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Nitric Oxide Modulation of Agonist-Evoked Intracellular Ca^{2+} Release in Neurosecretory PC-12 Cells: Inhibition of Phospholipase C Activity via Cyclic GMP-Dependent Protein Kinase I

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SUMMARY

Nitric oxide is a signaling molecule involved in events crucial to neuronal cell function, such as neurotransmitter release, gene transcription, and neurotoxicity, i.e., a number of processes in which a key role appears to be played by increases in intracellular Ca^{2+} concentration. In the neurosecretory/neuronal cell line PC-12, we have investigated the role of nitric oxide in the modulation of Ca^{2+} release from intracellular stores elicited by activation of three different receptors coupled to phosphatidylinositol-4,5-bisphosphate hydrolysis, i.e., the purinergic P_{2U}, muscarinic M3, and bradykinin B_2 receptors. The results obtained show that nitric oxide donors have an inhibitory effect on agonist-evoked Ca^{2+} release. This effect is not due to nitric oxide-induced modifications of Ca^{2+} storage, because the total releasable Ca^{2+} pool, measured as the radioactivity released by thapsigargin and ionomycin in cells loaded at equilibrium with $^{45}Ca^{2+}$, was unchanged. In contrast, nitric oxide donors decreased agonist-evoked inositol-1,4,5-trisphosphate generation and total inositol phosphate accumulation. Similarly, nitric oxide inhibited total inositol phosphate accumulation stimulated by either aluminium fluoride or Ca^{2+}. All of these effects were mimicked by the cGMP analogue 8-bromo-cGMP. When cells were incubated with nitric oxide synthase inhibitors, the results observed were opposite those produced by nitric oxide donors. All of the effects of nitric oxide were abolished when cells were treated with the cGMP-dependent protein kinase I inhibitor KT5823. Furthermore, KT5823 mimicked the effects of nitric oxide synthase inhibitors. We conclude that nitric oxide and Ca^{2+} signaling pathways are interconnected in PC-12 cells. Modulation of inositol phosphate generation and Ca^{2+} release by nitric oxide appears to be exerted primarily at the level of phospholipase C functioning and to be mediated by the activation of cGMP-dependent protein kinase I.

NO is a short-lived, highly reactive radical, originally identified as both a mediator in vasodilation (1) and an active agent in macrophage cytotoxicity (2). In addition, NO has been shown to function as a neuronal messenger involved in crucial physiological events such as neurotransmitter release (3), long-term potentiation (4), and gene transcription (5, 6), as well as in the pathophysiological events underlying neurotoxicity (7, 8). Another key messenger, Ca^{2+}, and its signaling pathways are deeply involved in these as well as many other neuronal processes (9). A detailed characterization of the relationship between NO synthesis and Ca^{2+} homeostasis would therefore be important to better understand the intricate network of intraneuronal signaling.

Among the multiple mechanisms through which Ca^{2+} exerts its actions in neurons, a relevant role is played by its release from intracellular stores. Such a process is triggered by the activation of membrane receptors coupled, via heterotrimeric G proteins of the G_q family (10), to PIP_2 hydrolysis with IP_3 generation. Previous studies on platelets and vascular smooth muscle cells have revealed that NO exerts an inhibitory action on agonist-evoked Ca^{2+} release from intracellular stores (11–14). This effect has been shown to be mediated by activation of soluble guanylyl cyclase (15), with increased cGMP formation, inhibition of PIP_2 hydrolysis,

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ABBREVIATIONS: NO, nitric oxide; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; PLC, phospholipase C; Tg, thapsigargin; Iono, ionomycin; SNAP, *S*-nitroso-*N*-acetylpenicillamine; L-NIO, L-*N*-(1-iminoethyl)ornithine; SNP, sodium nitroprusside; D-NAME, N^{ω}-nitro-D-arginine methyl ester; L-NAME, N^{ω}-nitro-L-arginine methyl ester; CCh, carbachol; BK, bradykinin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; KRH, Krebs-Ringer-HEPES; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; IP, inositol phosphates.

reduced IP_3 generation (16–18), and ultimate impairment of Ca^{2+} release from the IP_3-sensitive Ca^{2+} stores. In Chinese hamster ovary cells, the inhibitory effect of cGMP has been demonstrated to be exerted through the activation of cGMP-dependent protein kinase I, an enzyme present in the cytosol of virtually all cell types (13, 19, 20). The precise molecular level at which and the mechanism by which the IP_3/Ca^{2+} signaling pathway is inhibited are, however, still unclear. In this respect, the only existing data are indirect observations in aorta smooth muscle cells (18) and platelets (12), suggesting that the ability of cGMP (and in turn of NO) to reduce PIP_2 hydrolysis could result from an inhibition of the interaction between activated G proteins and PLC(s).

In the present study we have investigated the mechanism through which NO modulates IP_3-induced Ca^{2+} release triggered by different membrane receptors coupled to the G protein/PIP_2 hydrolysis signal transduction pathway. In particular, we have focused on Ca^{2+} release subsequent to activation of the purinergic P_{2U} receptors (21), the muscarinic M3 receptors (22), and the BK B_2 receptors (23) in PC-12 cells. This cell line, which originated from a rat pheochromocytoma (24), is commonly used as a neurosecretory/neuronal cell model. The results obtained demonstrate for the first time that the inhibitory effect of NO on Ca^{2+} release is exerted primarily at the level of PLC function and is mediated through activation of cGMP-dependent protein kinase I.

