The Atrial Natriuretic Peptide Receptor (NPR-A/GC-A) Is Dephosphorylated by Distinct Microcystin-sensitive and Magnesium-dependent Protein Phosphatases*

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Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), found in the atria and ventricles of the heart, respectively, are cardiac hormones that counterbalance the renin-angiotensin-aldosterone system (1, 2). Acutely, they decrease blood pressure by (i) increasing renal sodium and water excretion, (ii) stimulating vascular vasorelaxation, and (iii) inhibiting aldosterone and renin secretion. Chronically, ANP inhibits the hypertrophy of cardiomyocytes (3, 4), whereas BNP inhibits pressure-induced ventricular fibrosis (5). ANP and BNP bind two distinct cell surface proteins known as the natriuretic peptide clearance receptor and NPR-A/guanylyl cyclase A (6–10). The clearance receptor consists of an extracellular domain, a single membrane-spanning region, and only 37 intracellular amino acids. It controls the local concentrations of natriuretic peptides through receptor-mediated internalization and degradation (11) and may signal through the heterotrimeric G proteins G₁ and/or G₃ (12). Experiments conducted on mice lacking NPR-A suggest that the known cardiovascular effects of ANP and BNP are mediated through this receptor (13, 14). However, a signaling function for the clearance receptor has therefore been observed by many laboratories (15–17), suggesting that this receptor may mediate some natriuretic peptide responses.

Natriuretic peptide receptor (NPR)-A is the primary signaling receptor for atrial natriuretic peptide and brain natriuretic peptide. Ligand binding to NPR-A rapidly activates its guanylyl cyclase domain, but its rate of cGMP synthesis declines with time. This waning of activity is called homologous desensitization and is mediated in part by receptor dephosphorylation. Here, we characterize two distinct NPR-A phosphatase activities. The serine/threonine protein phosphatase inhibitor, microcystin, inhibited the desensitization of NPR-A in membrane guanylyl cyclase assays in the absence of magnesium. EDTA also inhibited the desensitization, whereas MgCl₂ stimulated the desensitization. Because the effects of microcystin and EDTA were additive, and microcystin did not block the magnesium-dependent desensitization, the targets for these agents appear to be distinct. Incubation of membranes at 37 °C stimulated the dephosphorylation of NPR-A, and microcystin blocked the temperature-dependent dephosphorylation. The addition of MgCl₂ or MnCl₂, but not CaCl₂, further stimulated the dephosphorylation of NPR-A, and microcystin failed to inhibit this process. The desensitization required changes in the phosphorylation state of NPR-A because the guanylyl cyclase activity of a receptor variant containing glutamate substitutions at all six phosphorylation sites was unaffected by MgCl₂, EDTA, or microcystin. Together, these data indicate that NPR-A is regulated by two distinct phosphatases, possibly including a member of the protein phosphatase 2C family. Finally, we observed that the desensitization of NPR-A in membranes from mouse kidneys and NIH3T3 cells was increased by prior exposure to atrial natriuretic peptide, suggesting that hormone binding enhances receptor dephosphorylation.

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The abbreviations used are: ANP, atrial natriuretic peptide; KHD, kinase homology domain; NPR, natriuretic peptide receptor; PP, protein phosphatase; BNP, brain natriuretic peptide; HGBP, Hepes glyceral protease inhibitor; IPB, Immunoprecipitation buffer; TBST, Tris-buffered saline with Tween.
In the continued presence of natriuretic peptide, the guanylyl cyclase activity of NPR-A wanes (24–27). This process is called homologous desensitization and is mediated, at least in part, by dephosphorylation of all six NPR-A phosphorylation sites, a process termed global dephosphorylation (27–30). Consistent with this model of desensitization, a “constitutively phosphorylated” version of NPR-A containing glutamate residues at all six phosphorylation sites (NPR-A-6E) to mimic the negative charge of phosphate is hormonally responsive and resistant to homologous desensitization (31). NPR-A is also desensitized in the absence of ANP or BNP by pressor hormones that antagonize the physiologic consequences of natriuretic peptides (32, 33). This process, called heterologous desensitization, may involve protein kinase C because pharmacological activators of protein kinase C, such as phorbol esters, mimic this response (26). The phorbol ester-dependent desensitization also is associated with the dephosphorylation of NPR-A (30). However, it differs from the homologous desensitization process in that only a single or small subset of the total phosphorylation sites is dephosphorylated (30). The identity of the residue(s) dephosphorylated in response to heterologous desensitization is currently unknown.

