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Nitric Oxide Regulates Cyclic GMP-Dependent Protein Kinase Phosphorylation in Rat Brain

Alaa El-Din El-Husseini, Christopher Bladen, Julie A. Williams, Peter B. Reiner, and Steven R. Vincent

Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, British Columbia, Canada

Abstract: Nitric oxide (NO) acts via soluble guanylyl cyclase to increase cyclic GMP (cGMP), which can regulate various targets including protein kinases. Western blotting showed that type II cGMP-dependent protein kinase (cGK II) is widely expressed in various brain regions, especially in the thalamus. In thalamic extracts, the phosphorylation of several proteins, including cGK II, was increased by exogenous NO or cGMP. In vivo pretreatment with a NO synthase inhibitor reduced the phosphorylation of cGK II, and this could be reversed by exogenous NO or cGMP. Conversely, brainstem electrical stimulation, which enhances thalamic NO release, caused a NO synthase-dependent increase in the phosphorylation of thalamic cGK II. These results indicate that endogenous NO regulates cGMP-dependent protein phosphorylation in the thalamus. The activation of cGKII by NO may play a role in thalamic mechanisms underlying arousal. **Key Words:** Cyclic GMP-dependent protein kinase—Nitric oxide—Thalamus—Cyclic GMP—Phosphorylation—Arousal.
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Cyclic guanosine 5′-monophosphate (cGMP) mediates the physiological actions of two distinct messengers in the nervous system, the atriopeptides and nitric oxide (NO). Atriopeptides act on a unique family of cell surface receptors that have a single transmembrane domain and an intracellular catalytic domain, corresponding to what has been termed the particulate guanylyl cyclases (Garbers, 1992). NO, which can diffuse freely across cell membranes, acts on the soluble isoforms of guanylyl cyclase, binding to the heme moiety of the protein, and thereby activating this enzyme (Ignarro, 1991). The resultant increases in cGMP are thought to underlie the physiological actions of the atriopeptides and NO.

There appear to be three main classes of intracellular targets for cGMP, cGMP-regulated phosphodiesterases, cGMP-gated ion channels, and cGMP-dependent protein kinases. By altering phosphodiesterase activity, cGMP can regulate the intracellular levels of cyclic AMP (cAMP) (Beavo, 1995). cGMP-gated ion channels are well studied in photoreceptors and olfactory epithelium and also occur in the brain (El-Husseini et al., 1995*b*; Yao et al., 1995). Finally, by activating specific protein kinases, cGMP may regulate the function of numerous substrate proteins (Lincoln and Cornwell, 1993).

Two distinct types of cGMP-dependent protein kinase have been identified. The type I kinase (cGK I) occurs in two isoforms, arising from alternative mRNA splicing (Sandberg et al., 1989). cGK I is present at high levels in smooth muscle, lung, and platelets. Immunohistochemical studies have demonstrated that in the brain, cGK I is highly expressed in Purkinje cells in the cerebellar cortex, together with soluble guanylyl cyclase (Lohmann et al., 1981; Furuyama et al., 1993). In contrast, NO synthase is expressed in the granule cells innervating the Purkinje cells via the parallel fibers, and in the inhibitory basket cells that provide a GABA input to the Purkinje cells (Vincent, 1996).

A second form, type II cGMP-dependent protein kinase (cGK II) was cloned from mouse brain (Uhler, 1993) and rat intestine (Jarchau et al., 1994) and is expressed in widespread areas of the brain, with particularly high levels observed in the thalamus (El-Husseini et al., 1995*a*). The major source of NO in the thalamus is the innervation from the brainstem, principally from the laterodorsal and pedunculopontine tegmental nuclei (Vincent and Kimura, 1992). These cells, which express very high levels of NO synthase, are known to be cholinergic and are thought to play a critical role in regulating thalamic activity (Steriade

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Address correspondence and reprint requests to Dr. S. R. Vincent at Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3.

