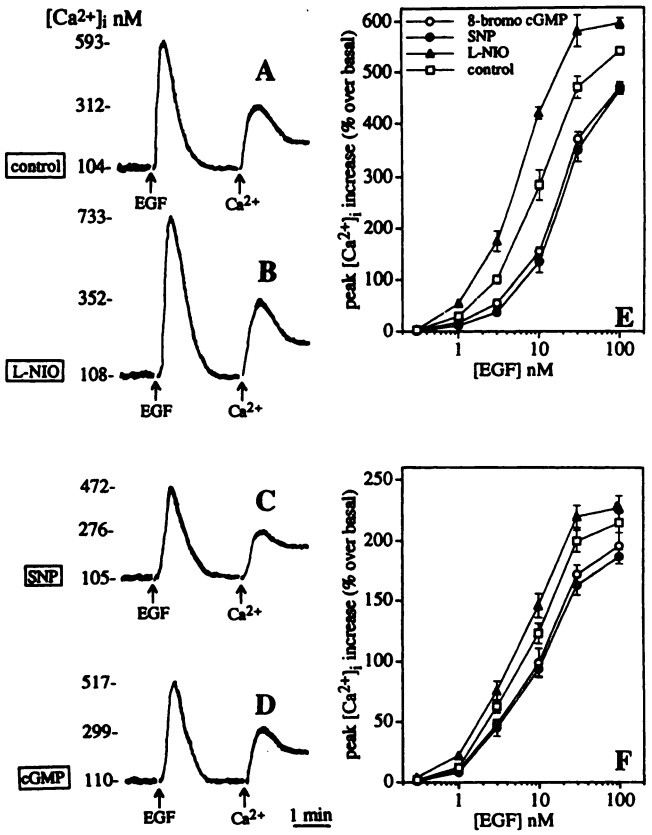

Fig. 2. Effects of pretreatment with L-NIO, SNP, and 8-Br-cGMP on EGF-induced Ca^{2+} release and influx. *Traces A–D*, Fura-2-loaded cells, suspended in Ca^{2+}-free KRH medium, challenged with EGF (30 nM) where indicated. Ca^{2+} (2 mM) was added to the medium after 2–3 min. *Trace A*, control cells. *Traces B–D*, effects of 15-min pretreatment with L-NIO (100 μM), SNP (30 μM), or 8-Br-cGMP (200 μM), respectively. *Numbers to the left*, $[Ca^{2+}]_i$ values. *Graphs in E and F*, concentration dependence of the two components of the EGF-induced Ca^{2+} response expressed as the percent increase of $[Ca^{2+}]_i$ measured at the peak of the response over basal resting $[Ca^{2+}]_i$ values. E, Ca^{2+} release from intracellular Ca^{2+} stores. F, Ca^{2+} influx from outside.

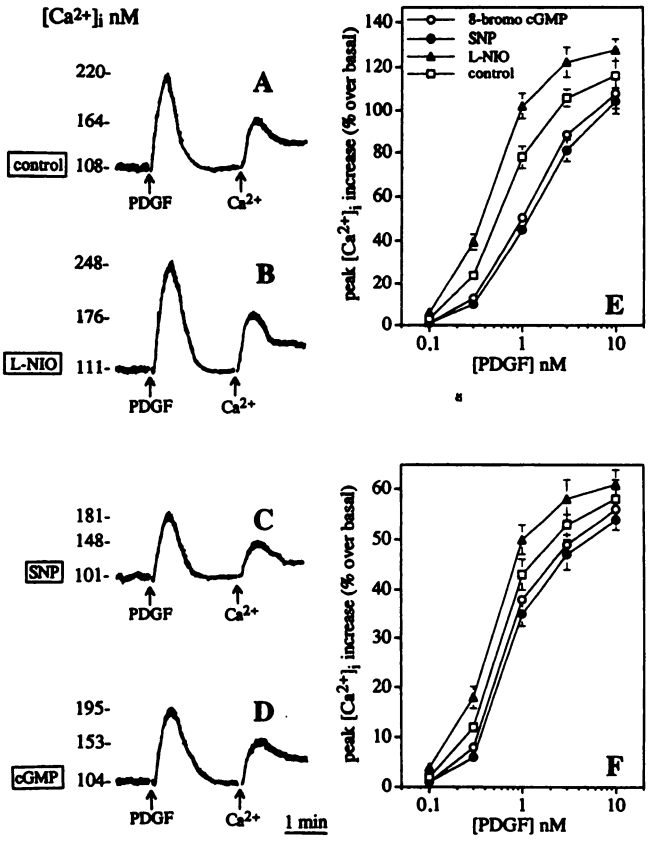

Fig. 3. Effects of pretreatment with L-NIO, SNP, and 8-Br-cGMP on PDGF-induced Ca^{2+} release and influx (experiment conditions were as for Fig. 2). Cells were challenged with PDGF (3 nM) and Ca^{2+} (2 mM) where indicated. *Trace A*, control cells. *Traces B–D*, effects of 15-min pretreatment with L-NIO (100 μM), SNP (30 μM), or 8-Br-cGMP (200 μM), respectively. *Numbers to the left*, $[Ca^{2+}]_i$ values. *Graphs*, concentration dependence of PDGF-induced Ca^{2+} release from intracellular stores (E) and influx from outside (F).

