Adenylyl Cyclase, a Coincidence Detector for Nitric Oxide*

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Adenylyl cyclase activity by NO.

Nitric oxide (NO) donors inhibit hormone- and forskolin-stimulated adenylyl cyclase activity in purified plasma membrane preparations from N18TG2 neuroblastoma cells. Northern blot analyses indicate that the predominant isoform of adenylyl cyclase in N18TG2 cells is the type VI. Our experiments eliminate all the known regulatory proteins for this isoform as possible targets of NO. NO decreases the V_max of the enzyme without altering the K_m for ATP. Occupancy of the substrate-binding site protects the enzyme from the inhibitory effects of NO, suggesting that the conformation of the enzyme determines its sensitivity. The inhibition is reversed by reducing agents, implicating a Cys residue(s) as the target for nitric oxide and an S-nitrosylation in the underlying modification. These findings implicate NO as a novel cellular regulator of the type VI isoform of adenylyl cyclase.

Nitric oxide (NO) has been attributed roles in a variety of cellular activities throughout the body. For example, NO has been implicated as a regulator of vasodilation, synaptic plasticity, and immune defense. In each case, however, NO has potentially deleterious effects should its production be disturbed, as occurs in excitotoxicity and ischemia (reviewed in Refs. 1–3). To understand the means by which NO achieves its paradoxical effects, and eventually to control its actions, attempts have focused on characterizing the enzymes responsible for NO production (NOS), identifying target molecules that are altered by NO, and specifying the underlying mechanism(s) by which NO alters those targets.

One target of NO is the soluble guanylyl cyclase. It is the subsequent increase in cGMP levels that is believed to mediate NO-induced vasodilation. Given that an activation of soluble guanylyl cyclase occurs almost universally in response to NO, changes in cGMP have received primary consideration as the mechanism by which NO acts. However, the multiplicity of NO actions are unlikely to be explained by a common mechanism and a number of laboratories have suggested alternative targets of NO. Generally, cellular components have been implicated as targets of NO based largely on the ability of NO or NO-releasing compounds to alter their activities in vitro. Thus, whether they are altered in intact cells in response to NO, or what the possible physiological consequences may be, remain speculative. Our approach to discerning how NO functions has been to examine its effects on intact cells. We have previously demonstrated that NO inhibits the production of cAMP pulses in Dictyostelium discoideum and does so independently of any changes in guanylyl cyclase activity (4, 5). The sum of the data indicated that NO specifically alters either a regulatory domain of the adenylyl cyclase itself or a distinct regulatory moiety. We have also observed that the addition of NO gas or NO donor compounds to cultures of N18TG2 neuroblastoma cells inhibits G_s-coupled and forskolin-stimulated cAMP accumulation (6).

NO-mediated inhibition of forskolin-stimulated cAMP production is unaltered by pretreating cells with pertussis toxin, implying that the inhibition did not involve G_i (6). Although experiments using intact cells were able to eliminate a number of potential targets that NO could modulate that would inhibit cAMP production, a number of possible targets still remained. In particular, NO has been proposed to inhibit GAPDH and in so doing limit the cellular levels of ATP (7). Similarly, NO-stimulated poly-(ADP-riboseylation) has been proposed to limit ATP production as a result of decreased availability of NAD (8). If such events were to occur in N18TG2 cells in response to NO, the cell’s ability to produce cAMP could be compromised. NO has also been shown to alter the activity of ion channels (9).

Because a number of adenylyl cyclase isoforms are regulated by Ca^{2+} (10), this could potentially influence the activity of the activity in N18TG2 cells. For example, the type V and VI isoforms are inhibited by Ca^{2+} by what appears to be the binding of the cation directly to the enzyme (11). Thus, to further address the mechanism by which NO regulates cAMP production in N18TG2 cells, and to identify the target of its actions, we have studied the effects of NO on adenylyl cyclase activity in purified plasma membrane preparations. In the present report, we identify the predominant isoform of adenylyl cyclase present in N18TG2 cells and characterize its inhibition by NO in purified plasma membranes. Our findings demonstrate a novel, isoform-specific regulation of adenylyl cyclase activity by NO.

