Cyclic AMP-dependent phosphorylation of neuronal nitric oxide synthase mediates penile erection

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Nitric oxide (NO) generated by neuronal NO synthase (nNOS) initiates penile erection, but has not been thought to participate in the sustained erection required for normal sexual performance. We now show that cAMP-dependent phosphorylation of nNOS mediates erectile physiology, including sustained erection. nNOS is phosphorylated by cAMP-dependent protein kinase (PKA) at serine(S1412). Electrical stimulation of the penile innervation increases S1412 phosphorylation that is blocked by PKA inhibitors but not by PI3-kinase/Akt inhibitors. Stimulation of cAMP formation by forskolin also activates nNOS phosphorylation. Sustained penile erection elicited by either intracavernous forskolin injection, or augmented by forskolin during cavernous nerve electrical stimulation, is prevented by the PKA inhibitor λ-NAME or in nNOS-deleted mice. Thus, nNOS mediates both initiation and maintenance of penile erection, implying unique approaches for treating erectile dysfunction.

Results

Electrical Stimulation of Penile Innervation Augments Phosphorylation of nNOS. We developed a C-terminal antibody that selectively recognizes P-nNOS. Immunoreactivity is absent in cells overexpressing nNOS in which S1412 is mutated to alanine (Fig. S1). We also developed an antibody selective for unphosphorylated nNOS (unP-nNOS) that shows decreased affinity for P-nNOS but can be used at higher concentrations to detect total nNOS. A commercial N-terminus antibody that recognizes total nNOS does not discriminate nNOS S1412 phosphorylation.

Using an established physiologic model of erectile function, we found electrical stimulation of the rat CN markedly increases P-nNOS immunoreactivity but not unP-nNOS in penile lysates (Fig. 1A). With 6-V stimulation, P-nNOS increases 15-27-fold following 1 or 5 min of stimulation, respectively (Fig. 1B and C). Direct application of 1- or 6-V electrical stimulation to the rat major pelvic ganglion (MPG) increases P-nNOS in the neuronal tissue by two- or ninefold (Fig. 1D and E).

nNOS is alternatively spliced (22-24). The alternatively spliced forms are designated nNOS-β and nNOS-γ, whereas the predominant wild-type nNOS is designated nNOSα (25-27). Alternatively spliced nNOS isoforms retain some catalytic activity but lack the N-terminal PDZ domain that links nNOS to PSD95 and NMDA-glutamate receptors (25, 28, 29). We wondered whether nerve stimulation would influence phosphorylation of S1412 in the alternatively spliced isoforms (Fig. 1F). We detect very low levels of phosphorylated nNOSβ and nNOSγ in the mouse MPG, with no effect of electrical stimulation. Interestingly, unphosphorylated nNOSγ is increased in nNOSα-/- mice, perhaps reflecting some compensatory response (30).

The antibodies to P-nNOS and unP-nNOS are suitable for immunohistochemistry. Both antibodies and the N-terminal nNOS antibody selectively stain MPG neuronal cell bodies and their processes in a pattern similar to the neuronal marker synaptophysin (Fig. 2). All three antibodies similarly stain cytosolic nNOS, with staining also evident at the plasma membrane.

Electrical Stimulation Provides Prolonged Activation of nNOS Phosphorylation Mediated by PKA. Physiologic depolarization-dependent increases in intracellular calcium that maximally activate nNOS are short lived, usually returning to baseline in seconds (31, 32). The increase of P-nNOS in rat MPG elicited by electrical stimulation is maintained for over a minute and then declines at 3 and 10 min (Fig. 3A and B), similar to other persistent calcium-dependent signaling processes (33, 34). Although the proportion of P-nNOS increases with electrical stimulation, total nNOS normalized to β-actin is not significantly altered. Our prior work showed distinct εNOS-dependent stimulation of erection by papaverine, with remarkably decreased intracavernosal pressure (ICP) responses in


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sequence surrounding nNOS S1412 corresponds to a PKA consensus site (Fig. 4A), and although another PKA consensus sequence is present at S370, direct phosphorylation of that site has not been reported. Full-length nNOS purified from transfected HEK293 cells can be selectively phosphorylated with $^{32}$P]ATP and PKA. The phosphorylation is abolished in nNOS-S1412D mutants (Fig. 4B and Fig. S1). Kemp and colleagues (37, 38) reported that both Akt and PKA phosphorylate eNOS at S1179, which is comparable to S1412 of nNOS and supports our findings.

