STUDIES OF NITRIC OXIDE SIGNAL TRANSDUCTION

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1998

(Submitted January 12, 1998)

Acknowledgement

I would like to thank my family and friends for their consistent support. In particular I would like to thank my mother, Pat Sullivan and my housemates over the years, especially Beth Miles who proofread this thesis. Thanks to the "Sullivan lab night shift," the undergraduates with whom I had the privilege to work, Stephen Wong and Barbara Novak. You made me realize the truth in the saying "The smartest people at Caltech, aside from the professors, are the undergrads." I would like to thank the members of my thesis committee, each of whom helped, in his or her own way, to educate me. Special thanks is reserved for Norman Davidson whose words of wisdom steered me through.

> For almost half an hour, there is only this still-life picture: the motor home under a cloudless sky, the rugged mountainside rising above it. Now and then a breeze stirs, but all the air seems to move at once, muffling sounds, preserving a strange hush. Insects clicking softly in the sagebrush. Inside, Lisa lies on her ravaged stomach. She will explain later that it is unwise at this point to sleep: The body begins to shut down. Instead, she meditates. In her mind she makes a list of all those reasons she should quit, the complaints of 127 horrific miles, every negative thought. When the list is as long as she can make it, she lights a tiny imaginary fire-and she burns it.
>
> The door opens. She is helped down to the pavement, and she turns to the mountain.
>
> "I'm going to get to the top."
>
> Los Angeles Times, Column One 8/23/97

Abstract

Nitric oxide (NO) is released by many cells of the body, acting both as a signaling molecule or as a cytotoxic agent. In order to further our understanding of the mechanisms by which NO may function in the nervous system to regulate synaptic function, the following topics have been studied: 1) The NO-stimulated ADP ribosylation of synaptosomal proteins. 2) The subcellular localization of the neuronal isoform of nitric oxide synthase (nNOS) when the enzyme was overexpressed in embryonic hippocampal neurons by gene transfer with a recombinant adenovirus. 3) The release of NO and its primary metabolite, nitrite, as assayed by an electrochemical NO meter, from cultured cells infected with recombinant adenovirus expressing either nNOS or eNOS. Furthermore, the effects of inhibiting membrane attachment of eNOS were examined. From these studies, in Chapter 4, we conclude that the known reaction rates of NO with O_2 do not account for the observed large ratio of nitrite to NO.

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Chapter 1

Introduction and experimental questions

Nitric oxide is an important signaling molecule in a number of tissues. Its history began with the discovery that it was the elusive endothelial derived relaxing factor (Furchgott 1988, Ignarro et al. 1988). It was later shown to be released, along with other free radicals such as superoxide, by macrophages as part of the immune response (Macmicking et al. 1997). Its production is necessary for some forms of learning (Kendrick et al. 1997) and activity dependent changes in synaptic strength, such as long-term potentiation (LTP), in multiple brain regions (Linden 1994; Schuman and Madison 1994; Zhuo et al. 1994; Lev-Ram et al. 1997).

Long-lasting enhancement of synaptic transmission in the hippocampus after stimulation was first described in the synapses between axons of the perforant path and the granule cells of the dentate gyrus (Bliss and Lomo 1973). Tetanic stimulation of the axons of the pyramidal neurons in area CA3 of the hippocampus results in "CA1 LTP," the persistent strengthening of the CA3 neurons' synapses onto the dendrites of the pyramidal neurons of area CA1 (Bliss and Collingridge 1993). LTP has been extensively studied as a cellular model of "spatial" or "declarative" learning and memory because it is similar to these phenomena in the following ways (Bliss and Collingridge 1993; McHugh et al. 1996; Tsien et al. 1996): LTP is cooperative meaning that there is a threshold of stimulation which will elicit it, below which there will be no effect on synaptic strength (McNaughton et al. 1978). LTP is associative; stimulation of multiple synapses increases the probability of strengthening a synapse which is not strong enough or which is weakly stimulated (McNaughton et al. 1978; Levy and Steward 1979). This property can result, in one example, from the voltage dependence of the NMDA receptor-channels which only allow calcium to enter a cell when sufficient depolarization relieves their blockade

by extracellular magnesium ion (Ascher and Nowak 1988). Stimulation of a weak synapse or weak stimulation of a strong synapse could cause calcium flux through NMDA receptor-channels only if the stimulus is coincident with stimulation of an adjacent synapse on the same neuron which provided sufficient depolarization to unblock the NMDA receptors. Another possible mechanism for associativity is the spread of signaling molecules, such as nitric oxide, from strongly activated synapses to less active nearby synapses (Schuman and Madison 1994). Finally, both LTP and memory can be long-lasting with LTP having been seen to continue for the lifetime of the experimental tissue *in vitro* (Abel et al. 1997), or days *in vivo* (Abraham et al. 1993).

Debate regarding the cellular mechanisms responsible for this synaptic strengthening continues. One of the few assertions that is accepted universally by workers in the field is that the initial signal that triggers CA1 LTP is the influx of calcium ions through the NMDA-type glutamate receptors on the CA1 neurons (Bliss and Collingridge 1993; Tsien et al. 1996). This calcium signal results in a variety of downstream effects, all of which likely either modulate or contribute to the complex cellular phenomenon of LTP. For example, calcium can cause enhancement of the formation of cAMP (Tang and Gilman 1992) and activation of calcium/calmodulin-dependent protein kinase II (CaM kinase II or CaMKII) (Kennedy et al. 1983).

Both cAMP and CaM kinase signal transduction pathways are postulated to be important for strengthening the postsynaptic side of the CA3-CA1 synapse by enhancing activation of glutamate receptors. Postsynaptically, there is also the intriguing possibility that the number of active synapses can be modulated (Liao et al. 1995). Multiple workers have presented evidence, however, which suggests that the synaptic strengthening that

occurs during LTP results at least in part from presynaptic changes that cause an increase in the probability of glutamate release for each action potential (Bekkers and Stevens 1990; Malgaroli and Tsien 1992; Bolshakov and Siegelbaum 1995). If there is a postsynaptic signal for the induction of LTP and a presynaptic locus of expression, there must be a signal that travels from the postsynaptic cell to the presynaptic cell, a "retrograde messenger," that tells the presynaptic cell to release more neurotransmitter. The messenger must be produced in response to calcium, and it must be able to pass from its site of production in the CA1 cell to its site of action, the adjacent CA3 presynaptic terminal. The best characterized retrograde messenger is nitric oxide, NO (or NO•), a reactive free radical gas (Schuman and Madison 1994; Feelisch and Stamler 1996). Its production by nitric oxide synthases (NOSs) in the brain is stimulated by calcium-calmodulin (Stuehr and Griffith 1992). Due to the fact that it is both non-polar and uncharged, NO diffuses through cells (Feelisch and Stamler 1996). Its reactivity creates a small radius of influence which allows spatially localized signaling (Schuman and Madison 1994) and eliminates the need for special enzymatic degradation pathways. The endothelial isoform of nitric oxide synthase (eNOS or NOS3) is present in CA1 pyramidal neurons (Dinerman et al. 1994; O'Dell et al. 1994) and the neuronal isoform (nNOS or NOS1) may be present in smaller amounts (Bredt et al. 1991; Chiang et al. 1994; Dinerman et al. 1994; O'Dell et al. 1994; Wendland et al. 1994).

NO production in CA1 pyramidal neurons has been shown to be necessary for the induction of CA1 LTP by multiple researchers, especially when weak, physiological, stimuli are used (Haley et al. 1993). Inhibitors of NOSs block the induction of LTP when applied to the entire hippocampal slice (O'Dell et al. 1991; Schuman and Madison 1991;

O'Dell et al. 1994) or when added intracellularly to CA1 neurons (Schuman and Madison 1991) and postsynaptic cultured hippocampal neurons (Arancio et al. 1996), but not when added intracellularly to presynaptic cultured hippocampal neurons (Arancio et al. 1996). Also, extracellular application of hemoglobin, which binds both NO and CO, blocks LTP (O'Dell et al. 1991; Schuman and Madison 1991). Further, application of NO directly to hippocampal neurons in culture causes an increase in spontaneous neurotransmitter release (O'Dell et al. 1991; Arancio et al. 1996). Thus all of the theoretical requirements for NO to be a retrograde messenger at the CA3-CA1 synapse have been met. It is produced in the postsynaptic cell and travels to the presynaptic cell where it enhances neurotransmitter release. Adenovirus mediated gene transfer experiments in rat hippocampal slices demonstrate that eNOS is the principal NOS isoform contributing to CA1 LTP (Kantor et al. 1996). This is consistent with the observation that eNOS is more abundant than nNOS in CA1 pyramidal cells (Dinerman et al. 1994; O'Dell et al. 1994). A further prediction based on the observation that eNOS is highly expressed in the stratum radiatum dendrites of the CA1 pyramidal neurons is that slices prepared from mice which lack this enzyme ("eNOS knockouts") will not have CA1 LTP. Mice lacking eNOS have deficits in CA1 LTP when some weak stimulation paradigms are used (Wilson et al. 1997), but when typical stimulation paradigms are used, the CA1 LTP is normal and is sensitive to NOS inhibitors (Son et al. 1996; Wilson et al. 1997). Mice which are mutant for both eNOS and nNOS have greatly reduced LTP, however (Son et al. 1996). Taken together with the immunocytochemical data, these genetic observations also support the view that eNOS accounts for the majority of the NO produced during the induction of CA1 LTP. The results with the eNOS knockouts can be explained by

assuming either that low endogenous levels of nNOS can produce sufficient NO in the absence of eNOS or that nNOS is upregulated in the mutants. Although still somewhat controversial (Liao et al. 1995), the consensus that has emerged from these studies by multiple researchers employing multiple methods is that the production of NO by eNOS in the postsynaptic CA1 pyramidal cells and activation of downstream effectors of NO in the CA3 cells are necessary for the induction of CA1 LTP especially when weak, physiological stimulation paradigms are used.

NO targets

There are at least two mechanisms through which NO can influence target proteins. Nitric oxide binds iron/heme in proteins and this binding activates soluble guanylyl cyclase (sGC, Southam and Garthwaite 1991). NO also reacts with free sulfhydryls, the best example being in the NMDA receptor/channel (Lipton et al. 1993). Activation of sGC is commonly used as a sensitive assay of NO formation. It was the first known downstream effector of NO (Gruetter et al. 1981) and remains the best characterized (Koesling et al. 1991). Activity of the sGC and its enhancement by NO have been suggested to have functions ranging from muscle relaxation (Gruetter et al. 1981) to CA1 LTP (Zhuo et al. 1994; Zhuo et al. 1994; Arancio et al. 1995, but see Schuman et al. 1994; Selig et al. 1996) to olfactory learning (Kendrick et al. 1997). The reaction of NO with free sulfhydryls in the NMDA receptor/channel reduces activity of the channel and is postulated to function as an important negative feedback loop to prevent excitotoxicity (Lipton et al. 1993). NO has been shown to activate other proteins such as cyclooxygenase (Salvemini 1997), it can influence transcription (Peunova and Enikolopov 1993) and it can activate mono-ADP-ribosyltransferases (ADPRTs) (Duman

et al. 1991; Williams et al. 1992; Schuman et al. 1994; Sullivan et al. 1997), which catalyze the covalent addition of a single ADP-ribose moiety from NAD^+ to an amino acid, usually a cysteine or arginine, of a substrate protein (Ueda and Hayaishi 1985).

The fact that inhibitors of the cGMP signal transduction pathway do not inhibit the induction of CA1 LTP in all laboratories (Schuman et al. 1994; Selig et al. 1996; Stamler et al. 1997) drove the search for other actions of NO in the hippocampus. The effect of NO on the NMDA receptor/channel is inhibitory and seems unlikely to be involved in NMDA-receptor-dependent-LTP, but nitrosylation of other proteins, such as those in the synaptic vesicle docking and fusion pathway (Meffert et al. 1996; Stamler et al. 1997) may enhance synaptic function. Increased cyclooxygenase activity caused by NO, resulting in increased metabolism of arachidonic acid into prostacyclin, thromboxane A2 and the prostaglandins may play a role in LTP (Salvemini 1997). An inhibitor of phospholipase A2, the enzyme that produces arachidonic acid, has been reported to inhibit the induction of LTP (Williams et al. 1989; O'Dell et al. 1991). This result, however, is also consistent with the involvement of platelet activating factor, another retrograde messenger candidate (Kato et al. 1994), in LTP.

Modulation of ADPRTs by NO has a great deal of potential as a signaling mechanism, given the diversity of the previously identified effects of ADPRTs. For example, ADP-ribosylation of G-proteins by bacterial toxins can either enhance or inhibit their activities (Gierschik 1992; Serventi et al. 1992) with the dramatic pathophysiological results seen in cases of cholera and pertussis. Furthermore, a substrate of cholera toxin, the G-protein alpha subunit Gs, is ADP-ribosylated by endogenous ADPRTs in the brain and this reaction is modulated by NO (Duman et al. 1991; Williams

et al. 1992). In addition, induction of LTP is blocked by ADPRT inhibitors (Schuman et al. 1994). At the same time, controversy had arisen because of erroneous claims of "NO-stimulated ADP-ribosylation" (McDonald and Moss 1993). Therefore I compared the sensitivity of endogenous ADPRTs to the inhibitors used in the physiology experiments and validated the enhancement of ADP-ribosylation by NO in the hippocampus using proper controls (Sullivan et al. 1997).

NOS enzymology

NOSs catalyze the two-step conversion of L-arginine into L-citrulline and NO with N-hydroxy-L-arginine as a stable intermediate (Griffith and Stuehr 1995). The first step, in which L-arginine is converted to N-hydroxy-L-arginine with the consumption of NADPH and molecular oxygen, is analogous to the even-electron oxidations catalyzed by the cytochrome P450 enzymes and likely proceeds by a similar mechanism (Griffith and Stuehr 1995). The mechanism of the second step, the conversion of N-hydroxy-L-arginine to L-citrulline and NO with consumption of ½ NADPH, is a somewhat unprecedented odd-electron oxidation using electrons donated from NADPH which is constrained to donate two electrons. The extra electron is thought to be stored in the flavins between enzymatic cycles (Griffith and Stuehr 1995).

The three identified isoforms of nitric oxide synthase (NOS) are produced by separate genes and can be grouped into two categories based on the regulation of their expression. The neuronal and endothelial isoforms are constitutively expressed in cells in which they occur (Stuehr and Griffith 1992). They are therefore termed the "constitutive isoforms." Their activity is controlled post-translationally by binding of calcium-calmodulin (Stuehr and Griffith 1992). Expression of the "macrophage" or "inducible"

isoform (iNOS, macNOS, or NOS2) is enhanced in macrophages by stimuli including the cytokines tumor necrosis factor α, interleukin 1 β, and interferon γ in addition to microbial products such as lipopolysaccharide (Devera et al. 1996). Once expressed, NOS2 is constitutively active and tightly binds calmodulin regardless of the presence of calcium (Stuehr and Griffith 1992).

Even though it is considered a "constitutive" isoform, the human gene for nNOS is composed of 29 exons and is spliced in a tissue- and development-specific manner to form a number of different transcripts (Silvagno et al. 1996; Brenman et al. 1997; Eliasson et al. 1997; Kolesnikov et al. 1997; Lee et al. 1997). All exons except exon 1 are coding and the full-length nNOS transcript, termed nNOSα, accounts for the majority of NOS activity in the mouse brain (Eliasson et al. 1997). One variant in mice, nNOSβ which lacks the PDZ domain (see below) contained in exon 2 but is catalytically functional (Brenman et al. 1996), is expressed in brain areas such as the ventral cochlear nucleus, pedunculopontine nuclei, striatum and cortex (Eliasson et al. 1997). In skeletal and cardiac muscle, 34 amino acids are inserted between the CaM and FMN binding domains in nNOSμ (Silvagno et al. 1996). The large number of serines in the insert suggests that this splicing event introduces phosphorylation sites (Silvagno et al. 1996). Interestingly, the discovery of multiple alternate untranslated versions of exon 1 suggests that tissue and development specificity may be achieved through the use of multiple promoters (Lee et al. 1997).

NOSs are dimeric iron/heme-containing proteins which require an unusually large number of accessory molecules and cofactors for maximal activity including tetrahydrobiopterin (THB), flavin adenine dinucleotide (FAD), flavin mononucleotide

(FMN) and NADPH (Stuehr and Griffith 1992; Griffith and Stuehr 1995; Feelisch and Stamler 1996). Structural models of NOSs depict all isoforms as having three structural/functional domains. The amino terminus contains an iron/heme-, tetrahydrobiopterin- and L-arginine-binding oxygenase domain, analogous to cytochrome P450, while the carboxy terminus contains a reductase domain, ~60% homologous to cytochrome P450 reductase, which binds FMN, FAD and NADPH (Griffith and Stuehr 1995; Masters et al. 1996). The two domains are separated by a calmodulin-binding domain which regulates enzymatic activity by controlling electron transfer between the other two domains (Abu-Soud et al. 1994). In the most probable reaction mechanism, an active oxygen species is generated by reducing molecular oxygen using electrons from NADPH with iron-heme as a catalyst (Feelisch and Stamler 1996). The flavins likely participate in electron transfer and facilitate production of the active oxygen species while tetrahydrobiopterin is essential for dimerization and may participate in the reaction, possibly by preventing feedback inhibition (Mayer and Werner 1995; Feelisch and Stamler 1996).

A crystal structure of the oxygenase domain of iNOS (NOS_{OX}) has recently been solved. Similarity between the three NOS isoforms (Griffith and Stuehr 1995) predicts that the structure should be representative of the other two. It has an overall structure similar to a "catcher's mitt" with heme clasped in a "palm-like" pocket at an exposed edge of the presumptive dimer interface (Crane et al. 1997). As expected, the configuration of the L-Arginine analogue aminoguanidine bound to the crystalized enzyme suggests that the terminal nitrogen of L-Arginine would be close enough to interact directly with heme-bound oxygen. The overall fold, heme location and heme

microenvironment of NOS_{OX} are different from the known cytochrome P450 structures, suggesting that these two enzyme families have arrived at the same catalytic mechanism through convergent evolution (Crane et al. 1997). While there is no crystal structure of the calmodulin-binding- or reductase- domains, there is a crystal structure of the aforementioned homologue of the reductase domain, cytochrome p450 reductase (CPR), one of the few proteins that also binds NADPH, FAD and FMN (Wang et al. 1997). The structure shows FAD and FMN closely apposed, allowing electrons to flow directly between the two flavins without any intervening amino acid(s). NADPH binds near FAD and residues which bind cytochrome P450 lie adjacent to FMN, providing a physical basis for electron flow between the pyridine nucleotide and heme of cytochrome P450. In the case of NOSs, cytochrome P450 is analogous to the oxidase domain and electron flow between the oxidase domain and CPR-like reductase domain is controlled by the calmodulin-binding domain.

Subcellular localization

One important difference between the isoforms is their subcellular localization. The iNOS isoform exists as a soluble enzyme (Hevel et al. 1991; Stuehr et al. 1991), eNOS is particulate (Forstermann et al. 1991; Pollock et al. 1991) and nNOS is approximately half soluble and half particulate in the cerebellum (Hecker et al. 1994). Both e- and nNOS have amino-terminal motifs which determine their cellular localization. Amino-terminal myristoylation of eNOS results in its nearly 100% membrane association (Forstermann et al. 1991; Pollock et al. 1991). This membrane localization has been demonstrated to be important for eNOS's ability to release extracellular NO (Sakoda et al. 1995) and for induction of CA1-LTP (Kantor et al. 1996).

The nNOS isoform contains an amino terminal PDZ domain which presumably accounts for both the fraction of enzyme that is present in the particulate fraction of brain homogenates (Hecker et al. 1994) and its punctate subcellular localization seen in some neurons (Aoki et al. 1993; Wendland et al. 1994; Brenman et al. 1996). Nonetheless, there are multiple examples of neurons that show strong nNOS immunoreactivity in their non-synaptic cytosol (Aoki et al. 1993; Wendland et al. 1994).