Experimental Procedures

Materials. Culture sera and media were purchased from GIBCO (Basel, Switzerland), fura-2, KT5823, Tg, and Iono from Calbiochem (San Diego, CA), and SNAP and L-NIO from Alexis (Laufelfingen, Switzerland). $^{45}Ca^{2+}$ and *myo*-[2-^{3}H]inositol were purchased from Amersham International, and SNP, D-NAME, L-NAME, CCh, UTP, BK, 8-bromo-cGMP, and the remaining chemicals were from Sigma-Aldrich (Milano, Italy).

PC-12 cell clone selection and culture. Isolation of PC-12 cell clones has been described elsewhere (23). For the purpose of the present study, PC-12 cell clone 64 was chosen. This particular clone is characterized by high responsiveness of the muscarinic receptor, in terms of $[Ca^{2+}]_i$ variations (25). As with other clones we have isolated, clone 64 does not express a functional nicotinic receptor and is also devoid of functional intracellular ryanodine receptors (23). In the present studies, PC-12-64 cells were routinely grown as described previously (25) and were used before week 10 after thawing. On the day of the experiment, cell monolayers were detached from Petri dishes by a gentle flow of KRH medium (125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 6 mM glucose, 0.8 mM L-arginine, 25 mM HEPES-NaOH, pH 7.4). Cells were washed three times by centrifugation and were resuspended in KRH medium. Viability in the presence or absence of the various drugs used was >95%, as assessed by the trypan blue exclusion test. Incubation with L-NAME, D-NAME, L-NIO, SNP, SNAP, 8-bromo-cGMP, or KT5823 did not alter cell viability.

$[Ca^{2+}]_i$ measurements. Cell monolayers, detached and washed as described above, were resuspended, loaded with the Ca^{2+}-sensitive dye fura-2 in KRH medium as described (25), and kept at 37° until use. Cell aliquots (4×10^6 cells) were finally transferred to a thermostat-equipped cuvette in a Perkin Elmer LS-5B fluorimeter (37°), where they were maintained with continuous stirring. Preincubations with the various drugs interfering with the NO pathway (L-NAME,D-NAME, L-NIO, SNP, SNAP, 8-bromo-cGMP, and KT5823) were carried out for 15 min at 37°, conditions previously demonstrated to be sufficient for maximal drug effects in PC-12 cells (6). One minute before addition of the Ca^{2+}-releasing agents (CCh, BK, Tg, or UTP), the samples were supplemented with excess EGTA (2 mM) (estimated $[Ca^{2+}]_o$, $<10^{-8}$ M), a condition known to leave only Ca^{2+} release from intracellular stores being detectable. Traces were recorded until the end of the fluorescence peak and were analyzed as described (23). Results shown are averages ± standard deviations of eight to 10 separate experiments.

$^{45}Ca^{2+}$ measurements. PC-12 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 extensively washed and resuspended in KRH medium. An aliquot of the suspension was immediately centrifuged, and the ensuing pellet was used for the measurement of total cell $^{45}Ca^{2+}$ content. The rest of the suspension was incubated at 37° in EGTA-containing, Ca^{2+}-free, KRH medium. At different times, aliquots of 1×10^6 cells were centrifuged, and the $^{45}Ca^{2+}$ recovered in the medium was assayed in a Beckman β counter (for additional details, see Ref. 26). Results shown are means ± standard deviations of four separate experiments.

IP measurements. Cells were labeled for 48 hr with 2 μCi/ml *myo*-[2-^{3}H]inositol in basal Eagle's diploid modified medium containing 7% inositol-free fetal calf serum and 7% inositol-free horse serum. They were subsequently detached, washed, and resuspended in KRH medium. The cell suspensions were preincubated for 15 min at 37° with the various drugs interfering with the NO pathway (see above), in the presence (for total IP measurements) or in the absence (for IP_3 measurements) of 20 mM LiCl. Aliquots of 1×10^6 cells were then challenged with either UTP, Iono, or AlF_4^- and the reaction was stopped at the indicated times by the addition of ice-cold formic acid (20 mM final concentration). Samples were kept on ice for 30 min and then centrifuged, and the supernantants were loaded onto anion exchange columns. Radioactive total IP or IP_3 was isolated by stepwise elution as described (27), and radioactivity was counted in a Beckman β counter. Results shown (means ± standard deviations of four to eight independent experiments) were all obtained in Ca^{2+}-containing KRH medium. Control experiments in which total IP and IP_3 production were stimulated in Ca^{2+}-free KRH medium gave similar results.

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 0.6 mM isobutylmethylxanthine, with or without L-NAME (300 μM). NO synthase activity was stimulated by exposing cell suspensions to UTP (100 μM) for 1 min at 37°. The reaction was terminated by addition of ice-cold trichloroacetic acid (final concentration, 7.5%). After ether extraction, cGMP levels were measured using a radioimmunoassay kit (Amersham) and were normalized to cellular protein levels, which were determined using the bicinchoninic acid assay (bicinchoninic acid protein assay reagent; Pierce). Results described are means ± standard deviations of three experiments.

All of the results shown below are from experiments in which L-NAME was used as a NO synthase inhibitor and SNP was used as a NO donor. Qualitatively similar results were obtained using the NO synthase inhibitor L-NIO and the NO donor SNAP. Experiments were also performed with the less active enantiomer of L-NAME, i.e.,D-NAME; the results obtained did not differ significantly from those obtained with the untreated control cells. Statistical analysis of the results was performed with the Student *t* test for unpaired data.