Early work by Ingebritsen and Cohen (34) classified serine/threonine protein phosphatases (PPs) into two general categories, based on their ability to dephosphorylate the α or β subunit of phosphorylase kinase. PP1 preferentially dephosphorylates the β subunit, whereas members of the PP2 family prefer the α subunit. Experimentally, PP1 can be differentiated from other phosphatases by its nanomolar sensitivity to the thermostable protein inhibitors 1 and 2. The PP2 family, which is not sensitive to inhibitor 1 or inhibitor 2, can be further subclassified into PP2A, PP2B, and PP2C. Neither PP1 nor PP2A requires divalent cations for activity, and both are sensitive to inhibition by 0.1–10 nanomolar concentrations of microcystin, okadaic acid, tautomycin, and calyculin A. PP2B is not affected by these compounds, requires calcium and calmodulin for activity, and is specifically inhibited by cymemythrine, cyclosporin, or FK-506. The distinguishing features of PP2C are that it requires relatively high concentrations of magnesium or manganese (−1.5 mM) for activity, and it has no known specific inhibitor. Several additional protein phosphatases, such as PP4 (also called PPX), PP5, PP6, and PP7, have recently been identified by molecular cloning techniques (35). PP4, PP5, and PP6 do not require divalent cations for activity and are inhibited by nanomolar concentrations of okadaic acid or microcystin, whereas PP7 requires magnesium for activity, is localized to the retina, and is insensitive to microcystin or okadaic acid (36, 37).

Little is known about the kinases and phosphatases that regulate NPR-A (7). Microcystin prevents the desensitization of NPR-A in membrane fractions (38), and experiments with the constitutively phosphorylated receptor variant (NPR-A-6E) indicated that the target of the microcystin-sensitive phosphatase is NPR-A itself (31). In this study, we provide evidence for a second distinct NPR-A phosphatase that has characteristics in common with PP2C.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rat ANP was from purchased from Peninsula Laboratories, Inc. (www.penlabs.com) or Sigma-Aldrich (www.sigma-aldrich.com). Microcystin-LR was purchased from Calbiochem (www.calbiochem.com). Microcystin-LR was purchased from Calbiochem (www.calbiochem.com) and was dissolved at a concentration of 400 μM. The aluminia resin used for cGMP purification was from Sigma. Protein A-agarose was from Pierce (www.piercenet.com). The horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody was from Amersham Biosciences (www.aphitech.com). [α-32P]GTP (NEG-006H) was from PerkinElmer Life Sciences. The data were graphed with GraphPad Prism for the Macintosh (www.graphpad.com).

**Cell Culture and Stable Cell Lines**—The majority of the studies presented in this report were conducted on the previously described 293-GC-A cell line (27), which we call 293-NPR-A here for consistency. Unlike many varieties of 293 cells that endogenously express NPR-A (25), the parental cell that was used to make this line does not endogenously express detectable levels of any natriuretic peptide receptor. We also used another stable cell 293 line called 293-NPR-A-6E in these studies. We made this cell line by transfecting the pCMV3-GC-A-6E construct, which encodes the rat cDNA for NPR-A that has all six of its phosphorylation sites mutated to glutamate (31), into the same parental cell line as described above. Cells were grown to 40–50% confluence in 10-cm dishes that had been precoated with 50 μg/ml poly-l-lysine. Twenty-four h later, the cells were transfected with 5 μg of the pCMV3-NPR-A-6E construct and 0.5 μg of pcDNA3.1-hygro plasmid (www.invitrogen.com) to convey hygromycin resistance using the BES+(N-N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid)-buffered calcium phosphate co-precipitation method. Forty-eight h later, the cells were switched to medium containing 100 μg/ml hygromycin to select for cells that had incorporated the resistance plasmid into their genome. After 10–14 days, individual colonies were isolated using cloning cylinders and tested for NPR-A-6E expression by guanylyl cyclase and Western blot analysis. The 293-NPR-A-6E cell line was established from a single colony and was used for all studies requiring NPR-A-6E.