To investigate the role of PKA in S1412-nNOS phosphorylation in the intact penis, we performed injections of forskolin (FSK), a potent and selective activator of adenylyl cyclase, beneath the rat MPG, and monitored P-nNOS in ganglion/CN preparations (Fig. 4C). Periganglial FSK elicits a substantial increase in neuronal P-nNOS, but the inactive derivative deoxy-forskolin (dFSK) does not. No change in P-nNOS is detected with dFSK treatment. We also examined penile P-nNOS levels following intracavernosal injection of FSK or dFSK (Fig. 4D). FSK triples penile P-nNOS, but dFSK has no effect. In contrast, FSK does not influence penile phospho-eNOS levels. The unexpected absence of increased P-eNOS in this preparation may reflect the shorter time course before tissue collection (30–90 s for FSK, and 5–7 min for papaverine). Thus, in the MPG and penile tissue P-nNOS immunoreactivity reflects PKA phosphorylation of nNOS at S1412.

**Neurally Evoked Penile Erection Is Mediated by PKA Phosphorylation of nNOS.** We explored whether PKA phosphorylation of nNOS is required for penile erection evoked by nerve stimulation. First we examined the influence of FSK. As little as 0.25 μg FSK injected intracavernosally in the mouse elicits a four- to fivefold increase in ICP, with only modest increase at higher doses (Fig. 5A and Fig. S2), but the inactive dFSK has no effect. The effect of low doses of FSK (0.25–1 μg) is markedly reduced in nNOS$^-$/− mice or following treatment with the NOS inhibitor 1-nitro-arginine-methylester (L-NAME). The increased ICP at high doses of FSK (2.5–5 μg) is not influenced by nNOS deletion or inhibition, consistent with known nNOS-independent actions of FSK mediating smooth muscle relaxation. The similar changes in ICP at higher doses of FSK suggest that different signaling pathways mediate low- and high-dose FSK effects and that the smooth muscle contractile apparatus is not modified in the nNOS$^-$/− mice. The FSK effect on penile erection is evident in measurements of both maximal ICP (Fig. 5A) and for the integrated total

![Fig. 1.](image)

**Fig. 1.** Electrical stimulation increases P-nNOS in the penis and MPG. (A) Electrical stimulation of the rat CN causes voltage-dependent increase in penile P-nNOS. (B) The electrically stimulated increase in P-nNOS is also time-dependent, but the total nNOSx detected with an N-terminal antibody is unchanged, showing that nNOS protein is stable during stimulation. (C) Quantification of P-nNOS in arbitrary units is performed by densitometry. Each bar represents mean ± SE of P-nNOS/unP-nNOS expressed relative to unstimulated sham control. **P < 0.05, ***P < 0.001 compared with sham. (F) Representative blots show differential phosphorylation of nNOS isoforms in wild-type and double nNOS/eNOS- (dNOS$^-$/−) deleted mice after direct electrical stimulation.

eNOS$^-$/− versus wild-type or nNOS$^-$/− mice. Papaverine dilates blood vessels, increasing flow and shear stress, which augments phospho-eNOS (12, 13) to produce penile erection. We investigated whether intracavernosal papaverine in the rat would also augment P-nNOS. Although papaverine increases phospho-eNOS (Fig. 3C), there is no change in nNOS phosphorylation (14). Although papaverine is a nonselective phosphodiesterase inhibitor, it does not increase cAMP production in penile tissue (35, 36). This finding suggests separate regulation of phosphorylation of eNOS and nNOS by distinct signaling pathways in normal erectile physiology.

We used a pharmacologic approach to identify the kinase that phosphorylates nNOS at S1412. Ten minutes after periganglionic injection of inhibitors, the rat MPG and CN were electrically stimulated for 1 min, whereupon the ganglion-nerve preparation was snap-frozen for subsequent analysis (Fig. 3D and E). Electrical stimulation elicits three- to fourfold augmentation of P-nNOS, which is prevented by treatment with PKA inhibitors H89 and PKA inhibitor peptide (PKAI). In contrast, wortmannin (Wrt) and LY294002 (LY), well-established inhibitors of PI3-kinase, fail to alter P-nNOS levels. The response of phospho-Akt to electrical stimulation is markedly different. Electrical stimulation fails to alter phospho-Akt levels, which are reduced by Wrt although not significantly decreased by LY. Surprisingly, H89 and PKAI significantly increase phospho-Akt.