The nNOS isoform's PDZ domain has been demonstrated to heteromultimerize with the second PDZ domain of PSD-95 (Brenman et al. 1996), which is present in the postsynaptic density (Cho et al. 1992); nNOS and PSD-95 immunoreactivity colocalize in multiple brain regions at the ultrastructural level (Brenman et al. 1996). Combinatorial peptide libraries have been used to show that nNOS's PDZ domain can bind a tripeptide, -D-X-V-COOH, which is present in the carboxyl-terminus of proteins such as glutamate and melatonin receptors (Stricker et al. 1997). The nNOS PDZ can bind the carboxyl-terminus of a newly identified protein called CAPON in a yeast two-hybrid system (Snowman et al. 1997). In efforts to compare the affinity of the interactions between nNOS and its various binding partners, binding of nNOS's PDZ to CAPON and the association of PSD-95's PDZ2 with the carboxy tail of the NMDA receptor subunit NR2B both compete favorably with heteromultimerization between the PDZ domains of nNOS and PSD-95 (Brenman et al. 1996; Snowman et al. 1997). The interaction between nNOS and PSD-95 was characterized using heterologous expression systems and immobilized proteins, which may allow weak interactions. Therefore, the high affinity of the association between NR2B and PSD-95 (Kornau et al. 1997) can be used to assess the relative physiological significance of the nNOS-PSD-95 interaction by introducing nNOS

into cells which express both NR2B and PSD-95, such as cultured hippocampal neurons (Kornau et al. 1995). A further motivation for these experiments comes from the block of CA1 LTP by highly specific NOS inhibitors in mice which lack eNOS (Son et al. 1996), suggesting that hippocampal expression of nNOS may be up-regulated in a compensatory manner in the eNOS knockouts. Therefore, I used an adenovirus to drive expression of nNOS in cultured hippocampal neurons, giving nNOS the opportunity to compete with physiological levels of binding partners including NR2B for PSD-95 binding. PSD-95 localizes to synapses in these cells (Kornau et al. 1995) and binding of nNOS to PSD-95 would result in punctate nNOS staining which coincides with synaptic markers. I find that staining for nNOS in Ad-nNOS infected neurons is mostly not associated with synapses, suggesting that interactions between PSD-95 and some non-nNOS binding partners is stronger than the interaction between nNOS and PSD-95, at least for the levels of the proteins which are present in hippocampal neurons.

The nNOS isoform contains sites for phosphorylation by PKA, PKC and CaM kinase (Brune and Lapetina 1991; Nakane et al. 1991; Bredt et al. 1992) and the effects of phosphorylation have been the subject of study by a number of labs. While there is still not a good consensus, phosphorylation by PKA or CaM kinase tends either to have no effect or to decrease NOS activity (Brune and Lapetina 1991; Nakane et al. 1991; Bredt et al. 1992) suggesting that phosphorylation may act as a negative feedback mechanism. Phosphorylation by PKC has been shown to enhance NOS activity, especially at low [Ca^{++}] (Nakane et al. 1991; Okada 1995, but see Bredt et al. 1992).

Metabolism of NO

The free radical NO has an elaborate chemistry in biological systems, making prediction and analysis of metabolites difficult. It can be oxidized or reduced to NO^+ and NO-, respectively. Production of NO^+ under physiological conditions is of interest because NO^+ reacts with free sulfhydryls very effectively (Ridd 1979) and this reaction can influence synaptic physiology (Pan 1996) in addition to the aforementioned effects on NMDA receptor function. Some have argued that the biochemistry of NO- is similar to that of NO due to experimental uncertainties resulting from the ease of interconversion between the two (Murphy and Sies 1991; Stamler et al. 1992; Fukuto et al. 1993). NO- does have a slightly different chemistry than NO, however, especially its conversion to N_2O after dimerization and dehydration ($k \sim 2 \times 10^9\ M^{-1}\ s^{-1}$ Stamler et al. 1992) and, possibly, its reaction with thiols to form hydroxylamine (NH_2OH) (Lipton et al. 1994).

Aside from its binding to metals (Southam and Garthwaite 1991) and its oxidation and subsequent reaction with free thiols (Ridd 1979), there are two well-characterized metabolic fates of NO. The first is the relatively slow reaction with O_2, known as autoxidation, to form nitrite, NO_2- (Ignarro et al. 1993; Feelisch and Stamler 1996), which can be further oxidized to nitrate by metalloproteins such as hemoglobin in the blood (Ignarro et al. 1993). There is also the suggestion of other, poorly characterized pathways for the conversion of NO to nitrite in biological systems (Kelm and Schrader 1990; Taha et al. 1992). The second metabolic pathway of NO is its reaction with the superoxide anion to form peroxynitrite, ONOO- ($k = 5 \times 10^9\ M^{-1}\ s^{-1}$ Feelisch and Stamler 1996). Peroxynitrite is very reactive and its chemistry is also complex. Direct measurements of the results of its decay show that it is converted to a mixture of nitrate

and nitrite in a pH- and temperature-dependent manner resulting in approximately 70-75% nitrate at physiological pH and 25°C (Lewis et al. 1995; Pfeiffer et al. 1997). In addition, peroxynitrite can react with tyrosine residues to form nitrotyrosine (Beckman et al. 1992) and it can nitrosylate thiols in a reaction which can result in NO formation (Moro et al. 1994).

These degradation and buffering mechanisms combine to create a steep spatial concentration gradient (Montague et al. 1991) and short half life of NO in biological systems which is widely assumed to be on the order of 4-6 sec (Kelm et al. 1988; Kelm and Schrader 1990; Schuman and Madison 1994). These mechanisms are advantageous since they allow spatially localized signalling which has been remarked to be present without specific degradation mechanisms (Crane et al. 1997). Nonetheless, the primary oxidation product of NO in aqueous solution, nitrite, is itself reactive and appears to be specifically degraded in hepatocytes (Kim and Lancaster 1993) and mechanisms for the conversion of NO into nitrite appear to be present in biological systems (see below).

NO release and NO diffusion through cells

The constitutive isoforms of NOS transduce calcium signals by producing NO, which must diffuse to its target; and the cellular site of origin of the NO differs between the two isoforms. The nNOS isoform is present in both cytoplasmic and membrane fractions (Hecker et al. 1994) while eNOS is almost exclusively membrane-bound (Forstermann et al. 1991; Pollock et al. 1991). Nitric oxide produced by cytoplasmic nNOS must travel through a considerable amount of cytoplasm to reach its target. While that situation may be acceptable, even necessary, if a target is in the same cell, it may decrease the efficacy of signaling if the target is in an adjacent cell. In the present work, I

measure the quenching effect of diffusion of NO through a cell's cytoplasm. The constitutive isoforms of NOS were overexpressed in CHO cells using adenoviral vectors and were stimulated by addition of calcium usually with the calcium ionophore A23187. Release of NO was directly measured using an electrochemical electrode and the primary metabolite of NO in aqueous solution, nitrite (Ignarro et al. 1993; Privat et al. 1997), was also measured. To control for variation in expression, production of NO and nitrite were normalized using the ability of homogenates of the endothelial cells to produce citrulline, the more stable product of the NOS reaction. The nNOS isoform releases less than half of the NO and nitrite that eNOS releases for a given amount of catalytic activity. It is assumed that the calcium ionophore creates a large, more or less uniform increase in intracellular calcium concentration. It was less clear whether the NOSs would respond equally to a given concentration of calcium. Measurements of the EC50 of the NOSs for calcium have varied between 100-400 nM (Bredt and Snyder 1990; Mayer et al. 1990; Schmidt et al. 1991; Silvagno et al. 1996) and between 50-300 nM (Mayer et al. 1989; Mulsch et al. 1989; Forstermann et al. 1990) for the neuronal and endothelial isoforms, respectively. These studies have suffered from the limitation that they have used high-affinity EGTA and/or EDTA calcium buffers. Therefore I used a BAPTA-based calcium buffer which has a Kd of 160 nM (Molecular probes product literature) for calcium and would buffer more reliably in the range of calcium concentrations of interest. I find that the isoforms have EC-50s that are indistinguishable at approximately 145-150 nM free calcium. Finally, pharmacological manipulation of the subcellular localization of eNOS made it possible to further test the hypothesis that the difference between the isoforms' abilities to release NO is caused by their different subcellular localizations. The drug DL-

α-hydroxymyristic acid has been shown to block protein myristoylation (Nadler et al. 1993) and cause the eNOS to become cytosolic (Kantor et al. 1996) and it decreased NO and nitrite release in my experiments.

While there are obviously many exciting and challenging avenues for research on the various aspects of nitric oxide signaling, I have endeavored to provide experimental bases for answers to the following five questions:

1) Does NO stimulate a hippocampal ADP-ribosyltransferase?
2) Does nNOS localize to synapses in Ad-nNOS infected cultured hippocampal neurons, which contain physiological levels of both PSD-95 and NR2B?
3) Does eNOS release NO to the extracellular medium more effectively than nNOS?
4) Do the NOSs differ in their abilities to release extracellular NO because of differences in membrane localization?
5) Does autoxidation of NO account for the amount of nitrite in the CHO cell medium?

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Chapter 2

Modification of hippocampal synaptic proteins by nitric oxide-stimulated ADP ribosylation

RESEARCH

Modification of Hippocampal Synaptic Proteins by Nitric Oxide-Stimulated ADP Ribosylation

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Abstract

Nitric oxide has been shown to be an important neuronal signaling molecule that participates in both behavioral and synaptic plasticity. To better understand the potential mechanisms by which NO regulates synaptic function, the ability of NO to stimulate the modification of synaptic proteins by ADP ribosylation was examined. Two NO donors, sodium nitroprusside and 3-morpholino-sydnonimine, stimulated the ADP ribosylation of proteins at apparent molecular masses of 42, 48, 51, 54, and 74 kD in hippocampal synaptosomes. This stimulation was likely owing to the production of NO by the donors; ADP ribosylation was not stimulated by non-NO decomposition products of sodium nitroprusside, and quenching of superoxide anion did not inhibit Sin-1-induced ADP ribosylation. Experiments using NAD^+ that was radiolabeled on the nicotinamide moiety demonstrated that the modification of proteins of molecular masses of 30, 33, and 38 kD are not true ADP ribosylation, whereas labeling of the 42-, 48-, 51-, 54-, and 74-kD proteins likely represent ADP ribosylation. Some of the substrates were brain specific (54 and 74 kD), whereas others (42 and 51 kD) were present in multiple nonbrain tissues.

Introduction

ADP ribosylation is the covalent attachment of an ADP-ribose moiety from NAD^+ to an amino acid of a substrate protein (Ueda and Hayaishi 1985). Examples of proteins that are substrates of this reaction include nuclear proteins such as histones and G proteins of the G_s of the $G_{i/o}$ families. G_s and $G_{i/o}$ are modified by cholera and pertussis toxins, respectively (Gierschik 1992; Serventi et al. 1992). G_s is ADP-ribosylated on an arginine residue, resulting in inhibition of its intrinsic GTPase activity, persistent association of GTP with the G protein, and constitutive activation of adenyl cyclase (Serventi et al. 1992). Conversely, ADP ribosylation of $G_{i/o}$ on a cysteine residue results in uncoupling of the G protein from its receptor (Gierschik 1992) and constitutive inactivation of the G protein. Histones are modified by an endogenous poly(ADP-ribosyl) synthetase (PARS) (Okayama et al. 1978) in response to DNA strand breaks (Cleaver and Morgan 1991). The diffusible gas nitric oxide (NO) stimulates PARS, possibly as a result of free radical-induced DNA damage (Zhang et al. 1994). These observations raise the possibility that ADP ribosylation may modify neuronal function. Recent evidence has suggested a role for endogenous ADP ribosyltransferases (ADPRTs) in synaptic plasticity. Blockade of ADPRT activity has been shown to inhibit the induction of long-term potentiation (LTP) (Schuman and Madison 1994), a form of activity-dependent synaptic plasticity in the hippocampus that may be involved in memory formation. In addition, LTP-inducing electrical stimuli modulate endogenous NO-stimulated ADP ribosylation in the rat hippocampus (Duman et al. 1993).

Inhibition of NO synthase, the enzyme that produces the diffusible gas NO, has also been demonstrated to inhibit LTP induction by some stimulation protocols (for review, see Schuman and Madison 1994). NO has a variety of downstream actions including stimulation of a soluble guanylyl cyclase to produce cyclic GMP (cGMP) (Southam

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LEARNING & MEMORY 3:414–424 © 1997 by Cold Spring Harbor Laboratory Press ISSN1072-0502/97 $5.00

and Garthwaite 1991) and *S*-nitrosylation of various proteins such as the NMDA receptor (Lei et al. 1992). In addition, NO stimulates an ADPRT in homogenates of the hippocampus (Schuman et al. 1994) and other brain areas (Williams et al. 1992). These observations suggest that release of NO may change synaptic efficacy, at least in part, by stimulating an ADPRT.

To begin to identify substrates of ADPRTs in the brain, and specifically in the synapse, the reaction was studied in vitro using rat hippocampal synaptosomes. Various synaptic proteins that have specific stimulation patterns, tissue distributions, and subcellular localizations were observed to be ADP-ribosylated. These results suggest that in addition to cGMP formation and *S*-nitrosylation, NO may modify synaptic function by stimulating the ADP ribosylation of the individual proteins associated with the synapse.

Materials and Methods

ADP-RIBOSYLATION ASSAYS

Male Sprague-Dawley rats (29–37 days old) were anesthetized with halothane and decapitated. Hippocampi were removed and placed into ice-cold artificial cerebrospinal fluid (119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSo_4$, 2.5 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11.0 mM glucose) gassed with 95% O_2 and 5% CO_2. Rat hippocampal synaptosomes were prepared by the method of Carlin et al. (1980). Following preparation, synaptosomes were flash-frozen in liquid nitrogen and stored at −70°C. The ADP-ribosylation reaction mixtures contained synaptosomal protein, NAD^+ labeled with either ^{32}P, ^3H, or ^{14}C at a final concentration of 25 μM NAD^+ in an ADP-ribosylation buffer containing 1 mM EDTA, 0.5 mM $MgCl_2$, 0.5% (vol/vol) Triton X-100, 10 mM isoniazid, 1 mM 3-acetylpyridine adenine dinucleotide (APAD), 0.1 mM 5′-guanylylimidodiphosphate (GPP[NH]P or GIDP) in a final reaction volume of 100 μl. The NO donors, sodium nitroprusside (SNP) and 3-morpholinosydnonimine (Sin-1), were prepared within an hour of use. The amounts of radioactivity and synaptosomal protein per sample were constant within an experiment, ranging between 4μCi and 9μCi of radioisotope and 80μg and 250μg of protein per sample between experiments. All stimulation data are expressed as a ratio to the nonstimulated (lacking NO) control value for a given experiment to normalize for differences in radioactivity and protein concentration between experiments. Percent inhibition data are expressed as a percentage of the amount of labeling of a protein in the presence of SNP, with the amount of labeling in the presence of SNP considered to be 0% inhibition. The percent inhibition is calculated, with the aid of ImageQuant analysis software as 100% − [100 × (integrated counts in a band in the presence of inhibitor)/(counts in presence of SNP)]. In approximately half of the experiments, 10 mM thymidine was substituted for 1 mM APAD. Because there was no appreciable difference between the results obtained with the two buffers, the data were combined. The reaction proceeded for 30 min at 37°C and was terminated by adding 1 ml of ice-cold 10% trichloroacetic acid (TCA) and centrifuging the samples at 12,300*g* for 10 min. The supernatant was aspirated; the pellet was washed once with ether and recentrifuged. Finally, the proteins were resolubilized by vigorous vortexing in a buffer containing 62 mM Tris-Hcl (pH 6.7), 0.3% SDS, and 20 mM DTT in a total volume of 100 μl. The samples were then subjected to SDS-PAGE (Laemmli 1970) and autoradiography. The superoxide quenching system consists of two enzymes: 50 U/ml of superoxide dismutase (SOD), which catalyzes the reaction $2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$, and 350 U/ml of catalase, which catalyzes $2H_2O_2 \rightarrow O_2 + 2H_2O$. For ^3H experiments, EN^{3}HANCE (NEN) was used to intensify the tritium signal.

WESTERN BLOTS

Proteins, labeled by ADP ribosylation as above, were transferred to nitrocellulose and then probed with GAP-43/B-50 affinity-purified rabbit polyclonal antisera (kindly provided by A. Beatte Oestreicher, University of Utrecht, The Netherlands). Primary antibodies were detected using goat anti-rabbit secondary antibodies conjugated to alkaline phosphatase (from Boehringer Mannheim).

TISSUE DISTRIBUTION

Tissues were dissected from halothane-anesthetized rats and placed in ice-cold sucrose buffer (containing 0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM PMSF, and 1 mg/liter of leupeptin) immediately following decapitation. The tissue were then homogenized and centri-

fuged for 10 min at 1400*g*. The supernatant was saved, flash-frozen in liquid nitrogen, and stored at −70°C. A total of 80 μg of protein was added to each experimental sample from each tissue.

SUBCELLULAR LOCALIZATION

Hippocampal synaptosomal proteins were ADP-ribosylated in ADP-ribosylation buffer, which contains 0.5% Triton X-100, as above. Postsynaptic density proteins were pelleted by centrifuging the samples at 183,000*g* for 2 hr (Carlin et al. 1980). Subsequently, the supernatant, which was enriched for cytosolic proteins, was aspirated and precipitated with TCA as above. Both the cytosolic and membrane proteins were resuspended as above in preparation for SDS-PAGE. Amounts of the two samples that corresponded to identical amounts of precentrifugation starting material were loaded onto adjacent lanes of SDS-PAGE gels. Protein concentration was determined by the methods of Lowry (Lowry et al. 1951) or Bradford (1976) and ^{32}P was quantified by liquid scintillation counting and PhosphorImager analysis. Statistical significance was determined by one-tailed sign test or *t*-test as indicated. Data are expressed as mean ± S.E.M.

MATERIALS

[^{3}H-adenine-2,8]NAD^{+} was from ICN; [4-^{3}H]nicotinamide^{+} NAD, [^{14}C-carbonyl]nicotinamide NAD^{+}, and [U-^{14}C]adenine NAD^{+} were from Amersham; Sin-1 was from Molecular Probes, sodium nitroferricyanide (SNP) was from Mallinckrodt and Sigma; potassium ferricyanide was from Fisher; SOD and catalase were from Sigma; molecular weight markers were from BioRad. All other reagents were from Sigma.

LAB ANIMALS

The experimental protocols for the use of laboratory animals were approved by the appropriate institutional review committee and meet the guidelines of the National Institutes of Health (NIH).

Results

BASAL AND NO-STIMULATED ADP RIBOSYLATION IN HIPPOCAMPAL SYNAPTOSOMES

Under control conditions, at least 10 different proteins in hippocampal synaptosomes of approximate molecular masses of 42, 46, 48, 50, 51, 54, 69, 74, 95, and 116 kD were labeled by ^{32}P-labeled NAD^{+} (Fig. 1, Control). Addition of the NO donors Sin-1 or SNP enhanced labeling of the proteins at 42, 51, 54, and 74 kD in a concentration-dependent manner (Fig. 1, lanes 2–4, 8–10). Maximal stimulation of these proteins was observed with SNP (5.0 mM; lane 4) and Sin-1 (10 mM; lane 10); SNP usually produced slightly greater stimulation than Sin-1. The fold stimulation by NO, relative to control labeling, is presented in Table 1. A prominent protein at 38 kD exhibited labeling that was stimulated more robustly by Sin-1 than by SNP. Its stimulation is also quantified in Table 1. The ADP ribosylation of the 46- and 50-kD proteins was frequently inhibited, rather than stimulated by NO. Stimulation of very faint proteins at 30, 33, 48, 95, and 116 kD was occasionally observed but was not consistent enough to quantify.