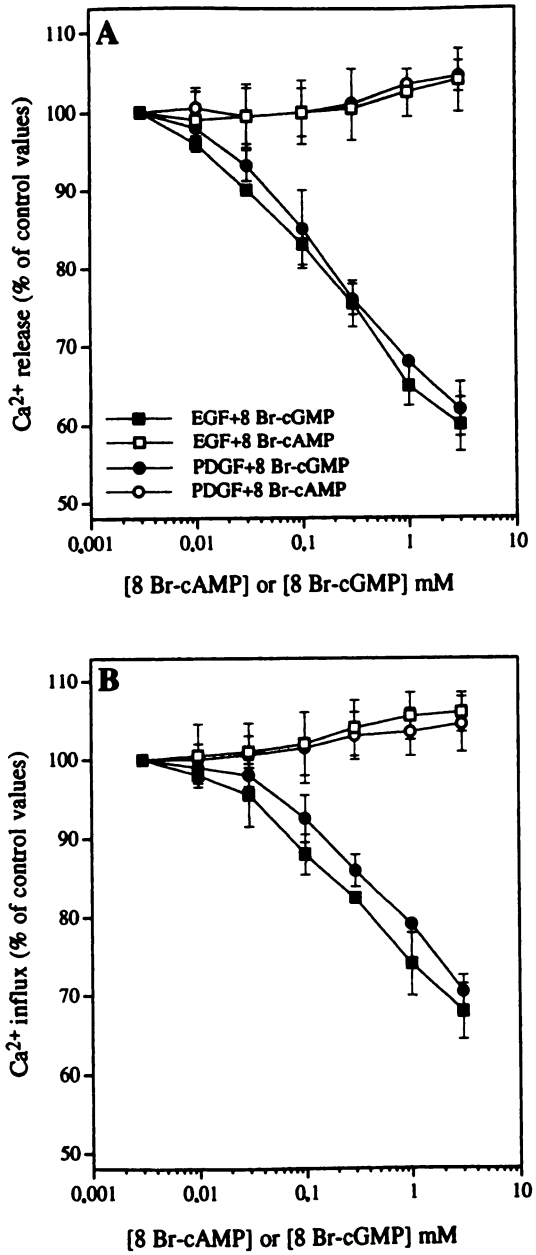

Fig. 4. Effects of 8-Br-cGMP and 8-Br-cAMP on growth factor-induced Ca^{2+} release and influx responses (experiment conditions were as for Fig. 2). *Graphs A and B*, effects of preincubation with increasing concentrations of either the cGMP or cAMP analogue on Ca^{2+} release and influx elicited by EGF (30 nM) or PDGF (3 nM) administration, respectively.

with EGF (Fig. 2) or PDGF (Fig. 3) without any pretreatment or after preincubation with L-NIO, SNP, or 8-Br-cGMP as above. In Ca^{2+}-free medium, pretreatment with L-NIO (100 μM) induced an increase in both EGF-and PDGF-induced Ca^{2+} release responses (Figs. 2 and 3, B and E) with respect to controls (Figs. 2 and 3, A and E), whereas SNP (30 μM) produced an inhibition (Figs. 2 and 3, C and E). When cell suspensions were preincubated with 8-Br-cGMP (200 μM), the effect observed was analogous to that of SNP (Figs. 2 and 3, D and E). Similar, although less evident, effects of L-NIO, SNP, and 8-Br-cGMP were observed when EGF- and PDGF-activated Ca^{2+} influxes were induced by introduction of Ca^{2+} (2 mM) into the medium at the end of the first fluorescence peak (Figs. 2 and 3, A–D and F).

The observation of analogous effects on Ca^{2+} release and influx responses after administration of either 8-Br-cGMP or SNP suggested that increased cGMP levels could account for NO effects, possibly via G kinase I activation. cGMP analogues are, however, known also to cross-activate protein kinase A (28, 29). To identify the role of the two cyclic nucleotides, the effects of the cAMP analogue 8-Br-cAMP were analyzed in parallel to those of 8-Br-cGMP. As shown in Fig. 4, preincubation of EGFR-T17 cells with increasing concentrations of the cGMP analogue yielded dose-dependent inhibition of both growth factor-induced Ca^{2+} release (Fig. 4A) and influx (Fig. 4B) responses. In contrast, preincubation with the cAMP analogue had only moderate effects, even at the higher concentrations used.

The amplitude of the Ca^{2+} influx subsequent to receptor activation is at least partially sustained by SDCs and therefore dependent on the degree of emptying of the intracellular Ca^{2+} stores (17). Thus, the facilitatory effect of L-NIO and the inhibitory effect of SNP and 8-Br-cGMP on the EGF- and PDGF-induced Ca^{2+} influxes, revealed by the experiments represented by Figs. 2–4, could result from a balance between the modulatory effects of these drugs on the growth

factor-induced Ca^{2+} release, on the one hand, and a direct effect on Ca^{2+} influx, on the other hand. The existence of the latter and its nature were therefore investigated.