The ability of PKA inhibitors to abolish the increase in P-nNOS with electrical stimulation strongly implies that physiologic phosphorylation of S1412 is uniquely mediated by PKA.

![Fig. 2.](image)
phosphorylation of nNOS-S1412 is distinct from phospho-eNOS regulation in penile erection. (A) Representative immunoblot shows sustained P-nNOS activity is not sufficient to produce erectile function with submaximal electrical stimulation and nNOSβ is not phosphorylated by PKA, so it cannot mediate the increased response after FSK treatment seen in wild-type animals expressing nNOSα.

**Discussion**

In the present study we demonstrate a major role for PKA phosphorylation of nNOS at S1412 in mediating penile erection. Using a highly specific antibody to phospho-nNOS-S1412, we showed that electrical stimulation of the CN markedly augments phosphorylation of nNOS in both MPG and penile tissue preparations. Moreover, cAMP activation of PKA is responsible for this phosphorylation, because FSK—which leads to generation of cAMP and PKA activation—markedly increases and PKA inhibitors decrease P-nNOS levels. FSK-mediated penile erection includes a component mediated specifically by nNOS, as the effect is similarly abolished by treatment with NOS inhibitors and in nNOSα−/− animals. In vitro studies have shown increased NO production at resting calcium levels with nNOS serine-1412 phosphorylation (40).

Previously we established two phases for penile erection. Phosphorylation of PKA mediates the persistent effect of FSK. With maximal electrical stimulation (16 Hz/4 V), ICP is similar to that elicited by modest stimulation plus FSK, although no additive effect is observed. nNOS deletion and l-NAME treatment markedly reduce penile erection with maximal stimulation. As we previously reported, nNOSβ mediates the NO-dependent change in ICP with maximal electrical stimulation in nNOSα−/− mice. However, nNOSβ activity is not sufficient to produce erectile function with submaximal electrical stimulation and nNOSβ is not phosphorylated by PKA, so it cannot mediate the increased response after FSK treatment seen in wild-type animals expressing nNOSα.
regulated event, this stimulation can only cause a brief increase in neuronal NO-dependent blood flow. This increased blood flow, however, activates endothelial PI3-kinase to stimulate Akt, phosphorylate and activate eNOS, and provide persistent NO production and sustained penile erection (14, 42, 43). Our findings here indicate that neuronal stimulation increases cAMP to activate PKA, which phosphorylates nNOS at S1412, stimulating nNOS catalytic activity (Fig. 6). This covalent phospho-modification can last substantially longer than the neuronal calcium transient and so, in coordination with phospho-eNOS, phospho-nNOS may contribute to sustained erection. We conjecture that activity dependent calcium-stimulated adenyl cyclase could mediate cAMP/PKA activation in the CN.

Ways in which alterations of nNOS and eNOS interact in coordinated neurovascular erectile physiology are not clear. Moreover, the influence of nNOS phosphorylation on its usual regulation by calcium/calmodulin is uncertain, although there is evidence that eNOS phosphorylation by Akt sensitizes it to the effects of calcium (13, 44, 45) and eNOS also associates with calmodulin. eNOS phosphorylation renders it more sensitive to resting intracellular calcium concentrations, providing a feed-forward augmentation of enzymatic activity. It is possible that nNOS phosphorylation by PKA likewise increases its sensitivity to resting calcium/calmodulin, thereby prolonging nNOS activation (40). It is known that Akt can phosphorylate nNOS at S1412. Our findings establish that PKA also directly phosphorylates and activates nNOS-S1412 in a physiologically meaningful way similar to endothelial PKA phosphorylation of eNOS-S1177 (37). Rameau et al. (17, 21) used immunofluorescent staining techniques to show that NMDA stimulation of cortical neuronal cultures enhances phosphorylation of nNOS at S1412 in specific and localized synaptic signaling. Conceivably, Akt and PKA phosphorylation of nNOS interact in a regulated fashion. Further studies will determine the coordinated regulation of stimulatory and inhibitory phosphorylation sites for cAMP-dependent penile NO production.

