Kalirin Inhibition of Inducible Nitric-oxide Synthase*

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Nitric oxide (NO) acts as a neurotransmitter. However, excess NO produced from neuronal NO synthase (nNOS) or inducible NOS (iNOS) during inflammation of the central nervous system can be neurotoxic, disrupting neurotransmitter and hormone production and killing neurons. A screen of a hippocampal cDNA library showed that a unique region of the iNOS protein interacts with Kalirin, previously identified as an interactor with a secretory granule peptide biosynthetic enzyme. Kalirin associates with iNOS in vitro and in vivo and inhibits iNOS activity by preventing the formation of iNOS homodimers. Expression of exogenous Kalirin in pituitary cells dramatically reduces iNOS inhibition of ACTH secretion. Thus Kalirin may play a neuroprotective role during inflammation of the central nervous system by inhibiting iNOS activity.

Nitric oxide (NO) mediates numerous physiological functions in the central nervous system, including neurotransmission, synaptic plasticity, and hormone secretion (1–5). In neurons that express nNOS, excitatory amino acids can act to mediate NMDA-type glutamate receptors, temporarily elevating intracellular calcium concentrations, resulting in transient synthesis of NO by nNOS. NO then acts as a neurotransmitter, diffusing into adjacent neurons. However, excessive NO production is neurotoxic and may be involved in diseases of the central nervous system. Various experimental systems demonstrate the neurotoxicity of NO. For example, NO derived from nNOS or NO donors can kill neurons in vitro (6, 7). Large amounts of excitatory amino acids generated during strokes can trigger neurons that contain nNOS to produce high concentrations of NO (8). Conversely, studies with nNOS null mice show that NMDA excitotoxicity is reduced, and cerebral infarct size is smaller in the absence of NO generated from NOS (9–11). In humans, NO derived from nNOS may play a neurotoxic role in strokes and in diverse neurodegenerative diseases, including Alzheimer’s disease, Huntington’s chorea, and amyotrophic lateral sclerosis. Neuronal NO is also found in a significant fraction of peripheral neurons, where it is thought to function as a neurotransmitter (12).

Another source of high concentrations of NO in the central nervous system is the iNOS isoform, which can be induced in neurons or microglia during inflammation (13–16). In contrast to nNOS, iNOS synthesizes large amounts of NO continuously, which can also be neurotoxic to neurons in vitro (17). Furthermore, cerebral infarct size is also reduced in iNOS null mice (18). The iNOS isoform is also expressed in experimental autoimmune encephalomyelitis, and NO inhibition reduces the severity of the disease (19–22). In humans, NO derived from iNOS may play a role in the pathogenesis of inflammatory disorders of the brain, such as AIDS dementia and multiple sclerosis (23–25), and may also contribute to the pathogenesis of Alzheimer’s disease (26). NO may also play a more subtle role during brain inflammation, interfering with physiological functions such as hormone secretion. Experimental evidence from neuronal cultures and animal models shows that NO can also inhibit specific secretory responses of neurons. For example, some but not all modes of stimulating hormone secretion from the pituitary are enhanced in the presence of NO inhibitors (27–36).

Because iNOS expression may lead to neurotoxicity in the brain, disrupting hormone secretion or killing neurons, we tested the hypothesis that proteins are expressed in the central nervous system which regulate iNOS activity, minimizing its neurotoxic effects. A yeast two-hybrid screen for hippocampal proteins that may bind to iNOS revealed that Kalirin interacts with iNOS.