Abbreviations used: 8-bromo-cGMP, 8-bromo cyclic GMP; cAMP, cyclic AMP; cGK I and II, type I and II cyclic GMP-dependent protein kinase, respectively; cGMP, cyclic GMP; ECL, enhanced chemiluminescence; EEG, electroencephalogram; NO, nitric oxide.

and McCarley, 1990). Indeed, the release of both acetylcholine and NO from these afferents to the thalamus varies with behavioral state, decreasing significantly in slow-wave sleep during which the electroencephalogram (EEG) is synchronized (Williams et al., 1994, 1997). These neurons are predicted to regulate cGMP production in their thalamic targets via the NO-dependent activation of soluble guanylyl cyclase.

There is limited information available regarding specific targets for the two types of cGMP-dependent protein kinases. cGMP-dependent protein kinase activity has been demonstrated in the brain (Nairn and Greengard, 1983) and many substrates show high specificity for these enzymes compared with other protein kinases (Wang and Robinson, 1995). This prompted us to examine the ability of NO to regulate cGMP-dependent protein phosphorylation in the thalamus, which expresses high levels of cGK II. Here, we show that cGMP regulates the phosphorylation of several proteins in the thalamus. In particular, activation of the NO/cGMP pathway stimulates the phosphorylation of its downstream target, the cGMP-dependent protein kinase cGK II, possibly as an initial step in regulating the activity of this protein kinase, which in turn may be required for the phosphorylation of other target proteins in the thalamus.

MATERIALS AND METHODS

Tissue preparation

All animal procedures were in strict accordance with the guidelines of the Canadian Council of Animal Care, and were approved by the local animal care committee. Adult male Wistar rats were obtained from the Animal Care Centre of the University of British Columbia.

Adult male Wistar rats were cervically dislocated, decapitated, and their brains removed so that the thalamus could be dissected free. Tissue was then transferred into ice-cold homogenizing buffer (1 g/1 ml) containing 50 m*M* HEPES-Tris, pH 7.2, 8% sucrose, 0.1 *M* NaCl, and 1 m*M* EDTA; 15 m*M* β-mercaptoethanol and 0.1 m*M* phenylmethylsulfonyl fluoride were added just before homogenization.

The tissue was homogenized, using a modified method of Ives et al. (1980), i.e., 10 strokes of a low-speed Teflon pestle, to ensure the integrity of cGMP-dependent protein phosphorylation. All homogenates were quickly spun down at 1,000 *g* at 4°C for 3 min, to remove cell debris, and the supernatants used immediately for phosphorylation experiments or stored at −70°C. Conditions used for the phosphorylation experiments were essentially those used by Cornwell et al. (1991) except that supernatants were solubilized for 30 min at 4°C by addition of 1% Triton X-100 and spun down at 100,000 *g* × 45 min at 4°C to obtain the solubilized extract.

Phosphorylation assays

In phosphorylation assays, 30-μl-aliquot samples were preincubated on ice with various drugs and nucleotides (times and concentrations outlined in Results). Protein phosphorylation was initiated by addition of an equal volume of a [γ-^{32}P]ATP phosphorylation cocktail (final concentrations: 25 m*M* HEPES, pH 7.2, 5 m*M* $MgCl_2$, 0.2 m*M* isobutylmethylxanthine, 100 μm EGTA, and 30 μm [γ-^{32}P]-ATP), and the reaction was allowed to proceed for 60 s at 4°C and then terminated with a 5× stop mix/sample buffer (Cornwell et al., 1991). The labeled proteins were then immediately denatured at 95°C for 5 min, run on a 10% slab or 5–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gradient gel and then either dried and exposed to x-ray film, or transferred to nitrocellulose.

Results of phosphorylation experiments were from at least three trials per treatment. All chemicals were from Sigma (St. Louis, MO, U.S.A.), except [γ-^{32}P]ATP and enhanced chemiluminescence (ECL) materials were from Amersham (U.K.); 7-nitroindazole was from Research Biochemicals (Natick, MA, U.S.A.).

Immunoblotting

Western blots were performed by using the Amersham ECL detection system with a rabbit antiserum raised against recombinant rat cGK II expressed in and purified from *E. coli* (Jarchau et al., 1994). The western blots were also exposed to x-ray film to compare the protein bands detected with ECL with those that were evident in the phosphorylation assay. A small aliquot of each sample was used to determine protein concentrations by using a modified Lowry assay.