FSK has herefore been thought to stimulate penile erection by activating PKA in smooth muscle of the penis (46–48), and that is also the likely mechanism in our higher-dose FSK experiments. Our findings suggest that FSK also acts by stimulating nNOS, enhancing the neurovascular coordination of sustained NO release and suggesting novel therapeutic approaches to erectile dysfunction. Current therapeutic agents selectively inhibit forms of phosphodiesterase that act primarily upon cGMP in the erectile smooth muscle. Our findings imply that drugs inhibiting the metabolism of both cAMP and cGMP will act synergistically by respectively enhancing NO generation and preventing cGMP degradation.

Materials and Methods

Reagents. FSK, dFSK, papaverine, Wt, LY, H89, PKAI, L-NAME were from Sigma-Aldrich; commercial anti-nNOS-S1412 antibody were from Abcam; nNOS N-terminus antibody, anti-total Akt, anti-phospho-Akt-S473 were from Cell Signaling Technology.

Animal Models. Male Sprague–Dawley rats (300–325 g; Charles River Breeding Laboratories) or 8- to 10-wk-old C57BL6/J (wild-type; Jackson Laboratories) or 8- to 10-wk-old C57BL6/J (wild-type; Jackson Laboratories), and nNOS^-/- mice (S.H.S. laboratory, Johns Hopkins University, Baltimore, MD) were anesthetized by intraperitoneal injection of ketamine (50 mg/kg)/xylazine (5 mg/kg). MPG and CN were identified/isolated via midline suprapubic incision for electrical stimulation of the CN. To monitor ICP in...
Fig. 6. A model integrating nNOS and endothelial NOS (eNOS) regulation in initiation and maintenance of erectile function. Initiating neural stimuli promote NO production by nNOS and nNOS sensitization by PPKA phosphorylation of S1412. Initial smooth muscle relaxation and increased penile blood flow stimulates PKA-Akt phosphorylation of eNOS-S1179. Phosphorylation and activation of nNOS and eNOS produces NO at resting intracellular calcium levels. Maintenance of maximum erectile response may be sustained by synergistic NO production from P-nNOS and P-eNOS. Dotted lines represent the proposed contribution of P-nNOS in this model.

Preparation of Protein Extracts, Western Immunoblot, and Phospho-Labeling. Samples were prepared as previously described (14). Briefly, frozen tissue was minced then homogenized (penis) or sonicated (MPG) in 8 vol of ice-cold buffer containing 50 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1% Triton X-100, and 1% (vol/vol) glycerol, with phosphatase and protease inhibitors [50 mM NaF, 5 mM sodium pyrophosphate/30 mM β-glycerophosphate (BGP)/1 mM sodium orthovanadate/2 μM staurosporine/phosphatase inhibitor mixture2 μg/mL aprotinin/10 μg/mL leupeptin/1 mM Pefabloc]. After centrifuging at 16,000 × g for 30 min, soluble protein was de-

mice, the penis was denuded of skin and right corpus cavernosum was pierced with 30-gauge needle attached to PE-50 tubing connected to a pressure transducer (Harvard Apparatus) as previously described (49). Response parameters were recorded using a data acquisition (DI-190; Dataq Instruments) and calculated using MATLAB software (Mathworks). All experiments were approved by the Johns Hopkins University Institutional Animal Care and Use Committee (IACUC).

Electrically Induced Penile Erection Studies. In anesthetized animals, electrical stimulation of penile erection was performed by placing a bipolar platinum electrode hook around the CN, as previously described (5). The electrode was attached to a Grass Instruments S48 stimulator. Stimulation parameters are indicated in results for various experiments. Typical maximum stimulation in rats was 16 Hz at 6 V with square-wave duration of 5 ms. Except for time-course experiments, duration of electro-stimulation was 1 min. In dephosphorylation experiments, rat CN was electically stimulated at maximal parameters for 1 min, then both the MPG and penis were collected 15 s to 10 min after termination of stimulation.

Electrophoresis of samples was performed by gel electrophoresis. Protein loading was determined by spectrophotometry. Gel images were scanned, and protein concentration was calculated using ImageJ software. Equal loading and protein integrity were confirmed by Coomassie blue staining. Blotting was performed as described above.


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