Kalirin is a cytosolic protein with nine spectrin-like repeats, a Dbl-homology domain that acts as a GDP/GTP exchange factor for Rac1, and pleckstrin homology and SH3 domains (see Fig. 1) (37, 38). Kalirin is closely related to two other proteins, Trio and UNC-73, which also possess spectrin-like motifs, Dbl homology domains, and pleckstrin homology domains (39, 40). Kalirin was initially identified by its ability to interact with the cytosolic domain of peptidylglycine α-amidating monoxygenase (PAM), an enzyme located in the trans-Golgi network and large dense core vesicles. PAM is a bifunctional type I integral membrane protein with both functional domains in the lumen of the secretory pathway; PAM is responsible for the α-amidation of neuropeptides and hormones, a modification essential for the biological potency of more than half of all the known bioactive peptides (41). Kalirin is expressed with PAM at high levels in cerebral cortex, piriform cortex, amygdala, hippocampus, and olfactory bulb. Although the precise role of Kalirin is...
unknown, it may serve to transduce signals between large dense-core vesicles and the actin cytoskeleton. Our data suggest a novel role for Kalirin in the regulation of hormone secretion by its effects upon iNOS.

**EXPERIMENTAL PROCEDURES**

**Antibodies, cDNAs, Vectors, and Reagents—**For immunoblotting and immunoprecipitation, the following antibodies were used: a monoclonal antibody raised against c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA) or prepared from hydridoma 9E10 cells (42); a polyclonal antibody raised against a murine iNOS peptide generated by us (43); a monoclonal antibody raised against murine iNOS (Transduction Laboratories), a polyclonal antibody to ENOS and nNOS (Transduction Laboratories), and a polyclonal antibody (JH2582) to Kalirin (spectrin repeats 4–7) generated by us. Standard cloning methods were used to join the Kalirin cDNA fragment identified using the yeast two-hybrid screen (encoding Kalirin amino acids 447–1124) in reading frame 3’ to the sequence of the c-Myc epitope and a Gly, linker (MEQKSEEEDLDNQGGG-Kalirin(447–1124)). This cDNA was cloned 3’ to the CMV promoter in the mammalian expression vector pSCEP to create pSCEP:myc-Kalirin(447–1124) (37, 38). The murine iNOS cDNA was generated as described (44). The (His)6-iNOS fusion protein was generated from a cDNA based on the pET vector from Novagen (45) and expressed in bacteria and then purified over a nickel resin (according to manufacturer instructions). Lipopolysaccharide (LPS) obtained from pig jejunal mucosa approximate molecular weights of substances in the eluted fractions.

**Cell Culture—**The interaction of Kalirin and iNOS was examined in non-transfected AT-20/16 cells, and in AT-20 cells stably expressing Kalirin(447–1124). The vector pSCEP:myc-Kalirin(447–1124) was transfected into AT-20 cells by lipofection. AT-20 cells stably expressing myc-Kalirin(447–1124) were first selected for hygromycin resistance (200 units/ml) and were then screened for expression of the Kalirin transcript by Northern blot analysis. Cells were maintained as described (37, 38). Induction of iNOS in cells was performed using IFN-γ (20 units/ml) and LPS (1 μg/ml) for 2–16 h (46, 47).

**Western Blot Analysis—**Cells were suspended in lysis buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5% of Triton X-100, 0.5% NaF, 50 mM Na3VO4, 1 mM leucine, and histidine) with 1 ml of a 50% suspension of protein A-Sepharose 4B (or goat anti-mouse agarose) for 4 h at 4 °C and then was washed three times with 1 ml of cold 20 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1 mM Na3VO4, 50 mM NaF, 1 mM EDTA, 0.2% Triton X-100, 0.2% PMSF. Protein extracts were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were incubated for 1 h with primary antibodies diluted 1:1000–1:50000 in blocking solution containing 3% bovine serum albumin, 0.1% Tween 20 in Tris-buffered saline and after washing were diluted 1:1000–1:5000 in blocking solution containing 3% bovine serum albumin, 0.1% Tween 20 in Tris-buffered saline and after washing were incubated for 1–2 h at room temperature with anti-eNOS and nNOS (Transduction Laboratories), and a polyclonal antibody to eNOS and nNOS (Transduction Laboratories), and a polyclonal antibody (JH2582) to Kalirin (spectrin repeats 4–7) generated by us. Standard cloning methods were used to join the Kalirin cDNA fragment identified using the yeast two-hybrid screen (encoding Kalirin amino acids 447–1124) in reading frame 3’ to the sequence of the c-Myc epitope and a Gly, linker (MEQKSEEEDLDNQGGG-Kalirin(447–1124)). This cDNA was cloned 3’ to the CMV promoter in the mammalian expression vector pSCEP to create pSCEP:myc-Kalirin(447–1124) (37, 38). The murine iNOS cDNA was generated as described (44). The (His)6-iNOS fusion protein was generated from a cDNA based on the pET vector from Novagen (45) and expressed in bacteria and then purified over a nickel resin (according to manufacturer instructions). Lipopolysaccharide (LPS) obtained from pig jejunal mucosa approximate molecular weights of substances in the eluted fractions.