Drug treatments

To inhibit endogenous NO production in neurons, rats were pretreated for 1 h with 20 mg/kg i.p. 7-nitroindazole, dissolved in canola oil with sonication. At this dose, this drug produces a large inhibition in neuronal NO synthase activity without affecting endothelial NO synthase (Babbedge et al., 1993). To examine the effects of exogenous NO on protein phosphorylation, aqueous NO was prepared in homogenization buffer as described by Feelisch (1991). In brief, 20 ml of buffer was sonicated for 10 min and then purged with argon for 40 min over ice to remove all free oxygen. It was then bubbled for 10 min with NO to produce a saturated solution of ~3 m*M*. Meanwhile, argon-treated solubilized protein samples from either control or 7-nitroindazole-treated rats were divided into two aliquots each and freshly prepared oxyhemoglobin (a potent NO scavenger) was added to one and an equal volume of buffer to the other. Saturated NO solution was then transferred by using a gas-tight syringe to the samples 1 min before phosphorylation. The final concentration of NO in the samples was 0.5 m*M* and the final concentration of hemoglobin was 0.6 m*M*.

To induce EEG synchronization, another group of animals was given 60 mg/kg sodium pentobarbital intraperitoneally, dissolved in 10% ethanol and 40% propylene glycol. Control animals were given an injection of vehicle.

Stimulation of NO release in the thalamus

Rats were anesthetized intraperitoneally with 1.5–2 g/kg urethane and implanted with two screw electrodes for EEG recording at 2 and 4 mm posterior to bregma and 2 mm from midline. Electrodes were held by an Amphenol strip connector and fixed to the skull with dental acrylic. A burr hole was drilled over the cerebellum for a concentric stimulating electrode, which consisted of a Teflon-coated silver wire inside stainless steel tubing (30 gauge). Electrodes were lowered at a 30° angle through the cerebellum to avoid the venous sinus and placed in the laterodorsal tegmentum (Kayama et al., 1992). Stimulation was applied for 5 min at 60 Hz (bipolar, sine wave pulses, 20–100 μA) for 10 s with 30-s intervals, to cause an increase in thalamic NO

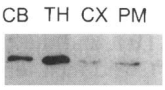

FIG. 1. Expression of cGK II in various brain regions. Western blots using a rabbit antibody to recombinant cGK II, with crude extracts taken from cerebellum (CB), thalamus (TH), pons medulla (PM), and cortex (CX), detected an 86-kDa protein corresponding to the intact cGK II protein.

release (Williams et al., 1997). In addition, animals were also given an injection of 7-nitroindazole or vehicle 1 h before stimulation.

RESULTS

The distribution of cGK II was examined in rat brain by using specific antibodies (Jarchau et al., 1994). Western blot analysis demonstrated that the intact 86-kDa cGK II protein was present in crude extracts of all four brain regions tested, with highest levels being found in the thalamus (Fig. 1). These results are consistent with our previous reverse transcriptase–PCR and in situ hybridization results indicating a widespread distribution of cGK II expression in the brain (El-Husseini et al., 1995*a*).

Because of the high expression of cGK II in the thalamus, cGMP-dependent protein phosphorylation was examined by using an in vitro protein phosphorylation technique as an initial step to identify targets of NO/cGMP in this brain region. The addition of exogenous cGMP (10 μM) increased the phosphorylation of several proteins when solubilized extracts from the thalamus were incubated with [γ-^{32}P]ATP and then separated on a 5–15% linear sodium dodecyl sulfate–polyacrylamide electrophoresis gel. An increase in the phosphorylation was noted in proteins of 120, 72, 42, 26, and 20 kDa (Fig. 2). When the phosphodiesterase-resistant, active analogue of cGMP, 8-bromo cyclic GMP (8-bromo-cGMP) (1 μM) was added to the assays, phosphorylation of these proteins was also dramatically increased. In addition, four other proteins at ~240, 100, 56, and 46 kDa were strongly phosphorylated (Fig. 2). Conversely, phosphorylation of some proteins, in particular one at 42 kDa, was dramatically reduced by the addition of 8-bromo-cGMP.

