[Frontiers in Bioscience 10, 1868-1880, May 1, 2005]

DYNAMICS OF CELLULAR NO-cGMP SIGNALING

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1. ABSTRACT

Despite its widespread biological importance, knowledge about the basic workings of the nitric oxide (NO) signaling pathway at the cellular level has been unsatisfactory. As reviewed here, recent findings have begun to rectify this deficiency. Elementary NO signals may be very discrete, being short lived (seconds or less), of low amplitude (peak concentration in the low nanomolar range), and confined to the immediate vicinity of the source (a micron or less). A more global signal may occur when many nearby sources are active simultaneously, though the amplitude appears to remain low. The properties of guanylyl cyclase (GC)-coupled NO receptors, for which a kinetic model is introduced, are well tuned to detect NO signals. The receptors can respond even to brief pulses of NO because they activate and deactivate with sub-second kinetics and they possess the appropriate sensitivity to low nanomolar NO concentrations. In some cells at least, the NO-evoked GC activity is very high, equivalent to the synthesis of up to 100 microM cGMP per second. The resulting shapes and sizes of cellular cGMP responses can vary considerably from cell to cell, however, which is likely to have repercussions for the selection of downstream pathways. The cellular diversity can be explained by variations in the rates at which the receptors desensitize and in the rates of cGMP hydrolysis by phosphodiesterases. There is a growing list of factors that may serve to modulate NO receptor function in cells, including Ca^{2+}, ATP, phosphorylation by kinases, and physical interactions with other proteins.

2. INTRODUCTION

Although there is now a vast literature about NO in health and disease, there remains a lack of clarity about many of the basic physiological properties of the signaling pathway, including the kinetics of NO release, the profiles of the resulting NO concentration in time and space, the numbers and types of receptors with which it combines, and the downstream signal transduction pathways. One reason for this deficiency is that NO is a difficult molecule with which to work, partly because it is unstable when used in experiments, and partly because NO lacks chemical specialization, prohibiting the pharmacological strategies that have been so successfully applied to other signaling systems. Another reason is that NO is difficult to measure at concentrations that are biologically active. Ignorance of the basics means that there have been few constraints on the way that experiments are conducted, for example, in the concentration, time and manner in which NO is applied to cells and tissues. This is a particular problem with NO because cells react to NO in diverse ways that depend on exactly these parameters.

The only established way in which NO acts physiologically on target cells is to stimulate guanylyl cyclase (GC), resulting in cGMP formation. The existence of "soluble" and "particulate" GC activities in homogenates of many different mammalian tissues, with the "soluble" type being preferentially activated by NO, goes back many years (1). Even then, it was shown that significant NO-evoked GC activity was also present in variable amounts in

membrane fractions, questioning the accuracy of the term "soluble". Knowing now that the GC-linked proteins in question are the natural receptors for endogenous NO, it is more useful to consider the signaling pathway within the general framework of ligand-receptor interactions, not least because doing so immediately provides a rich resource of theoretical and practical understanding that can readily be applied to NO-cGMP signaling. It also helps to highlight where current deficiencies lie. Accordingly, the proteins are here called "GC-coupled NO receptors", which reflects their function and the fact that, like all other receptors, they are equipped with a ligand binding site and transduction domain whose output (cGMP in this case) serves to trigger changes in cell function.

As with all biological mechanisms, knowledge of the underlying dynamics is essential for understanding how they work and why they might malfunction, and here an attempt is made to assemble the information available on the dynamics of NO-cGMP signaling. It should be made clear at the outset, however, that the information is very incomplete, particularly when compared with, say, fast synaptic transmission, where the sequence of events between arrival of an action potential at the presynaptic nerve terminal and the response of the postsynaptic receptors to the released transmitter can be described in quantitative detail (2).

3. CELLULAR NO RELEASE

During the last decade, a great deal has been learnt about the biochemistry of NO synthesis from studies of the purified NO synthase (NOS) enzymes. These are complex proteins found in three main forms called neuronal (nNOS), endothelial (eNOS) - both being active constitutively - and inducible (iNOS), which can be expressed in numerous cell types (prototypically in macrophages) when subjected to immunological challenge. All three isoforms generate NO from L-arginine but have distinct functional and structural features (3,4). Despite the biochemical progress, there remains uncertainty about what constitutes a physiological (or pathological) NO signal received by a target cell.

3.1. Amplitude of NO signals

The early development of an NO-sensitive microelectrode (5) capable of registering NO concentrations in subcellular dimensions initially gave hope that the issue would be rapidly resolved. This electrode design has now been applied to several different cell types and the results indicated that, following appropriate stimulation (e.g. exposure of endothelial cells to bradykinin), the NO concentration rises within a minute or so to approximately 1 microM (5,6). That this order of magnitude was physiological appeared to be reinforced by studies of the purified GC-coupled NO receptor, for which the potency of NO was reported to be around 250 nM (7). Some researchers still consider this range physiological, despite evidence to the contrary.

First, the readings with this electrode may be compromised by non-specificity, since it can detect various other compounds, including tyrosine, catecholamines and ascorbate (5,8,9). More recent attempts to measure NO release from the endothelium in blood vessels using other electrodes found only low nanomolar concentrations, the signals often being barely distinguishable from the background noise (10,11). That the NO concentration generated by endothelial cells only reaches this order of magnitude accords with earlier studies of NO produced by columns of endothelial cells grown on beads, where up to 20 nM NO was registered using a hemoglobin-based biochemical assay (12). In brain tissue, NO is typically released from axons or from dendrites when NMDA receptors are activated by the neurotransmitter glutamate (13). Measurements using a specially designed electrode on the surface of electrically-stimulated brain slices detected only low nanomolar NO concentrations or below (14,15), values that are compatible with the concentrations measured electrochemically *in vivo* (16) or in human neuronal cultures (17) after stimulation with NMDA, and with those estimated on the basis of cGMP measurements in brain slices exposed to NMDA (18).

