Atrial Natriuretic Peptide Induces Natriuretic Peptide Receptor-cGMP-dependent Protein Kinase Interaction*

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Circulating natriuretic peptides such as atrial natriuretic peptide (ANP) counterbalance the effects of hypertension and inhibit cardiac hypertrophy by activating cGMP-dependent protein kinase (PKG). Natriuretic peptide binding to type I receptors (NPRA and NPRB) activates their intrinsic guanylyl cyclase activity, resulting in a rapid increase in cytosolic cGMP that subsequently activates PKG. Phosphorylation of the receptor by an unknown serine/threonine kinase is required before ligand binding can activate the cyclase. While searching for downstream PKG partners using a yeast two-hybrid screen of a human heart cDNA library, we unexpectedly found an upstream association with NPRA. PKG is a serine/threonine kinase capable of phosphorylating NPRA in vitro; however, regulation of NPRA by PKG has not been previously reported. Here we show that PKG is recruited to the plasma membrane following ANP treatment, an effect that can be blocked by pharmacological inhibition of PKG activation. Furthermore, PKG participates in a ligand-dependent gain-of-function loop that significantly increases the intrinsic cyclase activity of the receptor. PKG translocation is ANP-dependent but not nitric oxide-dependent. Our results suggest that anchoring of PKG to NPRA is a key event after ligand binding that determines distal effects. As such, the NPRA-PKG association may represent a novel mechanism for compartmentalization of cGMP-mediated signaling and regulation of receptor sensitivity.

The natriuretic peptides (NPs)¹ are produced by the heart, the vasculature, and the kidney and are an ancient family of polypeptide hormones that regulate mammalian blood volume and blood pressure. More recently, the ability of NPs to modulate cell proliferation (1) and cardiac hypertrophy (2) has been demonstrated. Physiologically important NP actions include natriuresis (3), vasodilation (4), and ubiquitous inhibitory actions such as inhibition of smooth muscle proliferation (1, 5), cardiac fibroblast proliferation (6), cardiomyocyte hypertrophy (2, 7–9), sympathetic tone (10), renin-angiotensin-aldosterone activation (11), and hypothalamic-pituitary-adrenal axis signaling (12–14). In disease states such as heart failure, NP actions may be limited by resistance to hormone effects (15–17) that are at least in part because of insensitivity of the receptor itself (18).

Natriuretic peptide binding to type I receptors (NPRA and NPRB) on target cells activates their intrinsic guanylyl cyclase activity, resulting in a rapid increase in cGMP. Diffusible cGMP acts as a second messenger primarily by stimulating PKG (19). PKG is the major mediator of cGMP-induced smooth muscle relaxation (20). Downstream NP effects that have been directly tied to activated PKG include modulation of the L-type calcium channel (21, 22) and cross-talk with heterologous receptors, such as G protein-coupled receptors (23, 24). Furthermore, there is recent evidence that the membrane-bound guanylyl cyclase, NPRA, but not soluble cyclases that are activated by nitric oxide, has potent effects on plasma membrane control of the calcium ATPase pump (25), suggesting that NO- and NP-mediated effects are compartmentalized in cells.

Although the NPRA cDNA was first cloned more than 13 years ago (26), its regulation remains poorly understood. In its prebound state, the NPRA receptor exists as a homodimer (27), but ligand binding alone is insufficient to induce cyclase activity. Rather, phosphorylation of six serine and threonine residues in the intracellular juxtamembrane-kinase homology domain makes the receptor susceptible to NP activation (28). The protein kinase that mediates receptor phosphorylation is unknown.