Results

Suspensions of fura-2-loaded PC-12–64 cells were incubated for 15 min at 37° in KRH medium with or without the NO synthase inhibitor L-NAME (300 μM), the NO donor SNP (100 μM), or the stable cGMP analogue 8-bromo-cGMP (200 μM). They were then supplemented with excess EGTA (see Experimental Procedures) shortly before being challenged

with increasing concentrations of either UTP, BK, or CCh, agonists of the P_{2U}, B_2, and M3 receptors, respectively (21–23). Under these experimental conditions $[Ca^{2+}]_o$ is very low and Ca^{2+} influx is precluded; agonist-induced changes in $[Ca^{2+}]_i$ can therefore be attributed to Ca^{2+} release from intracellular stores (13, 23, 25). Pretreatment of PC-12-64 cells with L-NAME potentiated Ca^{2+} release induced by all three receptor agonists, whereas SNP exerted an inhibitory effect with respect to untreated control cells (Fig. 1). When the cell suspensions were preincubated with 8-bromo-cGMP, the effect observed was analogous to that of SNP (Fig. 1).

The facilitatory action of L-NAME and the inhibitory effects of SNP and cGMP were similar in extent, regardless of which receptor agonist was used to elicit intracellular Ca^{2+} release and independently of the amount of cation released (Fig. 1). This finding suggests at least two hypotheses (not necessarily alternative), (i) that the effect of NO on Ca^{2+} release in PC-12-64 cells is exerted somewhere along the common signal transduction pathway of PIP_2 hydrolysis-coupled receptors, as previously suggested for other cell types (12, 18), or (ii) that NO somehow interferes with the ability of PC-12 cells to store up Ca^{2+}, possibly by reducing the pumping activity of the endoplasmic/sarcoplasmic reticulum Ca^{2+}-ATPases or by increasing basal leak from the stores. In PC-12 cells, exchangeable Ca^{2+} is known to be stored in three major Ca^{2+} pools, (i) the IP_3-sensitive Ca^{2+} store, which is a subcompartment of the Ca^{2+} pool contained in the endoplasmic reticulum (this pool is also sensitive to ryanodine, provided that the cells express the corresponding receptor) (23), (ii) an endoplasmic reticulum-located Ca^{2+} pool that is insensitive to IP_3, and (iii) a large, as yet unidentified, pool that can be discharged by Ca^{2+}-specific ionophores (26).

To investigate whether NO could somehow affect Ca^{2+} storage in these pools, the experiments described in Fig. 2 were performed. PC-12-64 cells were loaded to equilibrium with $^{45}Ca^{2+}$, pretreated with L-NAME (300 μM) or SNP (100 μM) as described above, and challenged in sequence with UTP (to release Ca^{2+} stored in the IP_3-sensitive Ca^{2+} store), then with Tg (an irreversible blocker of the endoplasmic/sarcoplasmic reticulum Ca^{2+}-ATPases that induces leakage and emptying of Ca^{2+} from the entire endoplasmic reticulum within a few minutes) (28), and finally with Iono (an electroneutral ionophore that releases Ca^{2+} from all stores except those with an acidic luminal environment) (26). As can be seen in Fig. 2A, pretreatment with L-NAME increased and pretreatment with SNP decreased the amount of $^{45}Ca^{2+}$ released by UTP (100 μM). In contrast, the amount of labeled cation released by the administration first of Tg (100 nM) and then of Iono (1 μM) was unchanged by either pretreatment. Moreover, no effect of either L-NAME or SNP was observed on the basal leak of Ca^{2+}, which was assayed for >25 min before the addition of the Ca^{2+}-releasing drugs. To further confirm these findings, Tg-induced Ca^{2+} release was directly assayed also in fura-2-loaded cells; again, no significant difference was observed in cells pretreated with L-NAME or SNP, in comparison with untreated control cells (Fig. 2B).

We then investigated whether the effects of L-NAME and SNP on Ca^{2+} release could result from a modulatory effect of NO on PIP_2 hydrolysis. To this end we measured total IP accumulation and IP_3 production induced by UTP, BK, or CCh in cells pretreated or not, as described above, with L-NAME (300 μM), SNP (100 μM), or 8-bromo-cGMP (200 μM). Fig. 3A shows the results obtained with UTP. Pretreatment (15 min at 37°) with L-NAME potentiated the total IP accumulation stimulated by increasing concentrations of the agonist (for an additional 15 min, in the presence of 20 mM LiCl), whereas both SNP and 8-bromo-cGMP exerted an inhibitory effect. Similar results were observed when the time course of IP_3 production, generated after administration of 30 μM UTP, was assayed (Fig. 3B). The results obtained with BK and CCh were consistent with those obtained with UTP (data not shown). These findings suggest that the effect of NO is directed somewhere along the signal transduction pathway common to the three receptors, upstream of PIP_2 hydrolysis and IP_3 generation.