Second, the potency of NO for its receptors has been revised upwards by about 2 orders of magnitude. In the earlier estimation (7), the NO concentrations were not measured. Recently, methods for delivering known, stable NO concentrations have been devised and, in the case of the purified receptor, half-maximal GC activity was observed at around 1 nM NO (19,20). When the same method was applied to intact platelets, it was found that downstream phosphorylation (through cGMP-dependent protein kinase) could be elicited by subnanomolar NO concentrations (half-maximal effect at 500 pM) during 1-min exposures (21).

At least during physiological signaling, therefore, it seems likely that basal NO release provides local concentrations of 100 pM or less and, on stimulation of NO synthase, NO in adjacent target cells may only rise to concentrations of a few nanomolar, at best. New microelectrode designs or other types of detectors are needed to record NO signals at individual sites of synthesis with precision and accuracy.

3.2. Shapes of NO signals

With concerns over the validity of the earlier electrode measurements (see above), predicting the patterns of NO release from cells of different types equipped with different NO synthases becomes mostly a matter of speculation. At one extreme, the NO signal may be very transient and spatially restricted. The nNOS isoform, for example, is activated by calmodulin when fully loaded with its cargo of 4 Ca^{2+}. By virtue of their mutual binding to postsynaptic density-95 protein, nNOS in the brain is held in close physical proximity to NMDA receptor channels (22) which are situated in the majority of excitatory synapses and readily conduct Ca^{2+} (23). When NMDA receptors are activated by transient release of glutamate in synapses, the resulting local profile of intracellular Ca^{2+} follows a time-course resembling that of the current passing through the membrane, rising to peak in about 50 ms and then falling back to baseline within about 500 ms (24).

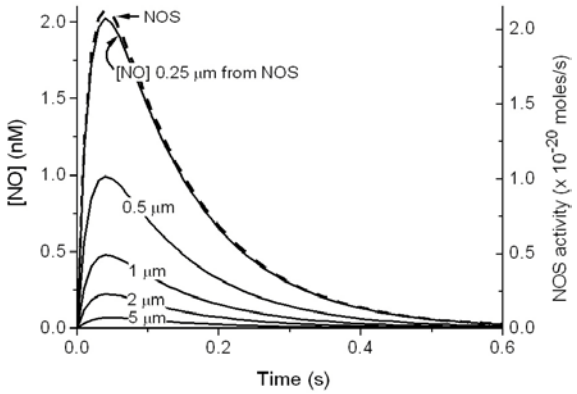

Figure 1. Simulation of the NO concentration profiles existing around a point source of NOS that, maximally, generates 20,000 molecules per second and whose activity follows the same time course of as that of Ca^{2+} in a dendritic spine following synaptic activation of NMDA receptors. The time course was modeled by a pulse function, i.e. NOS = $S(1-\exp(-k_1.t).\exp(-k_2.t)$, where S is the source strength, taken to be 3.4 x 10^{-20} moles/s, and the constants k_1 and k_2 are 50 s^{-1} and 8 s^{-1} respectively. The resulting NO profiles at different distances (indicated on each curve) were calculated assuming free 3-dimensional diffusion (28).

Removal of Ca^{2+} leads to rapid unbinding of the first 2 Ca^{2+} from the Ca^{2+}/calmodulin-nNOS complex, which causes arrest of NOS activity with a half-time of less than 70 ms. Unbinding of the remaining 2 Ca^{2+} is at least 10-fold slower and dissociation of calmodulin from nNOS slower still, with an estimated half-time of 7 s (25). Hence, a scenario for efficacious NO generation would be in a recently-activated synapse where calmodulin with 2 Ca^{2+} bound is already associated with nNOS. Synaptic NMDA receptor activation should then result in almost contemporaneous binding of the final two Ca^{2+} and subsequent NO production. Assuming that NOS activity follows the same time-course, that the NOS molecules in this scenario approximate to a point source, and that they can generate a maximum of 20,000 NO molecules/s at steady-state, the NO concentration profiles within close radii would closely mirror the synaptic NMDA receptor-mediated Ca^{2+} response (figure 1). The absolute concentrations achieved, however, would be very distance-dependent because of the rapid dispersal of NO by diffusion. In the example considered, the concentration falls from a peak of 2 nM at 0.25 μm from the source, down to 70 pM at 5 μm. Consequently, such an NO signal would be expected to echo the temporal features of the input signal but it would only have the power to activate receptors in the immediate vicinity of the source (say, within a 1-micron radius), implying that it may be synapse-specific. The same would apply to a point source of longer duration. With repetitive high-frequency synaptic activity of the type that leads to long-term changes in synaptic function, the temporal summation of NMDA receptor-mediated Ca^{2+} currents (26) and, presumably, NOS activity (15) could result in a build-up of more distant NO concentrations to levels capable of activating receptors. Alternatively, should the source strength be lower than assumed, summated activity may even be required for the NO concentration in the immediate locality to rise to the active range. These scenarios may be central to the role of NO-cGMP signaling in synaptic plasticity, whose initiation depends on periods of high-frequency synaptic stimulation (27).

The other extreme is where there are multiple sources of NO within a given tissue volume that become active roughly simultaneously. In this case, the individual NO signals could summate over distance and time, resulting in a local "cloud" of NO. The amplitude and duration of this type of signal would be critically shaped by the rate of breakdown of NO by the tissue (28). Clearly, such a global NO signal would convey different information from that localized to single synapses, but it has attractive features for a conceptually different role in synaptic plasticity, as well as in more global functions, such as the regulation of blood flow (29). The most pertinent experimental data are recordings of the NO concentrations achieved following electrical stimulation of a dense nNOS-containing fiber tract, namely the parallel fibres in the cerebellum (14). The summed NO concentration registered on the surface of the tissue (using a 300 μm diameter electrode) was found to be highly dependent on the rate of stimulation, rising maximally to a few nanomolar within 1-4 s and, on termination of the stimulation, falling back to baseline within a few seconds. Simultaneous activity in whole bundles of nerve fibers is unlikely to occur under physiological conditions but the result indicates that the NO concentration remains low even under conditions where multiple NO sources are switched on at the same time.