We have previously reported that ANP inhibits cardiac hypertrophy through cGMP/PKG-mediated activation of the ERK signaling cascade at the level of MEK (9) but could not demonstrate a direct interaction between PKG and MEK. PKG substrates are membrane-bound (29), cytosolic (30), and intranuclear (31). In an attempt to identify novel proteins that could be candidates for linking PKG to MEK, we used a cytosolic yeast two-hybrid system employing PKG as bait. We found that PKG directly interacts with NPRA. These results were initially quite surprising, because PKG was previously thought to regulate only downstream ANP targets. However, it has been demonstrated previously that PKG is a serine/threonine kinase capable of phosphorylating NPRA in vitro (32). We report for the first time the regulation of NPRA by PKG.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Studies—Screening for PKG-interacting proteins was done using a commercially available system (CytoTrap, Stratagene, La Jolla, CA) that identifies protein-protein interactions in the yeast cytoplasm. Rather than relying on transcriptional activation to detect interactions, the RAS signal transduction cascade is initiated by recruitment of bSOS to the plasma membrane in a temperature-sensitive mutant yeast strain, cdc25H, by virtue of the interaction of its bait fusion partner with a myristoylated prey protein, which allows growth.

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¹ The abbreviations used are: NP, natriuretic peptide; PKG, cGMP-dependent protein kinase; NO, nitric oxide; ANP, atrial NP, PIPES, 1,4-piperazinediethanesulfonic acid; SNP, S-nitroso-N-acetylpenicillamine; HEK, human embryonic kidney; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase.
² This paper is available on line at http://www.jbc.org.

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at 36 °C. A bovine PKG Iα cDNA was cloned in-frame into the hSOS bait plasmid. A human heart cDNA library in the pMyr plasmid (Stratagene) containing 7.4 × 10^6 independent clones was cotransfected with the pPKG-hSOS expression vector into competent cdc25H cells, which were grown for 4 days at 25 °C on minimal glucose plates. Colonies were isolated and tested for galactose-dependent growth at 36 °C. Plasmids were extracted from three initially positive colonies, and the inserts were sequenced before retransformation in cdc25H cells together with pPKG-hSOS. Conventional yeast transformation and manipulation protocols were used. Cells were replica-plated onto either galactose- or galactose-minimal medium containing relevant amino acids, according to the manufacturer’s protocols.

**Immunocytochemistry—HEK-NPRA and HEK293 control cells** were incubated in 4-well chamber slides at a density of ~100,000 cells/cm² in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Invitrogen). After fixation in 3.7% formaldehyde, cells were permeabilized with 0.3% Triton X-100 in phosphate-buffered saline for 10 min and blocked with 1% horse serum, 0.2% bovine serum albumin in phosphate-buffered saline. Fixed slides were incubated with a primary antibody mixture containing 0.1 μg/ml rabbit anti-PKG I (Stressgen) or rabbit anti-Ark (Santa Cruz Biotechnology) and 0.7 μg/ml mouse anti-FLAG M2 (Sigma). The immunogen used to generate the anti-PKG I antisera has no homology to PKG II making cross-reactivity extremely unlikely. Cells were blocked for 1 h and then for an additional hour with fluorescein isothiocyanate or Cy5-conjugated donkey secondary antibodies (Jackson Laboratories); cells were then mounted with DRAQ5 (Biozone, Reading, UK) for 1 h. Fluorescent imaging was performed with a Leica DMRA upright fluorescence microscope, and images were acquired with a Hamamatsu ORCA2 CCD camera.

**Cardiac Cell Culture**—The care of all animals used in this research was in accordance with institutional guidelines. Ventricular cardiac cells from 1–2-day-old Harlan Sprague-Dawley rats were prepared as described previously (33). Ventricles were dissected free from atria and quartered. Myocytes were dissociated in trypsin and DNase I and incubated in 4-well chamber slides at a density of 400,000 cells/chamber (Invitrogen). Cardiac cells were treated identically, except fixed cells were incubated with a 1:1000 dilution of rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 1 h. Fluorescent imaging was performed with a Leica DMR upright fluorescent microscope, and images were acquired with a Hamamatsu ORCA2 CCD camera.