The NIH3T3 cell line expressing NPR-A was previously described as 3T3-NPR-A here for consistency.

**Membrane Preparation**—Ten-cm plates of stably transfected cells were washed twice with 5 ml of ice-cold phosphate-buffered saline and then scraped off the plate in 0.5 ml of HGBP and the protease inhibitors pepstatin (1 μg/ml), leupeptin (10 μg/ml), and aprotonin (10 μg/ml). In some experiments, the protease inhibitors listed above were replaced with 180 μM AEBSF-(4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), 17 μM bestatin, 2.9 μM pepstatin A, 860 μM EDTA, and 2.2 μM E-64 (Sigma protease inhibitor mixture, P-8465). The suspended cells were then sonicated for 1 s with a Misonix Sonifier cell disrupter at 4 °C and centrifuged at 20,000 × g for 20 min at 2 °C. The resulting membrane pellet was resuspended in HGBP at a protein concentration between 2 and 4 mg/ml as determined by the BCA protein assay (Pierce). These membranes were either assayed for guanylyl cyclase activity immediately or dispersed in 0.5-ml aliquots and frozen at −80 °C.

**Metabolic Labeling**—293-NPR-A cells were washed twice with ice-cold phosphate-buffered saline and then scraped off the plate in 0.5 ml of HGBP and lysed by passing them through a 22-gauge needle 10 times. The resulting membranes were pelleted in HGBP at a protein concentration between 2 and 4 mg/ml as determined by the BCA protein assay (Pierce). These membranes were either assayed for guanylyl cyclase activity immediately or dispersed in 0.5-ml aliquots and frozen at −80 °C.

**Guanylyl Cyclase Assays**—Cyclase assays were conducted at 37 °C in the presence of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 50 mM NaCl, 0.5 mM isobutyl methyl xanthine, 0.1 mM bovine serum albumin, 5 mM creatine phosphate, 0.1 μM creatine phosphokinase, 1 mM GTP, 1 mM ATP, 1 μM ANP, and ~10 μCi of [32P]GTP. In some assays, 1 mM EDTA and 1 μM microcystin were included as phosphatase inhibitors. The reactions were stopped with 0.5 ml of 110 mM ZnOAc followed by 0.5 ml of 110 mM NaCO3 and then centrifuged at 2000 × g for 10 min at 2 °C to precipitate [32P]GTP. The [32P]GMP produced was purified by allowing the supernatant from the previous step to co-precipitate with 0.5 g of neutral alumina resin that had been acidified with 5 ml of 1 N perchloric acid. The samples were allowed to enter the resin and then washed with 10 ml of perchloric acid followed by 10 ml of water. The cGMP was eluted with 5 ml of 200 mM freshly prepared ammonium formate. The amount of [32P]GMP in the eluate was quantitated by the method of Cerenkov in a Beckman 8301 scintillation counter. For cyclase assays performed on mouse kidney
membranes, [32P]GTP was omitted, and the reactions were stopped with 0.4 ml of 50 mM sodium acetate buffer containing 5 mM EDTA followed by boiling for 5 min. The amount of cGMP in this mixture was estimated by radioimmunossay according to the manufacturer’s instructions (PerkinElmer Life Sciences; www.lifesciences.perkinelmer.com).

