Phosphorylation-Dependent Regulation of the Guanylyl Cyclase-Linked Natriuretic Peptide Receptor B: Dephosphorylation Is a Mechanism of Desensitization[†]

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Received September 16, 1997

ABSTRACT: C-type natriuretic peptide (CNP) binds the guanylyl cyclase-linked natriuretic peptide receptor B (NPR-B) and stimulates marked elevations of the intracellular signaling molecule, cGMP. Here, the essential role of phosphorylation in the hormonal activation and deactivation of this receptor is described. Exposure of NIH3T3 fibroblasts overexpressing NPR-B (3T3−NPR-B) to CNP resulted in time-dependent decreases in both subsequent CNP-dependent cGMP elevations in whole cells and hormone-dependent guanylyl cyclase activity assayed in crude membranes. NPR-B isolated from resting 3T3−NPR-B cells was phosphorylated on serine and threonine residues, and exposure to CNP resulted in a time-dependent dephosphorylation and desensitization of the receptor. Immunoblot analysis and guanylyl cyclase activity assayed with the general activators Mn^{2+} and Triton X-100 indicated that these reductions were not due to receptor degradation. Tryptic phosphopeptide mapping analysis suggested that CNP treatment caused a complete dephosphorylation of approximately one-half of the NPR-B population. In vitro dephosphorylation of crude 3T3−NPR-B membranes with purified protein phosphatase 2A was highly correlated with losses in CNP- but not Mn^{2+}- and Triton X-100-dependent guanylyl cyclase activity. Taken together, these data indicate that the catalytic activity of NPR-B is tightly coupled to its phosphorylation state and that dephosphorylation is a mechanism of desensitization.

Natriuretic peptides are a family of hormones which regulate varied physiologic functions including body fluid homeostasis, vascular smooth muscle tone, and cell proliferation (*1*−*3*). Atrial natriuretic peptide (ANP[1]), the first of three natriuretic peptides discovered to date, is secreted from atrial myocytes in response to increased arterial pressure and counteracts increased volumes by stimulating natriuresis, diuresis, and vascular relaxation (*2, 3*). Brain natriuretic peptide (BNP) was initially purified from brain but is primarily expressed in the heart (*3*). It elicits similar physiological responses as ANP, although under certain pathological conditions, such as congestive heart disease, it is released in much higher concentrations (*3*). C-type natriuretic peptide (CNP) is also found in brain but is present in much higher concentrations in endothelial cells and seminal plasma (*4, 5*). All three natriuretic peptides elicit hypotensive effects in animal bioassays; however, CNP is less potent in stimulating renal sodium and water excretion (*3*). Both ANP and CNP have been shown to decrease cell proliferation (*1, 6*), and transforming growth factor β has been shown to dramatically stimulate the expression of CNP mRNA (*5*).

Three natriuretic peptide-binding proteins have been reported. The natriuretic peptide clearance receptor consists of an extracellular ligand-binding domain, a single membrane spanning region, and a 37 amino acid intracellular tail (*7*). It is expressed in most tissues and binds all three natriuretic peptides with similar affinities (*2, 8*). Its primary function is to clear natriuretic peptides from the circulation (*9*), but it may also signal through heterotrimeric GTP-binding proteins to inhibit adenylyl cyclase (*10*) and/or activate phospholipase C (*11*−*13*). The two other natriuretic peptide-binding proteins, natriuretic peptide receptor A (NPR-A) and natriuretic peptide receptor B (NPR-B) (also known as guanylyl cyclase A and B or GC-A and -B, respectively), are members of the particulate guanylyl cyclase family which catalyze the synthesize of cGMP in response to peptide ligand stimulation (*14, 15*). NPR-A consists of an extracellular hormone-binding domain (approximately 40% identical to the extracellular domain of the natriuretic peptide clearance receptor), a single hydrophobic transmembrane span, an intracellular protein kinase−homology domain (KHD), and a carboxyl-terminal guanylyl cyclase catalytic domain (*14, 16*). NPR-A binds and is stimulated by ANP and is only slightly less sensitive to BNP (*8, 17, 18*). It does not respond to CNP at physiological concentrations (*17, 18*). NPR-B exhibits the same structural topology as NPR-A and is approximately 40% and 78% identical in the extracellular and intracellular regions, respectively (*19, 20*). NPR-B is selectively stimulated by physiological concentrations of CNP, but not by ANP or BNP (*17, 18*). Expression patterns for NPR-A and NPR-B show considerable overlap, but some tissues appear to predominately express one receptor. For example, a growing body of evidence suggests that NPR-A

[†] During the completion of these studies, I was supported as a research associate by the Howard Hughes Medical Institute.

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[1] Abbreviations: ANP, atrial natriuretic peptide; CNP, C-type natriuretic peptide; DMEM, Dulbecco's modified Eagle's medium; KHD, kinase homology domain; NPR-A, natriuretic peptide receptor A; NPR-B, natriuretic peptide receptor B; PAGE, poly(acrylamide) gel electrophoresis; SDS, sodium dodecyl sulfate.

S0006-2960(97)02303-9 CCC: $15.00
Published on Web 02/06/1998

is the major natriuretic receptor in the kidney (*21*) and that NRP−B is the principal signaling receptor in the brain (*22*, *23*). Since the majority of natriuretic peptide actions can be mimicked by cell-permeable cGMP analogues, NPR-A and NPR-B are thought to be the primary signaling receptors for these hormones (*2*).