When both Sin-1 and SNP decompose spontaneously in aqueous solution, they release NO and other products. SNP decomposes into $[Fe(CN_5)]^{2-}$ and NO, and Sin-1 releases a superoxide anion (O_2^-) before liberating NO (Southam and Garthwaite 1991). For this reason, the stimulation of ADP ribosylation resulting from addition of either SNP or Sin-1 could be caused by products other than NO. To determine whether the SNP stimulation is owing to NO or another SNP product, old SNP that had been exposed to light at room temperature for 12 hr was added to the reactions. Considering the short half-life of NO in aqueous conditions (Garthwaite and Boulton 1995), this solution should contain primarily $[Fe(CN_5)]^{2-}$. This NO-depleted SNP did not stimulate ADP ribosylation is synaptosomes (Fig. 1, lane 5). Similarly, the addition of potassium ferricyanide, a compound that is structurally similar to SNP but that lacks NO, did not stimulate ADP ribosylation (Fig. 1, lane 6; similar results were obtained in seven additional experiments). The question of whether superoxide production by Sin-1 contributes to the stimulation of ADP ribosylation was also addressed. A superoxide quenching system, consisting of 50 U/ml of SOD and 350 U/ml of catalase (Garthwaite et al. 1988), was added to a reaction mixture containing 1 mM Sin-1. If superoxide anion contributes to the Sin-1 stimulation, then the addition of SOD/CAT should decrease the labeling. This was only observed for the 38-kD protein (Fig. 1, lane 12), suggesting that superoxide contributes to the stimulation of this protein but does not modulate the other proteins. Similar results were obtained in ex-

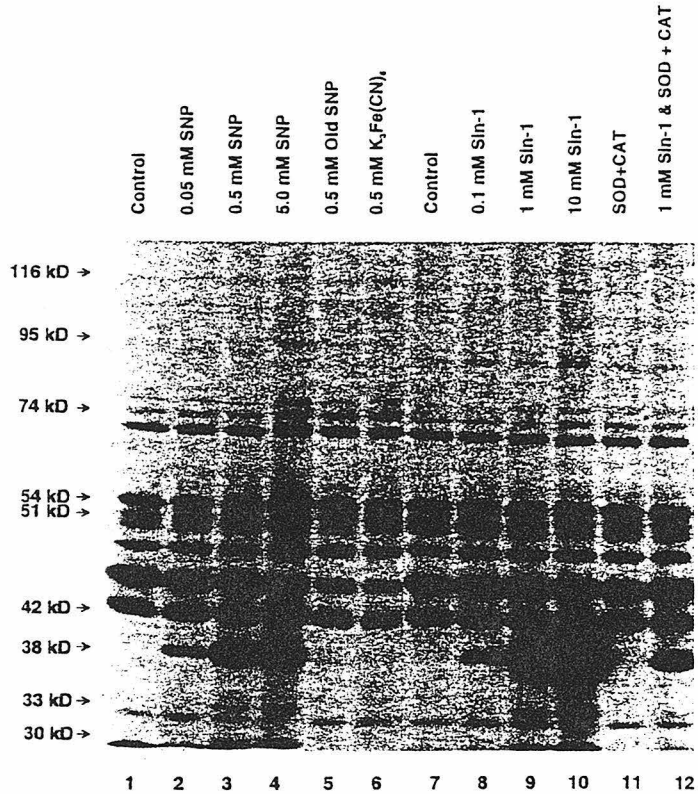

Figure 1: Basal and NO-stimulated ADP ribosylation of proteins in hippocampal synaptosomes. Hippocampal synaptosomal proteins were ADP-ribosylated as described in Materials and Methods in the presence of ^{32}P-labeled NAD^+. Two NO donors, SNP (lanes *2–4*) and Sin-1 (lanes *8–10*), were added to the reaction mixtures at the indicated concentrations resulting in the enhanced ADP ribosylation of proteins at 38, 42, 51, 54, 74, 95, and 116 kD. The addition of light-inactivated SNP (old SNP, lane *5*) or potassium ferricyanide ($K_3Fe(CN)_6$, lane *6*) did not stimulate the ADP ribosylation of substrates. The addition of a superoxide quenching system (lanes *11,12*) to 1 mM Sin-1-treated reactions reduced the stimulation of the 38-kD protein but did not significantly affect the other substrates.

periments in which 10 mM Sin-1 was used with the superoxide quenching system (data not shown; $n = 5$).

The sensitivity of the NO-stimulated ADP ribosylation to nicotinamide and novobiocin, inhibitors of ADPRTs (Rankin et al. 1989), was also exam-

Table 1: *Summary of NO donor effects on ADP ribosylation of hippocampal substrates*

Molecular mass (kD)	Sin-1 (mM) fold stimulation			SNP (mM) fold stimulation		
	0.1	1	10	0.05	0.5	5.0
38	2.37* (7/8)	20.58* (8/8)	149.36* (8/8)	2.43* (8/8)	12.85* (11/11)	25.68* (13/13)
42	0.97 (3/9)	1.06 (6/9)	1.29* (9/9)	0.99 (3/8)	1.00 (4/12)	1.25 (10/14)
51	1.14 (6/9)	1.27* (8/9)	1.42* (7/8)	0.96 (3/9)	1.30* (11/12)	1.70* (14/14)
54	1.06 (4/8)	1.07 (4/8)	1.18 (5/7)	0.96 (3/8)	1.04 (7/11)	1.44* (13/13)
74	1.04 (4/8)	1.12* (7/8)	1.27* (7/8)	0.97 (3/9)	1.17 (9/12)	2.00* (14/14)

The values shown are the mean-fold stimulation in the presence of NO donor relative to control. (A value of 1 indicates no stimulation). The values in parentheses indicate the number of experiments in which any stimulation was observed; the mean values were calculated including all individual experiments. Significance at $P < 0.05$ is indicated by an asterisk (*).

ined. Nicotinamide (10 mM or 100 mM) reduced the NO donor-stimulated ADP ribosylation of the 42-, 46-, 51-, and 54-kD proteins (mean percent inhibition of labeling in the presence of 0.5 mM SNP by 10 mM or 100 mM nicotinamide, respectively: 42 kD, 24.0 ± 0.06% or 52.1 ± 0.13%; 46 kD, 19.8 ± 0.09% or 69.0 ± 13.3%; 51 kD, 23.4 ± 14.4% or 56.8 ± 18.1%; 54 kD, 16.3 ± 13.8% or 54.3 ± 21.9%; $n = 3$ for all conditions) (Fig. 2). Novobiocin (0.5, 1, or 2 mM) also attenuated the NO-stimulated modification of the 42-, 46-, 51-, 54-, and 74-kD substrates (mean percent inhibition of labeling in the presence of 0.5 mM SNP by 0.5 mM or 2 mM Novobiocin, respectively: 42 kD, 13.0 ± 7.0% or 20.7 ± 7.2%; 46 kD, 6.6 ± 10.9% or 35.3 ± 11.9%; 51 kD, 10.6 ± 19.6% or 48.1 ± 5.6%; 54 kD, 12.8 ± 12.0% or 74.4 ± 6.2%; 74 kD, 0.0 ± 19.7%, or 56.9 ± 5.3%; n = at least 3 for all conditions). The sensitivity to other inhibitors of ADP ribosylation could not be determined owing to independent effects of the solvents.

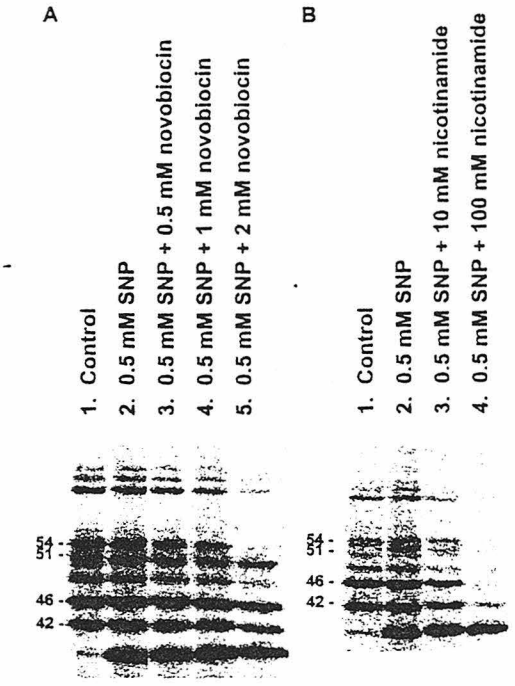

Figure 2: NO-stimulated ADP ribosylation of substrates is attenuated by inhibitors of mono ADP ribosylation. Shown are sample PhosphorImager images of experiments that show the clearest example of the sensitivity of NO-stimulated ADP ribosylation to two inhibitors of ADP ribosylation, novobiocin, and nicotinamide. (*A*) Novobiocin produced a concentration-dependent inhibition of SNP-stimulated substrates at 42, 46, 51, 54, and 74 kD. (*B*) Nicotinamide produced a concentration-dependent inhibition of SNP-stimulated substrates at 42, 46, 51, and 54 kD.

DOES THE MODIFICATION OF THE SUBSTRATES REPRESENT TRUE ADP RIBOSYLATION?

The protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was originally reported to be a substrate of NO-stimulated ADP ribosylation (Brüne and Lapetina 1989, 1990; Duman et al. 1991; Dimmeler et al. 1992; Kots et al. 1992; Zhang and Snyder 1992). Subsequent studies (McDonald and Moss 1993), however, have shown that a novel NAD^+ metabolite that includes the nicotinamide moiety of NAD^+ in addition to the ADP-ribose moiety is added to GAPDH. The physiological relevance, if any, of this autocatalytic (Dimmeler et al. 1992; Kots et al. 1992; Zhang and Snyder 1992) reaction is unknown. Does the modification of hippocampal proteins described here reflect true ADP ribosylation? To address this issue, NAD^+ was utilized, which had been radioactively labeled on the nicotinamide moiety with either ^{14}C or ^{3}H. The nicotinamide group is not added to the protein in an authentic ADP-ribosylation reaction (Ueda and Hayaishi 1985). Little labeling of proteins was observed when [4-^{3}H]nicotinamide NAD^+ was added to synaptosomal protein in a reaction mixture lacking NO donors (Fig. 3, lane 1; data not shown; n=3. In the presence of 1 mM Sin-1 (Fig. 3, lane 2), however, there was labeling of the proteins at 30, 33, and 38 kD, demonstrating that their labeling does not represent true ADP ribosylation. Lanes 3 and 4 of Figure 3 show the results of samples that were treated the same as those in lanes 1 and 2, respectively, except [^{3}H-adenine-2,8]NAD^+ was used. As expected, the pattern of labeling was similar to the experiments where ^{32}P-labeled NAD^+ was used. Similar experiments using ^{14}C-labeled NAD^+ yielded essentially the same results (data not shown; n = 2).

TISSUE DISTRIBUTION AND SUBCELLULAR LOCALIZATION OF THE SUBSTRATES

TISSUE DISTRIBUTION

Various rat tissues including spleen, penis, skeletal muscle, heart, and liver were homogenized

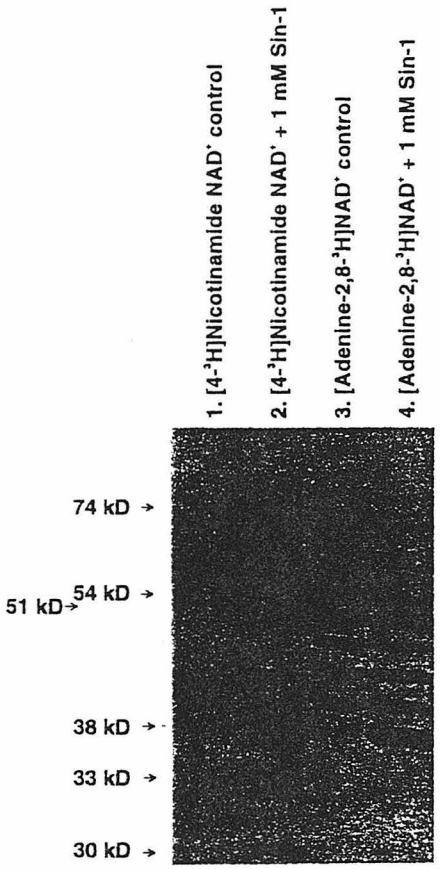

Figure 3: The majority of hippocampal substrates are modified by ADP ribosylation. Control reactions conducted in the presence of NAD^+ labeled on the nicotinamide moiety ([4-^{3}H]nicotinamide NAD^+) did not result in the detectable labeling of any substrates (lane *1*). The addition of 1 mM Sin-1 to nicotinamide-labeled NAD^+ reactions resulted in the labeling of proteins at 30, 33, and 38 kD, indicating that these substrates are not modified by true ADP ribosylation (lane *2*). In a separate set of reactions using NAD^+ labeled on the adenine moiety ([^{3}H-adenine-2,8]NAD^+), a different set of proteins of molecular masses of 42, 46, 48, 51, 54, and 74 kD were labeled under control conditions (lane *3*). The lack of labeling of these same proteins in lanes *1* and *2* indicates that these proteins are labeled by true ADP ribosylation.

and labeled by ADP ribosylation as described in Materials and Methods ($n = 3$). Hippocampal homogenate and homogenate made from forebrain (including the hippocampus) were also examined ($n = 3$). The same amount of protein was used for each tissue sample, permitting an analysis of the brain and tissue specificity of the various substrates by comparing the absolute amounts of labeled protein across samples. An example of the patterns of labeling observed for the rat spleen, penis, and skeletal muscle are presented in Figure 4B with the pattern from hippocampal synaptosomes shown in Figure 4A as a reference. The quantified data are presented in Table 2. Overall, the pattern of labeling in the various tissues was similar to that in the brain, with some notable differences. Although it is unclear whether the labeled proteins observed in the various tissues are identical, proteins of 38, 42, and 51 kD appeared in all of the tissues examined.

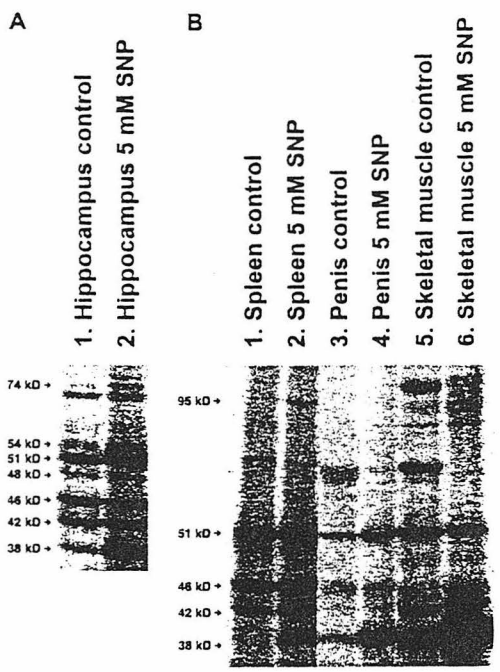

Figure 4: ADP-ribosylation substrates are present in both neural and non-neural tissue. (*A*) Control (lane *1*) and SNP-stimulated ADP ribosylation (lane *2*) in hippocampal synaptosomes are shown for reference. (*B*) ADP-ribosylation substrates in various non-neural tissues. In spleen homogenate (lanes *1,2*), ADP ribosylation of 38-, 42-, 46-, 51-, and 95-kD proteins were frequently enhanced by SNP. In penis homogenate (lanes *3,4*) ADP ribosylation of 38-, 42-, and 51-kD proteins were frequently enhanced by SNP. In skeletal muscle homogenate (lanes *5,6*) ADP ribosylation of 38-, 42-, and 51-kD proteins were frequently enhanced by SNP; in addition, there was substantial labeling of the 38-kD protein in the absence of the NO donor.

Table 2: *Summary of NO-stimulated ADP ribosylation in hippocampal and other tissues*

Molecular mass (kD)	Hippocampal homogenate	Forebrain homogenate	Penis	Skeletal muscle	Heart	Spleen	Liver
38	5.10*	1.92*	12.47*	12.22*	12.61	2.10	10.48*
	1.47	2.30	0.89	14.21	0.99	0.79	0.61
42	1.64*	1.13	1.29	1.49	1.27	1.79	2.16*
	1.31	2.05	0.37	2.94	0.52	1.13	0.41
51	1.24	0.86	3.00*	1.37*	1.16	0.84*	1.10
	1.79	2.23	0.38	1.91	4.51	2.66	2.71
54	—	—	—	—	—	—	—
74	0.82	—	—	—	—	—	—
	6.70						

The tissue distribution of basal and NO-stimulated ADP ribosylation are shown for five different proteins in various tissues. The top number of each pair represents the average-fold stimulation in the presence of 5 mM SNP, relative to control levels of ADP ribosylation in the same tissue, as in Table 1. The bottom number of each pair represents the fold enrichment of that band in that tissue compared to hippocampal synaptosomes. This bottom number was calculated as the ratio of the amount of the band in the indicated tissue, without NO donor, to the amount of labeling of the band at the same molecular mass, also without NO donor, in hippocampal synaptosomes. A value of 1.00 indicates no difference. (–) No labeling was observed. Asterisks (*) indicate significance at the $P < 0.05$ levels.

Each of these proteins was stimulated by 5 mM SNP in at least one of the tissues examined. A 46-kD protein was seen in all of the brain samples as well as in the liver and spleen, and a faint 44-kD protein appeared in skeletal muscle, heart, and penis. The 54- and 74-kD proteins were present in all of the brain samples, although the high background in the brain homogenates made them difficult to quantify. They were not, however, discernable in any of the other tissues.

SUBCELLULAR LOCALIZATION

The enrichment of the enzyme activity and the substrates in various subcellular fractions were quantified by conducting subcellular fractionation either before or after the NO-stimulation ADP-ribosylation reaction. In each experiment the amount of radioactivity incorporated into a particular molecular weight band was quantified by PhosphorImager analysis. When tissues were fractionated before the ADP-ribosylation reaction, the labeling of proteins at apparent molecular masses of 42, 46, and 51 kD was enriched in hippocampal synaptosomes compared with hippocampal homogenate (Table 3). When hippocampal synaptosomes were further separated into postsynaptic density (PSD) (Carlin et al. 1980) and non-PSD fractions, labeling of the 46- and 51-kD proteins was significantly enriched in the non-PSD fraction compared with the PSD (Table 3). Because the distribution of the ADPRT enzyme in the synaptosome, PSD, and cytosol fractions relative to the homogenate is not known, it is unclear whether the observed differences result from a differential distribution of the substrates, the enzyme, or both. However, when the synaptosome fraction is separated into PSD and non-PSD fractions after the ADP-ribosylation reaction, most of the labeled proteins are associated with the non-PSD fraction compared with the PSD (data not shown), suggesting that most of the substrates of ADP ribosylation are not tightly associated with the PSD either before or after modification.

SUBSTRATES

The possibility that one of the ADP-ribosylation substrates is the growth-associated protein-43 (GAP-43; B-50), which has been reported by others to be ADP-ribosylated (Coggins et al. 1993; Philibert and Zweirs 1995), was examined. GAP-43 runs in SDS-PAGE gels at between 43 and 50 kD depending on the density of the acrylamide polymer matrix (Gower and Rodnight 1982; Oestreicher et al. 1984; Benowitz et al. 1987). Proteins were radioactively labeled by ADP ribosylation using ^{32}P-labeled NAD^+ in the absence (Fig. 5, lane 3) or presence (Fig. 5, lane 4) of 5 mM SNP (exactly as those in Fig. 1, lanes 1 and 4, respectively). They were

Table 3: *ADPRT activity in the presence of 5 mM SNP is preferentially associated with certain subcellular fractions*

Molecular mass (kD)	Synaptosome (fmoles/mg per min)	Homogenate	Synaptosomes PSD (fmoles/mg per min)	Synaptosomes non-PSD (fmoles/mg per min)
42	2.93 ± 0.14*	1.40 ± 0.10	2.49 ± 0.18	2.29 ± 0.02
46	1.49 ± 0.10*	0.70 ± 0.08	0.74 ± 0.04	1.24 ± 0.05*
51	1.86 ± 0.04*	1.21 ± 0.10	0.71 ± 0.02	1.12 ± 0.04*

Numbers represent NO-stimulated incorporation of radioactivity in fmoles/mg per min for each molecular mass, calculated using PhosphorImager analysis. Statistical comparisons were made for each molecular mass comparing synaptosome and homogenate or PSD and cytosol. Asterisks (*) indicate significance at the $P < 0.05$ level; $n = 3$ for all conditions.

then transferred to nitrocellulose and probed with an affinity-purified GAP-43 antibody. The GAP-43 immunoreactivity (Fig. 5, lanes 1,2) comigrates with the 48-kD ADP-ribosylated protein (lanes 3, 4). The stimulation of this protein was quantified indicating that the 48-kD protein is modestly stimulated by 5 mM SNP (20 ± 3%; $n = 5$) and by 10 mM Sin-1 (29 ± 6%; $n = 5$).

Discussion

NO STIMULATES ADP RIBOSYLATION OF PROTEINS IN HIPPOCAMPAL SYNAPTOSOMES

The present study demonstrates the ADP ribosylation of several synaptic proteins, complementing previous studies (Duman et al. 1991; Williams et al. 1992). Stimulation of ADP ribosylation of proteins of apparent molecular masses of 74, 54, 51, 48, and 42 kD resulted from the addition of either of two different NO donors. As the two donors are structurally dissimilar compounds with different decomposition products, the observed stimulation was likely owing to the release of NO. This is further supported by experiments that demonstrated that ferricyanide and light-inactivated SNP did not stimulate ADP ribosylation (Fig. 1, lanes 5,6). In addition, the experiments in which 1 mM or 10 mM Sin-1 was combined with the superoxide anion quenching system of SOD/CAT showed that the production of superoxide anion did not contribute to the stimulation of ADP ribosylation by Sin-1.