Characterization of the Ca^{2+} influx response to EGF and PDGF. The first experiments were aimed at establishing whether activation of growth factor receptors resulted in the opening of SDCs only (activated by the emptying of intracellular stores) or of additional Ca^{2+} influx pathway(s) such as SMOCs, as already demonstrated for G protein-coupled receptors (e.g., see Refs. 16, 30, 31). To this end, a protocol was designed (31) in which SDC opening was fully activated, before growth factor administration, by the selective sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase blocker Tg (17), which causes complete depletion of the IP_3-sensitive Ca^{2+} stores (32). When added to EGFR-T17 cell suspensions bathed in the Ca^{2+}-free KRH medium, Tg (30 nM) yielded a large $[Ca^{2+}]_i$ increase due to extensive release of stored Ca^{2+}. Further release of the cation by subsequently applied high doses of EGF (100 nM), PDGF (10 nM), or the P_{2u} agonist ATP (100 μM) (11), administered alone or in combination, was completely prevented (Fig. 5A). When Tg (30 nM) was applied in the Ca^{2+}-containing KRH medium, it induced a biphasic $[Ca^{2+}]_i$ response that after 5–6 min reached a long-lasting plateau (Fig. 5, B and C) resulting from the balance between SDC-mediated influx and the activation of Ca^{2+} extrusion mechanisms, mainly the plasma membrane Ca^{2+}-ATPases (10, 31, 33). When, in these conditions, EGF (100 nM) and PDGF (10 nM) were applied 7 min after Tg administration, they both induced small, yet consistent increases in $[Ca^{2+}]_i$ over the plateau level, indicating the ability of both growth factors to induce influx of Ca^{2+} through mechanism(s) in addition to SDC (Fig. 5, B and C). Preincubation of the cells with ω-conotoxin (300 nM), verapamil (10 μM), or a mixture of the two did not affect the Ca^{2+} influx response to Tg, EGF, and PDGF, thus excluding involvement of voltage-operated channels of both the N- and L-types (data not shown). In a second set of experiments, parallel aliquots of EGFR-T17 cells were resuspended in Ca^{2+}-free KRH medium, treated with Tg (30 nM) for 10 min, and subsequently challenged or not challenged with either EGF (100 nM) or PDGF (10 nM) 1 min before Ca^{2+} (2 mM) addition. As can be seen (Fig. 5, D–F), on Ca^{2+} addition, both EGF- and PDGF-treated cells showed $[Ca^{2+}]_i$ increases that not only were distinctly higher but also exhibited faster initial rates than cells treated with Tg alone (4.7 ± 0.23; 4.1 ± 0.36 and 2.5 ± 0.16 nM/sec, respectively). This latter finding is in agreement with previously reported data indicating that the kinetics of Ca^{2+} influx through SMOCs are faster than those through SDCs (16, 31).

Pharmacology of the Ca^{2+} channels activated by Tg, EGF, and PDGF. The dual nature (SDCs and SMOCs) of the EGF- and PDGF-operated Ca^{2+} influxes was also determined through study of the permeability to Mn^{2+}, revealed by quenching of the Fura-2 signal, and the sensitivity to LU52396, a newly discovered inhibitor of Ca^{2+} channels with high selectivity for SDCs (34). EGFR-T17 cell aliquots were suspended in a slightly modified Ca^{2+}-free KRH medium (see Experimental Procedures) and then exposed to Tg (30 nM) 4 min before the addition of Mn^{2+} (25 μM), a time sufficient to achieve complete depletion of the intracellular Ca^{2+} stores and maximal SDC activation (Fig. 5A). Tg induced a marked increase of the quenching rate with respect to untreated cells (from 1.61 ± 0.12% to 7.25 ± 0.78% min^{-1}; compare Fig. 6A with Fig. 6, B and C). When either EGF (100 nM) or PDGF (10 nM) was subsequently applied to these preparations, a further increase in the Mn^{2+} quenching rate was observed (to 9.50 ± 0.87% and 8.98 ± 0.73% min^{-1}, respectively), indicating that additional cation influx was stimulated (Fig. 6, B and C) and that EGF- and PDGF-operated SMOCs were permeable to Mn^{2+}. It is of importance that similar quenching rate values were measured when EGF or PDGF was added at the same time as Tg (9.53 ± 0.82 and 9.00 ± 0.85, respectively; data not shown).

Treatment of the cell suspensions with LU52396 (10 μM; 5 min), a condition in which Ca^{2+} influx through SDCs is almost completely blocked but SMOC activity is only moder-

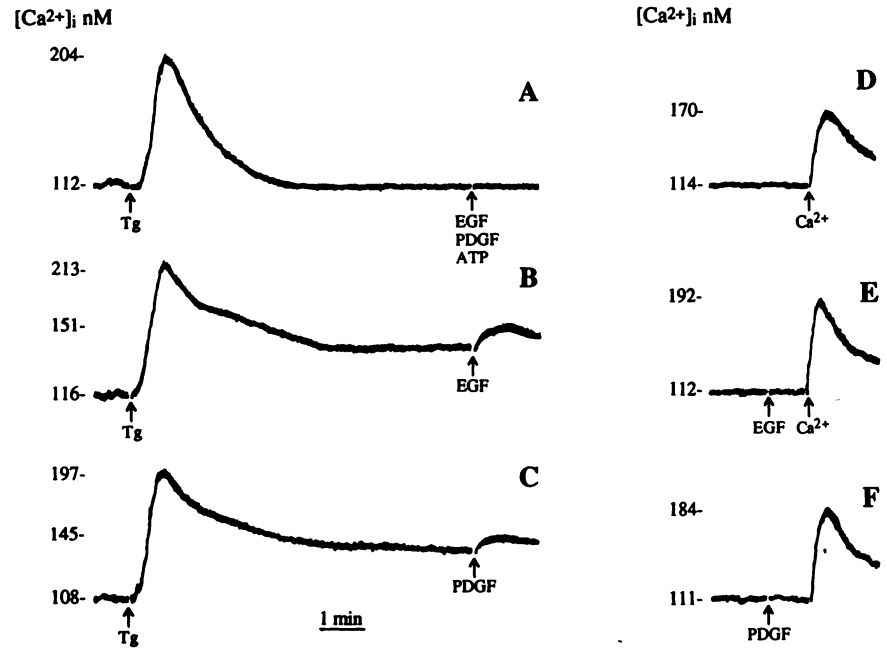

Fig. 5. Characterization of EGF- and PDGF-induced Ca^{2+} influx. *Traces A–C*, Fura-2-loaded cells suspended in Ca^{2+}-free (A) or Ca^{2+}-containing (B and C) KRH medium stimulated with Tg (30 nM) where indicated. EGF (100 nM; B), PDGF (10 nM; C), or a mixture of EGF, PDGF, and ATP (100 μM; A) was added 7 min after Tg. *Traces D–F*, Fura-2-loaded cells suspended in Ca^{2+}-free KRH medium incubated with Tg (30 nM) for 10 min and subsequently for 1 min without (D) or with EGF (100 nM; E) or PDGF (10 nM; F) before the addition of Ca^{2+} (2 mM), as indicated.