An understanding of the mechanisms by which natriuretic peptide receptors are activated is emerging. In addition to natriuretic peptides, ATP is required for maximal stimulation (*24*−*28*). This is presumably an allosteric effect mediated by the KHD since nonhydrolyzable adenine nucleotide analogues effectively substitute for ATP, and deletion mutants lacking the KHD are not stimulated by natriuretic peptides and ATP (*29*, *30*). NPR-A also has been shown to exist in a higher order structure in the absence of ANP, which suggests that hormonal activation is not mediated by inducing dimerization as has been demonstrated for some members of the protein tyrosine kinase class of receptors (*31*−*33*). Furthermore, it is likely that NPR-B can form higher ordered structures both with itself and NPR-A, since it can be immunoprecipitated with noncross-reacting antibodies directed against NPR-A (*31*).

The mechanisms involved in the attenuation of these activated receptors have received little attention. Although short (<1-h) ANP exposures do not appear to significantly down-regulate NPR-A receptor numbers (*34*), prior ANP treatment markedly reduces hormone-dependent guanylyl cyclase activity (*35*). The explanation for this apparent paradox appears to be linked to changes in the receptor's phosphorylation state. NPR-A is phosphorylated on serine and threonine residues in resting cells, and ANP exposure results in the dephosphorylation of the receptor with kinetics that parallel desensitization (*36*−*38*). In addition, NPR-A can be heterologously desensitized in the absence of ANP. This process is also correlated with dephosphorylation; however, unlike homologous desensitization, it requires protein kinase C and may involve different phosphorylation sites (*38*).

In contrast to NPR-A, there is little or no published data on either the mechanism of homologous desensitization of NPR-B or its possible regulation by phosphorylation. Therefore, I have investigated these processes in NIH3T3 cells expressing large numbers of NPR-B molecules because this system allowed the direct measurement of receptor levels as well as possible posttranslational modifications. The data presented in this study suggest that the hormone-dependent guanylyl cyclase activity of NPR-B is modulated by changes in its phosphorylation state and that dephosphorylation is a mechanism of desensitization.

EXPERIMENTAL PROCEDURES

Production of Stable Cells. The mammalian expression vector pBPV−NPR-B was constructed by removing the full length rat NPR-B cDNA from the Bluescript GC-B plasmid (*20*) by simultaneous digestion with the restriction endonucleases *Xho I* (5′) and *Not I* (3′). It was then ligated into the similarly digested bovine papilloma virus-based expression vector pBPV purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). At 50% confluence, mouse NIH3T3 fibroblasts (ATCC 1658) were transfected with 4.5 μg of pBPV−NPR-B, 0.5 μg of pWL-neo (a plasmid-conferring neomycin resistance), and 50 μL of cationic liposomes in a total volume of 100 μL according to the manufacturer's protocol (lipofectin-Gibco/BRL). Resistant colonies were subsequently isolated with 6-mm cloning cylinders and transferred to 24-well plates. A single clone (3T3−NPR-B) was used for all subsequent studies. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 100−200 μg/mL biologically active G-418 in an atmosphere of 95% air and 5% CO_2 at 37 °C.

Whole Cell Stimulations. 3T3−NPR-B cells plated in 12-well dishes 2 days before stimulation were rinsed with 1 mL of serum-free DMEM and incubated in the same medium for 2 h in an atmosphere of 5% CO_2 and 95% air at 37 °C. The dishes were then placed on a slide warmer maintained at 37 °C. The medium was aspirated and replaced with 1 mL of DMEM with or without 1 μM CNP. After the designated time of incubation, the medium was aspirated and the cells were washed. The cells were then incubated for 3 min with 1 mL of DMEM containing 0.4 mM 1-methyl-3-isobutylxanthine and 1 μM CNP. The stimulation was stopped with 1 mL of 1 N perchloric acid. Cyclic GMP was purified from the acidified cell extracts by column chromatography and quantitated by radioimmunoassay as described by Domino et al. (*39*).

Preparation of Crude Membranes. Ten-centimeter plates of 3T3−NPR-B cells at approximately 50% confluence were washed with 10 mL of DMEM and then incubated with 5 mL of serum-free DMEM in an atmosphere of 5% CO_2 and 95% air at 37 °C for 1 h. The cells were then incubated with 5 mL of DMEM in the presence or absence of 1 μM CNP for up to 20 min under similar atmospheric conditions. The cells were then washed three times with 10 mL of phosphate-buffered saline, scraped off the plates in 0.75 mL of HGPB (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 20% glycerol, 50 mM NaCl, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 μg/mL pepstatin), sonicated with a Branson sonifier cell disrupter at 4 °C, and centrifuged at 15800*g* for 20 min at 2 °C. The resulting membrane pellet was resuspended in HGPB at a protein concentration of approximately 1.5−2.5 mg/mL as estimated by the BCA protein assay (Pierce Chemical Company, Rockford, IL).

Guanylyl Cyclase Assays. All guanylyl cyclase assays were at 37 °C in the presence of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 50 mM NaCl, 0.25 mM 1-methyl-3-isobutylxanthine, 0.1% BSA, 5 mM creatine phosphate, 6−10 units/assay creatine phosphokinase, 1 mM GTP, and 0.1−0.2 μCi of α-^{32}P-GTP. Four millimolar $MgCl_2$, 1 mM ATP, and 1 μM CNP or 1% Triton X-100 and 3 mM $MnCl_2$ were also included in the reaction mixtures. Assays were initiated by the addition of a solution of the above reagents to approximately 50 μg of crude membrane protein in a total volume of 0.1 mL. Cyclic GMP accumulation was analyzed as described by Domino et al. (*39*).