4. GUANYLYL CYCLASE-COUPLED NO RECEPTORS

4.1 General features.

The receptors are alpha/beta-heterodimers of which there are currently two known isoforms, alpha1beta1 and alpha2beta1. The NO binding site is a prosthetic heme group anchored to a histidine group on the beta-subunit (30). When studied in a cell-free environment, both isoforms are similarly sensitive to NO, half-maximal activity of the associated GC activity occurring at about 1 nM (20). Other properties, such as the maximal GC activity and pharmacological properties also appear very similar (31,32) but whether this applies when they are in their natural cellular environment is unclear. The two receptors have a differing cellular distribution (33-35) and, at the subcellular level, may also have different locations because alpha2beta1, which is abundant in brain, binds to proteins that are enriched in synapses, such as postsynaptic density-95 protein (36). The other isoform, alpha1beta1 may be cytosolic in part but there is evidence that it too may become cell membrane-associated under conditions of raised intracellular Ca^{2+} (37). Membrane association may depend on binding to proteins such as heat-shock protein 90 (38). In homogenates, varying proportions of total NO-activated GC activity are found in membrane fractions (39): for example, in rat platelets, this component amounts to 60 % of the activity in the cytosol whereas, in the cerebellum,

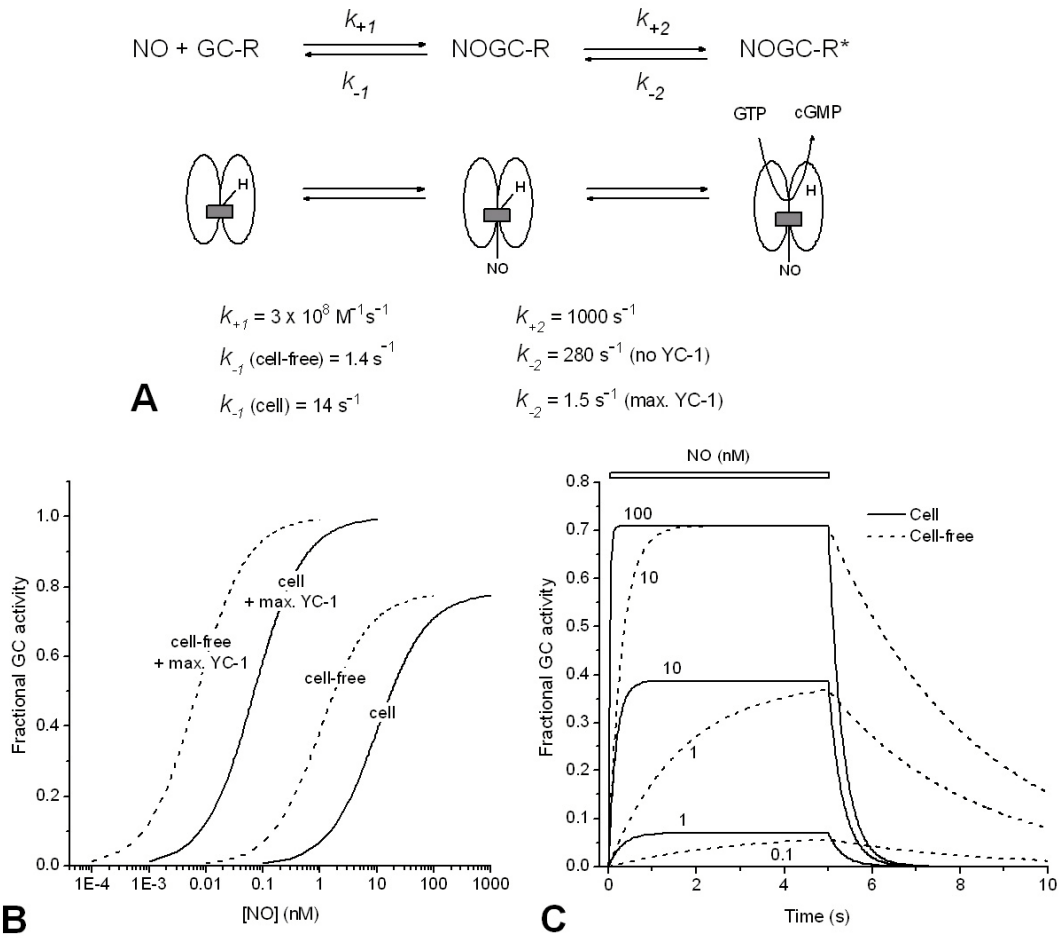

Figure 2. Kinetic model for GC-coupled NO receptors. (A) The theory, together with values assigned to the rate constants; H signifies the histidine residue that links the protein to the heme NO binding site in the inactive conformation. (B) Predicted steady-state concentration-response curves for receptor activation by NO in intact cells and in cell-free preparations (as indicated) without (solid lines) or with (dashed lines) a maximally active concentration of the allosteric activator YC-1. (C) Predicted profile of GC activity during a 5 s exposure to different NO concentrations (indicated on each curve) for the receptor in cells (solid lines) and in a cell-free environment (dashed lines).

it is half this amount (20). Based on studies in homogenates of heart, membrane localization was originally reported to sensitize the receptor to NO (37) but this was later found to be an artefact apparently resulting from the applied NO being consumed by reaction with myoglobin in the cytosol fraction, but not in the membrane fraction (20). In homogenates of platelets and cerebellum, NO was equipotent on the receptors in cytosol and membranes (20). The biological significance of the shuttling of alpha1beta1 receptors to the membrane (and, presumably, back again) is unknown. The linkage between α2β1 receptors and membrane-associated synaptic proteins may position them within close range of the sites of NO release, which would be particularly important if NO signaling operates in discrete spatial dimensions (see above). Conceivably, by placing the receptor closer to an NO source, attachment of the alpha1beta1 isoform to the outer cell membrane could perform a similar function. Even in subcellular dimensions, this positioning could make the difference between the receptors being accessible to a local NO-producing cell and being out of range (figure 1).