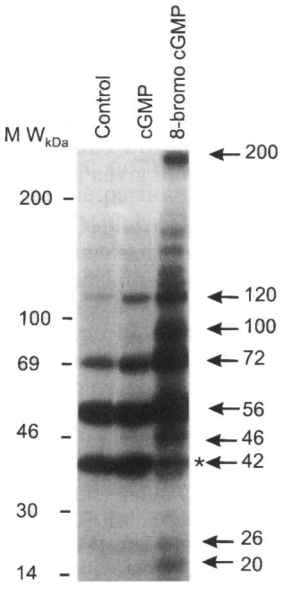

FIG. 2. cGMP-dependent protein phosphorylation in the brain. Solubilized thalamic extracts were incubated for 10 min with either buffer (Control), 10 μM cGMP, or 1 μM 8-bromo-cGMP before phosphorylation in the presence of [γ-^{32}P]ATP. The phosphorylation of several proteins was enhanced (indicated by arrows) on addition of cGMP or 8-bromo-cGMP. In contrast, the phosphorylation of a 42-kDa protein was reduced on addition of 8-bromo-cGMP (indicated by a star). Sizes of the molecular mass markers (MW) are indicated on the left.

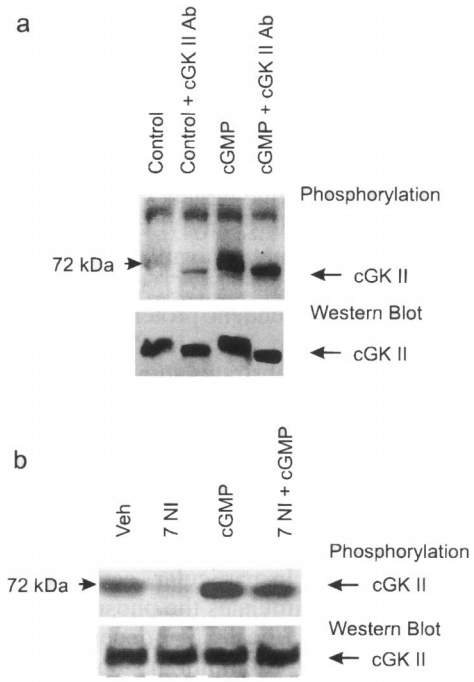

FIG. 3. Regulation of the phosphorylation of cGK II in the thalamus. **a: Top:** Solubilized thalamic preparations were incubated either with buffer as a control (lanes 1 and 2) or with 10 μM cGMP (lanes 3 and 4). Samples in lanes 2 and 4 were incubated for 30 min on ice with cGK II antibodies (cGK II Ab) before addition of [γ-^{32}P]ATP. **Bottom:** Western blot analysis shows that the phosphorylated 72-kDa protein corresponds to the major proteolytic product of cGK II. Preincubation with cGK II antibodies results in a shift of the phosphorylated 72-kDa protein (top and bottom panels, lanes 2 and 4). **b:** Reduction of cGK II phosphorylation in rats injected with the neuronal NO synthase inhibitor 7-nitroindazole (7 NI). **Top:** One hour before decapitation, rats were injected with either canola oil as a vehicle (Veh) or with 20 mg/kg 7 NI. Solubilized thalamic extracts were then incubated with [γ-^{32}P]ATP in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of cGMP. Phosphorylation of the 72-kDa protein that represents cGK II was reduced in animals treated with 7 NI, but cGK II phosphorylation was restored on cGMP addition. **Bottom:** Western blotting using cGK II specific antibody shows that the phosphorylated 72-kDa protein is cGK II.