**Figure 1. Interaction of NPRA and PKG in yeast.** Cdc25H cells were transformed with the indicated plasmids and plated on glucose minimal medium supplemented with the appropriate amino acids. Three independent transformant clones harboring prey plasmids encoding NPRA, RIT (a Ras-related GTPase (46)), and an unknown protein (3d) were grown at 25 °C, indicating successful plasmid co-transformation (left panel), replica-plated onto appropriately supplemented galactose minimal plates, and grown at 36 °C (right panel). Both the NPRA and RIT-containing chimeras grew at 36 °C when co-transformed with the PKG-SOS plasmid, but the RIT chimera also grew with the insertless pSOS vector, indicating PKG-independent activation. Clone 3d did not grow at 36 °C.

**Figure 2. PKG is present at the cell membrane.** A. PKG kinase activity is present in detergent-containing cell membrane lysates from HEK-NPRA cells but not control cells. NPRA immunoprecipitates of lysates from control cells or cells transfected with a bovine PKG Iα expression vector were assayed for PKG activity. Data are expressed as percent increase from reactions without cGMP (mean ± S.E. of three independent experiments, each performed in triplicate). B. PKG protein is present in cell membrane lysates of HEK-NPRA but not control cells (HEK293). HEK-NPRA and control cells were transfected with the PKG expression vector and then treated for the indicated times with 1 μM ANP before preparation of detergent-free lysates (upper panel). PKG is present in cytosolic lysates of both HEK-NPRA and control cells (middle panel). NPRA protein is present in cell membrane lysates of HEK-NPRA but not control cells (lower panel). Lysates underwent SDS-PAGE electrophoresis and were immunoblotted with anti-PKG or NPRA antibody (images are representative of three separate experiments).
centrifugation, the supernatant was added to a chromatography column (Bio-Rad 731–1550) containing 1.0 g of dry neural alumina resin (Sigma A9003) acidified with 5 ml of 1N perchloric acid. The column was washed sequentially with 20 ml of 1N perchloric acid and 20 ml of water. [32P]cGMP was eluted into scintillation vials with 10 ml of 200 mM ammonium formate and counted using a Beckman LS 6500 liquid scintillation counter.

RESULTS

NPRA and PKG Associate in Yeast—To identify potential PKG partners, we screened a human heart cDNA library using a cytosolic yeast two-hybrid system (see “Experimental Procedures”). Employing PKG as bait, the single positive clone identified corresponded to an ~300-bp cDNA fragment of NPRA encoding the entire C-terminal cyclase domain and part of the so-called hinge region (35) (Fig. 1).

NPRA and PKG Associate in Mammalian Cells—Experiments were carried out in HEK293 cells stably transfected with an expression vector encoding FLAG epitope-tagged NPRA (27) (HEK-NPRA). HEK-NPRA cells exhibit a hormone-stimulated generation of cGMP, whereas untransfected HEK293 cells express minimal endogenous NPRA (28). Using FLAG antibody, it was not possible to co-immunoprecipitate levels of PKG from HEK-NPRA membranes that were detectable by Western immunoblotting, presumably because detergent solubilization disrupted the NPRA-PKG association. Attempts to release NPRA from membrane lysates while maintaining PKG association using a variety of concentrations of both ionic and non-ionic detergents were unsuccessful. However, these same immunoprecipitates clearly contained low levels of PKG based upon highly sensitive PKG activity assays (Fig. 2A). Because NPRA is a transmembrane protein and membrane localization of PKG had not been previously reported, we prepared detergent-free cell membranes were assayed for kinase activity in the presence of cGMP. Data are expressed as percent increase from reactions at the control time point (mean ± S.E. of two identical independent experiments, each performed in triplicate). C, PKG inhibition blocks ANP-induced endogenous PKG translocation. HEK-NPRA cells were treated with ANP (1 μM) with or without the PKG inhibitor KT5823 (1 μM, Acros/Fisher). Images are representative of three independent experiments.

2 M. Chinkers, personal communication.
NPRA/PKG Interaction

### Fig. 4. ANP is required to maintain membrane localization of PKG. Untreated HEK-NPRA cells (top panel) show diffuse cytosolic PKG staining. After 30 min of ANP treatment (1 μM) PKG is localized to the plasma membrane (middle panel). Within 5 min of incubation in ANP-free medium, PKG membrane staining of PKG is lost (bottom panel). Images are from a single experiment and are representative of two independent experiments. Experimental procedures were as described in the legend to Fig. 3.