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EXPERIMENTAL PROCEDURES

SNP Pretreatment—Sucrose gradient purified plasma membranes (12) were resuspended in buffer A (50 mM NaHepes, pH 8, 3 mM MgCl_2, 1 mM EDTA) at 0.5 mg/mL. Protein determinations were done according to Bradford (13). Unless indicated otherwise, membranes were preincubated with 3 mM SNP for 30 min on ice. Other reagents were present as described in the individual experiments. Where indicated, membranes were washed free of any additions by diluting them 10-fold in ice-cold buffer A at 0.5 mg/mL.

Adenylyl Cyclase Activity—Adenylyl cyclase activity was assayed by incubating membranes at 30 °C for 20 min and monitoring the conversion of [α-32P]ATP to [γ-32P]cAMP at 30 °C (12, 14, 15). The standard reaction mixture contained 50 mM NaHepes, pH 8, 1 mM EDTA, 10 mM magnesium, 0.1 mM EGTA, and 100 mM NaCl. The reaction was started by the addition of 40 mM forskolin. The accumulation of cAMP was measured by conversion to [γ-32P]cAMP by the addition of 2 mM [γ-32P]ATP to the reaction mixture. The reaction was stopped by the addition of 55% (v/v) ethanol and 0.05% (v/v) of a mixture of adenosine 3′,5′-[(γ-32P)]monophosphate and adenosine 5′-[(γ-32P)]monophosphate. The reaction was then mixed with 100 mM dithiothreitol, 0.1 M EDTA, and 8 M urea and 30% (v/v) sucrose, layered on an 18% (w/v) sucrose gradient, and the gradient was centrifuged at 28,000 rpm for 16 h at 4 °C in a SW28 rotor. After centrifugation, the gradient was read on an Ozyme 1000 imaging system and the activity was plotted as a function of the gradient density.

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§ The abbreviations used are: NO, nitric oxide; CaM, calmodulin; G_s, guanine nucleotide-binding protein that stimulates adenylyl cyclase; G_i, guanine nucleotide-binding protein that inhibits adenylyl cyclase; PDE, phosphodiesterase; NOS, nitric oxide synthetase; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; SNP, sodium nitroprusside; PGE_1, prostaglandin E_1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol; AMP-PNP, adenosine 5′-[β,γ-imino]triphosphate.
NO Regulates Adenylyl Cyclase

RESULTS

NO Inhibits Adenylyl Cyclase Activity in Purified Plasma Membrane Preparations—Plasma membranes were preincubated with increasing concentrations of SNP, after which they were assayed for forskolin-stimulated adenylyl cyclase activity. Forskolin stimulated activity was also measured in the presence of GTP and will be discussed in a later section. As seen in Fig. 1, preincubation of membranes for 30 min with SNP attenuated the response of adenylyl cyclase to forskolin and did so in a concentration-dependent manner. Some inhibition was observed when membranes were preincubated with 0.1 mM SNP while maximum inhibition occurred when SNP was present at approximately 3 mM. The maximum inhibition varied between 50 and 75% with different membrane preparations, a range of inhibition similar to that seen with intact cells (6).

Although in some experiments we did observe an inhibition of basal enzyme activity, in most cases this inhibition was minimal (see figure legends for values in different experiments).

To confirm that the inhibition of forskolin-stimulated adenylyl cyclase activity represented the action of NO and not another product of SNP, we preincubated membranes with a chemically distinct NO donor, SNAP. SNAP also inhibited adenylyl cyclase stimulation by forskolin in a concentration-dependent manner. In general, both NO donors were equally effective in their ability to do so (data not shown). Other experiments indicated that the effects of NO donors were unaltered when membranes were preincubated in the presence of Trollox (0.1 mM) or superoxide dismutase (375 units/ml) plus/minus catalase (100 units/ml) (data not shown). It would appear that NO, rather than an oxidative product, is responsible for the inhibition of adenylyl cyclase activity.

The finding that the effects of NO in intact cells (6) can be faithfully reproduced in purified plasma membrane preparations limits the possible targets of NO to components in those preparations. Experiments using intact cells had indicated that changes in cGMP or PDE did not mediate the inhibition of cAMP accumulation in response to hormone or forskolin (6). Although we do not expect soluble guanylyl cyclase to be present in our membrane preparations, we confirmed the lack of involvement of cGMP by adding cGMP to the adenylyl cyclase reaction mixture. No changes in enzyme activity were observed (data not shown). Activation of PDE cannot account for the decrease in adenylyl cyclase activity in response to NO in purified membrane preparations because assays are performed in the presence a PDE inhibitor specific for the low Km cAMP-PDE isoform that is present in N18TG2 cells (18), and an internal [3H]cAMP standard is used to normalize for any loss of newly generated [32P]cAMP.