4.2, Kinetics of receptor activity

4.2.1. In a cell-free environment

Studies on the purified alpha1beta1 isoform indicated that, as with other ligand-binding heme proteins such as hemoglobin and myoglobin, NO binds to the heme site on GC-coupled receptors at a near diffusion-limited rate, the rate constant being greater than 10^7-10^8 $M^{-1}s^{-1}$ at sub-physiological temperatures (40,41). At 37 °C, and in the presence of substrate (GTP), deactivation of GC activity on removal of NO occurs with a half-time of 2-5 s (42,43), a range that accords with the rate of dissociation of NO from the NO-receptor complex under similar conditions (44).

The classical del Castillo-Katz scheme provides a minimal model for receptor function (45,46). Rate constants can be assigned to the various steps (figure 2A), with the values constrained to provide a realistic simulation of the rates of NO binding and receptor deactivation, the potency of NO, and the effect of allosteric activators (see below). According to this model, the NO dissociation

constant (given by the ratio of rate constants for NO binding and unbinding, k_{-1}/k_{+1} in figure 2A) is 4.7 nM. The efficacy (the ratio of forward-to-backward rate constants for the transition of the NO-receptor complex to its active form, k_{+2}/k_{-2} in figure 2A) is low (3.6, giving about 77 % of maximal receptor activity) compared with values typically found for ligand-gated ion channels (≥10). This is necessary to accommodate the fast deactivation rate (a reduction in the value of the constant k_{-2} increases the efficacy but slows the deactivation rate) and to enable increased efficacy (i.e. an increased maximal response to NO) observed in the presence of the allosteric activator YC-1 (47,48) or its more potent analogues BAY 41-2272 (49) and BAY 41-8543 (50). These allosteric activators decrease the deactivation rate, the half-time increasing from 2-5 s to several 100 s maximally (42,43). Slowing deactivation has the effect of increasing the potency of NO although by precisely how much is not known because it has not yet been measured under steady-state conditions using known NO concentrations. A reduction in the value of k_{-2} from 280 s^{-1} to 1.4 s^{-1} gives approximately the required deactivation rate (half-time = 335 s) and predicts a 140-fold decrease in the EC_{50} for NO, from 1 nM to 7 pM (figure 2B). The reduction in k_{-2} also increases the efficacy to 670, enabling 100 % receptor activity (47,48). For a pharmacological agent to stabilize a receptor in its activated state is not a novel concept; for example, such an action contributes to the action of the general anesthetic propofol on $GABA_A$ receptors (51) and of the antiparasitic agent ivermectin on ionotropic ATP receptors (52). At the molecular level, there must be separate YC-1-bound states of the receptors, but identification of those states and details of the resulting receptor kinetics are needed before this allosteric site can be incorporated realistically into the reaction scheme.

4.2.2. In cells

In their cellular environment, at least *in vitro*, GC-coupled NO receptors display several differences compared with the cell-free situation. First, measurement of the initial rate of cGMP accumulation in rat platelets gave an EC_{50} for NO of 10 nM compared with 1.7 nM when the same batches of platelets were lysed (21). The latter value is similar to the value of about 1 nM found for the purified protein (see above) and the former is essentially identical to the estimated potency of NO for relaxing vascular smooth muscle (53). Second, on removal of NO, the deactivation rate in a suspension of brain cells was about 10-fold faster than reported for the purified protein (54). The lower potency of NO and faster deactivation rate in cells should be mechanistically linked and can be modeled simply by increasing the rate of unbinding of NO from the NO-receptor complex by a factor of 10 (figure 2A,B). The explanation for these changes in cells is unknown but a functional advantage would be that the kinetics of NO signal transduction is speeded up. At higher NO concentrations (10 nM range), steady-state GC activity would be achieved with a half-time of 20-30 ms, which accords with experimental results (54). Even with the reaction rate being slowed at low agonist concentrations, steady-state activity would be established within about a second (figure 2C). When NO disappears, the activity stops with a half-time of 200 ms, matching experimental findings (54). Thus, the rates of activation and deactivation are both 10-fold faster than in cell-free preparations, which allows the receptor in cells to respond more faithfully to brief NO transients than it would do otherwise, albeit at the expense of a lowered NO sensitivity.

The third main difference in the behavior of the receptor in cells is that it desensitizes, meaning that the associated GC activity becomes reduced with time during continued exposure to NO (55) whereas, in a cell-free environment, the GC activity remains constant provided that the NO concentration remains steady (18-20), or is saturating (1). Desensitization of GC-coupled NO receptors was first described in suspensions of cells from the cerebellum, in which non-neuronal cells (astrocytes) were found to be the responsive cell type (55), and has subsequently been found in neurons from another part of the brain (56), and in platelets (21) and so is likely to be a general property of the protein in cells. The most comprehensive analysis was conducted in rat platelets, which suggested that there are three components. About 80 % of GC activity is subject to a type of desensitization which varies in rate and extent depending on the degree of stimulation. In cells that have low phosphodiesterase activity, the residual 20 % also becomes lost when cGMP rises to high levels. Lastly, starting within a few seconds of exposure to NO, there is a gradual reduction in the net capacity for cGMP accumulation, a phenomenon that can occur even at NO concentrations too low to evoke other types of desensitization. The mechanisms in all cases are unclear but they appear to be related to GC activity or cGMP levels (21,56). The influence of desensitization on the shaping of cGMP signals is discussed below (section 6). It is interesting to note here that one non-intuitive consequence of desensitization in cells with low PDE activity is that the potency of NO increases with time of exposure, because desensitization imposes a ceiling on how much cGMP can be generated. Thus, an EC_{50} for NO of 2 nM found after a 2 min exposure in normal cerebellar astrocytes (57) is entirely compatible with the value being 10 nM at the start (21).