Metabolic Labeling. Basal 3T3−NPR-B cells approximately 50% confluent were washed twice with 5 mL of phosphate-deficient DMEM (D-DMEM) and then changed to 97% D-DMEM, 3% dialyzed fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, and 1−2 mCi/mL [^{32}P]orthophosphate

(NEN). Cells were labeled in an atmosphere of 5% CO_2 and 95% air at 37 °C for 4−12 h.

Preparation of Polyclonal Antiserum Z658. The peptide G-G-R-K-G-P-P-G-L-L corresponding to the 10 carboxyl-terminal amino acids of rat NPR-B was conjugated to purified peptide derivative (Serumstaatensinstitut, Copenhagen, Denmark) and subcutaneously injected in the presence of complete Freund's adjuvant into rabbit Z658. Rabbit Z658 was then boosted four times with the same peptide in incomplete Feund's adjuvant and bled 10−14 days after each injection.

Immunoprecipitations. Metabolically labeled cells were incubated with or without 1 μM CNP for 15 min, and then crude membranes were prepared as described above except the cells were sonicated in phosphatase inhibitor buffer (HGPB containing 10 mM $NaPO_4$ (pH 7.0), 0.1 M NaF, 1 mM Na_3VO_4, 80 μM β-glycerol phosphate, and 0.1 μM okadaic acid). Equal amounts of membrane protein were solubilized in 0.6 mL of immunoprecipitation buffer (phosphatase inhibitor buffer containing 1% Triton X-100) and incubated on ice for 15 min. Insoluble matter was pelleted by centrifugation at 435 000*g* for 15 min at 2 °C. The supernatant fluid was then cleared by incubation with a 1/100 dilution of preimmune serum at 4 °C for 1 h. Immunoglobulin complexes were removed by incubation with 50 μL of a 50% solution of protein A agarose (Pierce Chemical Company) for 30 min at 4 °C followed by centrifugation at 15 800*g* for 2 min at 4 °C. The solution was transferred to a second tube and incubated for 1 h with a 1/200 dilution of Z658 antiserum. The immunocomplexes were precipitated with protein A agarose as described above and washed three times with 1.0 mL of immunoprecipitation buffer containing 0.1% SDS and 1% sodium deoxycholate. The immunocomplexes were dissociated by incubation with SDS sample buffer at 100 °C for 5 min and fractionated by reducing SDS−PAGE employing 4.5% stacking/8% resolving gels.

Immunoblot Analysis. NPR-B was isolated as described above and then electroblotted to a poly(vinylidene difluoride) (Immobilon-P) or nitrocellulose membrane for 12−16 h at 15 V and 4 °C in a buffer containing 50 mM tris(hydroxymethyl)aminomethane, 380 mM glycine, 0.1% SDS, and 20% (v/v) methanol. Following autoradiography, the amount of $^{32}PO_4$ associated with NPR-B was quantitated with an AMBIS radioanalytic imaging system (AMBIS Systems, Inc., San Diego, CA). The membrane was then blocked for 1 h in TBST (20 mM tris(hydroxymethyl)aminomethane, 500 mM NaCl, and 0.05% poly(oxyethylene) sorbitan monolaurate, pH 7.5) containing 5% nonfat dry milk, washed three times for 5 min with TBST, and then incubated with Z658 antiserum diluted 1/500 in TBST containing 1% nonfat dry milk for 1 h at 25 °C. The membrane was washed three times for 10 min with TBST and incubated for 45 min at 25 °C with an affinity-purified goat anti-rabbit IgG directed antibody conjugated to horseradish peroxidase (Tago, Inc., Burlingame, CA) diluted 1 to 25 000 in TBST containing 1% nonfat dry milk. The membrane was then washed once for 15 min and twice for 5 min in TBST. The NPR-B antibody complex was detected by chemiluminesence using the ECL western blot detection system from Amersham Life Sciences (Arlington, IL).

Phosphoamino Acid Analysis. $^{32}PO_4$-labeled NPR-B was immunoblotted to Immobilon-P as described, cut out of the membrane, and hydrolyzed in 5.7 N HCl at 110 °C for 2 h. The resulting phosphoamino acids were separated together with exogenously added phosphoserine, phosphothreonine, and phosphotyrosine by two-dimensional high-voltage electrophoresis as described by Boyle et al. (*40*) and visualized by ninhydrin staining followed by autoradiography using Kodak XRP-1 film.

Phosphopeptide Mapping. Phosphopeptide mapping was performed essentially as described by Boyle et al. (*40*). Briefly, NPR-B immunoprecipitated from $^{32}PO_4$-labeled cells treated in the presence or absence of CNP was fractionated by SDS−PAGE and transferred to Immobilon-P as described above. The membrane was then exposed to film to localize NPR-B. The corresponding band was then cut out and incubated with 0.5% poly(vinylpyrrolidone) (average molecular weight of 360 000) dissolved in 0.1 M acetic acid for 30 min at 37 °C. The membrane fragments were then washed five times with water and two times with 50 mM $(NH_4)_2CO_3$, pH 8.0. Five micrograms of TPCK-treated trypsin was added to each sample, which was then incubated for 4 h at 37 °C. Five more micrograms of trypsin was added, and the tube was incubate overnight at 37 °C. The remaining $(NH_4)_2CO_3$ was removed by repeated lyophilization using a rotory evaporator. The phosphopeptides were dissolved in a small volume of distilled water and spotted on 70-μm-thick cellulose plates (Kodak). The peptides were then separated in the horizontal dimension by high-voltage electrophoresis (1000 V) for 30 min in 2% ammonium carbonate, pH 8.9. The plate was dried for 1 h, and the phosphopeptides were separated in the vertical dimension by ascending chromatography in a buffer consisting of *n*-butyl alcohol, pyridine, glacial acetic acid, and water (94:62:19:75). The phosphopeptides were visualized by exposing the plates to Kodak XRP-1 film for 1 week at −80 °C with an intensifying screen.