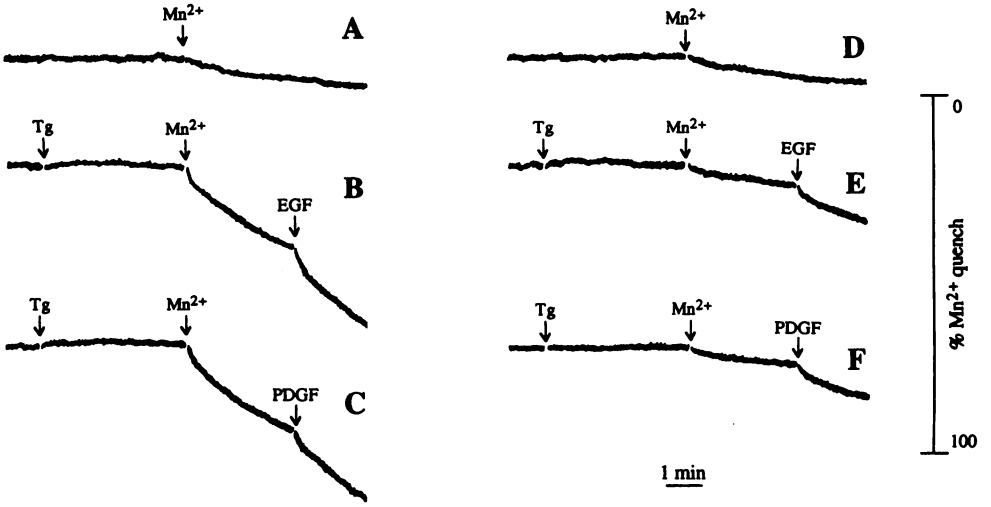

Fig. 6. Pharmacological analysis of EGF- and PDGF-induced Mn^{2+} influx. Fura-2-loaded cells suspended in Ca^{2+}-free medium incubated for 5 min in the presence (D–F) or absence (A–C) of LU52396 (10 μM) and then challenged (B, C, E, and F) or not (A and D) with Tg (30 nM) 4 min before the addition of Mn^{2+} (25 μM). Cells were subsequently challenged with EGF (100 nM; B and E) or PDGF (10 nM; C and F), where indicated. *Vertical bar to the right* specifies the maximum percent Mn^{2+} quenching.

ately reduced (34), had no effect *per se* (Fig. 6D) but resulted in almost complete blunting of the Tg-induced Mn^{2+} quenching of the Fura-2 signal (Fig. 6, E and F), which remained at values similar to those of Tg-untreated cells (Fig. 6, A and D). In contrast, when EGF (100 nM) or PDGF (10 nM) was added, increased Mn^{2+} permeability was still observed (Fig. 6, E and F). Such an increase was smaller (3.80 ± 0.33% and 3.29 ± 0.24% min^{-1}) than that generated in cells not exposed to the SDC inhibitor (Fig. 6, B and C).

Effects of NOS inhibitors and NO donors on the Ca^{2+} channels activated by Tg, EGF, and PDGF. NO stimulates the soluble guanylyl cyclase activity and increases the levels of intracellular cGMP. Because the latter is a well known activator of plasma membrane Ca^{2+}-ATPases (23, 35), its effect on Ca^{2+} influx cannot be studied with accuracy with $[Ca^{2+}]_i$ measurements. Mn^{2+}, however, is not a substrate for the Ca^{2+} ATPases (23, 33). The Mn^{2+} quenching protocol was therefore chosen to investigate the effects of NO on SDCs and growth factor-activated SMOCs. Preincubation of EGFR-T17 cell suspensions with L-NIO (100 μM) before Tg (30 nM) administration decreased the quenching effect not only of the latter drug but also of either EGF (30 nM) or PDGF (3 nM) when administered subsequently (Fig. 7, B and C, respectively). On the contrary, preincubation with SNP (30 μM) increased the Mn^{2+} quenching rate of all drugs, an effect mimicked by 200 μM 8-Br-cGMP (Fig. 7). Similar experiments were performed with another NOS inhibitor, L-NAME (200 μM); its less active enantiomer, D-NAME (200 μM); and the NO donor SNAP (30 μM). Pretreatment with L-NAME and SNAP yielded results qualitatively similar to those observed with L-NIO and SNP, respectively, whereas D-NAME had no appreciable effect. Quantitative analysis of the results obtained is presented in Table 1. As can be seen, the effects of NO modulating drugs on EGF- and PDGF-induced Mn^{2+} influxes were not a mere consequence of the effects they had on the influx elicited by previously administered Tg; NOS inhibitors and NO donors had an active role in inhibiting or increasing, respectively, the influx of Mn^{2+} specifically activated by both growth factors (Table 1).

Because the SDC influx response depends on the degree of depletion of Ca^{2+} stores, we checked whether NO modulating drugs had an effect on the latter parameter. To yield quantitative results, EGFR-T17 cells were loaded to equilibrium