Finally, there is a growing list of endogenous factors that may interact with the receptors in cells. It was reported several years ago that Ca^{2+} inhibited NO-activated GC (58) and that physiological concentrations of ATP inhibited supernatant GC activity in heart homogenates (59). More recently it has been found that, for Ca^{2+}, a high affinity component covers the physiological Ca^{2+} concentration range of 10 nM-1 μM (IC_{50} = 150 nM) and results in up to about 50 % loss of GC activity, with an additional low affinity component appearing at higher Ca^{2+} concentrations (60). Inhibition by ATP is half-maximal at about 1 mM (61), which is lower than the usual cellular concentration (about 3 mM). Hence ATP, and possibly Ca^{2+}, could tonically inhibit receptor activity in cells and changes in their concentrations could modify ongoing cGMP synthesis, although experiments designed to address this possibility directly have not yet been performed. There is also no information on the dynamics of the inhibition, or on whether it is activity-dependent. Another small molecule

able to inhibit NO-evoked GC activity in physiological concentrations (10 μM range) is inositol hexakisphosphate (62) although, again, its significance as a modulator of NO receptors in cells remains to be investigated. Receptor function is also potentially modifiable by alterations in the phosphorylation by protein kinase C (63), cAMP-dependent protein kinase (64) or cGMP-dependent protein kinase (65), or by interaction with proteins such as heat shock proteins 70 (66) or 90 (38), a chaperonin-containing polypeptide subunit (67), and a GTP-binding protein known as AGAP1 (68). An understanding of the molecular architecture and functioning of GC-coupled NO receptors seems to be emerging after a prolonged gestation.

5. cGMP HYDROLYSIS BY PHOSPHODIESTERASES

Most of the 11 known PDE families can hydrolyze cGMP, those with the highest affinity being PDE1, 2, 3, 5, 6, 9, 10 and 11 (69). PDE4, which has low affinity (> 100 microM) for cGMP (but high affinity for cAMP, about 1 microM), can also contribute to cGMP hydrolysis in those cells that accumulate cGMP to high levels (70). Several of these PDEs (types 2, 5, 6, 10 and 11) contain so-called GAF domains that, in the case of PDE2 and 5 at least, bind cGMP resulting in an enhancement of catalytic activity. The increase in activity comprises both an increase in the affinity of the catalytic domain for cGMP and an increase in the limiting rate of cGMP hydrolysis (71,72). The activity of PDE1, on the other hand, is augmented several-fold by Ca^{2+}-calmodulin.

Discerning quantitatively how PDEs hydrolyze cGMP in intact cells is complicated by several factors, including the practical difficulties of measuring rapid rates of cGMP hydrolysis, the presence of multiple PDE isoforms and, by analogy with cAMP, a possible localization of the signal transduction pathway to discrete subcellular microdomains whose PDE activity may differ from that in the bulk cytoplasm (73). Nevertheless, analysis has been permissible in three different cell types that have particularly advantageous properties.

First, non-neuronal cells isolated from the cerebellum possess high levels of GC-coupled NO receptors, but have a remarkably low PDE activity (the operative isoforms being identified pharmacologically as PDE5 and PDE4), which means that exposure to NO causes large increases in cGMP, calculated to be up to 800 microM. The rate of cGMP hydrolysis can be followed by stopping cGMP synthesis. The progress of cGMP breakdown by these cells obeyed simple Michaelis-Menten kinetics and it took many minutes for basal cGMP to be restored (55,56,70). Subcellular compartmentation of cGMP signaling does not appear to be a complicating factor in these cells, since rapid fixation in the 100-ms time-frame followed by cGMP immunostaining indicated that cGMP rose uniformly within the cytoplasm (54).

Neuroblastoma cells are large enough to impale with a cGMP-sensing microelectrode enabling a continuous readout of the intracellular cGMP concentration (74). On exposure to NO, cGMP normally rose to reach steady-state levels in the low micromolar range and, when NO was removed, cGMP fell back to baseline levels within about a minute, signifying a low PDE activity. Activation of muscarinic acetylcholine receptors on the cells, however, switched the PDE into a persistently more active form to the extent that subsequent cGMP responses to NO were severely curtailed. The mechanism, which also appeared to be present in cultures of sympathetic neurons, involved a non-selective PDE isoform (types 1, 2, 4 and 5 were excluded) whose activity became enduringly augmented as a result of phosphorylation events triggered by Ca^{2+} (75). It will be important to identify the PDE at work here, and to determine if a similar long-term switch in activity takes place in other cells.

Lastly, detailed kinetic studies of PDE activity changes have been carried out in rat platelets *in vitro*. These cells offer the advantages of being pure and of being small enough to fit the dimensions of a microdomain (about 1 micron). Furthermore, unlike in their human counterparts (55), cGMP following exposure to NO in rat platelets is hydrolyzed by a single PDE isoform, PDE5 (21). The limiting PDE activity amounted to an astonishing 70-100 microM cGMP hydrolyzed per second, which is 6000- to 9000-fold higher than in cerebellar astrocytes (see above). Under resting conditions, the activity was much lower, presumably because the cGMP-binding GAF domains on PDE5 were largely vacant. On exposure to 10-50 nM NO, giving 50 % or more of maximal activity of GC-coupled receptors (and similar proportions of maximal cGMP accumulation) it was deduced that the PDE5 catalytic activity became enhanced 30-fold with a half-time of about 5 s. Considering the magnitude of the cGMP response (intraplatelet cGMP levels rose to 75-150 microM within 5 s) and the submicromolar affinity of the GAF domain for cGMP (71), this is likely to correspond to the maximum rate at which PDE5 activity becomes enhanced in cells. At relatively low rates of cGMP accumulation (with low NO concentrations), the rate of PDE5 enhancement appeared to slow. A half-time of 14 s fitted the data when the platelets were exposed to 3 nM NO but the measurements here may be complicated by some of the cGMP being bound to its receptors, such as PKG (76).