In Vitro Dephosphorylation of NPR-B. Crude membranes were prepared from ^{32}P-labeled or unlabeled 3T3−NPR-B cells as described above except the cells were sonicated in HGPB instead of phosphatase inhibitor buffer. The catalytic subunit of protein phosphatase 2A was purified from bovine heart as described by Mumby et al. (*41*) and was estimated to be approximately 95% pure. Phosphatase assays were initiated by the addition of 25 μL of approximately 13.5 μM protein phosphatase 2A catalytic subunit (dissolved in 50% glycerol, 25 mM Tris (pH 7.5), 1 mM EDTA, and 1 mM dithiothreitol) to 225 μL of membranes (∼1.5 mg/mL), vortexed, and placed in a 37 °C water bath. At the indicated times, 40-μL aliquots were removed from the reaction mixture, transferred to tubes containing 50 μL of 10 μM okadaic acid dissolved in HGPB, vortexed, and placed on ice. Twenty-five microliters of each final reaction mixture was immunoprecipitated and fractionated by SDS−PAGE, and the ^{32}P content of NPR-B was quantitated using the AMBIS radioanalytic imaging system. Fifteen microliters was used for each guanylyl cyclase activity determination.

RESULTS

3T3-NRP-B Cells Are Desensitized by CNP Exposure. Mouse NIH3T3 fibroblast is known to express endogenous NPR-B (*4*). However, to facilitate studies on the direct modification of the receptor, the full length rat NPR-B cDNA

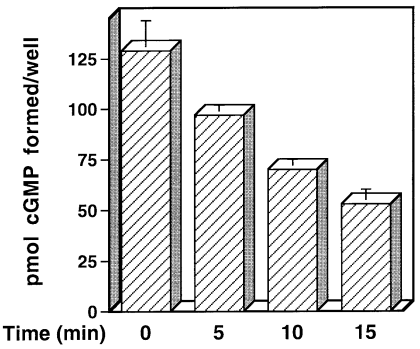

FIGURE 1: Exposure of 3T3−NPR-B cells to CNP causes time-dependent reductions in subsequent CNP-dependent cGMP elevations. 3T3−NPR-B cells were incubated with DMEM containing 1 μM CNP for the time periods indicated. The cells were then washed once with DMEM and stimulated for 3 min with DMEM containing 1 μM CNP. Cyclic GMP concentrations were determined as described in the Experimental Procedures section. Cyclic GMP concentrations of basal cells were approximately 0.2 pmol/well. The vertical bars centered over each column represent the standard error of the mean obtained from triplicate wells which were assayed in duplicate.

was stably overexpressed in these cells to create the 3T3−NPR-B cell line. In these cells, the particulate guanylyl cyclase activities determined in the presence of MnGTP and 1% Triton X-100 were approximately 10 nmol of cGMP/mg/min compared to the mock transfected cells which yielded approximately 0.15 nmol of cGMP/mg/mim under idenitical assay conditions. Therefore, the particulate guanylyl cyclase activities in the 3T3−NPR-B cell line can be unequivocally attributed to NPR-B and not another guanylyl cyclase, since their activity levels are more than 50-fold higher than those of the parental cell line.

Incubation of 3T3−NPR-B cells with 1 μM CNP elevated intracellular cGMP concentrations over 500-fold (Figure 1). However, prior exposure resulted in time-dependent reductions in subsequent CNP-dependent responses to less than 42% of the initial values, indicating that the hormone-dependent cGMP elevations were diminished by the continuous presence of the peptide (Figure 1). Thus, the CNP response in the 3T3−NPR-B cells adheres to the classical definition for homologous desensitization or tachyphalaxis (*42*).

To determine whether the reduced cGMP elevations could be explained, in part, by reductions in the ability of the cells to synthesize cGMP, guanylyl cyclase activity was measured in crude membranes isolated from 3T3−NPR-B cells which had been previously incubated for 15 min in the presence or absence of 1 μM CNP (Figure 2). When assayed with CNP, ATP, and $MgCl_2$ (conditions that result in maximum hormone-dependent stimulations),[2] guanylyl cyclase activity determined in the CNP-treated preparation (desensitized) was markedly diminished compared to the untreated preparation (control) (Figure 2, upper panel). The effect was most dramatic at earlier time points, with the desensitized membranes generating only 33% of the control activity. The rate of cGMP production in both preparations declined with time but was more pronounced in membranes from the control cells. In contrast, guanylyl cyclase activity determined in the presence of 1% Triton X-100 and $MnCl_2$ (conditions that