To further localize the site of NO inhibition, experiments

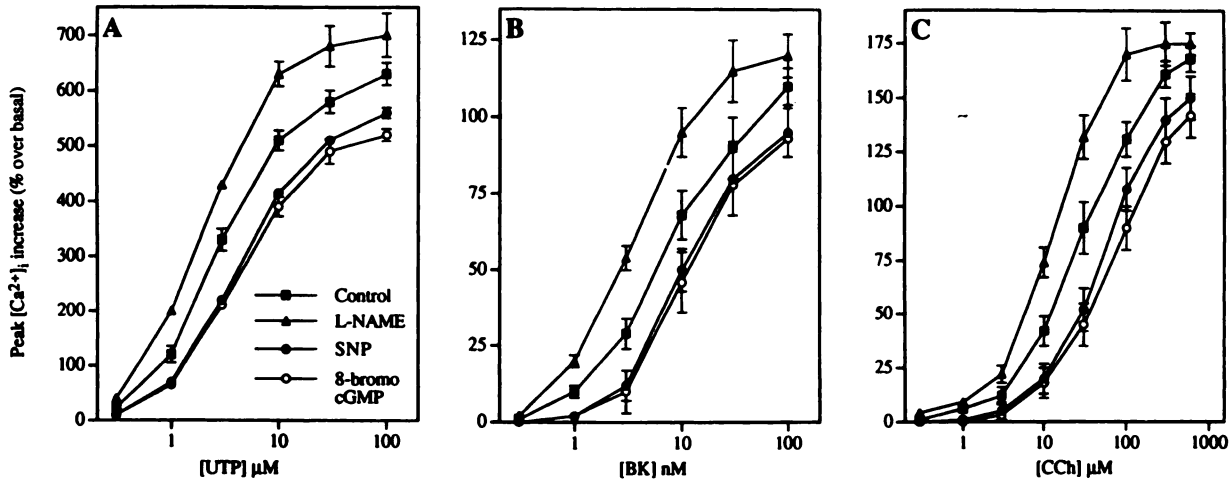

Fig. 1. Effects of L-NAME, SNP, and 8-bromo-cGMP on agonist-evoked Ca^{2+} release. Fura-2-loaded PC-12–64 cell suspensions were incubated for 15 min at 37° in KRH medium alone (*Control*) or supplemented with L-NAME (300 μM), SNP (100 μM), or 8-bromo-cGMP (200 μM). Cell aliquots were then challenged, in Ca^{2+}-free KRH medium, with increasing concentration of either UTP (A), BK (B), or CCh (C). Values are expressed as percentage increase over basal resting $[Ca^{2+}]_i$ (on average, 110 ± 25 nM). The graphs show the results of eight to 10 experiments (means ± standard deviations). Statistical analysis of the results showed that the differences observed between cells treated with L-NAME or SNP (or 8-bromo-cGMP) and controls were significant in all experiments ($p < 0.01$), whereas those observed between SNP- and 8-bromo cGMP-treated cells were not.

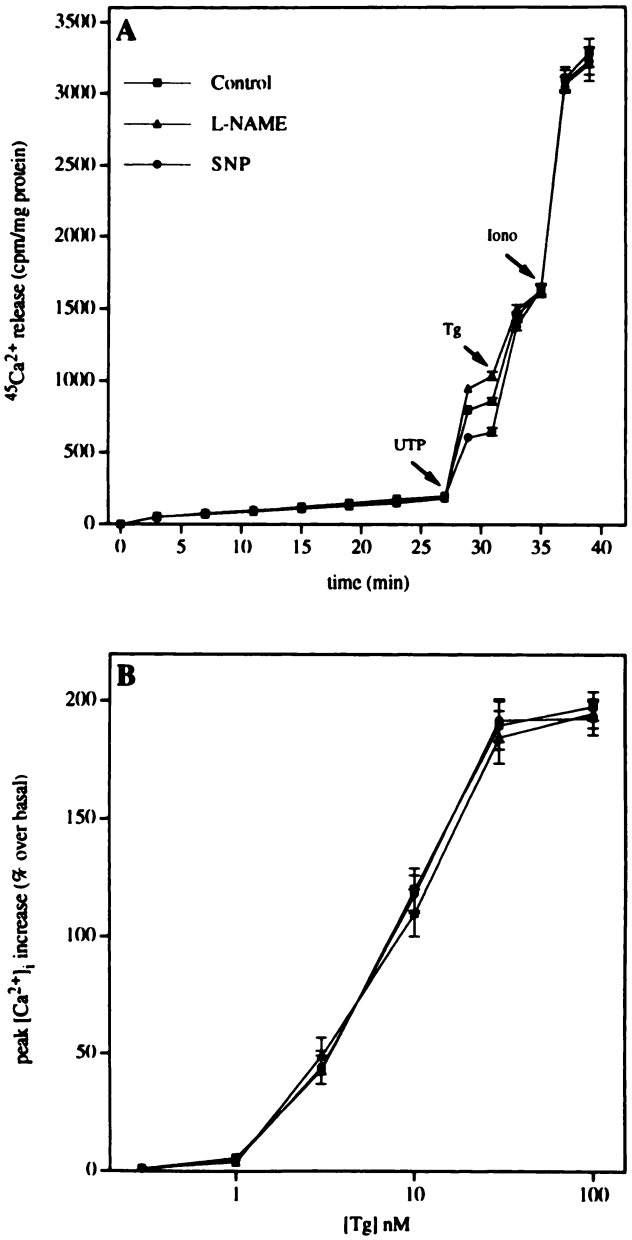

Fig. 2. Effects of L-NAME and SNP on the release of Ca^{2+} from the various intracellular Ca^{2+} pools. A, PC-12-64 cells were loaded at equilibrium with $^{45}Ca^{2+}$, as described in Experimental Procedures, and were then incubated for 15 min at 37° in KRH medium alone (*Control*) or supplemented with L-NAME (300 μM) or SNP (100 μM). After addition of excess EGTA (Ca^{2+} free-medium), basal $^{45}Ca^{2+}$ leak was analyzed before challenge of the cells by sequential addition of UTP (100 μM), Tg (100 nM), and Iono (1 μM) as indicated. Results illustrated are the averages ± standard deviations of four experiments. The differences observed in the amount of $^{45}Ca^{2+}$ released by UTP in controls, compared with L-NAME- and SNP-treated cells, were statistically significant ($p < 0.001$). B, Fura-2-loaded PC-12-64 cells that had been preincubated for 15 min with KRH medium alone or supplemented with L-NAME or SNP, as in Fig. 1, were challenged, in Ca^{2+}-free KRH medium, with increasing concentrations of Tg. The graph shows the mean ± standard deviation of percentage $[Ca^{2+}]_i$ increases over basal levels in eight experiments. The differences between cells treated with L-NAME or SNP and controls were not statistically significant ($p > 0.33$).