In solubilized thalamic extracts, the labeling of a 72-kDa protein was enhanced by the addition of cGMP and 8-bromo-cGMP (Fig. 3a). Previous studies on rat intestine have demonstrated that the 86-kDa cGK II is largely cleaved to a protein of ~72 kDa that is autophosphorylated (de Jonge, 1981; Jarchau et al., 1994). Western blotting was therefore performed to determine if the phosphorylated 72-kDa thalamic protein was cGK II. Immunoblotting, using a cGK II spe-

cific antibody, confirmed that the phosphorylated 72-kDa protein corresponds to the major proteolytic fragment of cGK II (Fig. 3a). Furthermore, in control experiments, a variety of protein kinase inhibitors including the cAMP-dependent protein kinase inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), the protein kinase C inhibitor bisindolylmaleimide, and the calmodulin inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) had no effect on the level of phosphorylation of cGK II (data not shown). These results indicate that the observed increase in the phosphorylation of cGK II was not due to the direct action of cAMP-dependent protein kinase, protein kinase C, or calmodulin-dependent protein kinase.

Next we examined if preincubation of solubilized thalamic extracts with cGK II antibodies (1:200 dilution) could block cGK II phosphorylation. Preincubation of solubilized thalamic extracts with cGK II antibodies resulted in a shift in the phosphorylated 72-kDa protein, but did not prevent its phosphorylation (Fig. 3a). Western blotting analysis using cGK II antibodies demonstrated that the shifted 72-kDa protein was cGK II (Fig. 3a).

We then tested the hypothesis that cGK II phosphorylation was dependent on endogenous NO levels. When solubilized extracts of the thalamus from animals that had received the neuronal NO synthase inhibitor 7-nitroindazole (20 mg/kg i.p.) were examined in the protein phosphorylation assay, several bands showed a distinct decrease in phosphorylation compared with that seen in vehicle-injected animals. In particular, a decrease in the phosphorylation of the 72-kDa cGK II band was observed (Fig. 3b). Such decreases in phosphorylation could arise either as a result of an increase in the endogenous phosphorylation of the protein, thus preventing its subsequent in vitro phosphorylation in the presence of [γ-^{32}P]ATP, or a decrease in the activity of endogenous cGK II, reflected in a reduced rate of phosphorylation in the presence of radioactive ATP. The latter interpretation appears to be correct, because addition of exogenous cGMP to the phosphorylation reaction increased the labeling of the proteins in the 7-nitroindazole-treated rats (Fig. 3b). We confirmed again, by western blotting analysis, that the 72-kDa phosphorylated protein represents cGK II (Fig. 3b).

We next determined whether exogenous NO could stimulate the phosphorylation of cGK II in a manner similar to cGMP. This was initially done in vitro, by adding exogenous NO to the phosphorylation assay. These experiments demonstrated an increase in cGK II phosphorylation in the presence of 0.5 m*M* exogenous NO (Fig. 4a). However, when the potent NO scavenger oxyhemoglobin (0.6 m*M*) was added to the buffer before the phosphorylation assay, exogenous NO was unable to produce an increase in cGK II phosphorylation (Fig. 4a). Furthermore, although cGK II phosphorylation was reduced by pretreating animals