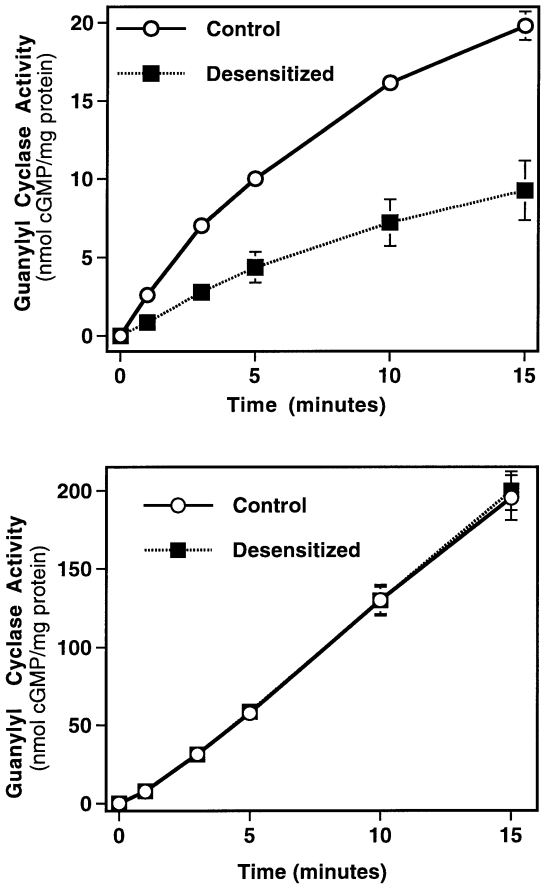

FIGURE 2: Incubation of NPR-B cells with CNP markedly reduces CNP/ATP/Mg^{2+}-dependent, but not Mn^{2+}/Triton X-100-dependent, guanylyl cyclase activity. 3T3−NPR-B cells were incubated with DMEM in the presence (squares) or absence (circles) of 1 μM CNP for 15 min, and then crude membranes were prepared and assayed for guanylyl cyclase activity in the presence of 1 μM CNP, 1 mM ATP, and 4 mM $MgCl_2$ (upper panel) or 1% Triton X-100 and 3 mM $MnCl_2$ (lower panel) for the indicated times as described in the Experimental Procedures section. The vertical bars within each symbol represent the range of values obtained from duplicate determinations.

stimulate particulate guanylyl cyclases to their maximal levels in the absence of ligand (*43*)) was linear with respect to time and was not affected by prior CNP treatment (Figure 2, bottom panel). Since the vast majority of the guanylyl cyclase activity in these cells can be attributed to NPR-B, these data clearly indicate that the amount of catalytically active NPR-B is not significantly reduced by prior CNP exposure. Therefore, the reductions in guanylyl cyclase activity observed in the presence of CNP, ATP, and $MgCl_2$ are not explained by receptor degradation.

NPR-B Is Phosphorylated on Serine and Threonine Residues in 3T3−NPR-B Cells, and CNP Exposure Causes Time-Dependent Receptor Dephosphorylation and Desensitization. Since CNP treatment resulted in a stable functional change to NPR-B, the possibility that the desensitization could be explained by a phosphorylation-dependent mechanism was investigated. NPR-B isolated from basal 3T3−NPR-B cells metabolically labeled with $^{32}PO_4$ was phosphorylated (Figure 3, ^{32}P autoradiograph). Phosphoamino acid analysis indicated that it contained primarily phosphoserine, lesser amounts of phosphothreonine, and no detectable phosphotyrosine (Figure 4). The addition of natriuretic

[2] Potter, L. R. Unpublished observations.

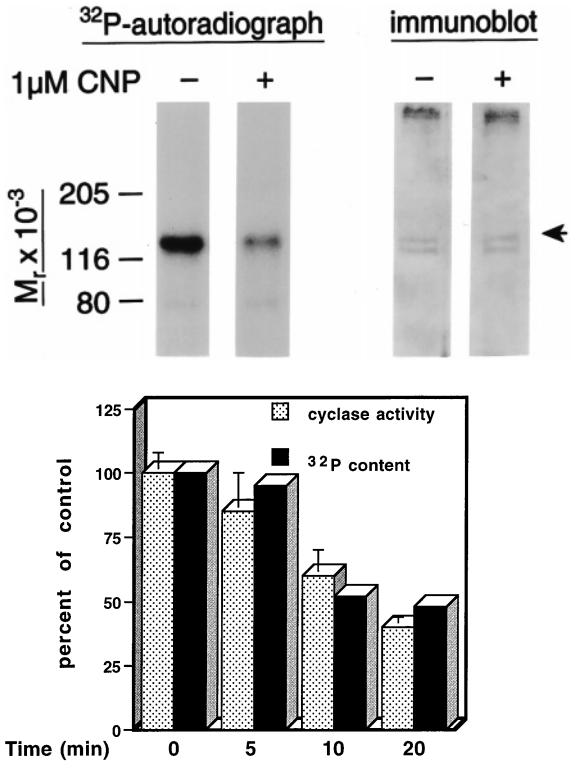

FIGURE 3: Whole cell CNP exposure results in a time-dependent dephosphorylation and desensitization of NPR-B. (top) CNP-dependent dephosphorylation of NPR-B. 3T3−NPR-B cells were metabolically labeled with DMEM containing 2 mCi/mL $^{32}PO_4$ for 4 h and then incubated with or without 1 μM CNP an additional 15 min. The cells were then lysed, and the resulting detergent extracts were adjusted to equal protein concentrations. NPR-B was immunoprecipitated from the extracts, fractionated by SDS−PAGE, electroblotted to Immobilon membrane, and visualized by ^{32}P autoradiography. The same membrane was subsequently immunoblotted with polyclonal antiserum directed against the carboxyl terminus of NPR-B, and the resulting immunocomplex was visualized by chemiluminescence. The arrowhead indicates the location of phosphorylated NPR-B. This experiment is representative of three separate experiments. (bottom) NPR-B dephosphorylation and desensitization are temporally correlated. Metabolically labeled 3T3−NPR-B cells were incubated in the presence of 1 μM CNP for the periods of time indicated, and then NPR-B was isolated as described above. The amount of radioactivity associated with the receptor was quanitated on a Molecular Dynamics phosphoimager (solid bars). In a separate experiment, 3T3−NPR-B cells were incubated in the presence of 1 μM CNP for the same periods of time, and then crude membranes were prepared and guanylyl cyclase determinations conducted in the presence of 1 μM CNP, 1 mM ATP, and 1 mM MgGTP as described in the Experimental Procedures section (hatched bars).