**Immunoprecipitation—**Cells were suspended in lysis buffer for 30 min on ice. Supernatants were recovered by centrifugation at 15,000 × g for 15 min, and 500 μl of supernatant was incubated with 10 μl of normal rabbit serum for 30 min and then incubated with 50 μl of protein A-Sepharose 4B for 30 min. Following centrifugation, 500 μl of supernatant was incubated for 3 h with primary antibodies and then with 40 μl of a 50% suspension of protein A-Sepharose 4B (or goat anti-mouse agarose) for 4 h at 4 °C and then was washed three times with 1 ml of cold 20 mM Tris-HCl, pH 7.4, 125 mM NaCl, 1 mM Na3VO4, 50 mM NaF, 1 mM EDTA, 0.2% Triton X-100, 0.2% PMSF. Protein extracts were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Membranes were incubated for 1–2 h at room temperature with antibodies to c-Myc, or iNOS (1:5000), washed, incubated for 1–2 h with goat anti-mouse or goat anti-rabbit antibody (1:10,000) coupled to horseradish peroxidase, and visualized with an ECL reagent (Amer sham Pharmacia Biotech). For the detection of iNOS and Kalirin complexes in vivo, (C57Bl6, 129)F2 wild-type and iNOS null mice from the other NOS isoforms. Screening of 2.6 × 106 yeast transformants was performed using antiserum Kathy, which detects ACTH (strain SYF526) were co-transformed by the lithium acetate method with various combinations of bait plasmid (pPC97 expressing a fusion protein of the Gal4-BD (binding domain) and portions of iNOS) and target plasmids (pPC86 expressing a fusion protein of the Gal4-AD (activation domain) and portions of Kalirin). Transformants were plated for 3 days at 30 °C on selective medium lacking tryptophan, leucine, and histidine. After 3 days, cells were assayed for β-galactosidase activity using ONPG as a substrate (nmol ONPG cleaved/min/mg protein measured at A420).

**Results**

We employed a yeast two-hybrid system to screen for potential binding partners of iNOS (49). The pPC86 library contained cDNA prepared from rat hippocampus (generous gift of Dr. A. Lahanan) (50). To prepare bait plasmids, murine iNOS cDNA sequences 1–546 base pairs or 1–210 base pairs) were prepared by the polymerase chain reaction and inserted 3’ to the cDNA for the Gal4 binding domain of plasmid pPC97. The resultant pPC97–iNOS bait plasmids encoded a fusion protein comprised of the Gal4 binding domain and an amino-terminal fragment of iNOS (amino acid residues 1–182 or 1–70). YRG-2 yeast cells were co-transformed with pPC97–I NOS and the pPC86-based library and grown on a selective medium lacking tryptophan, leucine, and histidine, or combinations thereof. Colonies that contain cDNA encoding target library proteins interacting with the bait fusion protein were identified by transcription of the HIS3- and lacZ-genes. A total of 2.6 × 106 yeast transformants were placed under selection. Plasmid preparations from β-galactosidase positive yeast colonies were isolated and retransformed into competent Escherichia coli DH5α cells.