Immunoprecipitation and SDS-PAGE—After exposure to the various phosphatase activators or inhibitors, the [32P]-labeled membranes were solubilized in 1 ml of IPB and rotated for 15 min with 50 μl of a 50% slurry of protein A-agarose at 4 °C. The extract was then cleared by centrifugation at 20,000 × g for 15 min at 2 °C. Eight hundred μl of the cleared extract from each treatment was incubated with 1 μl of polyclonal antiserum from rabbit 6326 with constant end-over-end tumbling for 14 h at 4 °C. Rabbit 6326 was immunized with the synthetic peptide KVRTYWLLGERGCSTRG that corresponds to the last 17 carboxyterminal amino acids of NPR-A, which was conjugated to keyhole limpet hemocyanin. Fifty μl of a 50% slurry of protein A-agarose was added to the extract and incubated for 1 additional h as described above. The protein A-immunocomplex was pelleted by low-speed centrifugation and washed three times with 1 ml of IPB. NPR-A was released from the agarose beads by boiling for 3 min in the presence of 50 μl of 2× reducing SDS sample buffer, fractionated by SDS-PAGE on an 8% resolving gel, and electroblotted to an Immobilon-P membrane using a Bio-Rad Trans-Blot semi-dry transfer cell (www.bio-rad.com).

Immunoblot Analysis—NPR-A was purified as described above. The Immobilon-P membrane was then blocked for 1 h in TBST containing 3% bovine serum albumin, washed three times for 5 min with TBST, and incubated with rabbit antiserum 6326 (1:10,000) in TBST containing 1% bovine serum albumin for 2 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 donkey anti-rabbit IgG-directed antibody conjugated to horseradish peroxidase (Amersham Biosciences) diluted 1:10,000 in TBST. The membrane was then washed four times for 5 min in TBST. The NPR-A antibody complex was detected by chemiluminescence using the ECL Western blot Detection System from Amersham Biosciences.

RESULTS

Desensitization of NPR-A in Membranes Is Mediated by Receptor Dephosphorylation—To investigate the relative contribution of receptor dephosphorylation to the homologous desensitization of NPR-A in membrane preparations, we measured guanylyl cyclase activity over time in membranes prepared from human epithelial kidney 293 cells stably expressing either wild-type NPR-A (293-NPR-A) or NPR-A-6E (293-NPR-A-6E). The latter receptor is responsive to stimulation by ANP and ATP but cannot mediate the homologous desensitization process, as indicated by our previous work (30). Together, they indicate that homologous desensitization requires the dephosphorylation of NPR-A, which is mediated in part by a microcystin-sensitive protein phosphatase.

Evidence for a MgCl2-dependent NPR-A Protein Phosphatase—To investigate the potential role of hormone binding and PP2C in the desensitization of NPR-A, we treated membranes with ANP or the PP2C activator, magnesium. In this two-stage assay, we incubated the 293-NPR-A membranes in the presence or absence of various agents at 37 °C or on ice for 30 min (first stage) and then performed a short 3-min guanylyl cyclase assay (second stage) in the presence of phosphatase inhibitors to assess the effects of the preincubation on the activity of NPR-A. In pilot experiments, we washed the membranes after the initial incubation to remove the modulating factors and found that the wash did not change our results. This indicates that the effects of the preincubation on the cyclase activity of NPR-A are due to stable modification within the membranes and are not mediated by “carry over” of the desensitizing agents into the cyclase assay. We found that the 37 °C incubation alone decreased the activity of NPR-A slightly more than 50% compared with the activity obtained from membranes incubated for the same time period on ice (Fig. 2). However, if the membranes were incubated at 37 °C in the presence of 1 μM microcystin, only a 15% decline in activity was observed. These
We then tested whether microcystin (MC) and EDTA/MgCl2 are distinct NPR-A phosphatases. The microcystin- and EDTA/MgCl2-sensitive NPR-A phosphorylation sites in the presence of protein phosphatase inhibitors for 3 min at 37 °C. The data suggest that the majority, but not all, of the temperature-dependent activity loss is due to protein dephosphorylation mediated by a microcystin-sensitive phosphatase. Because we speculated that ANP binding might result in a conformational change that would expose the NPR-A phosphorylation sites to a protein phosphatase (7), we tested whether the presence of ANP in the preincubation mixture would stimulate desensitization (Fig. 2). We found that prior ANP exposure only slightly inhibited the activity of NPR-A in these 293 cell membranes. In contrast, MgCl2 dramatically reduced NPR-A activity, and the divalent metal ion chelator, EDTA, increased cyclase activity. These data suggest that ANP binding does not noticeably increase the dephosphorylation rate of NPR-A in these 293 cell membranes and that a member of the PP2C or PP7 family dephosphorylates NPR-A because members of these families are the only known serine/threonine phosphatases that require magnesium for activity (37).