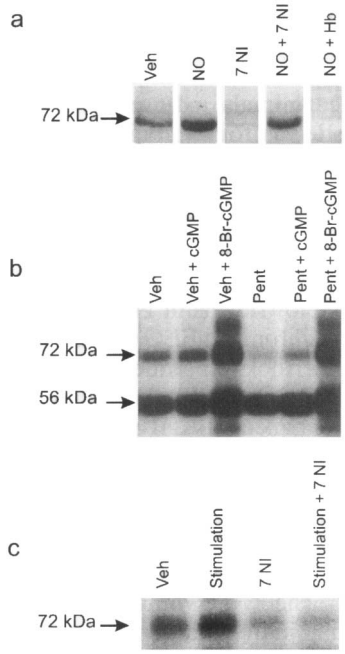

FIG. 4. NO and pentobarbital treatments modulate the phosphorylation of cGK II. Rats were injected 1 h before decapitation with either canola oil as a vehicle (Veh), or with 20 mg/kg 7-nitroindazole (7 NI) (a and c), or with 60 mg/kg pentobarbital (Pent) (b). **a:** Solubilized thalamic extracts were incubated with [γ-^{32}P]ATP in the absence (lanes 1 and 3) or the presence of exogenous NO (lanes 2, 4, and 5). Addition of NO enhanced cGK II phosphorylation (lane 2), whereas 7 NI treatment (lane 3) reduced basal levels of cGK II phosphorylation. Addition of NO restored cGK II phosphorylation in extracts of 7 NI-treated animals (lane 4). Addition of hemoglobin (Hb) blocked NO-dependent cGK II phosphorylation. **b:** Pentobarbital (Pent) treatment, which induces synchronized EEG, inhibits cGK II phosphorylation (lane 4) when compared with control animals injected with canola oil as a vehicle (lane 1). The inhibition of cGK II phosphorylation by pentobarbital was reversed by the addition of cGMP (lane 5) or 8-bromo-cGMP (lane 6). **c:** Activation of NO release in the thalamus enhances cGK II phosphorylation. Electrical stimulation of mesopontine tegmentum, which evokes NO release in the thalamus, was performed as described by Williams et al. (1997). Activation of NO released in the thalamus enhanced cGK II phosphorylation (Stimulation; lane 2), compared with control animals injected with the vehicle canola oil (Veh; lane 1). Preatment with 7 NI reduced basal levels of cGK II phosphorylation (7 NI; lane 3) and blocked the response to electrical stimulation (lane 4).

with the NO synthase inhibitor 7-nitroindazole, phosphorylation was again increased when these same samples were then treated with NO (Fig. 4a). This indicates that the effect of 7-nitroindazole on protein phosphorylation was due to the inhibition of NO production in the thalamus.

Our recent study showed that NO is released in the thalamus in a state-dependent manner, with the lowest rates occurring during slow-wave sleep when the EEG is synchronized (Williams et al., 1997). To test the hypothesis that cGK II phosphorylation may be regulated in a parallel manner, rats were treated with 60 mg/kg sodium pentobarbital, which is known to induce

EEG synchronization (Arduini and Arduini, 1954), before performing the protein phosphorylation assays. When extracts of thalamus were examined from pentobarbital-treated animals, a decrease in the phosphorylation of cGK II was found (Fig. 4b). The labeling of cGK II was restored when exogenous cGMP or 8-bromo-cGMP was added to this preparation (Fig. 4b), indicating that the reduction of cGK II phosphorylation was due to an inhibition of endogenous cGMP production. This decrease in cGK II phosphorylation is consistent with the low levels of NO released in the thalamus during synchronized EEG (Williams et al., 1997).

We also undertook experiments in vivo to stimulate endogenous NO release in the thalamus to examine if this could enhance cGK II phosphorylation. In the thalamus, the high concentrations of cGK II, together with the NO synthase-containing inputs it receives from the mesopontine tegmentum, suggest that cGK II may play a role in signal transduction pathways regulating thalamic arousal mechanisms. Electrical stimulation of the NO synthase containing thalamic afferents arising in the laterodorsal tegmentum induces endogenous NO production in the thalamus (Miyazaki et al., 1996; Williams et al., 1997). We have now found that electrical stimulation of the laterodorsal tegmentum also increased cGK II phosphorylation (Fig. 4c). This indeed appears to be dependent on NO production, because this effect was abolished by pretreatment with 7-nitroindazole (Fig. 4c).

DISCUSSION

The mechanisms whereby NO affects neuronal function are not yet clear. The results of the present study suggest that NO may exert many of its effects on brain tissue through the activation of cGMP-dependent protein kinases. Our autoradiographic results clearly show that the phosphorylation of many proteins was regulated by cGMP. Addition of exogenous cGMP or the active analogue 8-bromo-cGMP to thalamic extracts resulted in the enhanced phosphorylation of numerous proteins.

More significant is the fact that our experiments indicate that endogenous NO production can regulate the phosphorylation of cGK II in the thalamus. Reducing the endogenous levels of NO with 7-nitroindazole reduced the phosphorylation of cGK II, whereas addition of exogenous cGMP or 8-bromo-cGMP to the phosphorylation assays restored the phosphorylation of this protein kinase. In addition, exogenous NO as well as electrical stimulation of the brainstem, which results in increased NO production in the thalamus (Williams et al., 1997), also stimulated the phosphorylation of cGK II in thalamic neurons. This could be prevented by pretreatment with 7-nitroindazole, indicating that NO synthase was required for this effect. Thus, activation of cGMP-dependent protein kinase appears to be an important downstream mediator for NO in the nervous system.