**ACTH Secretion Assay—**For secretion experiments using LPS and IFN-γ, identical wells of cells were treated for 12 h with control medium or medium containing 50 ng/ml LPS and 10 units/ml IFN-γ; for treated cultures, LPS and IFN-γ were added fresh throughout the secretion experiment. Cells were transferred to basal medium (Dulbecco’s modified Eagle’s medium-air with 2 mg/ml bovine serum albumin, 0.1 mg/ml lima bean trypsin inhibitor, 1 μg/ml insulin, 0.1 μg/ml transferrin) for the secretion experiments and equilibrated in release medium for sequential 30-min periods. Supernatants were harvested from cells exposed to basal media, 100 mM corticotropin releasing hormone (Sigma) or 1 mM BaCl2 (51–53). Cell extracts were prepared using 5 N acetic acid with protease inhibitors, lyophilized, and then dissolved in radiommun assay buffer with inhibitors. ACTH radioimmunassays were performed using antiserum Kathy, which detects ACTH biosynthetic intermediate and ACTH but does not detect intact pro-opiomelanocortin (54).

**RESULTS**

We used the yeast two-hybrid system to search for proteins that might associate with iNOS (49). Yeast expressing a fusion protein consisting of the amino acids 1–182 of iNOS and the Gal4 DNA binding domain (BD) were transformed with a library of plasmids encoding fusion proteins consisting of rat hippocampal cDNAs and the Gal4 activation domain (AD) (50). The amino-terminal region of iNOS was selected as a potential target of interacting proteins because it is not homologous to the other NOS isoforms. Screening of 2.6 × 106 yeast transformants isolated cDNA encoding the protein Kalirin in Table 1. Our yeast two-hybrid screen also showed that KSHV Kaposi’s sarcoma-associated antigen, tel-related protein, and homologues to a serine/threonine kinase, and bicaudal D may also interact with the amino terminus of iNOS. These interactions have not been confirmed by in vivo testing, yet.

**Kalirin Interacts with iNOS in Vitro—**To define more pre-
Specific interaction between iNOS and Kalirin in the yeast two-hybrid assay

*S. cerevisiae* (strain SFY526) were co-transformed by the lithium acetate method with various combinations of bait plasmid (pPC97 expressing a fusion protein of the Gal4 binding domain and portions of iNOS) and target plasmids (pPC86 expressing a fusion protein of the Gal4 activation domain and portions of Kalirin). Transformants were plated for 3 days at 30 °C on selective medium lacking tryptophan, leucine, and histidine. After 3 days, cells were assayed for β-galactosidase activity by liquid culture assay method using ONPG as a substrate (nmol ONPG cleaved/min/mg of protein measured at A₄₂₀ (μ)). Assays were performed in triplicate.

| Bait       | Target        | His+ | β-galactosidase |
|------------|---------------|------|-----------------|
| iNOS-(1–70) | Kalirin (570–753) | +    | 35 ± 4.3        |
| iNOS-(1–182) | Kalirin (570–753) | +    | 32 ± 3.9        |
| iNOS-(70–220) | Kalirin (570–753) | –    | 6.7 ± 1.2       |
| iNOS-(1–182) | Kalirin (570–753) | –    | 4.3 ± 0.8       |
| iNOS-(1–70)  | Kalirin (570–753) | –    | 5.4 ± 1.5       |
| Fos        | Jun           | +    | 8.5 ± 6.8       |
| iNOS-(1–182) | Jun           | –    | 6.2 ± 1.6       |
| Fos        | Kalirin (570–753) | –    | 4.8 ± 1.1       |
| iNOS-(1–70)  | Jun           | –    | 3.9 ± 0.9       |

Fig. 1. Domains of iNOS and Kalirin. For iNOS, amino-terminal Hook Domain (dimerization segment), heme binding domain (HEME), calmodulin binding domain (CAL), flavin adenine mononucleotide binding domain (FMN), flavin adenine dinucleotide binding domain (FAD), nicotinamide adenine dinucleotide phosphate binding domain (NADPH) are shown. The Kalirin interaction domain of iNOS identified in this study is also indicated. For Kalirin, spectrin motifs (ovals), Dbl-homology domain (DH), pleckstrin homology domain (PH), and SH3 domain are shown.