In sum, knowledge of how cGMP-hydrolyzing PDEs behave in different cells is very limited. Even so, it is striking the extent to which the net PDE activity varies from cell to cell, and the short- and long-term modifications in activity that can occur in a stimulus-dependent manner. Both factors will have repercussions on the amplitudes and shapes of cellular cGMP responses to NO (see below). The potentially important implications of this plasticity on cell function remain to be investigated.

6. THE SHAPING OF CELLULAR cGMP SIGNALS IN RESPONSE TO NO

Despite innumerable publications during the past 3 decades on the changes in cGMP levels taking place in many different cells and tissues, most have limited value because the changes were not followed with respect to time, or the time intervals selected were inadequate to

resolve the underlying kinetics, or they were carried out in the presence of PDE inhibitors. Fortunately, there have been a sufficient number of exceptions to indicate that different cell types transduce NO signals in diverse ways. For instance, platelets (77), mast cells (78) and a neuroblastoma cell line (79) generated a transient, spike-like response peaking within a minute or less. In liver (80) and tracheal smooth muscle (81) the response was also transient, but slower. In heart (82) and in cells isolated from the cerebellum (13), cGMP accumulated on exposure to NO in a hyperbolic fashion, resulting in sustained plateau cGMP concentrations. One difficulty in interpreting these different shapes was that the applied NO concentrations were unknown and uncontrolled and so changes in NO concentration could have influenced the time-courses although, in some cases, it was clear that PDE activity contributed importantly to the shape observed (e.g. 80). By contrast, in cerebellar astrocytes, which generate a sustained cGMP plateau, inhibition of PDE had a minor effect on the rate and extent of cGMP accumulation during the initial minutes of NO exposure (55). The shape of the cGMP response was instead governed overwhelmingly by desensitization of the GC-coupled NO receptors. The differing shapes and sizes of cGMP responses presumably impact on the selection of downstream pathways, which range from kinases at submicromolar cGMP levels to ion channels in the low-to-mid micromolar range. In addition, the PDE activity would be expected to determine the extent to which NO signals arriving at different times can be integrated by the recipient cell. The levels of cGMP in cells with low PDE, such as cerebellar astrocytes, may serve as a readout of the net neuronal activity in the area over time scales of seconds-minutes.

6.1. Rat platelets as a model system

The first requirement for analyzing the kinetics of cellular cGMP responses in a more meaningful way is that the agonist (NO) is applied in known concentrations that remain constant for the duration of the experiment. In addition, to avoid diffusional delays and problems with the consumption of NO as it diffuses through tissues, the cells should be in direct and uniform contact with the applied NO concentration. The only study hitherto carried out under this degree of rigor was on rat platelets (21) but the findings with these cells, which possess high levels of both GC-coupled NO receptors and PDE5, provide a model that is useful in trying to understanding the behavior of other cell types, or at least of microdomains therein.

Under normal conditions in rat platelets, exposure to NO gives rise to very rapid increases in cGMP, peaking in 2-5 s and then falling at a similar speed. Assuming a uniform intracellular distribution of cGMP, the maximum cGMP concentration attained in the platelets is in the region of 100-150 μM. As the NO concentration was reduced, the amplitude became smaller but the general shape was maintained. This profile is explained by the influence of two factors: desensitization of the GC-coupled NO receptors, which varies in rate and extent depending of the NO concentration, and enhancement of PDE5 activity at a rate that is fixed at the higher NO concentration (10 nM and above) but becomes progressively slowed at lower NO concentrations (see figure 3 legend for values). The simulation depicted in figure 3A replicates the experimental data from the platelets and figure 3B illustrates the ways that the underlying time-dependent changes in the GC and PDE activities, which were measured or deduced from the experimental data, combine to shape the cGMP responses at two sample NO concentrations.

There are two obvious advantages of combining a desensitizing profile of GC activity with an augmentation of PDE activity with time. First, it is economical because desensitization reduces GTP consumption once high cGMP levels have been attained and because, without it, the cells would need twice as much PDE to bring cGMP levels down from their peak at the observed rate. Second, it permits a transient, but symmetrical, cGMP response to be generated over a wide range of NO concentrations. Without receptor desensitization (but keeping the PDE kinetics the same), the cGMP responses would become progressively briefer as the NO concentration is lowered, which may not allow sufficient time for cGMP to trigger downstream pathways: cGMP-dependent phosphorylation of vasodilator-stimulated phosphoprotein in the platelets, for example, is only just detectable after a 2-s exposure to a near-maximal NO concentration (21). With solely PDE activity varying, the peak cGMP would also be lowered (disproportionately more at the lower NO concentrations) which would reduce the effective potency of NO by a factor of 3 (EC_{50} = 30 nM instead of 10 nM).