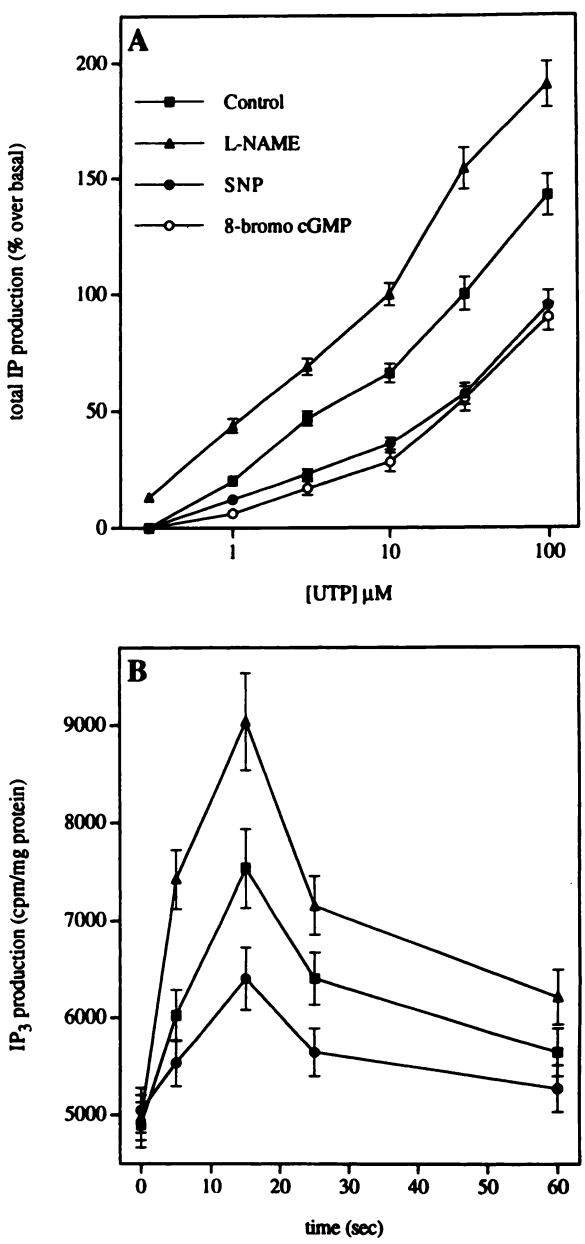

Fig. 3. Effects of L-NAME, SNP, and 8-bromo-cGMP on UTP-induced total IP accumulation and IP_3 generation. PC-12-64 cells that had been labeled with 2 μCi/ml *myo*-[2-^{3}H]inositol were incubated for 15 min at 37° in KRH medium alone (*Control*) or supplemented with L-NAME (300 μM), SNP (100 μM), or 8-bromo-cGMP (200 μM), as described in Experimental Procedures. A, Total IP accumulation measured in cells challenged with increasing concentrations of UTP for 15 min in the presence of 20 mM LiCl. The average basal radioactivity of total IP, here and in the experiments of Figs. 4, 5, and 6, was 15 ± 2.3 × 10^3 cpm/mg of protein. No appreciable difference in basal radioactivity was observed between cell preparations treated or not with L-NAME, SNP, or 8-bromo-cGMP. B, IP_3 generation induced by 30 μM UTP, monitored for 60 sec after UTP addition. Results are expressed as percentage increase of radioactivity over basal levels (A) or as cpm/mg of protein (B). Graphs show the results obtained in eight independent experiments (averages ± standard deviations). Analysis of the results of A and B showed a statistically significant difference between the cells treated with L-NAME or SNP (or 8-bromo-cGMP) and the controls ($p < 0.005$). The differences observed between SNP- and 8-bromo-cGMP-treated cells were not statistically significant ($p > 0.1$).

similar to those of Fig. 3A were performed using AlF_4^- as the stimulant. AlF_4^- has been demonstrated to activate PLCs and to induce PIP_2 hydrolysis by binding directly to virtually all G proteins, thus bypassing agonist receptors (29). As shown in Fig. 4A, *left*, pretreatment with L-NAME (300 μM) potentiated total IP accumulation stimulated by 20 mM AlF_4^-, whereas both SNP (100 μM) and 8-bromo-cGMP (200 μM) were inhibitory. Similar results were obtained when the cells were not preincubated with the NO-modulating drugs before AlF_4^- addition but were challenged for 15 min with AlF_4^- alone or together with L-NAME, SNP, or 8-bromo-cGMP (Fig. 4A, *right*).