A variety of cGMP binding proteins are present in the brain (Lincoln and Cornwell, 1993; El-Husseini et al., 1995*a*,*b*; Bladen et al., 1996). It has been known for many years that in the brain, cGK I is present selectively only in the Purkinje cells of the cerebellum (Lohmann et al., 1981). In contrast, recent in situ hybridization studies have demonstrated that cGK II is widely expressed in brain tissue and may therefore be the major neuronal target of cGMP (El-Husseini et al., 1995*a*). Indeed, the increase in cGMP-dependent protein phosphorylation observed in the present study is reflected in the NO-dependent phosphorylation of the cGMP-dependent protein kinase. Autophosphorylation on ligand binding is an initial step in the regulation of many protein kinases and has been described previously for cGMP-dependent protein kinases (Francis and Corbin, 1994). Autophosphorylation of cGK I and both the full-length (86 kDa) and the proteolytic fragment (72 kDa) of intestinal cGK II has been noted (de Jonge, 1981; French et al., 1995). In our experiments, using extraction methods containing relatively high salt and detergent concentrations, a 15-kDa N-terminal peptide appears to have been cleaved from cGK II. This limited proteolysis did not prevent the phosphorylation of the 72-kDa protein or the ability of the cGK II antibody to recognize it. Thus, we hypothesize that the observed NO/cGMP-dependent phosphorylation of cGK II may represent the autophosphorylation of this protein kinase. In agreement with our studies on the rat brain, previous studies of rat intestine have found that a 72-kDa cleavage product is the predominant autophosphorylated form of cGK II, and that the proteolytic cleavage that yielded this form could not be inhibited by a variety of protease inhibitors (de Jonge, 1981). Autophosphorylation of cGK I in the cerebellum leads to a dramatic increase in the affinity of the kinase for cAMP (Foster et al., 1981; Landgraf et al., 1986); however, the functional consequences of cGK II autophosphorylation are not yet known.

Preincubation of the thalamic extracts with the cGK II antibodies before the phosphorylation reaction resulted in a slight shift in the mobility of cGK II. This might be due to a blockade of some of the potential phosphorylation sites on the kinase by the bound antibody, as has been found previously for rat intestinal cGK II, using antibodies against the lung enzyme (de Jonge, 1981). Other phosphorylation sites must still have been accessible, however, as the 72-kDa protein was labeled by the phosphorylation reaction even in the presence of the antibody. Such a change in phosphate incorporation would result in changes in size, charge, and conformation of the protein that could explain the mobility shift.

It is likely that cleavage of the 86-kDa, full-length cGK II protein to yield the 72-kDa protein occurs at the same site seen with the intestinal enzyme, which would yield a fragment beginning at Pro^{139} (Vaandrager et al., 1997). Such a cleavage would separate

the phosphate-acceptor site of the predicted pseudosubstrate inhibitory region, as well as the potential autophosphorylation site at Ser^{117}, from the remaining catalytic protein (Jarchau et al., 1994). It is possible that phosphorylation may direct cleavage of the 86-kDa protein, because we only detected the 72-kDa protein after the in vitro phosphorylation reactions. Further potential cGMP-dependent phosphorylation sites exist in the 72-kDa fragment, including Ser^{152}, Ser^{153}, and Ser^{420}, and Thr^{160} and Thr^{676}. In addition, phosphorylation of Thr^{516} is known to be crucial to the activity of cGK I (Feil et al., 1995), and this site, Thr^{609}, is conserved in cGK II. Cleavage of cGK II to a 72-kDa fragment would also remove the N-terminal myristoylation site required for the membrane association of cGK II (Vaandrager et al., 1996), as well as a potential coiled-coil domain (amino acids 44–86) that might be involved in homodimerization or other protein–protein interactions. Indeed, previous study on cGK II in the intestinal brush border has indicated the importance of an N-terminal 15-kDa fragment in anchoring the protein to the cytoskeleton (de Jonge, 1981).