Kalirin interacts with iNOS in cells and in Mice—We next examined the ability of iNOS and Kalirin to interact in vitro. Bacterial expression vectors were constructed which express a (His)₆-iNOS fusion protein (45) or a glutathione S-transferase-Kalirin-(amino acid residues 447–1124) fusion protein. Purified (His)₆-iNOS fusion protein interacts with purified glutathione S-transferase-Kalirin-(447–1124) fusion protein in vitro (Fig. 2). This interaction was demonstrated by co-immunoprecipitation of GST-Kalirin with an iNOS antibody and by the binding of iNOS to GST-Kalirin immobilized on glutathione-agarose resin.

Fig. 2. Complexes of iNOS and Kalirin in vitro. (His)₆-iNOS, GST-Kalirin-(447–1124), and an irrelevant target, GST-midkine (93), were expressed and purified from bacteria. Various combinations of pure proteins were incubated together as indicated. A, immunoprecipitation with antibody to iNOS and immunoblot with antibody to GST. B, purification with glutathione-agarose and immunoblot with antibody to iNOS. Only iNOS and Kalirin fusion proteins are capable of interaction. (The experiment was repeated twice with similar results.)

Expression of iNOS. Kalirin is expressed only in AtT-20 cells that are transfected, and iNOS is expressed only in cells that are treated with LPS and IFN-γ, as detected by immunoblotting (Fig. 3A). Kalirin and iNOS also associate in these cells, as shown by co-immunoprecipitation (Fig. 3B). Most of the Kalirin-(447–1124) and most of the iNOS appear to be associated with each other.
We then examined the interaction of iNOS and Kalirin in mice. The availability of mice lacking iNOS provides a control for the specificity of this interaction. Kalirin is expressed in the brains of wild-type mice and iNOS null mice (Fig. 4A). Native Kalirin-(1–1899) has a molecular mass of approximately 210 kDa when expressed in mouse brains or in AtT-20 cells. The recombinant Kalirin-(447–1124) fragment expressed in AtT-20 cells and used in the other figures has a molecular mass of approximately 73 kDa. As expected, LPS injected intraperitoneally into wild-type and iNOS null mice induces iNOS expression only in wild-type mice (Fig. 4A). Complexes of endogenous Kalirin and endogenous iNOS are detected by immunoprecipitation in brain extracts of LPS-treated wild-type mice, but not in brain extracts of LPS-treated mice lacking iNOS (Fig. 4B) (55). Thus iNOS and Kalirin interact in a variety of experimental systems.

Specificity of Kalirin Interaction with NOS Isoforms—To test the specificity of the Kalirin interaction with NOS isoforms, we analyzed mouse brain extracts for the interaction of Kalirin and nNOS or eNOS. Lysates from brains of wild-type mice treated with LPS as above were fractionated by SDS-PAGE and probed with antibody to nNOS, showing that nNOS is expressed in mouse brain (Fig. 4C). Lysates from these brains were then immunoprecipitated with antibody to Kalirin, fractionated by SDS-PAGE, and probed with antibody to nNOS. No nNOS was co-immunoprecipitated with Kalirin. SDS-PAGE and analyzed by immunoblotting with antibody to Kalirin, or immunoprecipitated with antibody to iNOS followed by immunoblotting with antibody to Kalirin or to iNOS. B, physiological complexes of iNOS and Kalirin in mice. Brain lysates were prepared from mice as above, and either immunoprecipitated with antibody to iNOS followed by immunoblotting with antibody to Kalirin, or immunoprecipitated with antibody to Kalirin followed by immunoblotting with antibody to iNOS. C, Kalirin does not interact with nNOS in vivo. Lysates from wild-type mice (lanes 1 and 2) or from iNOS null mice (lanes 2 and 4) were fractionated by SDS-PAGE (lanes 1 and 2) or immunoprecipitated with antibody to kalirin, fractionated by SDS-PAGE (lanes 3 and 4), and then probed with an antibody to nNOS. D, Kalirin does not interact with eNOS in vivo. Samples were analyzed as in panel C using an eNOS antiserum. The experiment was repeated in three wild-type and three iNOS null mice with similar results.