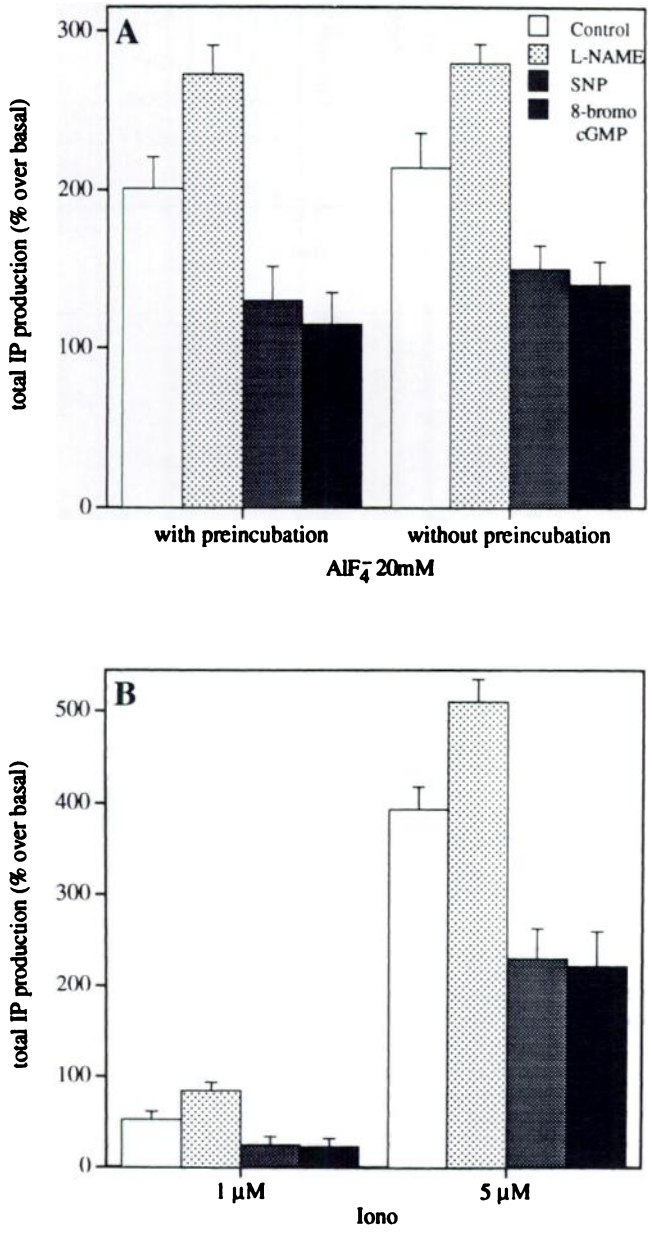

Fig. 4. Effects of L-NAME, SNP, and 8-bromo-cGMP on total IP accumulation induced by AlF_4^- (A) and Iono (B). Experimental details were as in Fig. 3A. A, *Left*, cells were preincubated for 15 min with or without L-NAME, SNP, or 8-bromo-cGMP before AlF_4^- stimulation; *Right*, preincubation was omitted, and PC-12-64 cells were incubated with AlF_4^- alone or together with L-NAME (300 μM), SNP (100 μM), or 8-bromo-cGMP (200 μM). The graphs show the mean ± standard deviation of percentage increases in total IP accumulation over basal levels from eight separate experiments. Analysis of the results showed the difference between the cells treated with L-NAME or SNP (or 8-bromo-cGMP) and the controls to be statistically significant ($p < 0.001$).

We next investigated total IP accumulation in cells in which PLC activity was stimulated directly. All PLC isoenzymes characterized so far are known to be activated by persistently high $[Ca^{2+}]_i$ (10^{-6} to 10^{-5} M) (30–33). The PLC activity in PC-12–64 cells, preincubated or not with L-NAME, SNP, or 8-bromo-cGMP as described above, was therefore stimulated with concentrations of Iono (1 or 5 μM, for 15 min) that gave rise to persistently high $[Ca^{2+}]_i$ (data not shown) (26). Also in this case, preincubation with L-NAME (300 μM) yielded higher levels of total IP accumulation, in comparison with untreated control cells, whereas SNP (100 μM) and 8-bromo-cGMP (200 μM) consistently reduced total IP accumulation, with both concentrations of Iono tested (Fig. 4B). Taken together, these findings strongly suggest that the primary site at which NO affects the Ca^{2+} release responses induced by receptor activation is PLC.

It is known that NO induces increases in cytosolic cGMP levels and can therefore act through cGMP-dependent protein kinase activation. Because the effects of SNP on $[Ca^{2+}]_i$ and IP accumulation were in all experiments paralleled by qualitatively similar effects when 8-bromo-cGMP was applied, we investigated whether, in our cells, the effect of NO on PLCs was exerted through cGMP formation and cGMP-dependent protein kinase activation. To this end we performed experiments in which PC-12-64 cells were preincubated with or without SNP (100 μM) together with KT5823 (10 μM), a widely used selective inhibitor of cGMP-dependent protein kinase I (20, 34). When the cells were preincubated with SNP together with KT5823, the inhibitory effect of SNP on UTP-, BK-, and CCh-induced Ca^{2+} release was almost completely abolished (Fig. 5A and data not shown). Similarly, the effect of SNP on total IP accumulation, triggered by 15-min stimulation with either UTP, Iono, or AlF_4^-, was inhibited to a great extent when the NO donor was applied together with KT5823 (Fig. 5B). These findings strongly suggest that the inhibitory effect of NO on PLC activity is mediated primarily by cGMPdependent protein kinase activation.

In additional experiments, KT5823 was administered alone or in combination with L-NAME. Fig. 6 shows the results obtained with UTP as a receptor agonist. As can be seen, the results obtained with PC-12-64 cells preincubated with KT5823 (15 min at 37°) mimicked those observed after a similar incubation with L-NAME (300 μM), when both UTP-induced Ca^{2+} release (Fig. 6A) and total IP production (Fig. 6B) were analyzed. When KT5823 and L-NAME were administered together, no additive effect was observed. Taken together, the findings of Figs. 5 and 6 strongly suggest the existence in PC-12-64 cells of ongoing feedback by which NO, presumably produced after increases in $[Ca^{2+}]_i$ induced by activation of cell surface receptors, modulates the signal transduction of membrane receptors coupled to PIP_2 hydrolysis. This hypothesis was further confirmed by the observation that intracellular cGMP formation, a marker of endogenous NO synthase activity, was increased by 1-min stimulation of PC-12-64 cells with UTP (100 μM), with respect to unstimulated control cells (9.32 ± 0.42 and 3.57 ± 0.27 pmol/mg of protein, respectively). The effect of UTP on cGMP formation was completely abolished when cells were preincubated (15 min at 37°) with L-NAME (300 μM).