THE OBSERVED LABELING IS THE RESULT OF ADP RIBOSYLATION

Given the controversies in the ADP-ribosylation literature surrounding the validity of claims that NO stimulates ADP ribosylation of proteins (McDonald and Moss 1993), it is perhaps most significant that we have demonstrated that in the

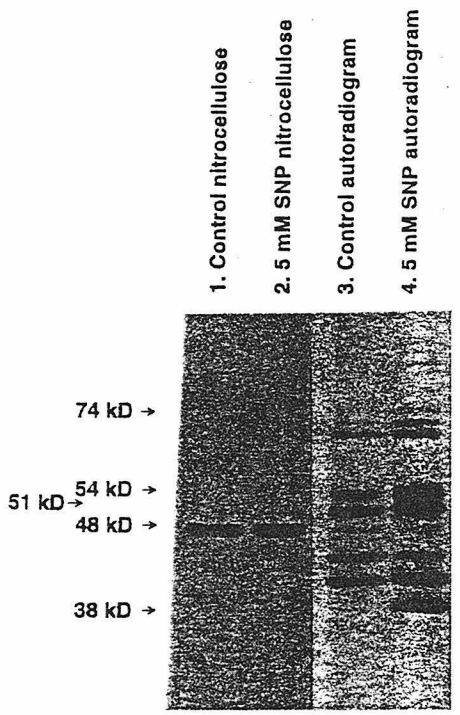

Figure 5: GAP-43 immunoreactivity comigrates with a 48-kD ADP-ribosylated substrate. GAP-43 immunoreactivity determined by Western blot analysis is shown in lanes *1* and *2*. The corresponding autoradiogram (lanes *3,4*) that was exposed to the nitrocellulose in lanes *1* and *2* shows the pattern of NO-stimulated ADP ribosylation, indicating that the GAP-43 immunoreactivity comigrates with the 48-kD protein.

presence of 1 mM Sin-1, the nicotinamide moiety was not added to the proteins at 74, 54, 51, 48, and 42 kD. It was, however, added to the prominently labeled 38-kD protein and the faintly labeled protein at 33 and 30 kD. These data suggest that the 30-, 33-, and 38-kD proteins were not the substrates of ADP ribosylation in the presence of 1 mM Sin-1, whereas the labeling of proteins at 74, 54, 51, 48, and 42 kD likely represents true ADP ribosylation.

TISSUE DISTRIBUTION AND SUBCELLULAR LOCALIZATION

Two proteins that appeared to be brain specific were those at 54 and 74 kD, both of which were absent from all of the non-neural tissues examined. Many of the proteins that were ADP-ribosylated in hippocampal synaptosomes, including those at 38, 42, 46, and 51 kD, appeared in other tissues as well. The ADP ribosylation of many of these proteins was stimulated by the NO donor SNP. These observations raise the possibility that ADP ribosylation may be a downstream effector of NO's participitation in immune responses or in smooth muscle relaxation. Lack of brain specificity of some of the substrates, however, does not necessarily imply that the proteins are not important for brain function. For example, Greengard and coworkers found that cAMP stimulated PKA to phosphorylate its regulatory subunit in the brain (Lohmann et al. 1980; Walter and Greengard 1981). The type II regulatory subunit is present in the heart, and its phosphorylation by PKA is stimulated by cAMP there as well (Lohmann et al. 1980). Thus, the substrates common to both neural and non-neural tissues may be important regulatory elements for signal transduction events.

The synaptosome preparation of Carlin et al. (1980) used in these studies is made up primarily of sealed-off presynaptic terminals with a PSD from a ruptured postsynpatic cell attached (Kennedy et al. 1990). The enrichment of many of the substrates in the non-PSD fraction suggests that the proteins may be presynaptic and further increases the likelihood that NO can act as a retrograde messenger.

IDENTITY OF THE SUBSTRATES

Others (Duman et al. 1991) have suggested that the 42- and 45-kD (in our experiments, 46-kD) proteins correspond to the isoforms of $G_{s\alpha}$. This conclusion was based on the observed similarities between the patterns of peptides in one-dimensional gels resulting from V-8 protease digestion of proteins that had been labeled with cholera toxin and those that had been labeled by endogenous ADPRTs. The present studies indicate that GAP-43 immunoreactivity comigrates with a 48-kD labeled protein that is stimulated by NO. This result is consistent with the reports of Zweirs and co-workers that GAP-43 is ADP-ribosylated in response to NO (Coggins et al. 1993; Philibert and Zweirs 1995). McDonald and Moss (1993) have shown previously that the 39-kD protein GAPDH shows stimulation properties similar to the 38-kD protein described here.

MAGNITUDE OF STIMULATION

The largest stimulation observed was a doubling of the labeling of the 74-kD band in the presence of 5 mM SNP compared with control. The stimulation observed for soluble guanylyl cyclase, arguably the best characterized downstream effector of NO, is orders of magnitude greater than that reported here for the ADPRTs (Southam and Garthwaite 1991). The difference suggests the possibility that soluble guanylyl cyclase might be the primary target of NO, whereas ADPRTs may play a modulatory role. The range of concentrations of NO donors that stimulate the two enzymes are similar, however, suggesting that both targets (guanylyl cyclase and ADPRT) may be stimulated upon physiological release of NO.

Another possibility is that NO acts synergistically with other, as-yet-unidentified second messengers to maximally stimulate the ADPRTs. The situation might be similar to that for protein kinase C (PKC). PKC isoforms are synergistically stimulated by multiple lipids, some of which have only modest effects when applied alone (McPhail et al. 1984; Bell and Burns 1991; Shinomura et al. 1991).

IMPLICATIONS FOR SYNAPTIC PLASTICITY

Our earlier experiments failed to support a role for cGMP as the downstream effector of NO during LTP (Schuman and Madison 1994), although others have suggested a role (Zhuo et al. 1994a,b; Arancio et al. 1995; but see Selig et al. 1996). The relative amount of NO-stimulated ADP ribosylation

demonstrated under the present experimental conditions, however, is modest for most substrates examined. This suggests that there may be additional routes by which NO influences synaptic function, one of which may be the direct nitrosylation of presynaptic vesicle proteins (Meffert et al. 1996). Electrophysiological studies of LTP have shown that inhibitors of ADPRT can block LTP (Schuman and Madison 1994). Might any of the NO-stimulated proteins observed in this study contribute to LTP? A previous study observed that prior LTP induction reduced the amount of SNP-stimulated ADP ribosylation that could be elicited (Duman et al. 1993). We attempted to determine whether the same inhibitors that prevented LTP had an effect on the NO-stimulated ADP ribosylation of the hippocampal synaptic proteins. We were unable to examine one of the ADPRT inhibitors used in the LTP studies, phylloquinone, as it required an organic solvent to dissolve it. Nonetheless, one inhibitor, nicotinamide, significantly inhibited the ADP ribosylation of the 42- and 51-kD proteins at concentrations similar to those that prevented LTP. Taken together with previous studies, these data show that NO released during the induction of synaptic plasticity may modify synaptic proteins by ADP ribosylation. Determining the identity of ADP-ribosylation substrates and how the ADP-ribosylation pathway may interact with other NO effectors and other signal transduction pathways will be the focus of future studies.

Acknowledgments

We thank A. Beate Oestreicher for generously providing the GAP-43 antibody and to Mary Kennedy for much advice and discussion. This work was supported by the NIH and by monies from the Alfred P. Sloan Foundation, John Merck Fund, and the PEW Charitable Trusts (all to E.M.S.).

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Received October 29, 1996; accepted in revised from January 23, 1997.

Chapter 3

Adenovirus-mediated expression of the neuronal isoform of nitric oxide synthase in cultured hippocampal neurons

Introduction

Nitric oxide is an important signaling molecule in multiple tissues (Barnes and Liu 1995; Fineman et al. 1995; Garthwaite and Boulton 1995; Wang et al. 1995). The "neuronal" isoform of nitric oxide synthase, nNOS or NOS1, contains an amino-terminal PDZ domain which is thought to be responsible for its partially particulate subcellular localization (Egberongbe et al. 1994; Hecker et al. 1994; Gerlach et al. 1995; Brenman et al. 1996). PDZ domains are present in a variety of cell junction-associated molecules from which the domain takes its name including the first PDZ protein identified, PSD-95, and its homologs at synapses (Cho et al. 1992), dlg-A in septate junctions (Woods and Bryant 1991) and ZO-1/-2 from tight junctions (Willott et al. 1993; Jesaitis and Goodenough 1994). The PDZ domain in nNOS binds specifically to the second PDZ domain of PSD-95 (Brenman et al. 1996), to syntrophin (Brenman et al. 1996), to PSD-93 (Brenman et al. 1996) and to the carboxy tail of a newly identified protein named CAPON (Snowman et al. 1997) There is also a suggestion that nNOS can bind a carboxyl-terminal domain DXV-COOH, which is present in glutamate and melatonin receptors (Stricker et al. 1997). The second PDZ domain of PSD-95 associates with the carboxy-terminal tSXV sequence found in the NMDA-type glutamate receptor NR2B (Kornau et al. 1995; Brenman et al. 1996) and potassium channels (Kim et al. 1995; Cohen et al. 1996). The picture that is emerging is one of promiscuous binding of the second PDZ domain of PSD-95. This proposed situation is quite different from the specific binding of PDZ domains in the inaD protein to components of the Drosophila phototransduction cascade such as channels, phospholipase C and protein kinase C (Chevesich et al. 1997; Tsunoda et al. 1997).

Biochemical fractionation of brain tissue reveals differences between the localization of endogenous PSD-95 and nNOS. PSD-95 is present primarily in particulate fractions such as the postsynaptic density (PSD) (Cho et al. 1992) while endogenous nNOS is present to varying degrees in both soluble and particulate fractions (Hecker et al. 1994; Gerlach et al. 1995). Microscopy also reveals differences between the localization of the endogenously-expressed proteins. Immunoreactivity for nNOS is present in both the cell body and the processes of neurons in humans (Egberongbe et al. 1994) and fish (Holmqvist and Ekstrom 1997) while PSD-95 is observed mostly in a punctate pattern along dendrites (Cho et al. 1992; Kornau et al. 1995; Apperson et al. 1996; Hunt et al. 1996). nNOS may also associate with intracellular organelles such as the endoplasmic reticulum (Wolf et al. 1992).

The interaction between PSD-95 and nNOS was initially characterized using yeast two-hybrid techniques, heterologous expression systems and immobilized proteins, all of which may allow weak interactions and none of which contain other proposed nNOS- or PSD-95-binding partners. Attempts have been made to compare the affinities of the interaction between nNOS and PSD-95 to the affinity of their interactions with their other binding partners using immobilized proteins (Brenman et al. 1996; Snowman et al. 1997). The studies have shown that the carboxyl tail of either CAPON or NR2B competes favorably with the PDZ-PDZ interaction between PSD-95 and nNOS.

The expression of nNOS in hippocampal pyramidal neurons has been controversial (Bredt et al. 1991; Chiang et al. 1994; Dinerman et al. 1994; O'Dell et al. 1994; Wendland et al. 1994) likely due to low endogenous levels of expression combined with the limited resolution of antibodies (Wendland et al. 1994). Recent genetic results

suggest that nNOS can play a functional role in these cells (Son et al. 1996). Mice that lack the endothelial isoform of the nitric oxide synthase, eNOS (eNOS (-/-) mice), still exhibit LTP that is blocked by NOS-specific inhibitors which do not distinguish between enzyme isoforms. LTP is severely impaired in mice that lack both eNOS and nNOS ("double mutants"), suggesting that either normal endogenous levels of nNOS can generate sufficient NO in the eNOS mutants for the induction of LTP or nNOS is up-regulated in a compensatory manner in the eNOS mutants. Because of the possibility of developmental compensation, the results of the LTP studies on eNOS (-/-) mice are not decisive as to whether nNOS occurs in CA1 pyramidal neurons in wild-type mice. Therefore, the subcellular localization of nNOS overexpressed in hippocampal pyramidal neurons is of potential interest for interpretation of the LTP results from the NOS mutants.

We report the use of adenoviral-mediated expression of nNOS in cultured hippocampal neurons, which contain PSD-95 along with physiological levels of its binding partners such as NR2B (Kornau et al. 1995), to estimate the relative strength of the interaction between PSD-95 and nNOS compared to the interaction between PSD-95 and its other binding partners. PSD-95 exhibits a punctate pattern of labeling in these cells with the puncta corresponding to individual labeled synapses (Cho et al. 1992; Hunt et al. 1996). If nNOS preferentially bound PSD-95, or another synaptically localized protein, in these cells its immunoreactivity would also be punctate with some of the nNOS immunoreactivity present in the puncta.

Methods

Virus construction

A bluescript (SK(-)) plasmid containing the rat brain nNOS gene cloned into the Eco R1 site was kindly provided by David Bredt (University of California, San Francisco). It contains 348 bp of 5' untranslated region (UTR), 3660 bp of coding region and 1037 bp of 3' UTR. It was subcloned into the Eco R1 site in the pAC cytomegalovirus (CMV) adeno transfer vector (Gomez-Foix et al. 1992) and its orientation was checked using restriction analysis. Just as for eNOS (Kantor et al. 1996), recombinant adenovirus containing nNOS was constructed by cotransfecting pAC plasmid with the right arm of AD5pAC I, digested with Xba I and Cla I, into HEK 293 cells (Ehrengruber et al. 1998). The virus contains a 2.7-kb deletion in the E3 region providing an additional cloning capacity needed for both nNOS and eNOS.

Plastic tissue culture dishes with glass-bottomed wells

Cover glasses (Assistant 22mm square glass) were sequentially washed in concentrated HNO_3, double distilled H_2O (ddH_2O), NH_4OH and finally ddH_2O. The cover glasses were attached to tissue culture dishes (35mm Corning 25000), which previously had a half-inch circle cut from the center, using as glue Sylgard 184 (Dow Corning), prepared as directed by the manufacturer. After being sterilized, 0.5mg/ml polyornithine in 150mM sodium borate (Mallinckrodt) was applied to the well region, being careful to avoid having the solution touch the plastic, for 1 hour. Finally the dishes were washed with sterile ddH_2O before addition of the cells.

Glial coverslips

Thermanox 25mm (Nunc) coverslips were sterilized and melted parowax (Bromar) was used to make three "feet" on their surfaces which would subsequently separate the glia from underlying neurons. The coverslips were coated with 0.1mg\ml polylysine for 1 to 12 hours at 36°C followed by two rinses with sterile ddH_2O. They were aspirated dry and 25,000-100,000 E17-E19 cells per coverslip were added in 0.5-1 ml of "settling medium" (neurobasal medium (Gibco) with 2% B27 supplement (Gibco), 25μM β-mercaptoethanol (Gibco), 25μM glutamate and 25μM glutamax (Gibco)) with 5% defined equine serum (Hyclone) to the coated footed side of the coverslip in a 35 mm dish. After 1-3 hours, 1.5ml more of the settling medium with serum was added. The glial coverslips were placed in an incubator and were fed by exchanging 1 ml of settling medium with serum for 1 ml of fresh equilibrated settling medium with serum, but without glutamate, at one week. Glia were grown until they were confluent.

Preparation of hippocampal neurons

A pregnant (E-17 to E-19) Wistar rat was anesthetized with $C0_2$. A quick cesarean-section was performed and the uterus was placed in an ice-embedded tissue culture dish. Brains were removed from the fetuses using forceps and briefly stored in ice-cold oxygenated "dissection solution" (Hanks' BSS (Gibco), 8mM $MgCl_2$ and 10mM Hepes). The hippocampi were removed and stored in ice-cold oxygenated dissection solution. Next, they were divided into thirds, transferred into dissection solution with 0.25% trypsin and 0.2mg\ml DNase and incubated at 36°C for 15 minutes. The hippocampal chunks were rinsed in fresh dissection solution. The pieces were transferred to a fresh tube and triturated in settling medium with serum by passing them through the

tip of a 1 ml pipettor 6-10 times. Next, the suspension was filtered through a 70 micron cell strainer (Falcon) and centrifuged for 2 minutes at 150 g. The supernatant was removed and the cell pellet was resuspended in 1 ml of settling medium (i.e., no serum) followed by dilution to a final concentration of 20 μg/ml laminin in settling medium. Between 7500 and 30,000 cells were then plated into the wells of glass-bottomed tissue culture dishes in a volume of 50 μl. After settling for approximately 1 hour, 2 ml of settling medium and a coverslip of glial cells were added. After 1 day, cultures were fed by means of a 1 ml exchange with fresh settling medium containing 2 μM cytosine β-D-arabinofuranicide (Sigma), giving a final concentration of approximately 1 μM. Cultures were maintained in 5% CO_2 with a humidity of 85%.

Infection

After 8-13 days in culture, the medium and glial cover slip in each dish were removed, saved and replaced with 400 μl glial conditioned medium containing from $5X10^7$ to $5X10^8$ PFU/ml Ad-nNOS. After two hours in the incubator, the Ad-nNOS containing medium was removed and the original medium, now containing 100 μM L-NMMA, an NO synthase inhibitor (McCall et al. 1991), was returned to each dish. The neurons were incubated for 20-28 hours to allow ample time for viral expression prior to staining and imaging.

Immunocytochemistry and imaging

In preparation for immunocytochemistry, the tissue culture medium was removed. Cells were washed with 4°C PBS (Irvine Scientific, Santa Ana, CA) for 5 minutes followed by a brief rinse with 4°C methanol. The cells were fixed in methanol for 15 minutes at –20°C with two subsequent 5 minute washes with 4°C PBS. Next the cells

were incubated in "block solution" (5% normal goat serum, 0.4% Triton-X100 in PBS) for one hour at room temperature. Afterward they were incubated with primary antibodies diluted in block solution, overnight at 4°C (anti-nNOS from Calbiochem; anti-synapsin and anti-PSD-95 kindly provided by Mary Kennedy (Caltech, Pasadena, CA) DeCamilli et al. 1983; Kornau et al. 1995). The primary antibody solution was aspirated and the unbound primary antibody was removed by two ten minute rinses in 0.25% BSA, 0.2% Triton-X100 in PBS. The cells were incubated with fluorescent-conjugated secondary antibodies diluted in block solution, at room temperature for one hour. Finally, the cells were washed twice for ten minutes with PBS, once with purified water, bathed in mounting medium (Sigma) and sealed using another glass cover slip and clear nail enamel. Fluorescence was measured using an inverted stage Bio-Rad MRC-600 confocal microscope system or a Zeiss Laser Scan microscope, both with an X63 oil objective. The condenser was intentionally misaligned to increase the contrast in the bright field images. Images were displayed using Adobe Photoshop 4.0. Synapsin was judged to be colocalized with nNOS either when the two proteins were enriched in exactly the same position (purple and green showing white in the colocalization) or when the proteins were enriched directly adjacent to each other (purple and green dots side-by-side).

CHO cells and analysis of protein and nucleic acid production

Chinese hamster ovary cells were cultured as described (Kantor et al. 1996). RT-PCR was performed using the Titan RT-PCR kit (Boehringer Mannheim) on RNA isolated from infected CHO cells using the RNeasy kit (Qiagen). Anti-eNOS was obtained from Transduction labs.

Results

Virus analysis

We verified the construction of an adenovirus containing the gene for the neuronal isoform of the nitric oxide synthase (Ad-nNOS) at the DNA, RNA and protein levels. Viral DNA, isolated from HEK-293 cells, was analyzed by PCR using two sets of primers, one which amplifies a 1 kb fragment from nNOS and one which amplifies a 500 bp fragment from the endothelial isoform of the nitric oxide synthase (eNOS). A 1 kb band with no 500 bp band results from PCR reactions which included DNA from HEK-293 cells infected with Ad-nNOS, while a 500 bp band but no 1 kb band appears in PCR reactions which included Ad-eNOS (figure 1a). Reverse transcriptase PCR reactions (RT-PCR) using RNA from similarly infected CHO cells with the same primers show analogous results (figure 1b). Western blots of proteins from Ad-nNOS or Ad-eNOS infected CHO cells show immunoreactivity for nNOS or eNOS, respectively, without evidence for the presence of the other isoform in either case (figure 1c).

Subcellular localization of proteins in E18 neurons

As previously reported (Cho et al. 1992), PSD-95 exhibits a punctate pattern of staining in mature E-18 hippocampal neurons at 1-2 weeks in culture (figure 2) that corresponds to individual synapses. The presynaptic marker synapsin also exhibits a punctate pattern of labeling (figure 4b). Both PSD-95 and synapsin are absent from the cytoplasmic region of the soma. Cytoplasmic staining for nNOS in Ad-nNOS infected neurons is strong, but is excluded from a circular region in the center of the cell, presumably the nucleus (figure 4a upper right cell). Cytoplasmic staining for nNOS in uninfected cells (figure 3 and figure 4 lower left cell) is similar to or less than levels of

staining observed in the nucleus of both infected and uninfected cells, suggesting that they are background. In the processes of Ad-nNOS-infected neurons, the nNOS staining is nonuniform, but is not preferentially associated with most synapsin-positive regions (figures 4d, 5 and 6). Typically 3-10% of cultured E18 hippocampal neurons show nNOS levels slightly above background at 8-12 DIC. These positive neurons have a morphology that is characteristic of GABA-ergic interneurons (Benson et al. 1994). In infected cultures, 30-90% of the neurons are strongly stained relative to both unstained neurons and their own nuclei. The majority of these positive neurons have pyramidal morphology (Benson et al. 1994).