### Fig. 5. ANP treatment recruits endogenous PKG to the sarcolemma of neonatal cardiac myocytes. Primary cells were grown in gelatin-coated slide wells and were either left untreated or stimulated with ANP (1 μM) for 1 h. Immunofluorescence microscopy demonstrated polymerized actin fibers (red). PKG (green) is localized at the cell membrane after ANP treatment. No immunofluorescence was seen in the absence of primary antibody (data not shown).

with ANP for 15 and 60 min, but not 5 min, demonstrated an apparent increase in membrane-associated PKG protein in the HEK-NPRA membranes only (Fig. 2B). PKG protein was present equally in cytosolic fractions of HEK-NPRA and control cells (Fig. 2B, middle panel), and NPRA protein was observed only in the HEK-NPRA membranes (Fig. 2B, lower panel). In additional experiments, ANP treatment for either 15 or 30 min resulted in a significant increase in PKG activity in crude membrane preparations of HEK-NPRA cells compared with untreated cells (Fig. 2B). Taken together, these data suggest that PKG is prebound to unliganded receptor and that ANP binding to NPRA recruits additional PKG to the cell membrane.

We next used immunofluorescence microscopy to verify the ANP-dependent co-localization of NPRA and endogenous PKG. After ANP treatment for 15, 30, and 60 min, we observed markedly increased staining of PKG at the membrane in HEK-NPRA cells (Fig. 3A) but not in control cells (data not shown). Furthermore, the PKG inhibitor KT5823 completely blocked the ANP-induced translocation of PKG to the plasma membrane (Fig. 3C), suggesting that activation of PKG is necessary for ANP-induced translocation. In parallel control experiments, cytosolic PKA immunofluorescence was readily detectable, but PKA did not migrate to the plasma membrane after ANP treatment indicating that the effect is specific for PKG (data not shown). Thus, even after prolonged exposure to the ligand for up to 60 min PKG membrane staining persisted. To determine whether the presence of NP was required to maintain membrane localization of PKG, cells were treated for 30 min, washed, and incubated in NP-free medium for different lengths of time. Within 5 min of incubation in NP-free medium, membrane staining was lost (Fig. 4). Because we initially identified the NPRA-PKG interaction by screening a heart cDNA library and to determine whether ANP-induced translocation of PKG also occurred in non-transfected primary tissue-derived cells, we examined cultured neonatal rat cardiomyocytes. In these primary cells, endogenous PKG migrated to the sarcolemma after ANP treatment (Fig. 5).

### PKG Increases Receptor Cyclase Activity—To determine whether ANP-dependent recruitment of PKG to the plasma membrane regulates NPRA function, we measured the effect of PKG on ANP-induced guanylyl cyclase activity (Fig. 6). To eliminate the possibility that receptor function was down-regulated as a result of receptor internalization, increased cGMP phosphodiesterase activity, or phosphatase action, studies were performed using isolated membranes prepared from HEK-NPRA cells using an array of phosphatase inhibitors. Furthermore, to increase the specificity of the reaction, the PKA inhibitor PKI was added to the lysates. In these in vitro experiments, PKG treatment alone had no effect on guanylyl cyclase activity whereas ANP increased cyclase activity by ∼70%. The addition of recombinant PKG to the ANP treatments, however, increased cyclase activity by ∼150%. Thus, the addition of PKG increased the modest effect of ANP alone by 2-fold.

To determine whether PKG translocation is a specific effect of ANP-induced NPRA activation or a general consequence of raising cGMP levels in the cell, we compared the effect of the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNP) to ANP treatment. Using fluorescence immunohistochemistry, we observed robust PKG translocation after ANP treatment. However, SNP treatment had no effect on the translocation of endogenous cytosolic PKG (Fig. 7A). PKG translocation was not observed in control HEK cells with either SNP or ANP treat-
ment (data not shown). To confirm that HEK-NPRA cells, in fact, contained activable soluble guanylyl cyclase, we measured cyclase activity in ANP- or SNP-treated cells. Soluble guanylyl cyclase activity was more than 2-fold higher in the SNP-treated cells compared with ANP-treated cells (Fig. 7B).