NO Inhibits Adenylyl Cyclase Activity without Altering the Regulation of G

Forskolin stimulates adenylyl cyclase by directly binding to the enzyme, and thus by-passes the hormone receptor-Gs protein signal transduction pathway. Although forskolin stimulation does not require GTP, it has been shown that Gs activation can lead to additional stimulation of the enzyme (19). The addition of GTP to the assay increased adenylyl cyclase activity above that seen with forskolin alone (Fig. 1). We generally observed a 15–20% increase in enzyme activity. This was true whether or not membranes had been pretreated with SNP. It would appear from this experiment that there is a population of Gs proteins in these membrane preparations that can be activated by the addition of GTP to enhance adenylyl cyclase activity and that SNP does not alter this ability.

Divalent cations have been shown to regulate the activation of Gs by effecting the GDP/GTP exchange in the absence of a hormone receptor stimulation (20, 21). Consistent with previously published data (20), Fig. 3 shows that the stimulation of adenylyl cyclase activity was linear with increasing concentrations of MgCl2 in the range of 3 to 75 mM. Also shown is that this linear enzyme stimulation was not altered in membranes that had been pretreated with SNP, although enzyme activity was significantly reduced. These data would suggest that the divalent cation regulatory site(s) was not modified by NO. In contrast, none of these effectors were able to elicit maximum enzyme activity.

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tivity was evaluated, the enzyme assay contained 10 of the types II, IV, and VII isoforms, for which stimulation of Gi VI isoform family (10, 23, 24). This contrasts with the behavior of forskolin were 1.2 and 1.7
branes were not significantly different (p
PGE1 (3
FIG. 2. Effect of SNP on the dose-response curve to hormone and forskolin. Membranes were preincubated with or without 3 mM SNP for 30 min after which time they were assayed for secretin (A), PGE1, or forskolin-stimulated adenylyl cyclase (C) activity at the indicated concentration of stimulators. When hormone stimulated activity was evaluated, the enzyme assay contained 10 \textsuperscript{-6} M GTP. For control and SNP-treated membranes, the EC50 values for secretin were 9.0 and 9.1 \times 10\textsuperscript{-7} M for PGE1, were 3.7 and 2.9 \times 10\textsuperscript{-7} M, and for forskolin were 1.2 and 1.7 \times 10\textsuperscript{-5} M. Unpaired t test analyses indicated that, in each case, the EC50 values for control and SNP-treated membranes were not significantly different (p < 0.05). The respective Vmax values, 96 and 26, 70 and 34, and 228 and 78 pmol/min/mg for control and SNP-treated membranes, were significantly different. The data are representative of three experiments.

N18TG2 Cells Express the Type VI Isoform of Adenylyl Cyclase—NO could attenuate adenylyl cyclase activity by targeting the enzyme directly or by altering the action of a distinct regulatory protein. To identify other possible regulatory proteins of the enzyme present in N18TG2 cells and to assess their role in this phenomenon, we identified the isoform present in N18TG2 cells. The likelihood that this would be a type V or VI isoform was already suggested by its biochemical properties. The lack of a Ca\textsuperscript{2+} regulation of CAMP production in N18TG2 cells (6) was confirmed when plasma membranes were incubated with calmidazolium and no change in adenylyl cyclase activity, or in the ability of NO to inhibit that activity, was seen. This would indicate that the enzyme is not of the type I, III, or VIII isoform family (10, 23, 24). The enzyme in N18TG2 cells is regulated by hormone receptors that stimulate via Gi and inhibit via G\textsubscript{i}, (12, 14, 15, 22), a characteristic of the type V, VI isoform family (10, 23, 24). This contrasts with the behavior of the types II, IV, and VII isoforms, for which stimulation of G
enhances cAMP production in response to G\textsubscript{i} stimulators (10, 23, 24). Finally, the adenylyl cyclase in N18TG2 cells is stimulated by forskolin, which distinguishes it from the type IX isoform (24). To confirm the biochemical data and to discern which member of the type V and VI adenylyl cyclase family is predominant in N18TG2 cells, we probed Northern blots of N18TG2 RNA with the cDNAs for the type V and VI isoforms. As seen in Fig. 4, a positive signal was observed when the blot was probed with the type VI cDNA (lane 4) but not when the type V cDNA was used (lane 3). As expected, no mRNAs corresponding to the type I and II isoforms were identified (lanes 1 and 2). If an isoform other than the type VI is expressed in these cells, it is present at a level that is undetectable biochemically or by Northern blot analyses and thus would not influence the interpretation of our data concerning the regulation by NO.