We hypothesized that Kalirin inhibits iNOS activity by affecting iNOS homodimerization, which is required for iNOS activity (56, 57). To test this hypothesis, cell lysates were prepared from either LPS- and IFN-γ-treated AtT-20 cells or from LPS- and IFN-γ-treated AtT-20 cells expressing Kalirin-(447–1124), and these lysates were fractionated on a gel filtration column. Aliquots were then electrophoresed on a denaturing gel. Immunoblotting of gel filtration fractions with antibody to iNOS suggests that most of the iNOS exists as a homodimer (M, 260,000) in cells without Kalirin (eluted in fractions 11–20 at approximately 300 kDa) (Fig. 6). The less abundant iNOS monomer eluted in fractions 27–30 at approximately 110 kDa. In contrast, in cells expressing Kalirin-(447–1124), most of the iNOS exists as an intermediate-sized form (fractions 18–23), probably representing iNOS/Kalirin heterodimers; some dimer was detected, but very little iNOS monomer was present in these cells (Fig. 6). Kalirin-(447–1124) eluted with iNOS in fractions 18–23, and trailed into later fractions. Thus Kalirin-(447–1124) reduces iNOS activity by more than 90% (Fig. 5A). Expression of Kalirin-(447–1124) reduces iNOS activity by more than 90% (Fig. 5A). Because expression of Kalirin-(447–1124) does not reduce the amount of iNOS protein expressed in response to IFN-γ/LPS treatment (Fig. 5B), we explored other mechanisms by which Kalirin could inhibit iNOS activity. Kalirin Inhibits iNOS Activity by Blocking iNOS Homodimerization—We then measured the effect of Kalirin on iNOS enzymatic activity. NOS activity was measured in AtT-20 cells and in AtT-20 cells expressing Kalirin-(447–1124); cells were either resting or treated with IFN-γ and LPS. As expected, treatment of AtT-20 cells with LPS and IFN-γ induces iNOS activity (Fig. 5A). Expression of Kalirin-(447–1124) reduces iNOS activity by more than 90% (Fig. 5A). Because expression of Kalirin-(447–1124) does not reduce the amount of iNOS protein expressed in response to IFN-γ/LPS treatment (Fig. 5B), we explored other mechanisms by which Kalirin could inhibit iNOS activity.
Kalirin inhibits iNOS homodimerization. AtT-20 cells were treated with IFN-γ and LPS for 16 h, and then cell lysates were fractionated on a Superdex 200 gel filtration column. Fractions of 1 ml were collected, resolved by SDS-PAGE, and immunoblotted with antibody to iNOS (A) or antibody to Kalirin (B). AtT-20 cells expressing Kalirin-(447–1124) were treated with IFN-γ and LPS for 16 h and then fractionated and analyzed in the same manner as above with antibody to iNOS (C) or with antibody to kalirin (D). The column was calibrated with the following standards: thyroglobulin (relative molecular weight $M_r$ 670,000) in fractions 8–12, gamma globulin ($M_r$ 158,000) in fractions 18–22, ovalbumin ($M_r$ 44,000) in fractions 32–40, myoglobin ($M_r$ 17,000), and cyanocobalamin ($M_r$ 1350). The experiment was repeated twice with similar results.