The Microcystin- and EDTA/MgCl2-sensitive Phosphatases Are Distinct—We then tested whether microcystin (MC) and EDTA were inhibiting the same protein phosphatase or different protein phosphatases. We reasoned that if they were inhibiting the same phosphatase, then the effects of adding both compounds together would not be significantly greater than adding saturating concentrations of either agent alone. On the other hand, if they were inhibiting two different phosphatases, then their effects would be additive. We observed the latter scenario (Fig. 3). Incubation with 0.5 mM EDTA or 0.5 μM microcystin alone increased the amount of cGMP formed by 2.1- and 2.6-fold, respectively. Incubation with both compounds together resulted in a 5.2-fold activation, which is slightly more than the 4.7-fold increase that would be predicted if the effects of the two reagents were exactly additive. In a different approach, we tested whether microcystin blocked the MgCl2-dependent desensitization of NPR-A. We found that it did not (compare 0.5 μM MC versus MC + MgCl2), again suggesting that the microcystin- and EDTA/MgCl2-sensitive NPR-A phosphatases are unique.

Microcystin and MgCl2 Increase and Decrease the Phosphorylation State of NPR-A, Respectively—Next, we examined whether microcystin and MgCl2 directly modulate the phosphorylation state of NPR-A (Fig. 4). To this end, we prepared membranes from 293-NPR-A cells that had been metabolically labeled overnight with [32P]orthophosphate and incubated them at 37 °C in the presence or absence of the indicated agents. After 30 min, we purified the receptors by immunoprecipitation and SDS-PAGE and measured their 32P content. We found that microcystin and MgCl2 increased and decreased the phosphorylation state of NPR-A similarly to the way they increased and decreased its guanylyl cyclase activity (Fig. 4, 32P-content). Likewise, the phosphorylation state of NPR-A isolated from membranes treated with MgCl2 and microcystin was higher than that of receptors isolated from membranes treated with MgCl2 alone but less than that of NPR-A isolated from membranes treated with only microcystin. Again, these phosphorylation data parallel the desensitization results. The reduced 32P signal was not explained by MgCl2-dependent proteolysis because Western blot analysis on the same membrane support used for the phosphate determinations revealed similar amounts of protein for each treatment (Fig. 4, Western). As expected, NPR-A isolated from the MgCl2-treated membranes migrated as a tighter band, which is similar to what was observed when NPR-A was dephosphorylated with the catalytic subunit of protein phosphatase 2A (27). Because MnCl2, but not CaCl2, also activates PP2C (34), we tested the ability of these two divalent cations to stimulate the dephosphorylation of NPR-A in the 32P-labeled 293-NPR-A membranes. We found that MnCl2, but not CaCl2, was as effective as MgCl2 in stimulating the dephosphorylation (Fig. 5). These results are consistent with the metal-dependent phosphatase being a member of the PP2C family but not the PP2B/calcineurin family because these phosphatases are activated by magnesium/manganese and calcium, respectively (35).

MgCl2 and EDTA Effects Require Changes in the Phosphorylation State of NPR-A—Although MgCl2 stimulated both the dephosphorylation and desensitization of NPR-A, this correlation does not prove that the desensitizing effects of the metal were mediated solely through receptor dephosphorylation. To address this question in a more definitive manner, we used...
membranes prepared from cells expressing NPR-A-6E. We reasoned that if the MgCl₂ was desensitizing NPR-A through receptor dephosphorylation, then a receptor that could not be dephosphorylated, such as NPR-A-6E, should be immune to the effects of magnesium. As in membranes containing the wild-type receptor (Fig. 2), incubation at 37 °C slightly reduced the guanylyl cyclase activity in 293-NPR-A-6E membranes. The thermal reduction was comparable to that observed in the 293-NPR-A membranes containing microcystin. Strikingly, MgCl₂ had absolutely no effect on the guanylyl cyclase activity of membranes prepared from the 293-NPR-A-6E cells (Fig. 6). Likewise, we saw no protective effect of EDTA on guanylyl cyclase activity in these 293-NPR-A-6E membranes (data not shown). These data indicate that the effects of MgCl₂ and EDTA on the wild-type receptor require changes in its phosphorylation state and are not mediated by other metal-dependent processes, such as proteolysis.