6.2. Detection of NO transients

The measurements carried out under steady-state conditions provide important kinetic parameters but NO is probably not normally maintained at constant concentrations in the vicinity of a target cell. One extreme situation would be when there is a "minimal" NO pulse of the type depicted in figure 1. Taking, as an example, the NO profile found 0.5 micron from the theoretical source, where the peak NO concentration (1 nM) occurs 40 ms after the start of NO synthesis, the receptor model (figure 2A) predicts that GC activity rises rapidly to reach a peak after 250 ms and then declines back to baseline by about 2 s (figure 3C). Assuming a GC activity similar to that found in rat platelets, but no PDE activity, cGMP would rise to a plateau of 1 microM after about 1 s. With a PDE activity similar to that existing in rat platelets at rest, cGMP would peak after 500 ms at about 0.4 microM, a concentration able to activate PKG (although a cGMP signal as brief as this has not been shown to do so). This simulation indicates that the NO-cGMP pathway can function over time-scales analogous to those operating during slow (biochemically- rather than ionotropically-mediated) synaptic transmission (83) and is consistent with smooth muscle relaxation, which depends on several downstream reactions, starting with a delay of 5 s after the arrival of an NO pulse (53). There is, in addition, scope for temporal summation of NO signals and, more so, of the resultant GC activity. Summation would serve to boost the cellular response to an extent that would depend on the frequency of NO pulses, and it may explain how nitrergic nerves relax smooth muscle effectively because, in these preparations, the degree of relaxation increases markedly with the frequency

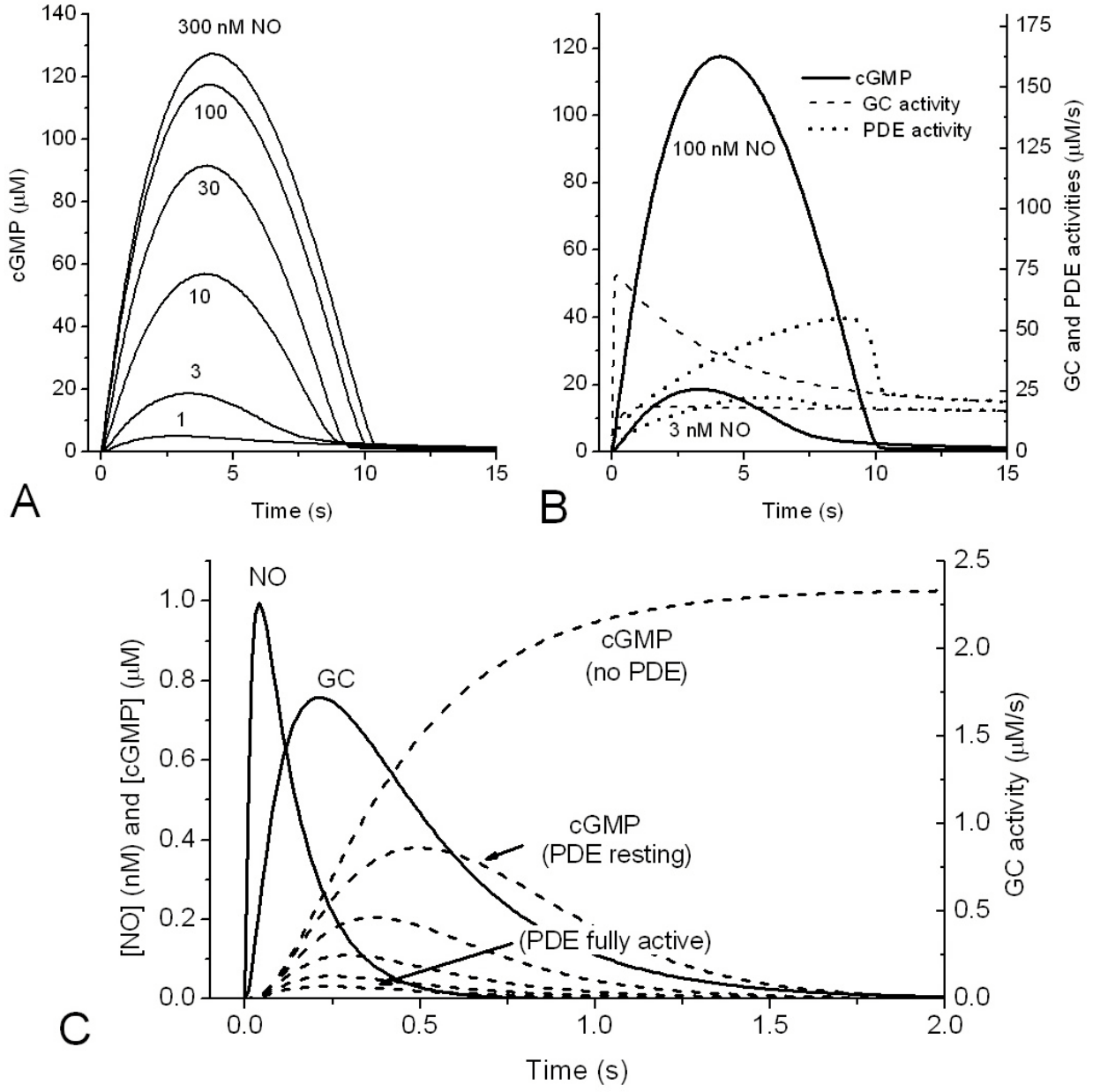

Figure 3. Detection and transduction of NO signals in cells. (A) Simulation of cGMP responses in rat platelets exposed to differing NO concentrations (indicated on each curve). The model used was as published before (21) except that NO receptor activity was simulated as in figure 2. It is assumed that GC activity comprises two components, one (limiting activity of 80 microM/s) that desensitizes and the other (limiting activity of 20 microM/s) that does not. The PDE activity (limiting activity at rest of 10 microM/s, K_m for cGMP of 5 microM) is assumed to become enhanced with time because of a 6-fold increase in limiting activity and a 5-fold reduction in K_m, giving a 30-fold increase in catalytic activity. The values of the rate constants for receptor desensitization and enhancement of PDE activity were chosen to match those experimentally measured or deduced where possible (respectively 0.25 and 0.15 s^{-1} at 100-300 nM NO, 0.17 and 0.15 s^{-1} at 30 nM NO, 0.07 and 0.12 s^{-1} at 10 nM NO, 0.01 and 0.05 s^{-1} at 3 nM NO, and 0 and 0.02 s^{-1} at 1 nM NO). (B) The underlying changes in GC (dashed lines) and PDE activity (dotted lines) giving rise to two sample cGMP profiles (solid lines) obtained at 100 nM and 3 nM NO in (A). (C) Transduction of an NO transient. The NO pulse was taken to be the one found 0.5 micron from the theoretical source in figure 1. This profile was then fed into the kinetic model used in (b) to predict the GC activity and the cGMP response (dashed lines) when PDE activity varies. The family of curves going from resting to fully active PDE correspond to those predicted to exist in platelets before (resting) and then 2, 5, 10, and 20 s (fully active) after the enhancement of PDE activity is evoked (rate constant of 0.15 s^{-1}).