Kalirin Associates with iNOS Monomers—In theory, Kalirin could associate with iNOS monomers, preventing iNOS homodimerization and eliminating NOS activity; or Kalirin could associate with iNOS homodimers, converting iNOS homodimers into monomers and thereby inhibiting NOS activity. To distinguish between these two possibilities, we performed experiments mixing cell lysates. Lysates were prepared from non-transfected resting AtT-20 cells, non-transfected AtT-20 cells treated with LPS and γIFN, resting AtT-20 cells expressing Kalirin-(447–1124), or AtT-20 cells expressing Kalirin-(447–1124) treated with LPS and IFN-γ. Pairs of these lysates were mixed together and either analyzed by immunoblotting or by immunoprecipitation with antibody to Kalirin followed by immunoblotting with the antibody to iNOS (Fig. 7). Kalirin can interact with iNOS if both proteins are produced in the same cell or if each protein is produced in different cells. However, because iNOS exists not only as homodimers but also as monomers in LPS/IFN-γ-treated AtT-20 cells, this experiment does not prove that Kalirin only interacts with iNOS monomers.

If Kalirin-(447–1124) only associates with iNOS monomers, then we would predict that addition of Kalirin-containing extracts to iNOS-containing extracts would not affect iNOS activity. This is indeed the case. Lysates were prepared from AtT-20 cells that were treated or not with LPS/IFN-γ, and mixed with lysates prepared from AtT-20 cells expressing Kalirin-(447–1124) that were treated or not with LPS/IFN-γ, and the NOS activity in the mixtures was assayed as above. Resting non-transfected AtT-20 cells have little NOS activity (113 ± 54 cpm/mg); resting AtT-20 cells expressing Kalirin-(447–1124) also have little NOS activity (527 ± 92). LPS/IFN-γ treatment resulted in increased NOS activity in non-transfected AtT-20 cells (4222 ± 125). Although iNOS activity was inhibited when iNOS and Kalirin were expressed in the same cells (481 ± 70), iNOS activity was not inhibited when Kalirin and iNOS were made in separate cells and the lysates were mixed (4661 ± 292).

To confirm that Kalirin-(447–1124) only associates with iNOS monomers, we mixed Kalirin-containing cell extracts with iNOS monomers or iNOS homodimers, which had been isolated by column chromatography of LPS-treated AtT-20 cell lysates, as in Fig. 6A. Mixtures of Kalirin-(447–1124) and iNOS dimers and mixtures of Kalirin-(447–1124) and iNOS monomers both contain Kalirin-(447–1124) and iNOS, as verified by immunoblotting. Co-immunoprecipitation of iNOS with antiserum to Kalirin was used to determine whether the proteins could interact; iNOS was detected in the Kalirin immunoprecipitate only when Kalirin-containing extracts were mixed with iNOS monomers (Fig. 8). These sets of experiments imply that Kalirin can only interact with iNOS monomers, and Kalirin cannot interact with iNOS homodimers.

Kalirin Reduces the Inhibitory Effect of iNOS upon ACTH Secretion—Other investigators have shown that NO inhibits the secretion of adrenocorticotropic hormone (ACTH) from the pituitary during systemic or central nervous system inflammation (9–11, 13, 19–25). To study the functional significance of the interaction between Kalirin and iNOS, we measured the ability of iNOS and Kalirin to alter ACTH secretion in AtT-20 cells. AtT-20 cells produce ACTH and β-endorphin from pro-opiomelanocortin, storing the peptide products in large dense core vesicles (58), and release of ACTH can be stimulated by secretagogues such as corticotropin-releasing hormone (CRH) and BaCl₂ (53, 59, 60).

We measured the ability of iNOS and Kalirin to alter ACTH secretion from non-transfected AtT-20 cells and from stably transfected AtT-20 cells expressing Kalirin-(447–1124). LPS...
and IFN-γ treatment decreases the basal release of ACTH from the non-transfected cells to 40%, compared with untreated non-transfected cells (Fig. 9). NO mediates this inhibition of ACTH secretion by LPS and IFN-γ, because pretreatment for 3 h with the NOS inhibitor nitro-arginine methyl ester (NAME) largely negates the effects of LPS and IFN-γ (data not shown). Similar to the effects of NAME, expression of Kalirin also blunts the effect of LPS and IFN-γ upon basal ACTH secretion (Fig. 9), presumably by inhibiting NO synthesis from iNOS. Stimulated secretion of ACTH was also reduced to half by LPS and IFN-γ treatment in the non-transfected cells. Kalirin expression also blocked this inhibitory effect of LPS and IFN-γ treatment on stimulated ACTH release (Fig. 9). Thus Kalirin reduces the inhibition of basal and stimulated secretion of ACTH in cells exposed to LPS and IFN-γ.