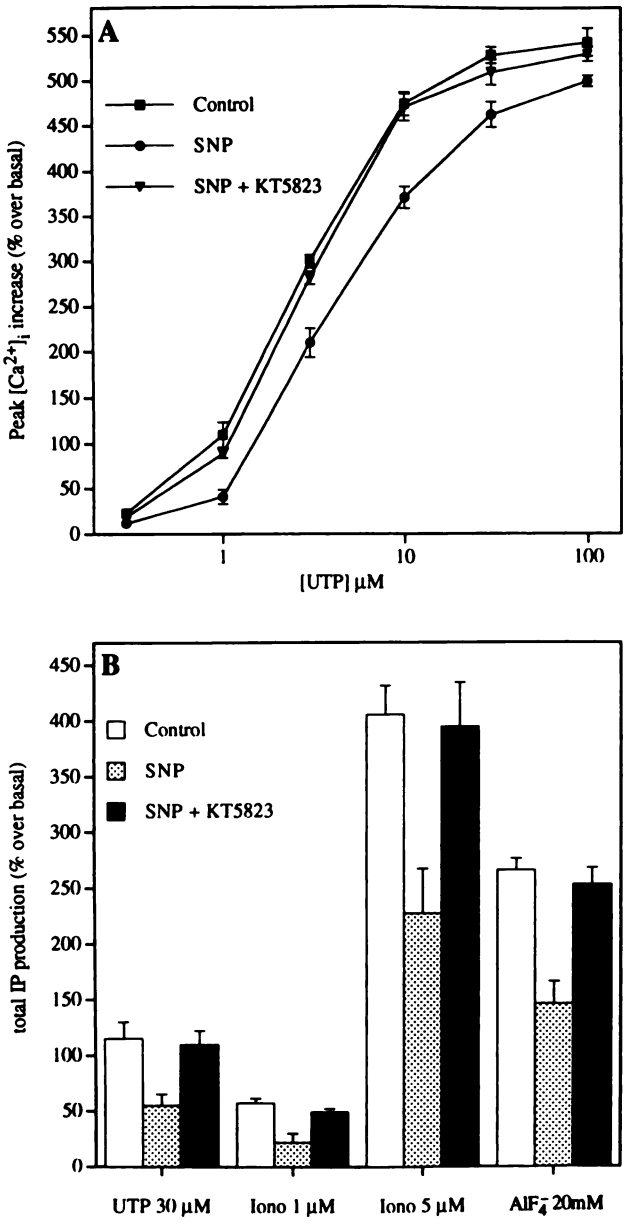

Fig. 5. Effects of the cGMP-dependent protein kinase inhibitor KT5823 on SNP-induced variations in $[Ca^{2+}]_i$ and total IP production elicited by UTP, Iono, and AlF_4^-. A, Fura-2-loaded PC-12-64 cells that had been preincubated for 15 min at 37° with KRH medium alone or supplemented with SNP (100 μM) or SNP plus KT5823 (10 μM) were challenged, in Ca^{2+}-free KRH medium, with increasing concentration of UTP. The graph shows the mean ± standard deviation of percentage increases in $[Ca^{2+}]_i$ over basal levels from nine experiments. B, PC-12-64 cells that had been labeled with 2 μCi/ml *myo*-[2-^{3}H]inositol were incubated for 15 min at 37° in KRH medium alone or supplemented with SNP or SNP plus KT5823, as in A, and were then challenged with UTP, Iono, or AlF_4^- at the indicated concentrations. Results shown are the mean ± standard deviation of percentage increases of radioactivity over basal levels from eight experiments. Statistical analysis of the results showed that the differences observed between control cells and cells treated with both SNP and KT5823 were not significant; the differences between control cells and those treated with SNP alone were significant ($p < 0.02$). Experimental details were as in Fig. 3A.