It was possible to also find examples of apparently immature synapses with less-punctate synapsin staining (Mary Kennedy, personal communication) in Ad-nNOS-infected cultures. In the example in figure 7, the synapses had faint nNOS staining. This neuron is weakly infected, as indicated by the small difference between the cytoplasmic and nuclear staining.

Discussion

Our results with Ad-nNOS infected cultured hippocampal neurons which overexpress nNOS show non-uniform, primarily non-synaptic nNOS staining in the processes with staining also present in the cell soma. These results are consistent with previous reports of endogenous nNOS staining (Aoki et al. 1993; Wendland et al. 1994). This pattern is different from staining for PSD-95, which is present postsynaptically (Hunt et al. 1996), and synapsin, a faithful presynaptic marker (DeCamilli et al. 1990), both of which consistently show very punctate staining in the processes with little or no staining in the cell body. After infection, the cultures were grown in the presence of a

NOS inhibitor for only one day. Due to the presence of the inhibitor, we assume that nNOS is not having any effects as a result of its enzymatic activity. Because the virus was only allowed to express for one day, we assume that effects due to prolonged overexpression of the viral transgene are absent.

These results show that binding of nNOS to its proposed synaptic binding partners, such as PSD-95, is not present in most mature synapses of hippocampal neurons. The interpretation that we favor is twofold. First, the expression levels of PDZ proteins appear to be similar to the endogenous expression of their binding partners. In other words, there is not a large pool of unbound synaptically-localized nNOS-binding-sites such as the second PDZ domain of PSD-95 waiting for a binding partner. Second, the affinity of the association between potential synaptically-localized nNOS binding partners, if present, and their endogenous ligand(s) is likely higher than the affinity of their association with nNOS. If their affinity for nNOS were highest, nNOS would displace the other molecules and localize at synapses. One possible higher-affinity ligand in the example of the second PDZ domain of PSD-95 is NR2B. In further support of the conclusion that the interaction between PSD-95 and nNOS is weak relative to other associations, biochemical purification of the postsynaptic density fraction does not result in enrichment of nNOS (Kachinsky et al. 1997), but such purification causes enrichment of both PSD-95 and NR2B (Kennedy 1997). A disruption of PSD-95's normal subcellular localization by exogenous nNOS is possible, but seems unlikely. PSD-95 contains three PDZ domains, only one of which has been shown to associate with nNOS (Brenman et al. 1996). In addition to an amino-terminal disulfide-linked multimerization

domain (Hsueh et al. 1997), ligands for the other two PDZ domains of PSD-95 (Kim et al. 1995; Niethammer et al. 1996; Irie et al. 1997) could sustain its normal localization.

It is possible that the percentage of a given molecule which is bound to a specific PDZ protein could be modified in a temporally- or cell-type-specific manner due to changes in the expression levels of binding partners or post-translational modifications such as phosphorylation (Cohen et al. 1996). Consistent with this hypothesis, there appears to be tissue-specific subcellular localization of nNOS in the marmoset brain with brain regions having different percentages of cytosolic NOS activity (Gerlach et al. 1995). The faint colocalization shown in apparently immature synapses shows that there are nNOS binding sites at some hippocampal synapses. Their immaturity combined with the lack of colocalization at most mature synapses may be indicative of competitive processes that underlie synapse formation. Molecules may arrive at synapses without binding partners and gradually, possibly even sequentially, find higher affinity binding partners until they and the synapse become mature.

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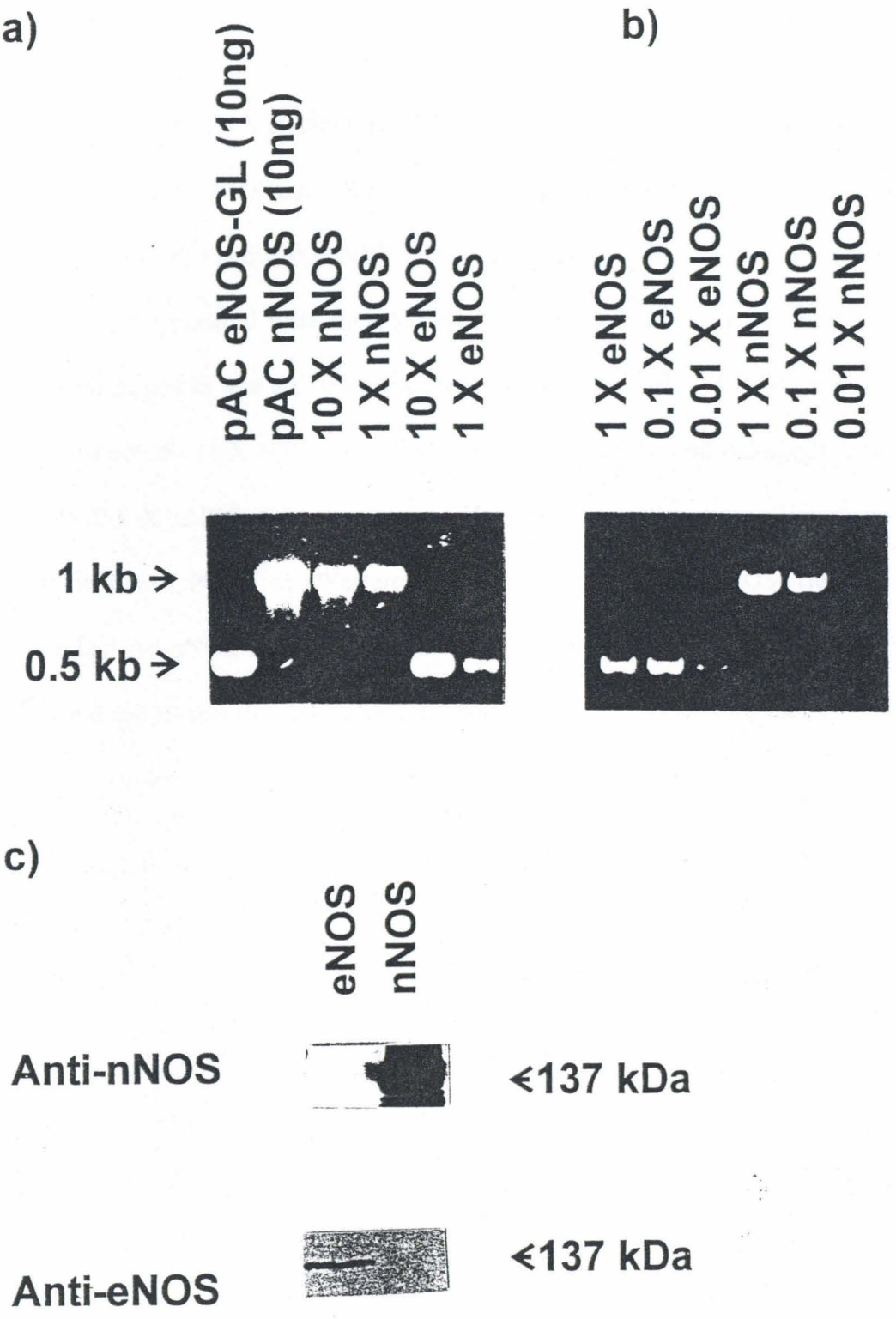
a)
pAC eNOS-GL (10ng)
pAC nNOS (10ng)
10 X nNOS
1 X nNOS
10 X eNOS
1 X eNOS
1 kb
0.5 kb
b)
1 X eNOS
0.1 X eNOS
0.01 X eNOS
1 X nNOS
0.1 X nNOS
0.01 X nNOS
c)
eNOS
nNOS
Anti-nNOS
<137 kDa
Anti-eNOS
<137 kDa

Figure 1 Analysis of viral DNA and the production of RNA and protein by a virus which contains the gene for the neuronal isoform of the nitric oxide synthase. a) PCR of dilutions of viral DNA isolated from HEK-293 cells which had been infected with either Ad-nNOS (10 X nNOS and 1 X nNOS) or Ad-eNOS (10 X eNOS and 1 X eNOS). pAC eNOS-GL (10 ng) and pAC nNOS (10 ng) are control PCR reactions in which 10 ng of the respective plasmid were included without any viral DNA. Primers amplify a 1 kb fragment in nNOS and a 500 bp fragment in eNOS. b) RT-PCR of dilutions of RNA from Ad-eNOS- (1 X eNOS, 0.1 X eNOS, 0.01 X eNOS) and Ad-nNOS-infected (1 X nNOS, 0.1 X nNOS, 0.01 X nNOS) CHO cells using the same primers used in the aforementioned PCR. c) Western blots of protein from Ad-eNOS- (left lane in both blots) and Ad-nNOS-infected (right lane in both blots) CHO cells using nNOS- (upper blot) and eNOS-specific (lower blot) antibodies.

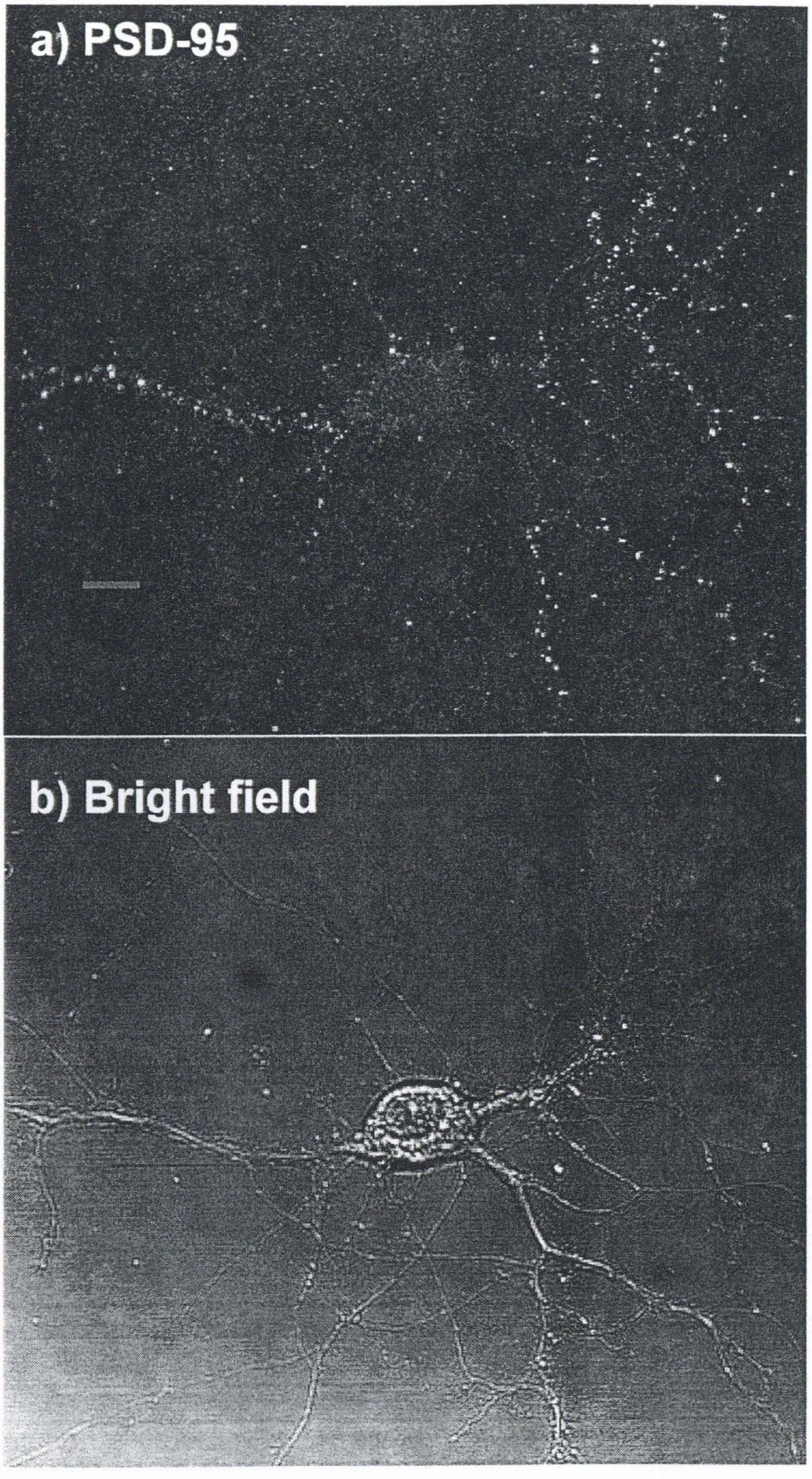
a) PSD-95
b) Bright field

Figure 2 Subcellular localization of PSD-95 in a mature cultured hippocampal neuron. a) Immunoreactivity for PSD-95 shown in white. b) Bright field image showing neuronal morphology. Scale bar = 10 μm. Cell was fixed at 14 dic.

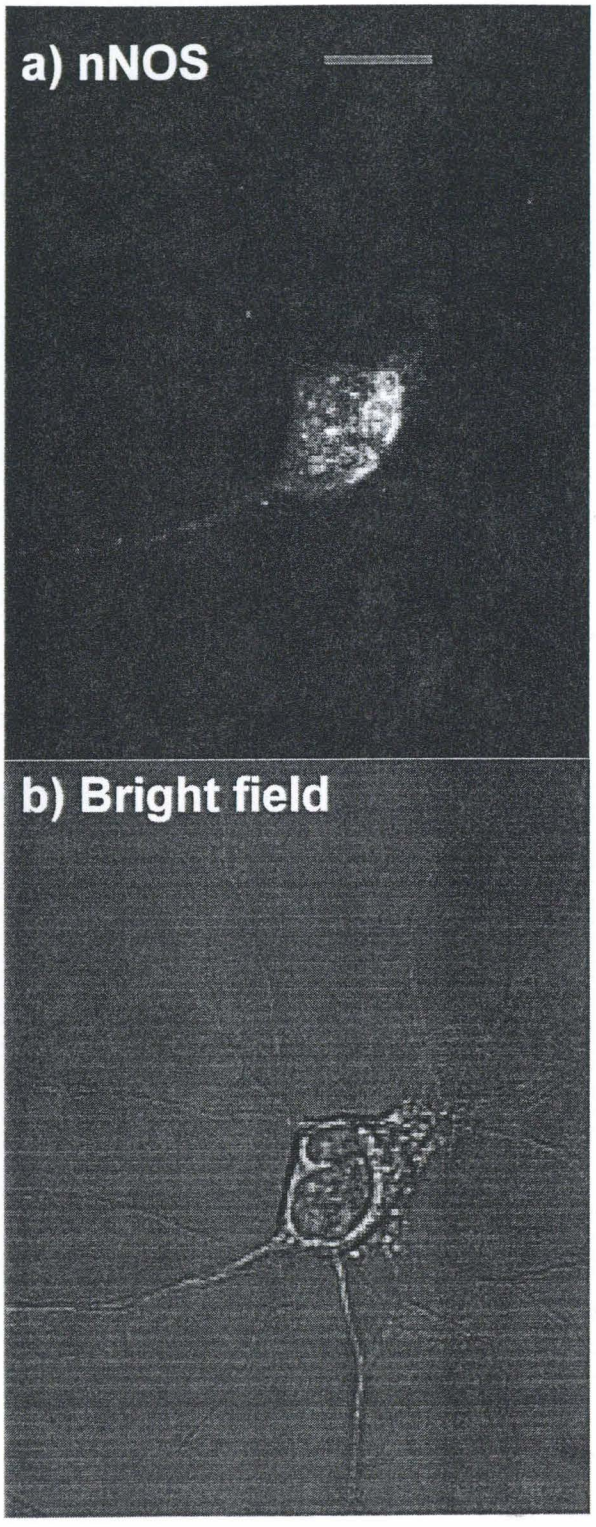
a) nNOS
b) Bright field

Figure 3 Subcellular localization of nNOS in an uninfected cell from an uninfected dish. a) Immunoreactivity for nNOS shown in white. b) Bright field image showing neuronal morphology. Scale bar = 10 μm.

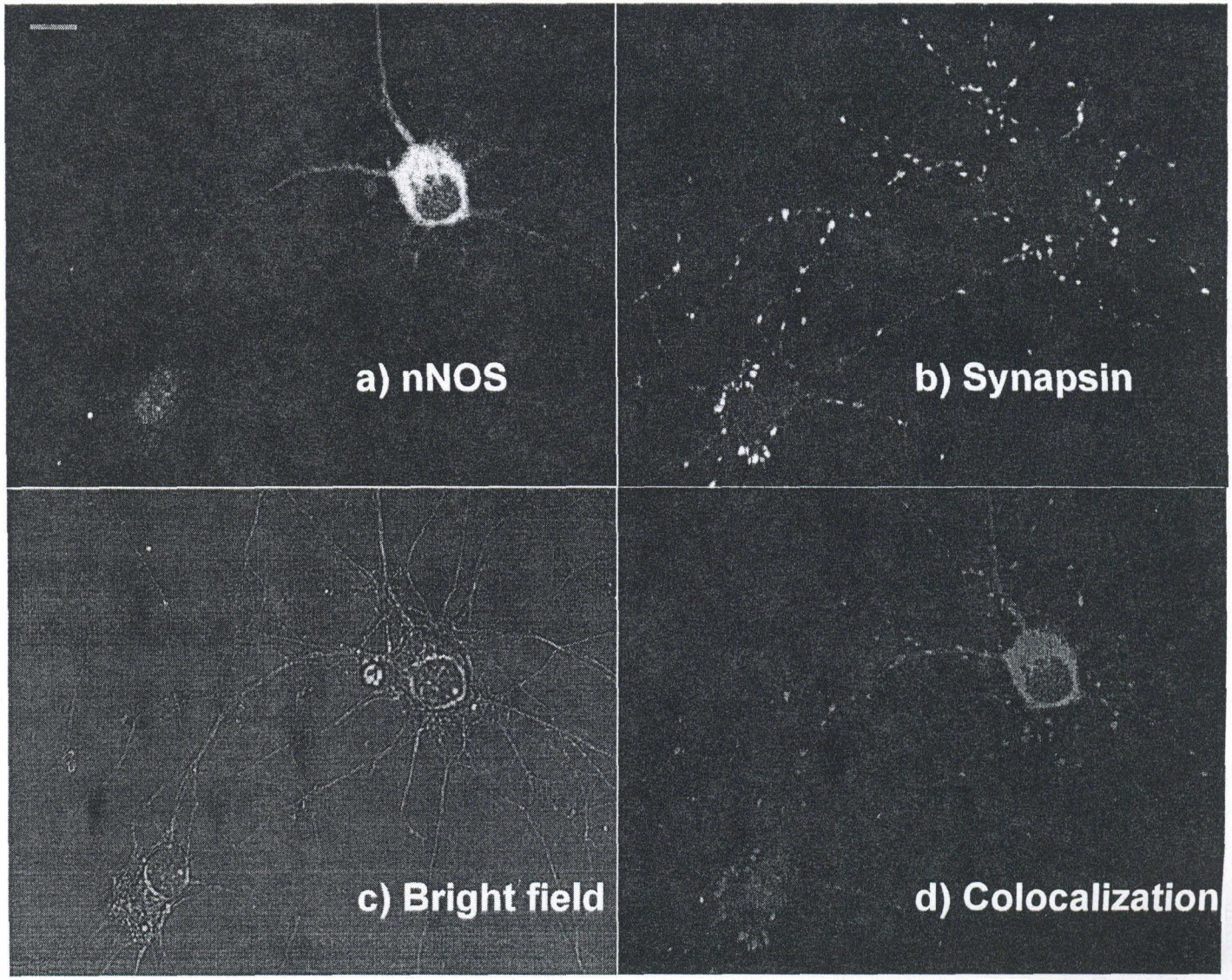
a) nNOS
b) Synapsin
c) Bright field
d) Colocalization

Figure 4 Subcellular localization of nNOS and synapsin in a mature infected (upper right) and an uninfected (lower left) cultured hippocampal neuron. a) nNOS staining in white b) synapsin staining in white c) bright field showing neuronal morphology d) nNOS and synapsin superimposed with nNOS in green and synapsin in purple. Scale bar = 10 μm. Cells were fixed at 9 dic.