Kinetic Analyses of the Effects of NO—We first preincubated membranes with SNP and determined forskolin-stimulated adenylyl cyclase activity at increasing concentrations of substrate. The results are depicted in Fig. 5. The kinetic analysis of the rate of substrate utilization indicated that the Km values did not differ between control and SNP-treated enzymes. The values were 0.48 \pm 0.12 and 0.49 \pm 0.091 mM for control and SNP-treated membranes, respectively. This would suggest that the apparent binding of substrate was not affected by the presence of NO. However, the Vmax value for SNP-treated membranes (622 \pm 44 pmol/min/mg) was reduced relative to that seen in control membranes (932 \pm 87 pmol/min/mg), indicative of a reduced rate of the enzyme conversion of substrate to product.

Although SNP inhibited adenylyl cyclase stimulation when membranes were preincubated with the NO donor, no inhibition was observed when SNP was added at the beginning of the assay. Fig. 6 shows that the extent to which SNP inhibits enzyme activity was dependent upon the length of the preincubation period. In that experiment, membranes were preincubated with SNP for the indicated times and then assayed for forskolin stimulated activity. Some degree of inhibition could be seen after a 10-min preincubation period while maximum inhibition occurred by 45 min. One conclusion of these observations is that SNP does not inhibit the adenylyl cyclase by altering a reagent in the assay. This was confirmed in other experiments in which SNP-treated membranes were washed.
free of the NO-donor prior to the assay. The degree of inhibition of forskolin-stimulated adenylyl cyclase activity was unaltered compared with unwashed membranes (also see Figs. 7-9).

The fact that SNP was ineffective when added at the time of the assay also raised the possibility that a component of the reaction mixture served to modulate the effects of NO. Types III, V, and VI adenylyl cyclases possess the consensus sequence for protein kinase A, and incubation of those isoforms with protein kinase A alters their activities. Added protein kinase C has also been shown to phosphorylate recombinant adenylyl cyclases, altering their activities (16, 23, 24). To test the hypothesis that a phosphorylation mechanism might alter the response to NO, AMP-PNP, which cannot serve as a kinase substrate, replaced ATP as the substrate for adenylyl cyclase. Whether AMP-PNP or ATP served as substrate, SNP-mediated inhibition was observed (data not shown), indicating that a phosphorylation event does not modulate the ability of NO to inhibit adenylyl cyclase.

We next addressed the possibility that occupancy of the substrate-binding site offered protection of the enzyme against the effects of NO. To do so, we determined if the presence of ATP during the preincubation period could limit the ability of SNP to inhibit the enzyme. In the experiment shown in Fig. 7, membranes were preincubated with or without 3 mM SNP in the absence or presence of the indicated compounds. After the 30-min preincubation, membranes were washed and resuspended in buffer as described under “Experimental Procedures.” Membranes were assayed for forskolin-stimulated (10−7 M) adenylyl cyclase activity. 100% was assigned to the value seen when membranes were preincubated in the absence of any additions (132 pmol/min/mg). Basal activities were 11.5 and 12 pmol/min/mg in control and SNP-treated membranes, respectively. The data are representative of five experiments. ANOVA and Tukey’s test indicated that the values for control, SNP plus AMP-PNP, and SNP plus ATP-pretreated membranes were not significantly different, but that they were different from the values for SNP and SNP plus GTP-pretreated membranes, and that the values for the latter two were not significantly different from each other.
substrate-binding site of the enzyme renders it less susceptible to the effects of NO.

Given these results, we examined if the conformation of the enzyme, as elicited by either forskolin or hormonal signaling, could also afford the enzyme some protection against the effects of NO. To do so, membranes were preincubated with SNP in the absence or presence of forskolin or PGE1. When membranes were preincubated with PGE1, GTP (10^{-4} M) was also present. The presence or absence of GTP did not alter the results. After 30 min membranes were washed and assayed for forskolin- or PGE1-stimulated adenylyl cyclase with 10^{-4} M forskolin present in the assay. Basal activity was 19 and 21 pmol/min/mg in control and SNP-treated membranes. The data are representative of three experiments.