peptide to the medium resulted in receptor dephosphorylation, with CNP exposure reducing the ^{32}P content of NPR-B to 48% of that associated with NPR-B isolated from control cells (Figure 3, ^{32}P autoradiograph). CNP treatment did not significantly affect the phosphoamino acid composition of the receptor (Figure 4). Immunoprecipitated NPR-B consisted of two closely migrating species when separated by SDS−PAGE (Figure 3, immunoblot). Based on analogy to NPR-A, the lower band is probably an incompletely glycosylated form of the receptor (*44*). Only the slower migrating species (completely glycosylated upper band) was phosphorylated (Figure 3, upper panel). Since immunoblot analysis of the same blot used for the phosphate determinations indicated that neither band was appreciably diminished by

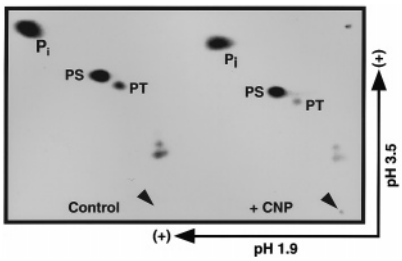

FIGURE 4: NPR-B is phosphorylated on serine and threonine residues in 3T3−NPR-B cells. $^{32}PO_4$-labeled NPR-B was isolated from 3T3−NPR-B cells incubated in the presence (+CNP) or absence (control) of 1 μM CNP for 15 min. The purified NPR-B was then hydrolyzed in 5.7 N HCl for 2 h at 110 °C, separated by two-dimensional electrophoresis, and visualized by autoradiography. P−S, P−T and P_i denote phosphoserine, phosphothreonine, and inorganic phosphate, respectively. The arrowhead indicates the origin of application.

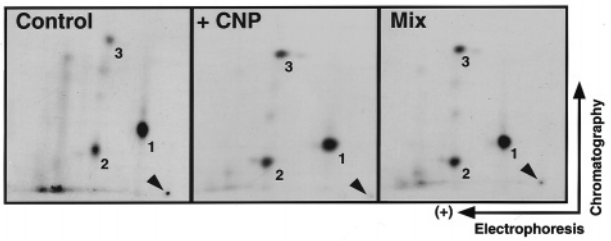

FIGURE 5: CNP treatment results in no apparent changes to the two-dimensional tryptic phosphopeptide maps of NPR-B. NPR-B was immunoprecipitated from $^{32}PO_4$-labeled 3T3−NPR-B cells treated with or without 1 μM CNP for 15 min, fractionated by SDS−PAGE, electroblotted to a nitrocellulose membrane, and digested with 10 μg of trypsin overnight at 37 °C. The tryptic phosphopeptides were dissolved in 5−10 μL of water, and an equal number of cpm (~1000) were added to the origin of each plate. Approximately 500 cpm from each sample were added to the origin of the "Mix" plate. The phosphopeptides were first separated in the horizontal dimension by high-voltage electrophoresis at pH 8.9. The plates were then dried and separated in the vertical dimension by ascending chromatography. The phosphopeptides were visualized by autoradiography using Kodak XRP-1 film and an intensifying screen.

CNP treatment (Figure 3, immunoblot), the decreased ^{32}P signal was not explained by losses in NPR-B protein. These data clearly demonstrate that whole cell CNP exposure reduces the phosphorylation state of NPR-B. I next asked whether the dephosphorylation and desensitization of NPR-B were temporally related. Incubation of 3T3−NPR-B cells with 1 μM CNP resulted in time-dependent decreases in the phosphate content of NPR-B, and these reductions correlated nicely with decreases in hormone-dependent guanylyl cyclase activities obtained from membranes of identically treated 3T3−NPR-B cells (Figure 3B). The $t_{1/2}$ for both dephosphorylation and desensitization was between 5 and 10 min.

CNP Incubation Does Not Markedly Change the Tryptic Phosphopeptide Maps of NPR-B. To determine if a single site or multiple sites were dephosphorylated in response to CNP treatment, two-dimensional NPR-B tryptic phosphopeptide maps were analyzed from cells incubated in the presence or absence of 1 μM CNP for 15 min (Figure 5). Maps of NPR-B isolated from resting cells revealed three major peptides. Maps of NPR-B isolated from cells that had been treated with CNP looked similar, if not identical, to maps of NPR-B isolated from resting cells. When an equal number of cpm from each phosphopeptide preparation were

mixed and fractionated together on the same plate, the major phosphopeptides comigrated. This suggests that the major phosphopeptides contained in each preparation are the same. Therefore, although CNP treatment causes more than a 50% loss in the amount of phosphate associated with the receptor (Figure 3), it yields phosphopeptide patterns that are indistinguishable from those obtained from NPR-B which was isolated from unstimulated cells. A possible explanation for these seemingly conflicting results is that NPR-B obtained from CNP-treated cells consists of two pools, one which is maximally phosphorylated and another which is completely dephosphorylated. The reason for the incomplete dephosphorylation may be a result of the abnomally high expression of the receptor in these cells which may perturb the stoichiometry between the receptor and phosphatase. Unfortunately, I cannot rule out the possibility that a specific phosphopeptide is dephosphorylated in response to CNP which is lost in the purification process. However, since the majority of the initial cpm were present at the end of the peptide purification process, the lost phosphopeptide(s) would have to represent a very small portion of the total pool.