DISCUSSION

Calmodulin was the first protein shown to interact with NOS (48); it is necessary for enzymatic activity of all NOS isoforms. Subsequently a variety of other proteins have been shown to interact with the constitutive NOS isoforms; syntrophin, post-synaptic density protein (PSD) 93, and PSD95 can each interact with the amino-terminal region of nNOS via a PDZ domain, localizing nNOS to specific subcellular regions of myocytes or neurons (5, 61, 62). The mammalian homologue of dynein light chain (PIN) can interact with nNOS, maintaining it in a monomeric form (63); and caveolin 1 and caveolin 3 can interact with eNOS, localizing eNOS to the caveolae of endothelial cells (64–72). Our report of the interaction between iNOS and Kalirin suggests a novel mechanism for regulation of iNOS, which was previously thought to be regulated primarily at the level of transcription (73).

Dimerization of NOS activates NO synthesis by permitting electron transfer between the reductase and oxygenase domains (74–76). Homodimerization of iNOS depends upon arginine, tetrahydrobiopterin and heme (77–84). Biochemical and crystallographic studies show that dimerization of iNOS involves the oxygenase domains of each iNOS monomer (83, 85, 86). A portion of the oxygenase domain of iNOS, amino acid residues 66–114, is particularly important for homodimerization (78). Crystallography shows that in this region, Cys-109 forms an interchain disulfide bond across the dimer interface (83). In our experiments using the yeast two-hybrid system, we found that Kalirin interacts with the initial 70 amino acids of the amino-terminal of iNOS. Because this region partially overlaps the iNOS dimerization domain, perhaps Kalirin prevents iNOS homodimerization by physically interfering with the amino-terminal iNOS dimerization region.

The fact that LPS and interferon treatment inhibits basal and stimulated ACTH secretion from AtT-20 cells is consistent with other studies which show that NO inhibits ACTH secretion (27–36). The effect of LPS upon the pituitary is complex: in the whole animal LPS is often reported to stimulate ACTH secretion (87). Some of the confusion about LPS and IFN-γ action could be because of time-dependent effects, because short term stimulation of ACTH secretion by IFN-γ is followed by prolonged inhibition of ACTH secretion in the longer treatment periods used in many studies and in this work (88). In the central nervous system, LPS induces iNOS which produces NO, which in turn can interact with specific protein components of the 20 S v-SNARE/t-SNARE complex, inhibiting exocytosis of synaptic vesicles (89). Here we present data showing that expression of Kalirin-(447–1124) inhibits the catalytic activity of iNOS, and thus reduces the ability of LPS and interferon to inhibit secretion of ACTH.

Kalirin interacts with other molecules in addition to iNOS that may be involved in the secretory process. Notably, Huntingtin associated protein-1 was also found to interact with the spectrin-like domains of Kalirin (90), as well as with p150^glued (91). We have also shown that Kalirin interacts directly with the protein kinase P-CIP2, which was originally identified as another interactor with the carboxyl domain of PAM (38) and as an interactor with stathmin, a regulator of microtubule depolymerization (92).

Expression of iNOS in the central nervous system is associated with a variety of inflammatory states, which can lead to neurotoxicity, apoptosis, and aberrant regulation of hormone release. Expression of iNOS and production of NO inhibit ACTH secretion by the pituitary. Our data show that Kalirin can inhibit iNOS activity in AtT-20 cells; in the central nervous system, Kalirin would preserve neuropeptide secretion during inflammation. In this manner, Kalirin may play a neuropro-
tective role, reducing neurotoxicity and restoring hormone and neuropptide secretion from large dense core vesicles.

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