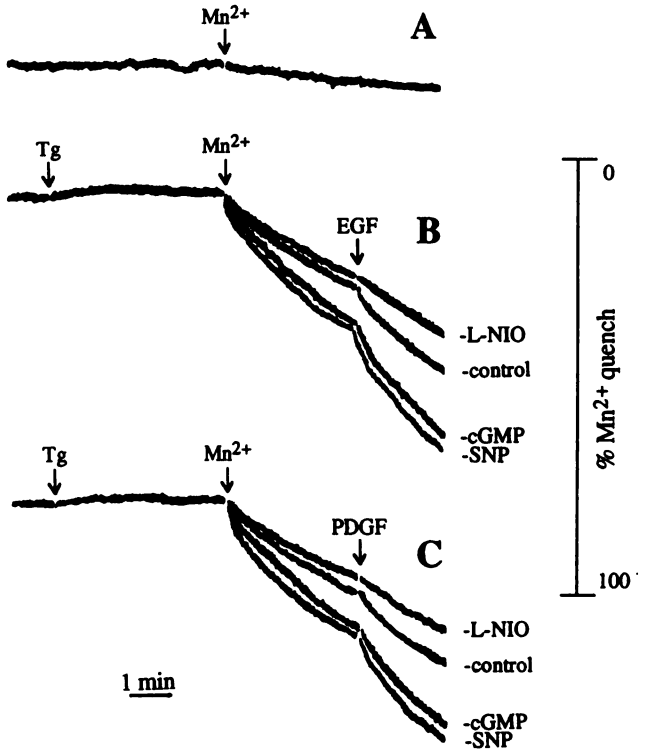

Fig. 7. Effects of L-NIO, SNP, and 8-Br-cGMP on Tg-, EGF-, and PDGF-induced Mn^{2+} influx. *Traces B and C*, Fura-2-loaded cells suspended in Ca^{2+}-free medium incubated for 15 min at 37° without (control) or with L-NIO (100 μM), SNP (30 μM), or 8-Br-cGMP (200 μM) and then treated with Tg (30 nM) 4 min before the addition of Mn^{2+} (25 μM). Cells were subsequently challenged with EGF (30 nM; B) or PDGF (3 nM; C), where indicated. *Trace in A*, basal Mn^{2+} influx in untreated, control cells. *Vertical bar to the right*, maximum percent Mn^{2+} quenching.

(72 hr) with $^{45}Ca^{2+}$, then incubated for 15 min at 37° with or without L-NIO (100 μM) or SNP (30 μM), and finally challenged with Tg (30 nM; 5 min). Under these conditions, Tg-induced $^{45}Ca^{2+}$ release was similar in cells preincubated with L-NIO or SNP compared with controls (1533 ± 35, 1518 ± 42, and 1489 ± 38 cpm/mg of protein, respectively).

Taken together, these findings strongly suggest that NO has a direct, facilitatory role not only on SDCs but also on the SMOCs activated by growth factor receptors.

TABLE 1

Quantitative effects of NOS inhibitors, NO donors, 8-Br-cGMP, and KT5823 on Mn^{2+} influx elicited by Tg, EGF, and PDGF

Treatment	Mn^{2+} quenching of Fura-2 fluorescence				
	Tg (SDC)	EGF (total influx)	PDGF (total influx)	EGF (SMOC)	PDGF (SMOC)
			% min^{-1}		
No pretreatment	7.25 ± 0.78	8.82 ± 0.54	8.38 ± 0.81	1.57 ± 0.09	1.13 ± 0.14
SNP (30 μM)	10.0 ± 0.98	13.5 ± 1.25	12.2 ± 1.17	3.49 ± 0.12	2.16 ± 0.19
SNAP (30 μM)	9.88 ± 0.44	13.0 ± 1.30	11.8 ± 1.09	3.12 ± 0.16	1.92 ± 0.18
8-Br-cGMP (200 μM)	9.72 ± 0.83	12.7 ± 1.16	11.6 ± 1.13	2.98 ± 0.87	1.88 ± 0.20
KT5823 (10 μM)	6.01 ± 0.53	7.00 ± 0.65	6.72 ± 0.53	0.99 ± 0.10	0.71 ± 0.07
L-NIO (100 μM)	5.98 ± 0.44	6.98 ± 0.70	6.70 ± 0.44	1.00 ± 0.07	0.72 ± 0.04
L-NAME (200 μM)	6.11 ± 0.60	7.20 ± 0.67	6.84 ± 0.55	1.09 ± 0.08	0.73 ± 0.05
D-NAME (200 μM)	7.23 ± 0.68	8.73 ± 0.79	8.40 ± 0.76	1.50 ± 0.12	1.17 ± 0.15
SNP + KT5823	7.02 ± 0.32	8.41 ± 0.71	8.01 ± 0.79	1.39 ± 0.07	0.99 ± 0.06
L-NIO + KT5823	6.00 ± 0.37	7.06 ± 0.58	6.68 ± 0.49	1.06 ± 0.09	0.68 ± 0.05

Fura-2-loaded EGFR-T17 cells were incubated in Ca^{2+}-free KRH medium for 15 min at 37° with the above treatments and then challenged with Tg (30 nM) 4 min before the addition of Mn^{2+} (25 μM). EGF (30 nM) or PDGF (3 nM) was added 3 min later. Mean Mn^{2+} quenching rate values were measured after Tg (SDC) or growth factor (total influx) administration. SMOC values are obtained by subtracting SDC values from total growth factor-induced influx. Results shown are mean ± standard deviation values of 8–12 experiments.