The Effects of NO on Adenylyl Cyclase Are Reversed by Reducing Agents—We previously demonstrated, in intact N18TG2 cells, that NO-mediated inhibition of cAMP accumulation in response to hormone or forskolin is readily reversible. Cells recover full ability to respond to these stimulators when incubated for 20 min in the absence of NO (6). As shown in Fig. 9, when SNP-treated membranes were washed free of SNP and then incubated for 20 min in buffer, forskolin-stimulated adenylyl cyclase activity remained inhibited. However, when washed membranes were incubated with DTT, forskolin-stimulated activity was recovered in a dose-dependent manner. Similar effects were observed when Cys was added instead of DTT. In contrast, cystine did not allow for recovery of forskolin-stimulated activity but was itself inhibitory to the enzyme. We have verified that neither 10 mM Cys nor DTT alter adenylyl cyclase activity when added directly to the assay. Thus, the NO-mediated inhibition of adenylyl cyclase activity in plasma membranes is reversible if an appropriate reducing agent is present. We also observed that the ability of DTT to reverse the effects of NO are rapid. When SNP-treated membranes were incubated for increasing times with 10 mM DTT prior to the determination of forskolin-stimulated adenylyl cyclase activity, maximum recovery was observed at 4 min, the shortest time point examined (data not shown).

**DISCUSSION**

Nine isoforms of the mammalian enzyme have been cloned, each with distinct regulatory properties, as revealed in reconstitution studies of the recombinant enzyme expressed in Sf9 cell membranes, or in co-transfection experiments (reviewed in Refs. 10, 23, and 24). With the understanding that each enzyme is uniquely regulated by a variety of factors, the view of a universal mechanism of adenylyl cyclase regulation has been replaced by the recognition that cell/tissue-specific changes in adenylyl cyclase activity require an understanding of the profiles of isoforms present in a particular cell. In the present study, we have identified the predominant isoform present in N18TG2 cells as being a type VI enzyme and have described its novel regulation by NO.

We had previously demonstrated that preincubation of intact N18TG2 cells with NO or NO donors attenuates their production of cAMP upon stimulation with PGE1, secretin, or forskolin (6). The effects of NO can be faithfully reproduced in purified plasma membrane preparations, limiting its possible targets to components in those preparations. (Ref. 6, and this work) eliminated guanylyl cyclase, PDE, Galpha, Gbeta, CaM, and protein kinases as NO targets. In so doing, we have eliminated the known regulators of the type VI isoform as targets of NO. That we are able to demonstrate the NO-mediated attenuation of adenylyl cyclase activity in plasma membranes also eliminates events such as changes in ATP levels or ion fluxes as possible mediators of the effects of NO on the enzyme. We conclude that NO functions either via an as yet unidentified novel regulator of the adenylyl cyclase in N18TG2 cells or that the enzyme itself is the target of NO.

It is of interest to note that another isoform of adenylyl cyclase may also be regulated by NO. Duhe et al. (25) reported that the Ca^{2+}/CaM regulation of recombinant type I adenylyl cyclase in membranes from Sf9 cells is inhibited by NO although basal enzyme activity is unaffected. The authors proposed that NO may function by oxidizing a Cys residue(s) at the CaM-binding site of the adenylyl cyclase. Vorhees et al. (26) had identified a region of the type I adenylyl cyclase that possesses CaM binding activity, a region that contains two Cys residues. Currently it is unknown if either, or both, of these residues is modified by NO, or if NO functions to inhibit CaM activation by modifying another region of the protein. Since experiments were restricted to analyses of Sf9 cell membranes, it also remains to be determined if NO regulates type I adenylyl cyclase.

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**Fig. 8.** Failure of forskolin or PGE1 to protect adenylyl cyclase from SNP. Membranes were preincubated with or without SNP. Where indicated, either 10^{-5} M forskolin or 10^{-5} M PGE1, was also present. When membranes were preincubated with PGE1, GTP (10^{-4} M) was also present. The absence or presence of GTP did not alter the results. After 30 min membranes were washed and assayed for forskolin- or PGE1-stimulated adenylyl cyclase with 10^{-4} M forskolin present in the assay. Basal activity was 19 and 21 pmol/min/mg in control and SNP-treated membranes. The data are representative of three experiments.