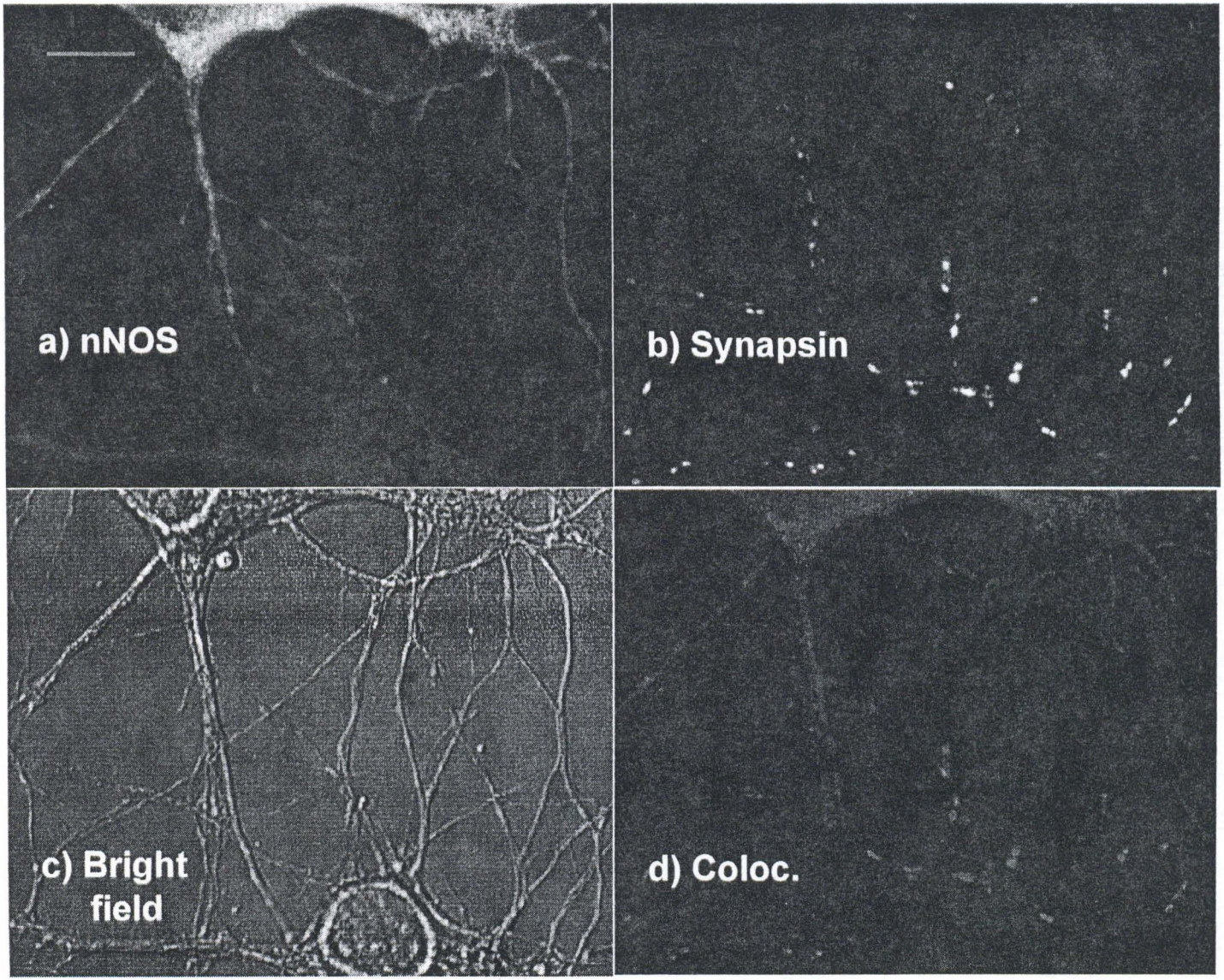
a) nNOS
b) Synapsin
c) Bright field
d) Coloc.

Figure 5 Subcellular localization of nNOS and synapsin in two infected neurons. a) nNOS staining in white b) synapsin staining in white c) bright field showing neuronal morphology d) nNOS and synapsin superimposed with nNOS in green and synapsin in purple. Scale bar = 10 μm.

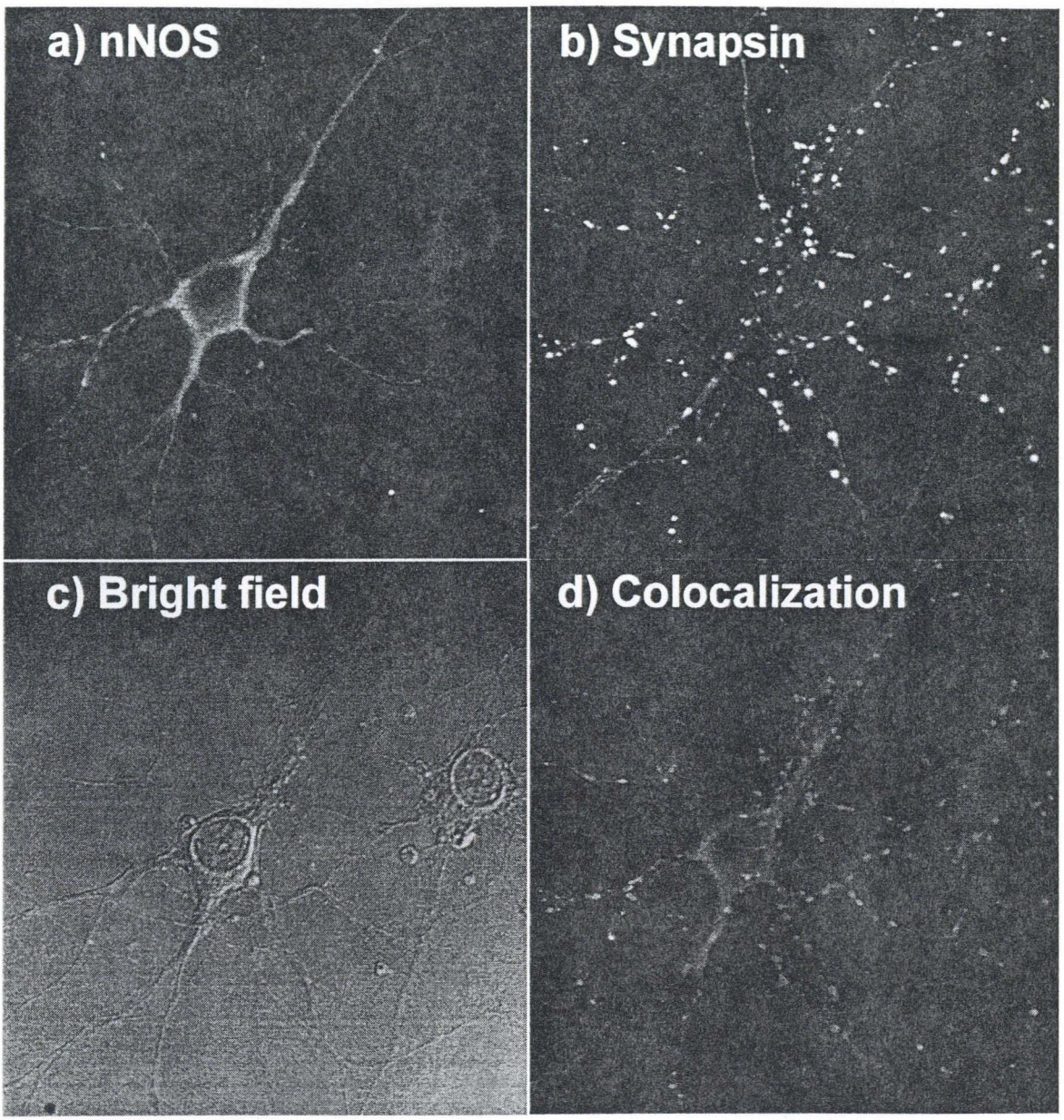
a) nNOS
b) Synapsin
c) Bright field
d) Colocalization

Figure 6 Subcellular localization of nNOS and synapsin in two infected neurons. a) nNOS staining in white b) synapsin staining in white c) bright field showing neuronal morphology d) nNOS and synapsin superimposed with nNOS in green and synapsin in purple.

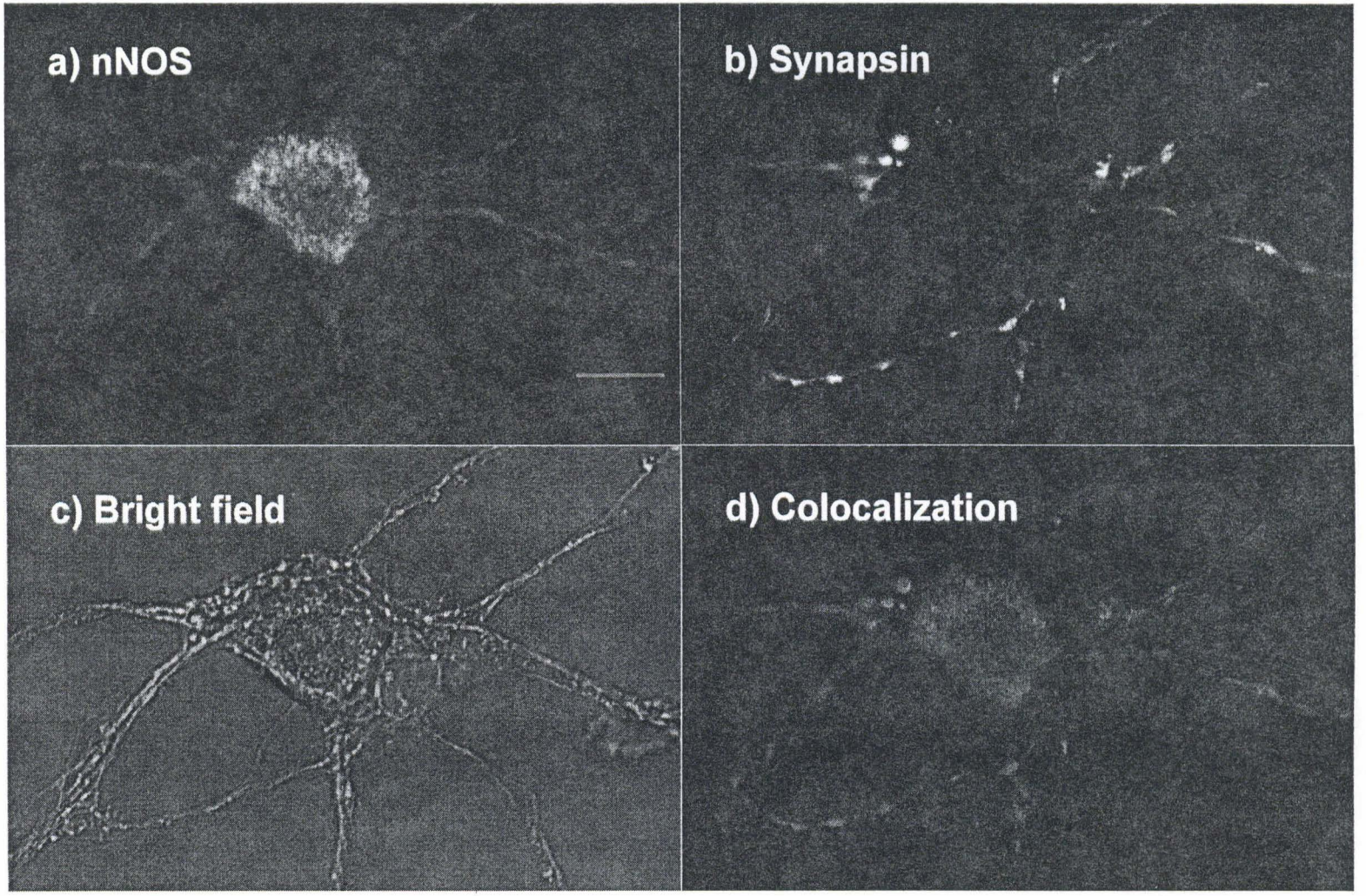
a) nNOS
b) Synapsin
c) Bright field
d) Colocalization

Figure 7 Subcellular localization of nNOS and synapsin in a weakly infected immature neuron. a) nNOS staining in white b) synapsin staining in white c) bright field showing neuronal morphology d) nNOS (green) and synapsin (purple) superimposed. Scale bar = 10 μm. Cell was fixed at 11 dic.

Chapter 4

Release and degradation of NO revealed by overexpression using adenoviral vectors

Introduction

Nitric oxide (NO) has a variety of biological functions including regulation of vascular tone (Garthwaite and Boulton 1995), long-term potentiation (Schuman and Madison 1994), long-term depression (Linden 1994; Zhuo et al. 1994; Lev-Ram et al. 1997) and synapse elimination at the neuromuscular junction (Wang et al. 1995). NO has been shown to influence multiple downstream effectors including activation of a soluble guanylyl cyclase (Gruetter et al. 1981; Southam and Garthwaite 1991), activation of an ADP-ribosyltransferase (Williams et al. 1992; Schuman et al. 1994; Sullivan et al. 1997) and modulation of redox sites on proteins including the NMDA receptor (Lei et al. 1992) and components of the synaptic vesicle release machinery (Meffert et al. 1996).

Most of the functions of NO as a second messenger require that it be released and diffuse to nearby cells through the extracellular medium (Schuman and Madison 1991; Schuman and Madison 1994; Arancio et al. 1996; Kendrick et al. 1997). It is therefore important to understand how the degree of intracellular localization of nitric oxide synthases (NOSs) affects release into the extracellular medium. The two NOS isoforms that are most relevant to neuronal function are the so-called endothelial form (eNOS) and the neuronal form (nNOS). Both of these isoforms are expressed in the nervous system (Bredt et al. 1991; Dinerman et al. 1994). A large portion of the expressed eNOS is localized in the plasma membrane because it is myristoylated (Liu and Sessa 1994; Busconi and Michel 1993; Kantor et al. 1996; Forstermann et al. 1991; Sakoda et al. 1995). Some nNOS is localized close to the cell surface because it contains an amino terminal PDZ domain (Brenman et al. 1996; Hecker et al. 1994; Gerlach et al. 1995). PDZ domains are present in a variety of molecules which lie close to the cell surface,

including the protein PSD-95, which is present in the postsynaptic density. The PDZ domain in nNOS binds specifically to the second PDZ domain of PSD-95 (Brenman et al. 1996). The second PDZ domain of PSD-95 also binds a carboxy-terminal consensus sequence, tSXV, which is present in the NMDA-type glutamate receptor NR2B (Kornau et al. 1995; Brenman et al. 1996) and potassium channels (Kim et al. 1995; Cohen et al. 1996).

To determine how intracellular localization of an NOS affects release of NO, we have overexpressed both eNOS and nNOS in CHO cells using recombinant adenovirus. Under these conditions, eNOS is largely expressed at the plasma membrane. However, by using the inhibitor of myristoylation, DL-α-hydroxymyristic acid (HMA) (Vaandrager et al. 1996), a uniform distribution of eNOS throughout the cytosol can be achieved (Kantor et al. 1996). When nNOS is expressed in CHO cells, it is uniformly distributed throughout the cytosol. We have accordingly measured the release of NO and of its primary metabolite nitrite (NO_2^-) for the membrane-localized eNOS and the two different cytosolic NOSs. Synthesis of NO by either NOS is stimulated by an increase in intracellular Ca^{++} concentration. Previous measurements of the calcium sensitivity of the NOSs in the hands of different investigators have given quite variable results, which are possibly due to the use of the high affinity calcium buffers EGTA and/or EDTA (Mayer et al. 1989; Mulsch et al. 1989; Bredt and Snyder 1990; Forstermann et al. 1990; Mayer et al. 1990; Schmidt et al. 1991; Silvagno et al. 1996). These reagents do not buffer well in the concentration range that is important. We have measured the stimulation of the enzyme isoforms as a function of calcium concentration using the reagent BAPTA, a lower-affinity calcium buffer which is effective in the range of calcium concentrations of

interest. We have determined that the enzyme isoforms are equally sensitive to calcium with an EC50 of approximately 145-150 nM.

The factors responsible for the degradation of NO in biological systems are not well understood. The best-characterized fate of NO, its reaction with molecular oxygen, is slow at physiological concentrations of NO ($t_{1/2}$ of 100 nM NO of tens of minutes, Kharitonov et al. 1994). In tissues, the half-life of NO is generally accepted to be on the order of 5 seconds (Schuman and Madison, 1994). We therefore determined whether the amount of nitrite accumulated in the CHO cell medium was due to air oxidation given the concentration of NO that was measured. The rate of production of nitrite in the medium bathing the CHO cells is faster than is predicted by air oxidation alone, demonstrating that mechanisms exist in CHO cells that convert NO to nitrite. The fact that CHO cells do not endogenously express NOS suggests that many cells possess similar mechanisms for eliminating the potentially cytotoxic free radical.

Methods

Viruses

The construction of Ad-eNOS, Ad-eNOS-GL (Kantor et al. 1996) and Ad-nNOS (Chapter 2, this thesis) have been described.

Tissue culture

Chinese hamster ovary (CHO) cells were maintained in culture as described (Kantor et al. 1996). One day prior to infection the cells were plated onto 35 mm tissue culture dishes. For subsequent recordings using the NO meter, the cells were plated at a density of approximately 2 X 10^5 cells/dish in unmodified dishes. For imaging, the cells

were plated at 1 X 10^5 cells/dish in plastic tissue culture dishes with glass-bottomed wells, which were prepared as described (Chapter 2, this thesis).

Adenoviral infection

CHO cells were infected with Ad-nNOS, Ad-eNOS or Ad-eNOS-GL diluted in 0.4-1.0 ml of Ham's F-12 medium with 10% fetal bovine serum for two hours. After this time, the total volume of medium was brought to 1 ml by addition of virus-free medium. The glass-bottomed dishes were processed for imaging at either 20-28 hours or at 44-52 hours, with similar results (not shown). The media in both the unprocessed dishes for imaging and the dishes for NO measurements were replaced with virus-free medium 20-28 hours after infection. Viral concentrations were selected for the NO release experiments by infecting with a range of concentrations of Ad-eNOS and Ad-nNOS, assaying the cells for the levels of release (not shown) and subsequently using the concentrations which yielded similar levels of release.

Immunocytochemistry

Cells were washed with 4°C PBS (Irvine Scientific, Santa Ana, CA) for 5 minutes followed by a brief rinse with 4°C methanol. The cells were fixed in –20°C methanol for 15 minutes at –20°C with two subsequent 5 minute washes with 4°C PBS. Next the cells were incubated in "block solution" (5% normal goat serum, 0.4% Triton-X100 in PBS) for one hour at room temperature. Afterward the cells were incubated in primary antibodies (anti-nNOS, Calbiochem), diluted in block solution, overnight at 4°C. The primary antibody solution was aspirated and the unbound primary antibody was removed by two ten minute rinses in 0.25% BSA, 0.2% Triton-X100 in PBS. The cells were incubated with fluorescent-conjugated secondary antibodies diluted in block solution, at

room temperature for one hour. Finally, the cells were washed twice for ten minutes with PBS, once with purified water, bathed in mounting medium (Sigma) and sealed using another glass cover slip and clear nail enamel. Fluorescence was measured using an inverted stage Bio-Rad MRC-600 confocal microscope system with an X63 oil objective. Images were displayed using Adobe Photoshop 4.0.

Nitric oxide, nitrite and nitrate measurements

Nitric oxide concentration was measured using an ISO-NO Mark II nitric oxide meter (World Precision Instruments, Sarasota, FL), calibrated according to the manufacturer's instructions. This instrument can detect 10 nM NO and gives linear readings up to concentrations of at least 1 μM. Nitric oxide release was measured from CHO cells in 35 mm tissue culture dishes which were initially bathed in 1 ml of "extracellular medium" (140 mM NaCl, 30 mM D-glucose, 5.4 mM KCl, 1 mM $MgCl_2$ and 10 mM HEPES pH 7.4). The electrochemical probe was placed at the surface of the medium, approximately 0.5 mm from the cells. After stabilization of the meter, 1 ml of extracellular medium with 4 mM $CaCl_2$ and 40 μg/ml A23187 (Molecular Probes) was added to the dish, yielding final concentrations of 2 mM and 20 μg/ml, respectively. The concentration of NO released was measured until it peaked and began to decrease after 10 to 15 minutes. After termination of the NO recording, the medium was mixed and 1 ml was removed for later analysis for nitrite. The remainder of the medium was removed and replaced with 0.3 ml "EDTA/EGTA buffer" (50 mM tris pH 7.0, 0.1 mM EDTA, 0.1 mM EGTA and protease inhibitor tablets (Boehringer Mannheim) and homogenized. The peak concentration of NO recorded in each dish was reported, normalized for the specific activity in the dish (see below).