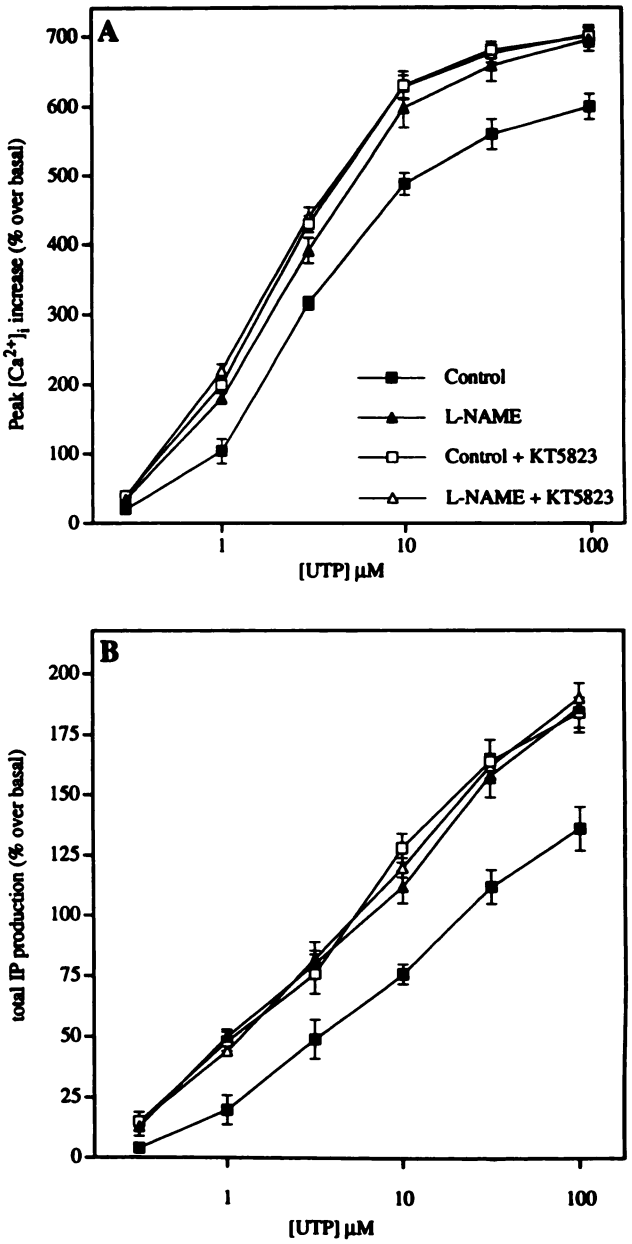

Fig. 6. Effects of the cGMP-dependent protein kinase inhibitor KT5823 on $[Ca^{2+}]_i$ variations and total IP production elicited by UTP in the presence or absence of L-NAME. A, Fura-2-loaded PC-12-64 cells that had been preincubated for 15 min at 37° with KRH medium alone or supplemented with L-NAME (300 μM), KT5823 (10 μM), or a combination of the two were challenged, in Ca^{2+}-free KRH medium, with increasing concentrations of UTP. The graph shows the mean ± standard deviation of percentage increases in $[Ca^{2+}]_i$ over basal levels from four experiments. B, PC-12-64 cells that had been labeled with 2 μCi/ml *myo*[2-^{3}H]inositol were incubated for 15 min at 37° with KRH medium alone or supplemented with L-NAME, KT5823, or both, as in A, and were then challenged with UTP. Results shown are the mean ± standard deviation of percentage increases of radioactivity over basal levels from four experiments. The differences between cells treated with L-NAME, L-NAME and KT5823, or KT5823 alone were not statistically significant. The differences between these groups and the controls were, in contrast, significant ($p < 0.01$). Experimental details were as in Fig. 3A.

Discussion

The present results document a regulatory role of NO on Ca^{2+} release in the neuroendocrine PC-12 cells, which was investigated by parallel analysis of the effects of NO synthase inhibitors and NO donors. The specificity of these results is demonstrated by the fact that, when the cells were preincu-

bated with NO synthase inhibitors, the effects observed were opposite those induced by treatment with NO donors. Moreover, this latter finding indicates that in PC-12 cells NO synthase is physiologically active and that endogenous NO plays a role in modulating the complex signaling response subsequent to receptor activation.

In this study we show that preincubation with NO donors inhibits Ca^{2+} release from intracellular stores elicited by three distinct membrane receptors and that this effect is accompanied by reductions in IP_3 generation and total IP accumulation. The effect of NO donors is qualitatively similar to that induced by the stable cGMP analogue 8-bromo-cGMP. These findings demonstrate the occurrence, in a classical nerve cell model, of NO and cGMP effects previously observed only in platelets and smooth muscle fibers. In these latter cells NO was demonstrated to exert its effect by increasing intracellular cGMP levels (11–14, 16–18). Until now, however, the site(s) of action of NO/cGMP in the signaling cascade leading to Ca^{2+} release had not been precisely identified.

When our cells were stimulated with AlF_4^-, a nonspecific activator of all known heterotrimeric G proteins (29), the inhibitory effect of NO on IP accumulation was maintained, suggesting an action distal to receptor-agonist binding and receptor-G protein coupling. In parallel, we observed the same inhibitory effect of NO when the PLC activity was directly stimulated by high $[Ca^{2+}]_i$. Effects of NO and cGMP similar to those we observed with AlF_4^- were previously reported in activated human platelets (12). In that case, however, the effect of NO/cGMP on IP generation disappeared when the PLC activity was stimulated directly, leading the authors to infer a site of action located at the G protein/PLC interface. Quite different is the situation in our neurosecretory cell model; although we cannot exclude an effect on G proteins, our results clearly document that the major inhibitory action of NO is on PLC activity.

Because all PLC isoenzymes can be activated by increasing concentrations of $[Ca^{2+}]_i$ (see Refs. 30–33 for PLC-β_1/PLC-β_2/PLC-β_3, PLC-γ_1, PLC-γ_2, and PLCδ_1, respectively), studies are in progress to elucidate whether the effect of NO is specific for the phospholipases that are believed to be activated by our receptors, i.e., the PLC-β forms (35), or also involves other PLC isoforms. In addition, it would be interesting to establish whether, among nerve cells, the role in PLC modulation is restricted to PC-12 cells or is more general. These studies could shed new light on the possible involvement of NO in critical phenomena of cell life, such as growth and differentiation. Based on studies in PC-12 cells, as well as in other cell types, a modulatory role for NO was indeed recently suggested (6, 36, 37).

Another important finding emerging from our data is that the NO effect reported herein is mediated through the activation of cGMP-dependent protein kinase I. This conclusion is supported by the observation that the specific enzyme inhibitor KT5823 (20, 34) counteracts the action of SNP at the level of both IP production and Ca^{2+} release. It might be suggested that at least part of the inhibitory effect of SNP on Ca^{2+} release can arise from a partial emptying of the IP_3-sensitive Ca^{2+} stores due to cGMP-dependent synthesis of cADP-ribose and subsequent ryanodine receptor opening (38). This is, however, unlikely, because the Ca^{2+} content of both the Tg- and Iono-sensitive pools was unaffected by 15-min incubation with SNP. Moreover, the particular PC-12 clone used in the present study (clone 64) is devoid of functional ryanodine receptors (23). Whether cGMP-dependent protein kinase I phosphorylates PLC(s) directly or phosphorylates a regulatory, as yet unidentified, protein remains to be established. The role of cGMP-dependent protein kinase I in NO physiological actions might go well beyond the regulation of Ca^{2+} homeostasis, because this enzyme is implicated in many other fundamental biological functions, such as growth control, modulation of immune response, Cl^- and K^+ channel opening, synthesis and secretion of hormones, and the control of neurotoxicity (20, 39).

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