The identification of substrate proteins for the cGMP-dependent protein kinases has yet to be directly ascertained (Wang and Robinson, 1995, 1997); however, certain likely candidates may be mentioned. The best-characterized cGK I substrate in brain is the G substrate, identified in Purkinje cells of the cerebellar cortex (Aswad and Greengard, 1981; Detre et al., 1984). A recent study has noted G substrate immunoreactivity is also present in neuronal fibers in the thalamus (Qian et al., 1996). This suggests that G substrate may be a potential target for cGK II as well, and that some of the actions of cGMP in thalamic neurons may be mediated through the hypothesized protein phosphatase inhibitor action of G substrate. Other potential targets include substrates for cAMP-dependent protein kinase, which are often readily phosphorylated by cGK, such as dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) (Tsou et al., 1993) and the transcription factor cAMP response-element binding protein (CREB) (Gamm et al., 1995). Phosphorylation of this protein would provide a direct mechanism for the regulation of gene expression by agents that increase cGMP (Gudi et al., 1996). Likewise, phosphorylation of neurotransmitter receptors (McDonald and Moss, 1994), calcium or potassium channels (Paupardin-Tritsch et al., 1986; Alioua et al., 1995), or rabphilin-3A (Fykse et al., 1995) would provide mechanisms for cGMP-dependent protein kinase to affect the electrical activity of target neurons as well as neurotransmitter release. In this regard, it is of interest that the hyperpolarization-activated cation current (I_h) in thalamic neurons has been shown to be modulated by NO and cGMP (Pape and Mager, 1992).

From a physiological point of view, it is of particular interest that changes in endogenous NO production and electrical activity in the thalamus can regulate the activity of thalamic cGK II. We have previously found that thalamic neurons express very high levels of cGK II mRNA (El-Husseini et al., 1995*a*), although they do not express NO synthase. Instead, they receive a major input from NO synthase-containing neurons in the brainstem reticular formation. These neurons, in the pedunculopontine and laterodorsal tegmental nuclei, appear to correspond to the cholinergic reticular activating system originally postulated by Moruzzi and Magoun (1949) and are well known to play a major role in regulating thalamic activity (Steriade and McCarley, 1990). It has previously been shown that the release of both acetylcholine and NO in the thalamus varies with behavioral state, decreasing significantly in slow-wave sleep during which the EEG is synchronized (Williams et al., 1994, 1997). In the present study, we have found that decreasing endogenous NO production with an NO synthase inhibitor resulted in a decrease in cGK II phosphorylation in the thalamus. A similar decrease in cGK II phosphorylation was found after pentobarbital administration causing EEG synchronization. Furthermore, electrical stimulation of the mesopontine tegmentum, which evokes endogenous NO production in the thalamus (Miyazaki et al., 1996; Williams et al., 1997), was associated with an increase in cGK II phosphorylation, which was decreased by pretreatment with an NO synthase inhibitor. The incomplete inhibition of cGK II phosphorylation by 7-nitroindazole may reflect incomplete NO synthase inhibition, but at the concentration used, the inhibition should have been at least 90% (Babbedge et al., 1993). Instead, a more likely explanation is that electrical stimulation of the laterodorsal tegmental neurons also evokes the release of atrial and brain natriuretic peptides, which are known to coexist with NO synthase and choline acetyltransferase in these neurons (Standaert et al., 1986; Saper et al., 1989). The release of these peptides would likely not be affected by 7-nitroindazole and they would thus still be available to act on particulate guanylyl cyclases to elevate cGMP and activate cGK II. Together these results suggest that the level of cGK II-dependent protein phosphorylation in thalamic neurons should vary with behavioral state. Indeed, NO has been shown to facilitate the responses of thalamic neurons to visual and tactile stimuli (Do et al., 1994; Cudeiro et al., 1996). Thus, it will be of particular importance to determine the target proteins for this kinase and the mechanisms whereby they regulate the properties of thalamic neurons.

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