The signal detected using the NO meter in infected CHO cells is the result of production of NO. It is not present in uninfected CHO cells and is sensitive to L-NMMA, an NO synthase inhibitor (not shown). The electrode responds to pure NO in water (not shown) and nitrite in 0.1 M KI/0.1 M H_2SO_4, a condition that stoichiometrically converts nitrite to NO (World Precision Instruments ISO NO Mark II product information) but not

to nitrite in water (not shown). Most of the experiments were performed with the surfaces of the dishes open to air. NO is approximately 15 times more soluble in the gas phase compared to the liquid phase (Feelisch and Stamler 1996) and escapes into the air quickly in stirred solutions (B.M.S. and Y.-X. L. unpublished observations). Therefore, we performed two experiments in which the abilities of e- and nNOS to release NO to the extracellular medium were measured with the surface area of the dishes covered using a floating lid. Since the medium in the CHO cell experiments is not stirred and since diffusion is relatively slow, these experiments yielded similar results to the experiments without the lid and the data were averaged together. Nitrite in the CHO cell medium was measured by addition of medium to a stirred solution bathing the ISO NO electrode with final concentrations of 0.1 M KI and 0.1 M H_2SO_4 in water. The nitrite produced under these conditions was divided by the time elapsed before termination of the recording and was recorded as the amount of nitrite produced per unit time by the CHO cells. The amount of nitrite measured in the extracellular medium in the absence of NO release, presumably due to minor contaminants in the constituent salts, was subtracted from the reported values. CHO cells which were infected for the experiments analyzing the effect of calcium concentration on NOS activity were not treated with ionophore and were homogenized in "BAPTA buffer" (1 mM BAPTA, 100 mM KCl, 10 mM MOPS pH 7.2). Nitrate was quantified by converting nitrate to nitrite using nitrate reductase from the fluorometric NOx assay kit (Cayman) and then analyzing nitrite as described above.

NOS specific activity measurements

Nitric oxide synthase activity was measured with the aid of the NOSdetect Assay Kit (Stratagene), with modification of the reaction conditions. L-[2,3,4,5-^{3}H] arginine

(Amersham) was purified using an anion exchange resin (Bio Rad AG-1-X8). All assays contained 200,000 cpm purified [^{3}H]arginine, 10 μM tetrahydrobiopterin, 5000 u/ml superoxide dismutase, 0.3 mM NADPH, 5 μM flavin-adenine-dinucleotide and 5 μM flavin-mononucleotide in buffer with a total reaction volume of 50 μl. For specific activity measurements, the buffer was EDTA/EGTA buffer and for the measurements of calcium sensitivity, the buffer was BAPTA buffer. Reactions were terminated by addition of 0.4 ml of "stop buffer" provided with the kit and the remainder of the analysis was performed as directed in the kit instructions. Background, determined in reactions using lysates from CHO cells which had not been infected, was subtracted from the raw activity measurements. Protein concentration was quantified by the method of Bradford (Bradford 1976). Specific activity is reported as background-subtracted cpm from the activity measurements divided by the protein concentration determined in the Bradford assay. NO and nitrite measurements for each dish were divided by the specific activity measurements for that dish to account for dish-to-dish variability. Data are reported as mean ± SEM.

Materials

DL-α-hydroxymyristic acid (HMA) was from Sigma. Reagents whose source is not indicated were acquired from a common commercial source such as Sigma and were of analytical grade or better. EC50s were calculated with the assistance of Microcal Origin 4.0.

Results

Subcellular localization of NOSs in infected CHO cells

The PDZ domain of nNOS is thought to associate with a limited number of binding partners (Brenman et al. 1996), which have not been shown to be present in CHO cells. As expected, aside from its absence from a large circular region in the center of the cells, presumably the nucleus, nNOS is present throughout the cytoplasm of infected CHO cells (figure 1, upper left). This pattern of staining is similar to that for eNOS+HMA (Kantor et al. 1996). In the absence of HMA, eNOS appears to be successfully myristoylated and is enriched in the plasma membrane of CHO cells (figure 1, lower left and Kantor et al. 1996).

NO and nitrite release from infected CHO cells

Chinese hamster ovary (CHO) cells were infected with adenovirus containing either nNOS or eNOS. The abilities of the NOSs to release NO to the extracellular medium were measured using two methods. Two days after infection the cells were treated with a solution containing 20 μg/ml of the calcium ionophore A23187 plus 2 mM Ca^{++}. The concentration of NO released into the medium was measured as a function of time with the NO detecting electrode placed approximately 0.5 to 0.75 mm above the cells. We also collected the medium after 10 min and measured the amount of nitrite and nitrate accumulated.

Figure 2 shows a typical curve for release of NO from eNOS infected cells as a function of time. For cells infected by Ad-eNOS, whether or not the dish was covered with a lid, the NO concentration increased to values typically between 40 and 100 nM over a period of 5-10 min until the reading was terminated. Since the primary end

product of oxidation of NO in aqueous solution is nitrite (Kelm and Schrader 1990; Ignarro et al. 1993; Privat et al. 1997), the homogenized solution at the end of the experiment was assayed for nitrite as described in Methods. In order to correct for differences in infection efficiencies between experiments, as well as for the possibility of different catalytic activities between the two NOS isoforms, the results were normalized by dividing by the catalytic activity of the lysed cells as assayed by the conversion of radiolabeled arginine to citrulline (see Methods). These data are shown in figure 3 and figure 4. We found that the levels of nitrate, another potential metabolite of NO, were considerably less than those for nitrite (data not shown, n=3). The data demonstrate that whether measuring peak NO concentration or total nitrite accumulation, the membrane localized eNOS gives much higher values than do the cytosolic species, nNOS or eNOS+HMA. An additional important point, shown in figure 5, is that the total nitrite accumulation is approximately 30 times greater than the peak NO levels.

Calcium sensitivity

In figure 6, we have compared the catalytic activities for lysates of eNOS- or nNOS-infected cells, as measured by the citrulline assay, as a function of Ca^{++} concentration. We have used BAPTA as a buffer, because it buffers well in the range of concentrations around the EC50s of the enzymes. We find values for the EC50 of 149± 9 nM for eNOS and 144 ± 10 nM for nNOS, i.e., substantially identical.

Discussion

The results reported above have implications for several important issues in understanding the physiology of NO and the two Ca/CaM-activated nitric oxide synthases, eNOS and nNOS, that are expressed in neural tissue.

NOx release as a function of NOS subcellular localization

Perhaps the most important result is the observation that extracellular release by an NOS is greater when the enzyme is localized at or close to the plasma membrane than when the enzyme is distributed uniformly throughout the cytosol (Liu et al. 1996). This conclusion is demonstrated by the data in figures 3 and 4. In the artificial situation of our experiments with the enzymes expressed by gene transfer into CHO cells, much of the eNOS is at the plasma membrane, but nNOS and eNOS+HMA are uniformly distributed throughout the cytosol. In physiological situations, a significant fraction of nNOS is anchored close to the cell surface by virtue of interactions with its PDZ domain (Brenman et al. 1996; Hecker et al. 1994; Gerlach et al. 1995). We presume that NO generated this close to the plasma membrane would escape to the extracellular medium as efficiently as NO released by myristoylated eNOS in the plasma membrane.

We observe that NO is efficiently converted to nitrite in the extracellular medium so that both NO and nitrite release are much higher for the membrane-bound eNOS than for the cytosolic NOSs, nNOS and eNOS+HMA. Thus, much of the NO generated intracellularly must be converted to some form which remains tightly bound in the cytosol. The diffusion coefficient (D) of free NO is about $3x10^{-5}$ cm^2 sec^{-1}. The average residence time for an NO molecule in the cytosol before it would escape to the cell surface is of the order of r^2/D, where r is the radius of the cell. For r=10 μm, this

calculated time is 0.03 sec. Therefore whatever reactions are responsible for the retention of NO intracellularly are taking place in a short period of time. NO_x is known to react with sulfhydryl groups of proteins and of free cysteine to form S-nitrosyl adducts (Stamler et al. 1997), with tyrosine groups to give nitrotyrosine (Beckman et al. 1992; Beckmann et al. 1994) and with superoxide to form peroxynitrite (Stamler et al. 1992; Feelisch and Stamler 1996; Pfeiffer et al. 1997). To our knowledge, there are insufficient rate and abundance data to enable a prediction of whether these reactions are major contributors to the intracellular conversion of NO to bound forms.

The ability of NOSs to release extracellular NO as a function of their subcellular localization is relevant to the biological function of nNOS, which is present in the cytosol to a significant degree throughout the brain (Hecker, 1994; Gerlach, 1995; Brenman, 1996; Egberongbe, 1994). The enzyme has also been shown to associate with intracellular organelles (Holmqvist and Ekstrom 1997), which might cause it to sediment with the particulate fraction but also to release less extracellular NO just as cytosolic nNOS. Cytosolic or intracellular-membrane-associated nNOS would have an impaired ability to release extracellular NO but might signal more effectively to targets within the same cell.

As a result of the use of the calcium ionophore A23187 in our experiments, the intracellular calcium concentration should be in the mM range in the CHO cells after stimulation. Physiological calcium influx is buffered very effectively in neurons by its extrusion through the plasma membrane and its uptake into intracellular stores. The resulting calcium gradients would further amplify differences in the ability of the NOSs

to be activated and, as a result, to release NO as a function of their subcellular localization.

NO degradation to nitrite

The second major conclusion from these studies is that the measured ratio of nitrite to NO in the extracelluar fluid is about 30:1 (figure 5). Specifically, we find a final nitrite accumulation of 1.6 μM in 2 ml of solution over the stimulated CHO cells expressing eNOS after 10 min. However, the typical concentration of NO, measured at approximately 750 μm from the cells, was approximately 50 nM. As discussed below, we believe that the known rate constants for the oxidation of NO by dissolved O_2 to give nitrite anion do not predict such a high degree of conversion. Therefore, there must be some other reaction that converts NO to NO_2^-.

The overall reaction between NO and O_2 in the concentration ranges of interest here is (Kharitonov et al. 1994):

(1) $2\ NO + O_2 \rightarrow 2\ NO_2$

(2) $2\ NO_2 + 2\ NO \rightarrow 2\ N_2O_3$

(3) $2\ N_2O_3 + 2\ H_2O \rightarrow 4\ NO_2^- + 4\ H^+$

Reaction (1) with the rate equation, $d[NO]/dt = 2k_1[NO]^2[O_2]$, is rate determining. For the overall reaction,

(4a) $4\ NO + O_2 + 2\ H_2O \rightarrow 4\ NO_2^- + 4\ H^+$

(4b) $-d[NO]/dt = d[NO_2^-]/dt = 4k_1[NO]^2[O_2]$

The value of the rate constant k_1 has been reported from several studies, ranging from $2.9x10^6$ (Goldstein and Czapski 1995) to $1.6x10^6\ M^{-2}\ sec^{-1}$ (Kharitonov et al. 1994).

First note that for a uniform concentration of NO of 50 nM, with the concentration of O_2 in air saturated water of 210 μM (Mayer et al. 1995), with k_1 taken as $2.9x10^6$, equation (4b) predicts a total concentration of NO_2^- after 10 min of 4 nM, rather than the observed value of 1.6 μM. This is an overly simplified analysis, but it serves to illustrate the important point.

In our reaction system, NO is produced at the cell layer and reacts with O_2 as it diffuses upwards. This results in a concentration gradient of NO with the highest concentration at the cell surface. Because of the second order dependence of reaction rate on NO concentration, the fractional rate of oxidation is greater the higher the NO concentration. This problem has been treated by a rigorous numerical solution of the partial differential equation which includes both NO diffusion and its reaction with O_2, with vertical distance and time (t) as the independent variables (Laurent et al. 1996). The model was designed to be applied to the generation of NO by the inducible NOS isoform expressed in activated macrophages in 35 mm tissue culture dishes. The goal was to correlate the NO concentration in the immediate vicinity of the cells from the rate of NO

generation by the cells, the diffusion coefficient of NO and the known kinetics of oxidation.

Predicted results for our experimental situation can be estimated by interpolation from the figures in Laurent et al. From figure 3 we estimate that approximately 0.4 of all NO released would be converted to NO_2^-; the final average concentrations should be approximately 1 μM NO and 0.6 μM NO_2^-. We therefore conclude that some additional mechanism for conversion of NO to NO_2^- is occurring.

Similar reports from other laboratories

Others have observed a rate of nitrite production from NO in biological systems which is greater than that predicted by air oxidation (Kelm and Schrader 1990; Lewis et al. 1995). In an example from macrophages (Lewis et al. 1995), which release large amounts of NO in addition to superoxide, the difference was attributed to the possible reaction of NO with peroxynitrite to form nitrite. The authors stated that they could not quantitatively evaluate their hypothesis since the rate of the reaction between peroxynitrite and NO had not been measured. Subsequently, it has been shown that the concentration of NO would have to be approximately 7.6 μM for the reaction to be as fast as the decomposition of peroxynitrite at physiological pH and temperature (Pfeiffer et al. 1997). The concentration of NO was approximately 1 μM in the macrophage experiments (Lewis et al. 1995). In our experiments, the average NO concentration was 42 ± 8 nM with accumulation of 1.5 μM nitrite and negligible nitrate. Peroxynitrite has been shown to decompose to a mixture of approximately 75% nitrate and 25% nitrite in the pH and temperature used in our experiments (Pfeiffer et al. 1997), and superoxide dismutase decreases the production of nitrate relative to nitrite in complex mixtures of

NO and superoxide (Lewis et al. 1995). If superoxide were reacting with a significant proportion of our NO, we would see appreciable nitrate formation. The smaller amount of nitrate in our experiments, relative to nitrite, combined with the low concentration of NO, suggest that peroxynitrite formation and either its subsequent reaction with NO or its decomposition to nitrite, do not account for the excess nitrite. Considering that the primary means of escape of superoxide from cells is thought to be through chloride channels and that the resting chloride conductance of CHO cells is small (Jun Li, personal communication) it is perhaps not surprising that effects of superoxide are absent. The other example of an increase of NO conversion to nitrite was from perfused hearts (Kelm and Schrader 1990). The conversion of NO to nitrite as it circulated through the heart was over 10 times faster than predicted by air oxidation.

Mechanism?

Is the reaction that converts NO to nitrite occurring inside the cells, at the cell surface or in the extracellular solution? It is likely not occurring in the extracellular solution; it is changed to a defined saline solution just prior to the experiments. Some intracellular conversion of NO to nitrite is possible. But intracellular nitrite is probably not escaping from the CHO cells. The only channels which would allow appreciable nitrite flux are chloride channels (Hille 1992) and, as mentioned previously, the resting chloride conductance of CHO cells is low. Given the absence of reports of calcium-activated chloride channels in CHO cells, we assume that they are also not present. A23187 is a selective divalent cation ionophore (Pressman 1976) and the specific transport of nitrite seems unlikely in cells that do not endogenously express NOS.

The potential mechanisms of this increased rate of conversion of NO to nitrite are open to speculation. Possibilities include enzymatic catalysis of the rate of free radical oxidation and some effect of the outer leaflet of the lipid bilayer. The increased solubility of NO and O_2 in lipids, specifically the lipid bilayer of the plasma membrane, might concentrate NO and O_2, facilitating their reaction. Kinetic analysis of the effect of lipids on the reaction between NO and O_2 would be useful. The reaction of NO with molecular oxygen is complex and is influenced by the concentrations of bases such as phosphate (Goldstein and Czapski 1995). It will be important to determine the effects of physiological concentrations of both lipids and base-catalysts on the autoxidation rate of low (nM) concentrations of NO. The results presented herein show that CHO cells are a good model system for future study on the nature of the factor(s) responsible for the degradation of NO in biological systems.

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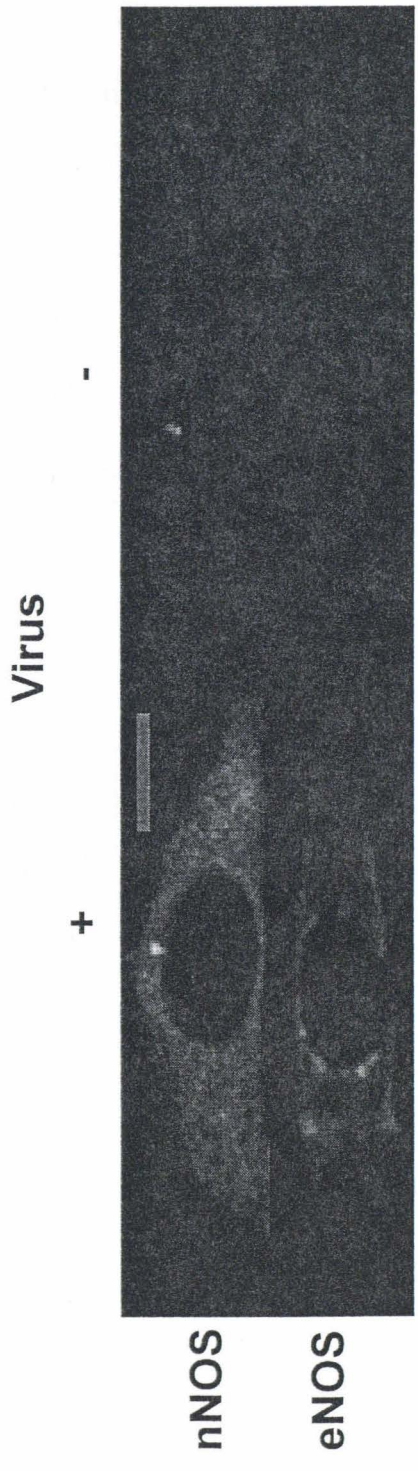
Virus
+
-
nNOS
eNOS

Figure 1 Subcellular localization of NOSs in infected CHO cells. CHO cells, infected with Ad-nNOS (upper left) show nNOS immunoreactivity evenly throughout their cytosol. Cell infected with Ad-eNOS-GL, a fusion protein containing eNOS and a version of green fluorescent protein, show a primarily plasma membrane localization of eNOS. Uninfected cells (nNOS upper right, eNOS lower right) show no staining at identical microscope settings to those used in the images to the left.

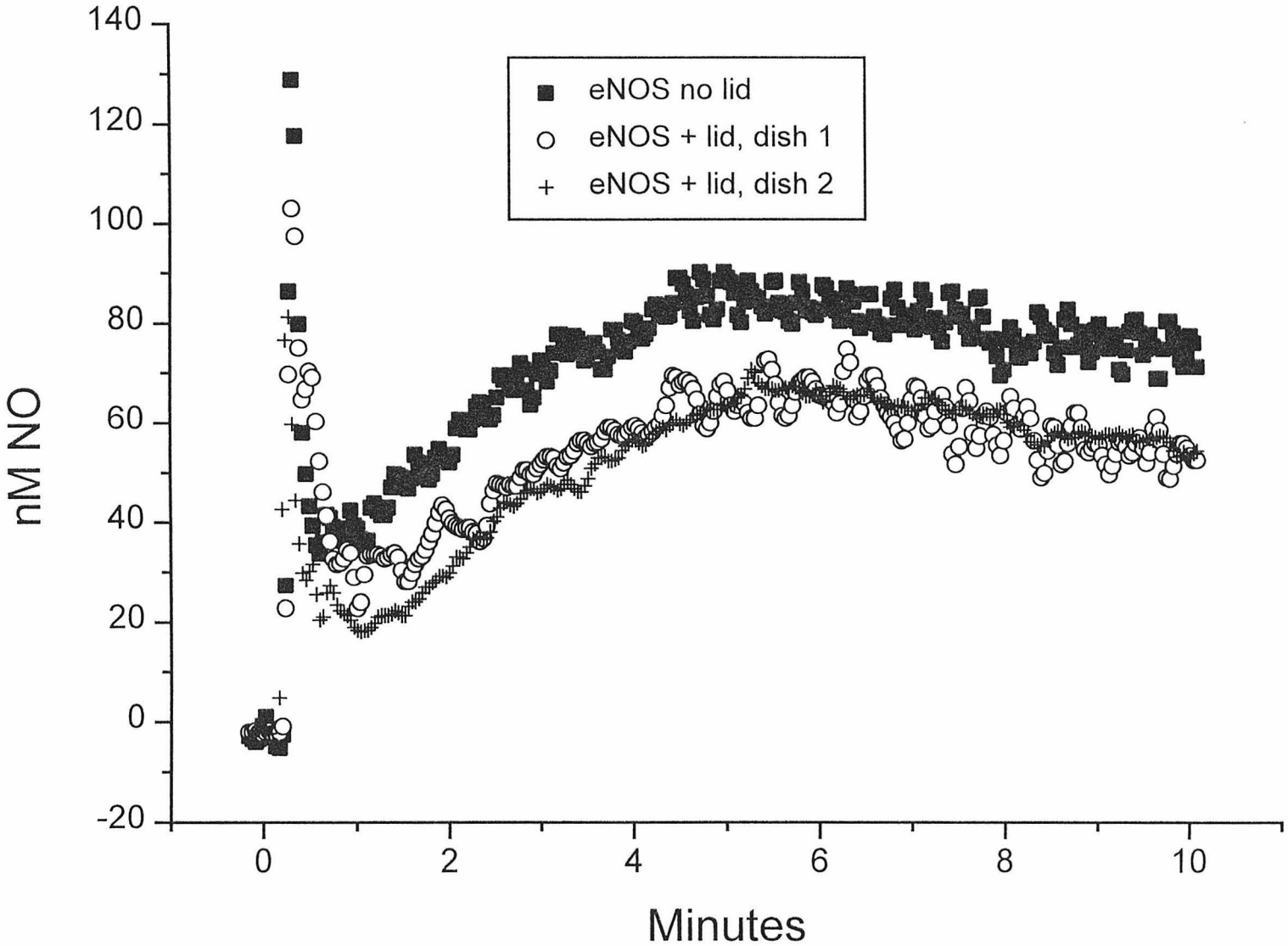
140
120
100
80
60
40
20
0
-20
nM NO
0
2
4
6
8
10
Minutes
eNOS no lid
eNOS + lid, dish 1
eNOS + lid, dish 2

Figure 2 Traces from Ad-eNOS-infected CHO cells stimulated as in figure 2 in the presence (+ lid) and absence (- lid) of a floating lid, showing similar kinetics. Final concentrations of 2 mM Ca^{++} and 20 μg/ml A23187 were added at time zero, with an associated transient. Peak [NO] was typically reached between 5 and 10 minutes. Peak amplitude varies between dishes due to differences in NOS infection/expression.