Pharmacological evidence for a role of G kinase I in the regulation of Ca^{2+} channels activated by Tg, EGF, and PDGF. The effects of SNP and SNAP on Ca^{2+} and Mn^{2+} influx were in all cases mimicked by 8-Br-cGMP administration. We therefore investigated whether, in our cells, the effect of NO on SDCs and SMOCs was exerted through cGMP formation and subsequent G kinase I activation. Experiments were set up in which EGFR-T17 cells were preincubated for 15 min with or without SNP (30 μM) plus KT5823 (10 μM), a selective inhibitor of the G kinase I (e.g., see Refs. 20 and 36). Under these conditions, the facilitating effects of SNP on Tg-, EGF-, and PDGF-induced Mn^{2+} influxes were completely abolished (Fig. 8 and Table 1). The effects of KT5823 were then compared with the effects of another selective G kinase inhibitor, Rp 8-Br-cGMP[S] (29), and to those of two protein kinase A inhibitors, KT5720 (36) and Rp 8-Br-cAMP[S] (29). As shown in Fig. 9, when EGFR-T17 cells were treated with SNP (30 μM) plus increasing concentrations of either KT5823 or Rp 8-Br-cGMP[S], Mn^{2+} influx induced by both Tg and growth factors was dose-dependently inhibited by each of these G kinase inhibitors. In contrast, the analogues more selective for protein kinase A, KT5720 and Rp 8-Br-cAMP[S], exhibited small effects only at high concentrations. This last result was unexpected because both protein kinase A blockers are known to exhibit some activity against G kinase (29, 36). It should be noted, however, that although weak, the effects of cAMP on Ca^{2+} influx appear to be opposite those of cGMP (see Fig. 4). Moreover, additional unspecific effects of the high concentrations of the drugs used, contributing to offset those due to inhibition of G kinase, cannot be excluded.

In additional experiments, KT5823 was administered alone or in combination with L-NIO. When EGFR-T17 cells were preincubated with KT5823 (10 μM; 15 min) the effect observed on the Mn^{2+} influx induced by Tg, EGF, and PDGF mimicked that observed after a similar incubation with L-NIO (100 μM) (Fig. 10 and Table 1). When KT5823 and L-NIO were administered together, no additive effect was observed (Table 1). The findings illustrated in Figs. 8–10 appear to be consistent with the possibility that the facilitatory effect of NO on SDCs and growth factor-activated SMOC influx is mediated by G kinase I activation.

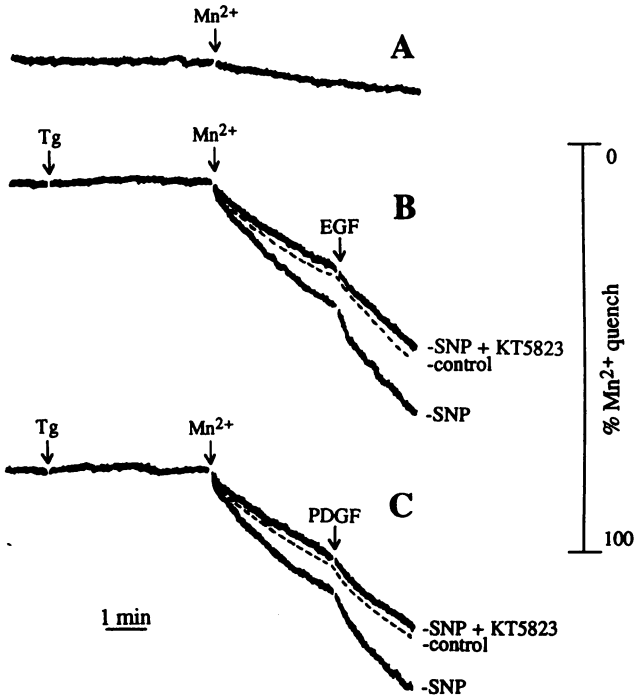

Fig. 8. Effects of SNP and KT5823 on Tg-, EGF-, and PDGF-induced Mn^{2+} influx. *Traces B and C*, Fura-2-loaded cells suspended in Ca^{2+}-free medium incubated for 15 min at 37° without (contol) or with SNP (30 μM) alone or in combination with KT5823 (10 μM) and then treated with Tg (30 nM) 4 min before the addition of Mn^{2+} (25 μM). Cells were subsequently challenged with EGF (30 nM; B) or PDGF (3 nM; C), where indicated. *Trace in A*, the basal Mn^{2+} influx in untreated, control cells. *Vertical bar to the right*, maximum percent Mn^{2+} quenching.

Effects of growth factors on cGMP formation. The results obtained with L-NIO and L-NAME in the $[Ca^{2+}]_i$ and Mn^{2+} influx experiments strongly suggest the existence in EGFR-T17 cells of a constitutive, presumably Ca^{2+}-dependent, NOS activity. The possibility therefore existed that growth factor-induced increases in $[Ca^{2+}]_i$ could enhance endogenous NO formation and that the gaseous messenger, acting via cGMP formation, could in turn act as a feedback modulator of growth factor-induced Ca^{2+} signaling. To test this hypothesis, intracellular cGMP accumulation was measured after stimulation of EGFR-T17 cells with either EGF or

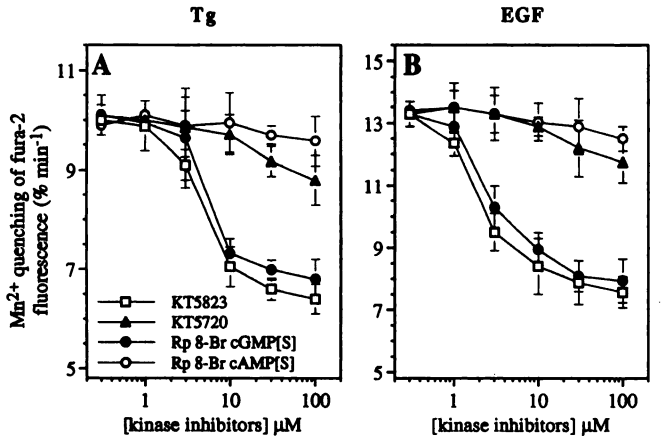

Fig. 9. Effects of G kinase I and protein kinase A inhibitors on Tg- and growth factor-induced Mn^{2+} influx responses (experimental conditions were as for Fig. 7). Cell suspensions were preincubated with SNP (30 μM) plus increasing concentrations of either one of the G kinase I inhibitors KT5823 and Rp 8-Br-cGMP[S] or the protein kinase A inhibitors KT5720 and Rp 8-Br-cAMP[S]. *Graphs A–C*, effects of the various inhibitors on Tg-, EGF-, and PDGF-induced Mn^{2+} influx, respectively.