**DISCUSSION**

We have demonstrated, both in yeast and in mammalian cells, a novel association of NPRA and PKG. Although the original finding in yeast was unexpected, the notion that PKG interacts with NPRA was compelling because PKG was already well established as a downstream component of the ANP-NPRA signal. The initial co-immunoprecipitation experiments did not demonstrate this interaction because the solubilization of membrane-bound NPRA with either ionic or non-ionic detergents presumably also reduced PKG binding. Nevertheless, highly sensitive PKG *in vitro* kinase activity assays of these same NPRA immunoprecipitates demon-
desensitization. Exclusion of PKG from its NPRA-binding sites results in defining the critical sequence or motif of the NPRA receptor in NPRA kinase activity (45). Although our results suggest that prebound PKG is the NPRA kinase and that ANP binding is necessary and sufficient for both recruitment to and maintenance of membrane-bound PKG. Our findings may also be of relevance to brain natriuretic peptide because it is nearly identical in structure to ANP, and both ANP and brain natriuretic peptide preferentially bind with high affinity to NPRA (36). Finally, this ligand-dependent process appears to regulate the intrinsic guanylyl cyclase activity of NPRA. Thus, NPRA-PKG interaction may play a role in determining receptor sensitivity.

We have demonstrated that ANP, but not the NO donor SNP, induces PKG translocation. These data suggest that PKG membrane recruitment is an effect of NPRA activation and not a general consequence of raising cGMP levels. Thus, soluble and particulate guanylyl cyclase appear to be compartmentalized with respect to PKG migration. How this observation relates to the regulation of diverse downstream PKG actions will require additional study. It is well established that both nitric oxide and NP's signal through cGMP generated by soluble and membrane-bound guanylyl cyclases, respectively. Most of the downstream actions of GMP (37) occur through its binding and subsequent activation of PKG, but cGMP also directly regulates ion channels (38–41) and phosphodiesterases (42). There is recent evidence that particulate, but not soluble, cytosolic guanylyl cyclase (39). Delay, R. J., Dubin, A. E., and Dionne, V. E. (1997) J. Membr. Biol. 162, 173–184. Fiedler, B., Lohmann, S. M., Smolenski, A., Linnenmuller, S., Piecke, B., Schröder, F., Molkentin, J. D., Dreyer, H., and Wollert, K. C. (2002) Circ. Res. 90, 430–435. Wiedema, K., Jahn, H., and Ketler, M. (2000) Exp. Clin. Endocrinol. Diabetes 108, 5–13. Levin, E. R., Gardner, D. G., and Samson, W. K. (1998) N. Engl. J. Med. 339, 321–328. Dreyer, J. G., and Garbers, D. L. (1994) Endocr. Rev. 15, 155–162. Covi, R. J., Atlas, S. A., and Laragh, J. H. (1988) Eur. Heart J. 9, 29–33. Ku, D. D., Gun, L., Dai, J., Acuff, C. G., and Steinheimer, M. E. (1996) Am. J. Physiol. 271, H2368–H2376.

Tsutamoto, T., Kanamori, T., Morigami, N., Sugimoto, Y., Yamaoka, O., and Dzau, V. J. (1994) J. Biol. Chem. 269, 25892–25899. Chinkers, M., and Wilson, E. M. (1992) J. Biol. Chem. 267, 10557–10561. Lincoln, T. M., Dey, N., and Sellak, H. (2001) J. Biol. Chem. 276, 11096–11105. Acknowledgments—We thank Michael Chinkers (University of Southern Alabama) for the HEK-NPRA cells, Thomas Lincoln (University of Alabama) for the PKG I expression vector, and Philip Stork, Richard Goodman, and Elizabeth Wilson (Oregon Health and Science University) for helpful discussions.