[nitrite] citrulline -1 minutes-1
1.0
0.8
0.6
0.4
0.2
0.0
eNOS
nNOS
eNOS+HMA

Figure 3 Comparison between the release of nitrite from membrane-bound (eNOS) and cytosolic NOSs (nNOS and eNOS+HMA). CHO cells were infected with either Ad-eNOS, Ad-nNOS or Ad-eNOS in the presence of the myristoylation inhibitor hydroxy-myristic acid (eNOS+HMA). Cells were stimulated as in figure 2. Nitrite concentrations released into the bath were measured and divided by the length of the recording. The NOS expression in each dish was measured in cell homogenates after the NO/nitrite release assay using an assay for the conversion of arginine to citrulline. The protein concentration in the homogenate of each dish, which reflects the number of cells present, was also measured. The values presented are ([nitrite]/time)/(citrulline/protein) ± SEM. Release from nNOS and eNOS+HMA CHO cells were normalized to the release from eNOS-infected cells (i.e., eNOS = 1) to allow comparison between experiments. n = 8, 6 and 3 for eNOS, nNOS and eNOS + HMA, respectively.

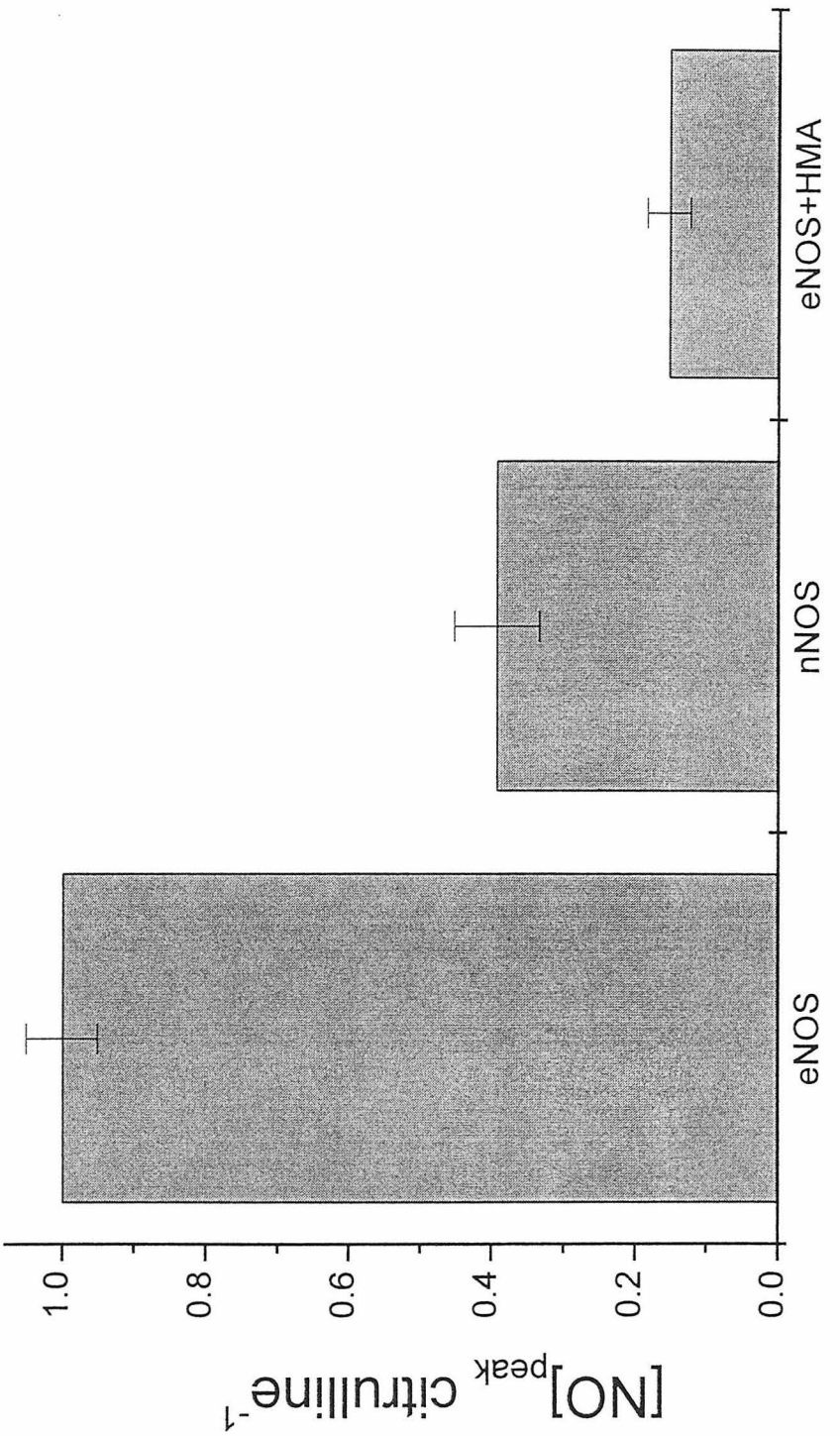
[NO]peak citrulline-1
1.0
0.8
0.6
0.4
0.2
0.0
eNOS
nNOS
eNOS+HMA

Figure 4 Peak NO release from CHO cells. Cells were stimulated as in figure 2. Peak NO concentration released by each dish was divided by the specific activity measurement (citrulline/protein) from that dish to control for differences in NOS expression among the dishes. The values presented are ($[NO]_{peak}$/(citrulline/protein) ± SEM. n = 14, 8 and 9 for eNOS, nNOS and eNOS+ HMA, respectively.

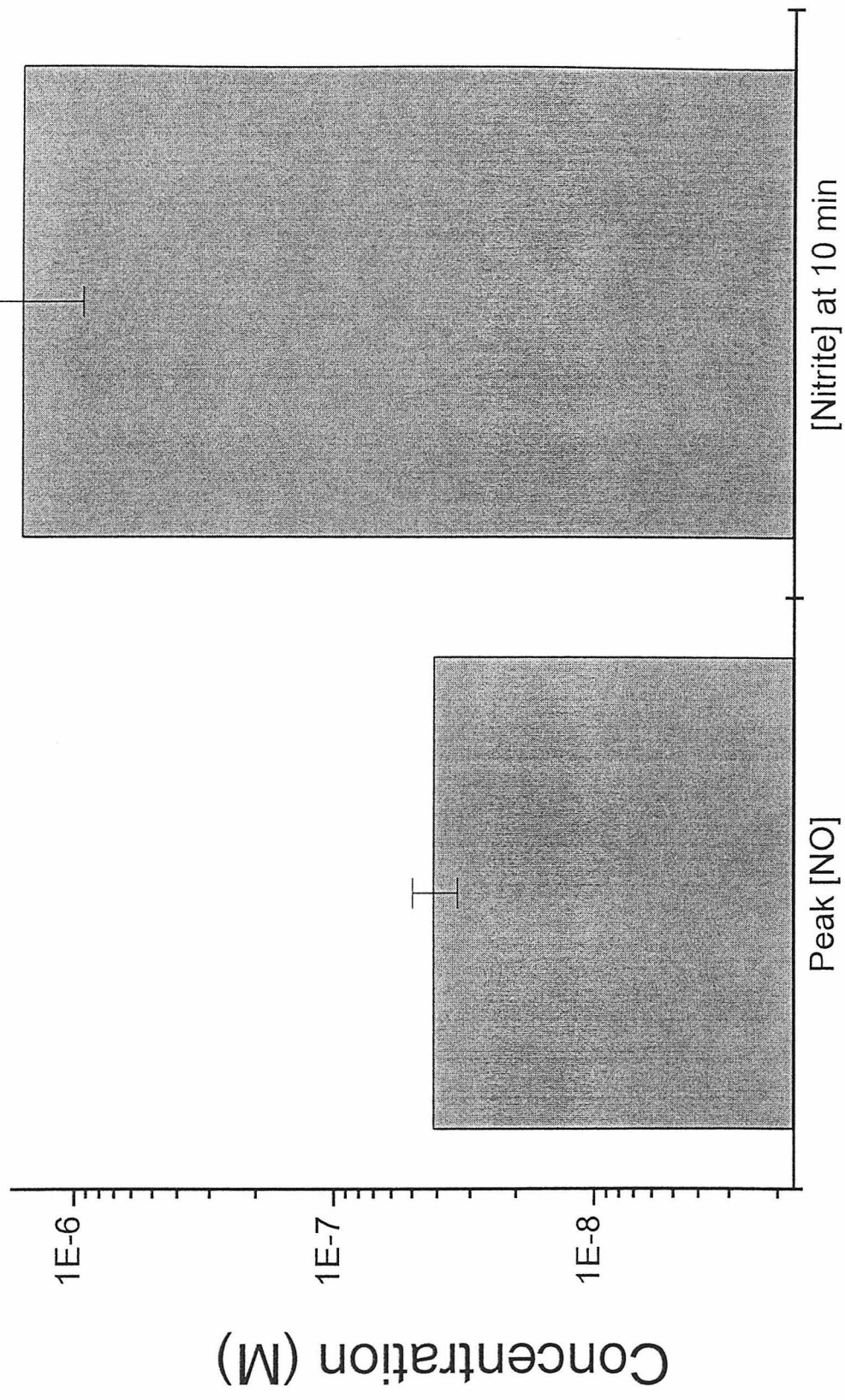
Concentration (M)
1E-6
1E-7
1E-8
Peak [NO]
[Nitrite] at 10 min

Figure 5 Peak NO and total nitrite concentrations produced by Ad-eNOS-infected CHO cells in the presence of the floating lid (see figure 6). n = 6 for both nitrite and NO.

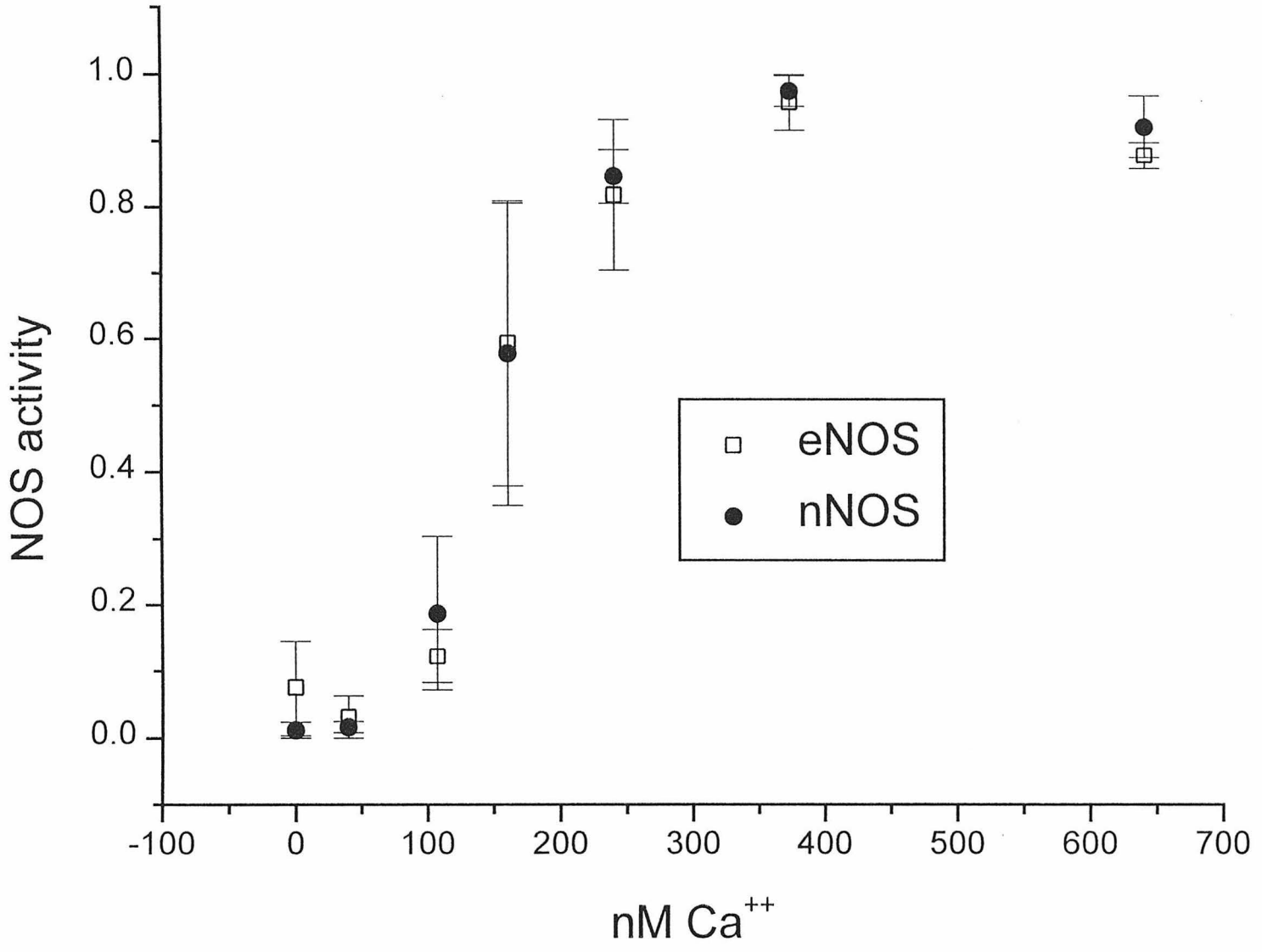
NOS activity
1.0
0.8
0.6
0.4
0.2
0.0
-100
0
100
200
300
400
500
600
700
nM Ca++
eNOS
nNOS

Figure 6 Calcium sensitivity of e- and nNOS determined using an assay for the conversion of radiolabeled arginine to citrulline. Data points (cpm) were normalized to the maximal rate of citrulline formation. n = 3 for each point.

Chapter 5

Concluding remarks

ADP-ribosylation

The ADP-ribosylation studies show that nitric oxide stimulates the ADP-ribosylation of multiple proteins in hippocampal synaptosomes. One significant aspect of this work is the demonstration that the reaction is, in fact, ADP-ribosylation. Initially the metabolic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was claimed to be a substrate of NO-stimulated ADP-ribosylation. Later it became apparent that the entire NAD molecule, not just ADP-ribose, was covalently added to the protein through an autocatalytic mechanism, not by ADP-ribosylation. A relatively simple control, performing ADP-ribosylation reactions using NAD in which the nicotinamide moiety is radioactively labeled, distinguishes between the multiple substrates of real ADP-ribosylation seen in my experiments and covalent addition of the entire nicotinamide moiety, as in the case of GAPDH. Since the nicotinamide moiety is not added to the substrate protein in an authentic ADP-ribosylation reaction, the protein bands which are labeled in these control reactions are not ADP-ribosylation. Not only did I see such a band whose molecular weight corresponds to that of GAPDH, I exposed the gels for so long (18 months in the longest example) that I also saw two faint bands with similar properties which had not been previously identified. Clearly, if there were any component of the stimulation of the other bands which was not ADP-ribosylation, it would have been evident in these experiments. The realization of the erroneous claims with respect to GAPDH made many researchers skeptical of studies of ADP-ribosyltransferases (ADPRTs). Hopefully my carefully controlled work will renew enthusiasm for research into the role of ADPRTs in brain function generally and hippocampal LTP specifically.

The second important conclusion of the ADP-ribosylation studies is the pharmacological similarity between ADP-ribosylation and LTP. The fact that nicotinamide blocks both at a similar concentration suggests that the reaction may be important for the induction of LTP.

Subcellular localization of exogenous nNOS in cultured hippocampal neurons

Two separate lines of evidence suggest that nNOS, expressed in cultured hippocampal neurons, should localize to synapses. The first is the presence of NOS-dependent LTP in eNOS -/- mice. Work done in collaboration between the Schuman and Davidson labs shows that the NOS isoform which is responsible for producing NO during normal LTP induction is eNOS. The presence of either low levels of endogenous synaptic nNOS or compensatory up-regulation of nNOS that localizes to synapses in the mutants are the most reasonable hypotheses. The second argument that predicts synaptic localization of nNOS in cultured hippocampal neurons is the demonstrated binding of the PDZ domain of nNOS to the second PDZ domain of PSD-95, which localizes to the postsynaptic density.

The majority of synapses on Ad-nNOS infected cultured hippocampal neurons do not contain levels of nNOS that are above background. A small number of synapses contain low levels of nNOS, but the synaptic staining for nNOS in these cells is small relative to the cytoplasmic staining in the cell body. Does this small amount of synaptic staining represent sufficient amounts of synaptic nNOS which, in the eNOS -/- mice, could generate enough NO for the induction of LTP? Probably not. The drug HMA, which blocks myristoylation of eNOS but has no effect on nNOS, completely blocks LTP, suggesting that endogenous levels of nNOS in CA1 pyramidal neurons are not sufficient

for the induction of LTP. If there is developmental compensation in the mutants which results in higher levels of nNOS, it also seems reasonable that the synaptic binding sites of nNOS would be up-regulated.

The lack of large amounts of synaptic nNOS staining is more useful for comparing the affinity of potential synaptically-localized binding sites, such as PSD-95, for nNOS compared to their affinities for their endogenous binding partner(s), such as NR2B in the case of PSD-95. The endogenous levels and affinities of PDZ proteins and their binding partners are well-matched. There is not a large excess of synaptically-localized nNOS binding sites waiting for a binding partner. Also the affinity of synaptically-localized nNOS binding sites, such as PSD-95, in hippocampal pyramidal neurons for their endogenous binding partners, such as NR2B, is greater than their affinity for nNOS. Otherwise, nNOS would displace them and localize to synapses. An interesting avenue for future study on PDZ domains is the possiblity of a dynamic role for PDZ domains, more specifically their modulation by phosphorylation.

NO release studies

There are two separate, but possibly related, main conclusions from the NO release work. First, NO generated intracellularly does not escape to the extracellular medium as well as NO generated at the plasma membrane. Second NO is converted to nitrite either in the extracellular space just outside the CHO cells or at the extracellular surface of the plasma membrane at a rate that is higher than predicted by air oxidation. It is interesting that these phenomena occur in cells that do not express NOSs endogenously, suggesting that they occur in many, if not all, cell types. It is perhaps useful to recall that NO is produced in or near all cells at various times. It is generated

either as a signaling molecule, in many cases of normal physiology, or as a cytotoxic agent, in cases of infection. Regardless of the reasons for its production, control of the concentration of NO is critical. The NO "signal" must be terminated and the NO "cytotoxin" needs to kill the bacteria with as small an effect on normal cells as possible. Mechanisms for the degradation of NO are therefore generally useful for eukaryotic cells and appear to be present. Degradation of a cellular messenger is a common mechanism for terminating its signal. One classical example is the cleavage of acetylcholine (ACh) by acetylcholinesterase in the neuromuscular junction. ACh is only released in the nervous system and the enzyme acetylcholinesterase is present in or near the cells where it is needed. Since NO is produced in or near all cells, low levels of the factor(s) responsible for its degradation may be present throughout the body.

The observation that nNOS in the brain is half soluble and half particulate is surprising in light of the fact that intracellular NOS releases much less NO than plasma-membrane associated NOS. While it is difficult to know where, exactly in the cell, the "soluble" nNOS is localized, it is probably not as close to the plasma membrane as myristoylated eNOS. Perhaps this soluble nNOS couples more effectively to release of calcium from intracellular stores or the NO that it produces signals to targets within the same cell.

Ca++ sensitivity

The constitutively expressed isoforms of NOS, e- and n-NOS, have an indistinguishable sensitivity to calcium with a half-maximal response at 145-150 nM Ca^{++}. This finding was facilitated by a small but significant technical advance. I used the low-affinity Ca^{++} buffer BAPTA, which buffers effectively in the range of calcium

concentrations of interest. In studies which used other buffers, the measurement of the Ca^{++} sensitivity of the NOSs varied from 60-300 nM. Since the resting calcium concentration in cells is typically below 100 nM, fine-tuning of the measurement is useful.