In Vitro Dephosphorylation of NPR-B Decreases CNP-Dependent Guanylyl Cyclase Activity. To test the hypothesis that dephosphorylation of NPR-B results in diminished hormone-dependent cyclase activity, crude 3T3−NPR-B membranes were incubated with purified protein phosphatase 2A catalytic subunit and then assayed for guanylyl cyclase activity. Phosphatase treatment decreased CNP-dependent activity by more than 90% but only reduced the activity assayed in the presence of Triton X-100 and $MnCl_2$ by 14% (Figure 6A). The dramatic loss in hormone-dependent activity was not due to thermal inactivation or a contaminating protease present in the phosphatase preparation since the control membranes were incubated at the same temperature and with the same amount of phosphatase as membranes treated with active phosphatase. The only difference between the two treatments was the presence or absence of the specific phosphatase inhibitor, okadaic acid. To further test the hypothesis that NPR-B is desensitized by dephosphorylation, the temporal relationship between receptor dephophosphorylation and desensitization was examined (Figure 6B). Incubation of crude $^{32}PO_4$-labeled 3T3−NPR-B membranes with purified protein phosphatase 2A catalytic subunit caused time-dependent decreases in the ^{32}P content of NPR-B, which closely paralleled reductions in the CNP-dependent guanylyl cyclase activity (Figure 6B). As in the previous experiment, the reductions were not explained by proteolysis since 30-min incubations of 3T3−NPR-B membranes with the phosphatase and the specific phosphatase inhibitor, okadaic acid, failed to decrease the ^{32}P content or guanylyl cyclase activity of NPR-B. These data strongly suggest that the CNP-dependent guanylyl cyclase activity of NPR-B is regulated by its phosphorylation state.

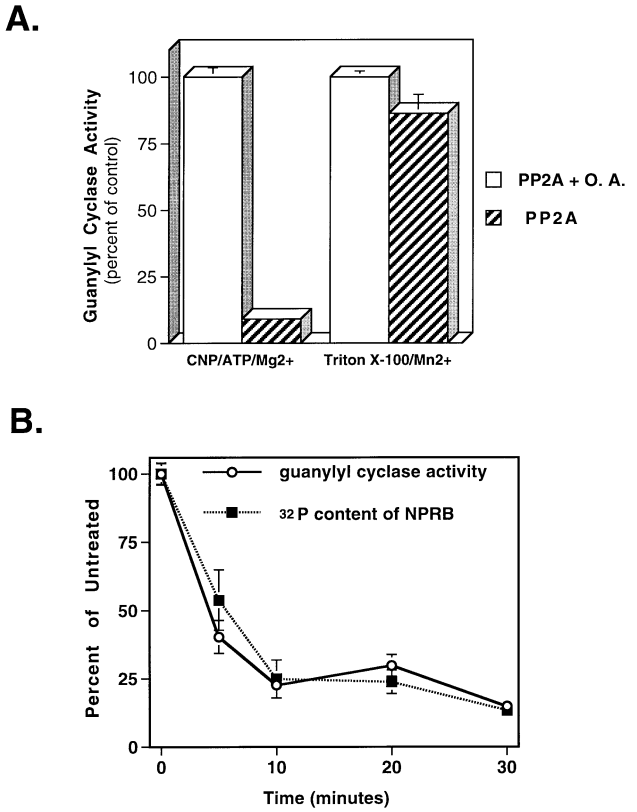

FIGURE 6: In vitro dephosphorylation of NPR-B correlates with decreases in CNP-dependent guanylyl cyclase activity. (A) Incubation of 3T3−NPR-B membranes with purified protein phosphatase 2A catalytic subunit markedly decreases CNP/ATP/Mg^{2+}-dependent, but not Triton X-100/Mn^{2+}-dependent, guanylyl cyclase activity of NPR-B. Equal portions of 3T3−NPR-B membranes were incubated with purified protein phosphatase 2A in the presence (open bars) or absence (hatched bars) of 10 μM okadaic acid for 30 min at 37 °C. The membranes were then assayed for guanylyl cyclase activity with the activators shown as described in the Experimental Procedures section. The vertical bars centered above each column represent the range of values obtained from two separate preparations, which were determined in duplicate. (B) Temporal correlation between in vitro dephosphorylation and desensitization of NPR-B. $^{32}PO_4$-labeled 3T3−NPR-B membranes were incubated with purified protein phosphatase 2A at 37 °C for the time periods indicated, and then the reaction was stopped by transferring the membranes to a buffer containing 10 μM okadaic acid on ice. Portions of each reaction were either immunoprecipitated, fractionated by SDS−PAGE and analyzed for NPR-B ^{32}P content by radioanalytic imaging (squares), or assayed for guanylyl cyclase activity for 2.5 min in the presence of CNP, ATP, and Mg^{2+} (circles) as described in the Experimental Procedures section. The vertical bars within the symbols indicate the range of duplicate determinations.