of nerve stimulation, typically over the range of 1-20 Hz (e.g. 84,85).

If, at the arrival of the NO pulse, the PDE activity is enhanced above its resting value, the cGMP response would be curtailed to progressively shorter durations and lower peaks (figure 3C). Thus, a prior switch-on of PDE activity (in the case of PDE5, by previous cGMP generation) could effectively switch-off responsiveness to NO. Should it occur, such tachyphylaxis might be long-lasting because the cGMP-induced enhancement of PDE5 activity can persist for tens of minutes, putatively because PKG-induced phosphorylation traps cGMP in the GAF domain (86-88). However, data from studies of different nitrergic pathways innervating smooth muscle show that the response to nerve stimulation does not fade when the stimuli are repeated at regular intervals of 1-2 min or less (e.g. 89). In an extreme example, nitrergic nerve-induced smooth muscle relaxation was sustained during up to 2 h of continuous stimulation (90). Also, NO produces repeatable relaxations of vascular smooth muscle (91). Hence, it appears that there is either no long-term enhancement of PDE5 activity in these tissues despite repeated (or continuous) activation of the signaling pathway, or there are compensatory devices, or the PDE5 activity is maximally enhanced throughout. If the latter, the elevated PDE activity clearly does not shut down NO-cGMP signaling in these preparations.

6.3. Extrapolation to other cells

While PDE5 is a principal cGMP-metabolizing enzyme in smooth muscle, PDE2 plays an important role in hydrolyzing NO-evoked cGMP responses in a number of brain areas including hippocampus, cortex and striatum (56,92-94). The activity of PDE2 is also enhanced when cGMP binds to its GAF domain (72), so it may function similarly to PDE5. The role played by other PDEs in regulating NO-induced cGMP responses in cells remains unclear.

The model described above is derived from measurements in rat platelets, which have high GC and PDE activities, but it is quite easy to simulate the shapes of cGMP responses to NO found in other cells by reducing the levels of GC or PDE and/or the kinetic parameters of receptor desensitization or PDE enhancement. Implicit in each simulation are predictions about how the amplitude and duration of the cGMP signal would change at different NO concentrations, or durations of NO exposure, but it is premature to consider these further here because there are almost no relevant experimental data. The only pertinent findings (albeit incomplete) are from cerebellar astrocytes, where cGMP accumulates hyperbolically to attain plateau levels that vary in amplitude with the applied NO concentration (55,57). This pattern, which is dictated largely by GC desensitization, is predicted for cells with a PDE activity too low for the rate of cGMP hydrolysis to exceed the rate of cGMP synthesis during exposure to active NO concentrations.

Finally, it has been suggested that a triphasic cGMP response (i.e. a transient followed by a sustained plateau) observed in vascular smooth muscle exposed to a saturating concentration of a nitrosothiol is a consequence simply of the coexistence of GC-coupled NO receptors and PDE5, because the shape could be replicated in human embryonic kidney cells transfected with the two proteins (95). However, this shape is not seen in rat platelets (which contain the two proteins in question) unless the PDE5 inhibitor sildenafil is present (21). Being a competitive inhibitor, sildenafil lowers the affinity of the enzyme for cGMP and the higher the sildenafil concentration (i.e. the lower the affinity), the higher the amplitude of the plateau becomes. A better explanation of a triphasic profile, and one that can readily be modeled, is that there is an additional PDE operating in smooth muscle with low affinity for cGMP. The same could apply to the transfected embryonic kidney cells because these cells natively express PDE4 (96) which, despite having an affinity for cGMP in the 100 μM range, would hydrolyze cGMP significantly at the prevailing concentrations (70).

7. CONCLUSIONS/FURTHER DIRECTIONS

Knowledge of how cells signal to each other through NO is still thin, especially when viewed in comparison with other intercellular chemical messengers, such as neurotransmitters. Contrary to the original concept, NO signaling may be confined to discrete spatio-temporal domains analogous to those operating at synapses. Progress here will depend on the development of new methods for detecting NO release in subnanomolar concentrations, in subcellular dimensions, and in real time. Understanding the operation of GC-coupled NO receptors has been transformed by new methods that enable NO to be applied in fixed concentrations and that allow receptor activity to be monitored within subsecond time scales. Studies carried out using these methods have revealed that the isolated receptors are about two orders of magnitude more sensitive to NO than previously thought and that, in cells, they switch on and off with subsecond kinetics, allowing them to act as dynamic NO detectors. They also have other properties that are not seen when the protein is out of its normal environment. The underlying mechanisms need to be sought. Finally, there is growing list of small and large molecules that could function to modulate the activity of the receptor in cells and/or localize it in discrete subdomains. Whether they do so or not requires investigation.

8. ACKNOWLEDGEMENTS

Research in the author's laboratory is supported by the Wellcome Trust and the Sir Jules Thorn Charitable Trust.

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Key Words: Nitric oxide, cGMP, Phosphodiesterase, Guanylyl Cyclase, Neurotransmission, Synaptic Plasticity, cGMP-Dependent Protein Kinase, Review

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