**Fig. 9.** Ability of reducing agents to reverse the effects of SNP. Membranes were preincubated with 3 mM SNP for 30 min. Membranes were then washed free of SNP and incubated for 20 min in the absence or presence of the indicated concentrations of DTT or Cys. Forskolin-stimulated (10^{-5} M) adenylyl cyclase activity was measured. The results are presented relative to the forskolin-stimulated adenylyl cyclase activity seen in membranes that had not been preincubated with SNP or reducing agent. The data are representative of three experiments.
in either membranes or in intact cells that normally express the enzyme and, if so, if this results in a change in its activation by Ca\(^{2+}\). This contrasts with the effects that we have observed in which NO inhibits the forskolin-stimulated cAMP accumulation, in both intact N18TG2 cells and in purified plasma membranes, and does so in a manner that is independent of any CaM regulation of the enzyme.

Recent studies of truncated soluble constructs of adenylyl cyclase have indicated that the enzyme exists in multiple conformational states, elicited by the binding of effectors such as Ga subunits, forskolin, or by occupancy of the ATP-binding site (27, 28). Our kinetic analyses indicated that the inhibition by NO reflects an altered V\(_{\text{max}}\) suggestive of the conformation of the enzyme is altered in a manner that precludes its optimal catalytic activity. The inhibitory effects of NO were not altered when membranes were preincubated with SNP plus either forskolin or GTP. Thus, the conformations of the enzyme that are achieved as a result of forskolin or Ga binding do not alter the ability of NO to inhibit adenylyl cyclase. Occupancy of the ATP-binding site, however, did afford the enzyme significant protection from the inhibition by NO. This would suggest that, under those latter conditions, the amino acid(s) targeted by NO is no longer available, either because it is sterically shielded upon ATP binding or because the enzyme conformation induced upon ATP binding now renders it unavailable. Several observations would suggest the latter to be the more reasonable interpretation. If the catalytic site contained the residues targeted by NO, then we would expect that basal cyclase activity would also be inhibited by NO. This was not generally the case. Given the homologies between the putative catalytic sites of the various isoforms, we would also expect other isoforms to be similarly regulated by NO. We note that the basal activity of the type I recombinant enzyme is not altered by NO (25), suggesting that, in their basal states, the type I and VI isoforms share a conformation that precludes susceptibility to NO. In contrast to the type VI isoform, it would appear that the type I isoform of adenylyl cyclase, in which residues that modulate CaM binding are susceptible to NO, exists in a conformation such that NO is not able to inhibit forskolin stimulation.

The finding that occupancy of the substrate-binding site of the enzyme protects it from inactivation by NO would indicate that optimal attenuation of cAMP production will occur when cell exposure to NO precedes stimulation of the enzyme by a Ga modulator. When adenylyl cyclase stimulation precedes, or is simultaneous with that of NO, the inhibitory effects of NO will be diminished. Our data indicate that the effects of NO will be modulated also by the ability of cells to reverse the inhibition of adenylyl cyclase. With intact cells, the inhibitory effects of NO on cAMP accumulation are readily reversed when cells are incubated in the absence of NO donors (6). In plasma membrane preparations, the removal of NO donor compounds did not alleviate the inhibition of adenylyl cyclase activity, suggesting that a stable modification had occurred. The NO-mediated inhibition, however, was reversed when membranes were washed free of NO donors and incubated in the presence of a reducing agent, either DTT or Cys, a more physiological agent. Such observations strongly implicate S-nitrosylation as the underlying mechanism of adenylyl cyclase inhibition (3). They also indicate that the ability of NO to regulate a cell’s production of cAMP will depend upon the cell’s redox potential. Over recent years, a large body of evidence has amassed to indicate that the cell’s redox potential will significantly alter a variety of signal transduction pathways involved in the control of cell growth and death (29). These data extend such observations to now include signal transduction via cAMP.

Given the readily reversible nature of the effects of NO, it is likely that the physiological consequences of NO exposure, with respect to cAMP production, will depend upon the levels of NO and its stability. If the source of NO results from the activity of a constitutive NOS, such levels will be low and transient. The resulting changes in a cell’s responses to Ga modulators would also be transient and thus more fitting of a homeostatic response. More chronic exposure, or higher levels of NO as produced by inducible NOS, would result in a longer lasting refractory period to Ga stimulators. In general, high levels of NO are believed to be toxic, contributing to the pathologies resulting from e.g., ischemic injury, heart failure, and a number of neurodegenerative diseases (reviewed in Refs. 1–3).