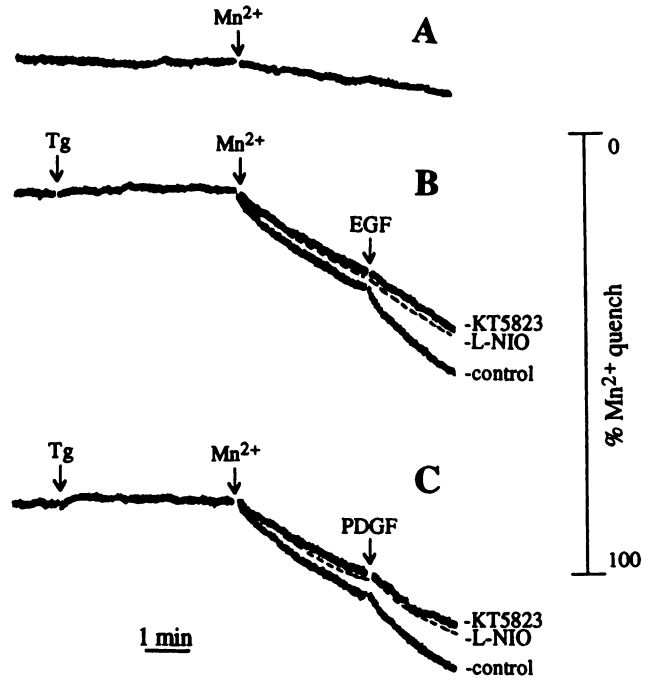

Fig. 10. Effects of L-NIO and KT5823 on Tg-, EGF-, and PDGF-induced Mn^{2+} influx. *Traces B and C*, Fura-2-loaded cells suspended in Ca^{2+}-free medium incubated for 15 min at 37° without (control) or with either L-NIO (100 μM) or KT5823 (10 μM) and then treated with Tg (30 nM) 4 min before the addition of Mn^{2+} (25 μM). Cells were subsequently challenged with EGF (30 nM; B) or PDGF (3 nM; C), where indicated. *Trace in A*, basal Mn^{2+} influx in untreated, control cells. *Vertical bar to the right*, maximum percent Mn^{2+} quenching.

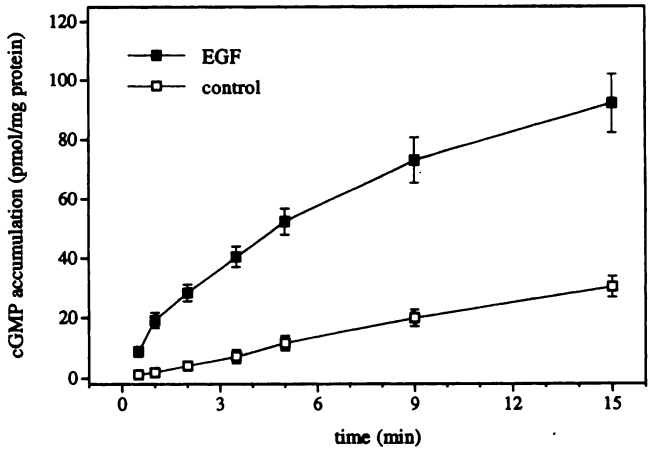

Fig. 11. Time course of cGMP accumulation elicited by EGF showing cGMP accumulation in EGFR-T17 cells preincubated for 15 min with 0.5 mM 3-isobutyl-1-methylxanthine and challenged for the indicated period of time with or without EGF (100 nM).

PDGF (100 and 10 nM, respectively) in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine and followed for up to 15 min. During the first 5 min of incubation, both growth factors enhanced cGMP accumulation with respect to controls, with an almost constant rate (average of 10.36 ± 0.27 and 8.97 ± 0.42 versus 2.10 ± 0.18 pmol/mg of protein/min, respectively). Thereafter, the rate of accumulation tended to level off progressively (Fig. 11). Clear stimulations were also observed with 3- and 10-fold lower concentrations of both growth factors, whereas with a further decrease, their effects became inappreciable. The NO dependence of cGMP formation induced by growth factors was demonstrated by the fact that it was almost completely prevented by preincubation of cells with 100 μM L-NIO (2.85 ± 0.26 and 2.46 ± 0.16 pmol/mg of protein/min for EGF and PDGF, respectively). In parallel experiments in which the cells were preincubated with L-NIO alone, cGMP formation was slightly reduced (1.29 ± 0.14 pmol/mg/min).

Discussion

The results of the present study document a modulatory role of NO in the regulation of $[Ca^{2+}]_i$ variations subsequent to EGF and PDGF receptor activation in NIH 3T3 cells. $[Ca^{2+}]_i$ responses elicited by the two growth factors are the result of multiple mechanisms: Ca^{2+} release from intracellular stores, the subsequent activation of SDCs, and the activation by both growth factors of an SMOC influx. All of the components of the $[Ca^{2+}]_i$ response appear to be modulated by NO, as demonstrated by the parallel analyses of the effects of NOS inhibitors and NO donors. The latter drugs were shown to exert, on the one hand, an inhibitory effect on intracellular Ca^{2+} release induced by EGF and PDGF receptor activation and, on the other hand, a stimulatory effect on both SDCs and SMOCs when investigated separately. Because, however, the degree of SDC opening is dependent on store depletion (which is inhibited by NO), the net effect on Ca^{2+} influx was a slight inhibition (see Figs. 2 and 3). The specificity of the present results is demonstrated by the observation that D-NAME had no appreciable effect, like SNP

when solubilized 24 hr before use, a time sufficient for the NO generated to fade away. In addition, when the cells were preincubated with NOS inhibitors, the effects observed were opposite those induced by treatment with NO donors. The latter finding, together with the observation that an NOS inhibitor, L-NIO, slightly decreased basal cGMP formation, indicates that in NIH 3T3 cells NOS is physiologically active, as suggested previously (37). Thus, endogenous NO may play a role in modulating the complex signaling response subsequent to growth factor receptor activation. All of the effects of NO donors were mimicked by 8-Br-cGMP, indicating cGMP to be the actual mediator of NO effects. In addition, studies with various specific inhibitors of either protein kinase A or G kinases exclude a role of the first enzyme and appear to be consistent with the involvement of the second enzyme. Attempts to reveal G kinase I in our cells by Western blot analysis have remained unsuccessful, however, since the levels of expression in NIH-3T3 cells appear to be low (<1 μg/mg of total cell protein[2]).