DISCUSSION

In this report, the mechanism of desensitization of NPR-B was investigated. NPR-B was found to be phosphorylated in vivo, and CNP treatment caused a temporally correlated dephosphorylation and desensitization of the receptor. The role of phosphorylation in the regulation of NPR-B activity was further investigated in vitro by treating 3T3−NPR-B membranes with purified protein phosphatase 2A catalytic subunit and measuring receptor phosphate and activity levels. As in whole cells, the level of CNP-dependent guanylyl cyclase activity was closely linked to the phosphorylation state of the receptor. Thus, the most direct conclusion that can be drawn from these data is that phosphorylation of NPR-B is required for maximal hormone-dependent guanylyl cyclase activity and that dephosphorylation is a mechanism of NPR-B desensitization. Although both the desensitization and the apparent dephosphorylation of NPR-B could possibly be explained by ligand-mediated receptor internalization and degradation, this does not appear to be a likely explanation for two reasons. One, CNP treatment does not significantly affect the detergent-stimulated guanylyl cyclase activity of

NPR-B, and two, CNP treatment does not significantly reduced the amount of NPR-B protein detected in these cells. However, I have observed that longer (>1-h) incubations of 293 cells overexpressing NPR-A or NPR-B with their cognate ligands result in decreased receptor numbers.[2] It is possible that dephosphorylation serves as a signal for this process. However, this hypothesis has yet to be tested.

Based on these data, the following working model is proposed for the homologous desensitization of NPR-B. In the basal state, NPR-B exists as a higher ordered structure (possibly a dimer) and is phosphorylated primarily on serine with lesser amounts of phosphate on the threonine residues. The location of the phosphorylation sites has recently been mapped to the kinase−homology domain.[3] Upon CNP binding, NPR-B is envisioned to undergo a conformational change which derepresses the catalytic domain. This is based on analogy with the NPR-A, which has been shown to be constitutively active when the KHD is removed (*29*, *30*). Therefore, it has been suggested that the KHD serves to repress the catalytic domain in the absence of hormone. Since the KHDs of NPR-A and NPR-B can be effectively exchanged (*30*), it is likely that NPR-B is regulated in a similar fashion. The exact role of ATP in this process in not known; however, it is thought to be required for maximal hormone-dependent activity (*28*). Within the KHD of the NPR-A, there is a stretch of amino acids with the sequence *GRGSNYG* which is similar to the known ATP binding motif *GXGXXG* (*45*). This region has been suggested to be involved in mediating the ATP effect on NPR-A, but no direct ATP-binding data have been presented to support this hypothesis. The corresponding ATP-binding region in NPR-B is LR*G*SSY*G*, which is quite divergent from the ATP-binding consensous sequence. However, mutation of this region to LR*A*SSY*G* has been shown to disrupt the ability of the receptor to respond to ligand, suggesting a significant functional role for this domain in hormone-dependent receptor activation (*28*).

After the hormone-induced change in conformation, the catalytc activity of the receptor is dramatically enhanced, presumably by bringing the two carboxyl-terminal catalytic domains of the dimer together. However, a secondary consequence of the conformational change is the exposure of previously inaccessible phosphoserine and/or phosphothreonine residues to a protein phosphatase activity. Once the NPR-B is dephosphorylated, it may be completely unable to respond to CNP. Whether a single site or muliple sites are dephosphorylated at this time is unclear. However, because CNP exposure does not significantly change the tryptic phosphopeptide maps, it is possible that the receptor is dephosphorylated completely. This is similar to the homologous desensitization of NPR-A where ANP treatment was shown to reduce the ^{32}P content of the receptor by more than 50% but did not have any noticeable effect on the resulting tryptic phosphopeptide maps (*38*). Why then does CNP treatment result in only a ∼50% decrease in receptor-associated phosphate if the receptor is being completely dephosphorylated? One possible explanation is that the NPR-B specific phosphatase acivity is limiting due to the high level of receptor expression in these cells. Therefore, only a subpopulation of the total pool of NPR-B is able to be dephosphorylated. If this is true, then one would expect a more dramatic desensitization response in cells expressing lower receptor levels. In fact, in the parental NIH3T3 cell line which expresses low levels of endogenous NPR-B, CNP treatment reduces the subsequent activity to approximately 15% of the untreated cells.[2] Unfortunately, I have not been able to successfully immunoprecipitate sufficient quantities of the endogenous receptor from $^{32}PO_4$-labeled cells to perform phosphopeptide mapping experiments.

In conclusion, this report describes for the first time the in vivo regulation of NPR-B by phosphorylation. Furthermore, it provides additional data to support the hypothesis that guanylyl cyclase-linked natriuretic peptide receptors are positively, and not negatively, regulated by phosphorylation, as has been suggested by some groups for NPR-A (*46*, *47*). Clearly, the next step in understanding this process will be the identification of the NPR-B phosphorylation sites. Once determined, it will presumably be possible to directly test the effect of dephosphorylation on the hormone-dependent guanylyl cyclase activity of this receptor.

[3] Potter, L. R., and Hunter, T. Unpublished observations.

ACKNOWLEDGMENT

This work was initiated and largely conducted in the laboratory of David L. Garbers in the Department of Pharmacology at the University of Texas Southwestern Medical Center. I am grateful to Dr. Garbers for his support and advice throughout the duration of these studies. I also thank Mike Chinkers for constructing the pBPV−NPR-B vector and Robert Estes and Marc Mumby for the generous supply of purified protein phosphatase 2A. Finally, I thank Tony Hunter for critically reading this manuscript and graciously allowing me to complete these studies in his laboratory.

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BI972303K