In light of their varied and complex modes of regulation, adenylyl cyclases have been proposed to serve as “co-occurrence detectors” (30), where the enzymes are activated upon varied hormones and then integrate these influences into a change in cAMP. The response to different signals could be synergistic, e.g. type II adenylyl cyclase responds to Ga stimulators in a manner that could be amplified by the simultaneous presence of βγ generated by Ga stimulators. Alternatively, the response to one signal may be attenuated by the presence of a second signal, e.g. the type V and VI isoforms are stimulated by Ga but inhibited by Ca\(^{2+}\) (10). Our data indicate that the type VI isoform of adenylyl cyclase predominant in N18TG2 cells is a coincidence detector for NO and that both the redox potential of the cell and the order of input signals will influence the ability of NO to regulate cAMP production.

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REFERENCES

1. Garthwaite, J. (1991) Trends Neurosci. 14, 60–67
2. Bredt, D. S., and Snyder, S. H. (1994) Annu. Rev. Biochem. 63, 175–195
3. Stamler, J. S. (1994) Cell 78, 931–936
4. Tao, Y-P., Najafi, L., Shipley, S., Howlett, A., and Klein, C. (1992) FEBS Lett. 314, 49–52
5. Tao, Y-P., Howlett, A., and Klein, C. (1996) Cell Signal. 8, 27–34
6. Tao, Y-P., Najafi, L., Shipley, S., Howlett, A., and Klein, C. (1998) J. Pharmacol. Exp. Ther. 286, 288–294
7. Dímmelser, S., and Brune, B. (1992) Eur. J. Biochem. 210, 305–310
8. Zhang, J., Dawson, V. L., Dawson, T. M., and Snyder, S. (1994) Science 263, 687–689
9. Zafull, P., Shepherd, G. M., and Barnstable, C. J. (1997) Curr. Opin. Neurobiol. 7, 404–412
10. Mons, N., and Cooper, D. F. M. (1995) Trends Neurosci. 18, 536–542
11. Scholich, K., Barber, A. J., Mullenix, J. B., and Patel, T. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5915–5919
12. Howlett, A. C. (1982) Mol. Pharmacol. 27, 429–436
13. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
14. Bisutti-Russell, M., and Howlett, A. C. (1987) Neurochem. Int. 11, 287–292
15. Howlett, A. C., Qualy, J. M., and Khachatrian, L. L. (1986) Mol. Pharmacol. 29, 307–313
16. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 191–193, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Siegel, L. L., and Bresnick, E. (1986) Anal. Biochem. 159, 82–87
18. Howlett, A. C. (1982) Mol. Pharmacol. 21, 664–670
19. Sutkowski, E. M., Tang W-J., Broome, C. W., Robbins, J. D., and Seamon, K. B. (1994) Biochemistry 33, 687–689
20. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 191–193, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Walz, M. A., Holt, I. L., and Howlett, A. C. (1987) Neurosci. Res. 17, 291–297
22. Matsuura, H., and Nirenberg, M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3472–3476
23. Iyengar, R. (1993) FASEB J. 7, 768–775
24. Sunahara, R. K., Dessauvage, C. W., and Gilman, A. G. (1996) Annu. Rev. Pharmacol. Toxicol. 36, 461–480
25. Duhe, R. J., Nielsen, M. D., Dittman, A. H., Villacres, E. C., Choi, E. J., and Storn, D. R. (1994) J. Biol. Chem. 269, 7290–7296
26. Verheir, T., Knoepf, L., Hofmann, F., Mollen, S., Pfeuffer, T., and Carafoli, E. (1993) Biochemistry 32, 6081–6088
27. Tesmer, J. J. G., Sunahara, R. K., Gilman, A. G., and Sprang, S. R. (1997) Science 278, 1907–1916
28. Tang, W-J., and Hurley, J. H. (1998) Mol. Pharmacol. 54, 231–240
29. Peunova, N., Kuzin, B., Roberts, I., O’Kane, C., and Enikolopov, G. (1996) Cold Spring Harbor Symp. Quant. Biol. LXXI, 417–426
30. Bourne, H. R., and Nicoll, R. (1993) Cell 10, 65–75