Ca^{2+} release from intracellular stores and the subsequent opening of SDCs are well known components of the intracellular response generated by growth factor receptor activation (9, 17). In contrast, SMOC activation, a widespread mechanism of Ca^{2+} influx described for G protein-coupled receptors (15–17, 30, 31), had never been investigated. Thus, to our knowledge, this is the first demonstration that activation of SMOCs occur on growth factor receptor stimulation.

Two important properties of growth factor-activated SMOCs emerge from our results. The first is the facilitatory effect exerted by NO on these channels. Indirect evidence suggested a similar role for NO in the activation of carbachol-induced Ca^{2+} influx in pancreatic acinar cells (23), whereas no detectable modulation was revealed on muscarinic-, thrombin-, and ADP-coupled SMOCs in PC12 cells and in platelets (19, 22). The second property is that both EGF- and PDGF-operated channels are permeable to Mn^{2+}, a characteristic of some (17, 19, 31) but not all (16, 17) SMOCs. These differences in sensitivity to NO and in Mn^{2+} permeability are not surprising. SMOCs comprise a highly heterogeneous family of channels that are still largely obscure in terms of regulation by second messengers, sensitivity to physiological modulators, and ion permeability (15, 17).

The facilitating effect of NO on Ca^{2+} influx through SDCs observed in EGFR-T17 cells confirms recent observations in pancreatic acinar cells (21). In both cases, the effect of NO was mediated by cGMP formation and G kinase I activation. NO was recently reported to increase tyrosine phosphorylation in murine fibroblasts by a cGMP-dependent mechanism (38). In addition, increasing evidence supports the notion that tyrosine kinases play a crucial role in the activation of SDCs in a variety of cell systems (e.g., see Ref. 39). Taken together, these observations suggest a possible pathway by which NO activates SDCs. This mechanism, however, might not be the only one activated by NO. In PC12 cells, NO has been reported to facilitate SDC independent of cGMP formation (22). In addition, indirect observations in human platelets have shown that nitrovasodilators exert an inhibitory rather than a stimulatory action on SDCs (19). Divergences in NO effects are in line with accumulating evidence suggesting heterogeneity of the SDCs in different cells (16, 17, 26), despite the fact that they sustain an almost ubiquitous Ca^{2+} influx system with activation strictly dependent on the emptying of intracellular Ca^{2+} stores, independent of the mechanism that triggered the emptying process (17).

The effect of NO on the other type of $[Ca^{2+}]_i$ increasing process stimulated by growth factors, i.e., Ca^{2+} release from intracellular stores, appears to be similar to that observed when Ca^{2+} release is elicited by activation of G protein-coupled receptors (18–20). In the case of G protein-coupled receptors, the mechanism of the inhibition was suggested to be mediated by G kinase activation (19) with inhibition of either the phosphatidylinositol-4,5,-bisphosphate hydrolyzing enzymes, the phospholipases Cβ (20), or the G protein/phospholipase C interaction (40). However, no direct phosphorylation at the level of phospholipases or G proteins have been reported. The phospholipase C isoenzymes stimulated at the growth factor receptors are not of the β but rather are of the γ family and therefore not activated by G protein interaction but rather by direct tyrosine phosphorylation (9, 10). It would be interesting to determine whether the effect of NO on the growth factor-induced Ca^{2+} release is mediated via inhibition of the ability of phospholipases Cγ to function, in analogy with what has been suggested for the Cβ isoenzymes.

The observations that growth factors, by increasing $[Ca^{2+}]_i$, can enhance endogenous cGMP formation, presumably via the activation of constitutive and Ca^{2+}-dependent NOS, add evidence to the notion that the signal transduction pathways involving NO and Ca^{2+} as second messengers are strictly and mutually interconnected at the physiological level. Increased NO generation by growth factors appears to be followed by a negative modulation of intracellular Ca^{2+} release, which might act as a feedback controller of the NO-generating pathway. The concomitant stimulation of Ca^{2+} influx through SDCs (and SMOCs), a process mainly aimed at correct refilling of the stores (16, 17), might ensure their ability to function when further stimulations are delivered.

In conclusion, the present pharmacological observations indicate that the role of NO in the control of Ca^{2+} homeostasis is broader than previously envisaged as it includes not only the processes activated by G protein-coupled receptors but also those activated by growth factor receptors. Most studies carried out on growth and differentiation have been focused primarily on the final effects of NO rather than on the underlying mechanisms. The present data, taken together with previous reports suggesting a role for Ca^{2+} in the control of cell proliferation (11–14), suggest a possible mechanism by which the regulatory role of NO could be mediated.

Acknowledgments

The continuous support and critical suggestions of J. Meldolesi and the technical assistance of G. Racchetti are gratefully acknowledged. We thank D. Pöhler and U. Walter for the Western blot analysis of G kinase expression. We also thank F. Clementi, A. Malgaroli, L. Vallar, G. Bagetta, and E. K. Rooney for helpful discussion and careful reading of the manuscript.

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