Microcystin and MgCl₂ Modulate NPR-A Activity in Mouse Kidney Membranes—Because the parental 293 cell line that was transfected to make the 293-NPR-A cells does not endogenously express NPR-A, and because our stable lines express higher than normal levels of these receptors, we were concerned that the desensitization of NPR-A in this system may differ from that observed in physiologic tissue. To address this issue, we investigated the desensitization of NPR-A in crude mouse kidney membranes because the kidney is an established target tissue for ANP and expresses high levels of NPR-A (39). We found that incubation of these membranes at 37 °C reduced the ANP-dependent activity of NPR-A by 50% and that microcystin blocked a little more than half of this loss, mirroring the results observed in the 293 membranes (Fig. 6). Incubation with MgCl₂ dramatically reduced NPR-A activity, and this reduction was not blocked by microcystin (Fig. 6, compare 1 μM MC versus 10 mM MgCl₂/1 μM MC). Again, these data are similar to what was observed in the 293 membranes and are consistent with the presence of two distinct NPR-A phosphatases: one that is magnesium-dependent but not inhibited by microcystin, and one that is magnesium-independent but sen-
Fig. 7. Mouse kidney membranes contain both MgCl₂-dependent and microcystin-sensitive NPR-A phosphatase activities. Mouse kidney membranes were incubated at 37 °C for 30 min in the presence or absence of the indicated agents. The effects of this incubation were then assessed by measuring guanylyl cyclase activities in the presence of Mg-GTP (basal, hatched columns) or Mg-GTP, ANP, and ATP (activated, solid columns). The bars centered over the columns represent the range of two samples that were assayed in duplicate. This experiment was repeated twice with similar results.

Fig. 8. ANP exposure reduces subsequent hormone-dependent guanylyl cyclase activity in membranes from 3T3-NPR-A cells. Membranes from 3T3-NPR-A cells were incubated with the indicated agents for 30 min at 37 °C. The effects of the preincubation were then assessed by measuring the ANP/ATP-dependent guanylyl cyclase activity of the membranes in the presence of protein phosphatase inhibitors for 3 min at 37 °C. The bars centered above each column represent the range of two determinations. This experiment was repeated at least three times with similar results.

Discussion

In this report, we have documented the dephosphorylation of NPR-A by two distinct protein phosphatase activities. One does not require magnesium for activity but is sensitive to microcystin/okadaic acid-like inhibitors and has been described previously by Foster and Garbers (38) as well as by our laboratory (31). The other phosphatase does require magnesium for activity but is not inhibited by microcystin and has not been described previously. The identity of the former phosphatase is not known, but based on its sensitivity to microcystin, PP1, PP2A, PP4, PP5, or PP6 are reasonable candidates (36, 37).

In contrast to the microcystin-sensitive phosphatase, potential candidates for the magnesium-dependent phosphatase are limited to members of two families, PP2C or PP7, because these are the only known serine/threonine phosphatases that require magnesium for activity and are not inhibited by microcystin. Based on tissue distribution, we can eliminate PP7 from consideration because its expression is restricted to the retina (40), whereas NPR-A expression is widespread. Hence, it is likely that the magnesium-dependent NPR-A phosphatase is a member of the PP2C family. Based on primary amino acid sequence similarity, there are seven known mammalian gene products that comprise the PP2C family: α, β, γ, δ, Wip, FIN13, and CaMKIIPase (Ca²⁺/calmodulin-dependent protein kinase II phosphatase). Based on their exclusive nuclear localization, it is unlikely that Wip and FIN13 are involved in the dephosphorylation of NPR-A (41, 42). Likewise, because of its disparate expression pattern compared with NPR-A (high in skeletal muscle and testis but low in kidney and lung), PP2Cγ seems an unlikely candidate. Similarly, the fact that manganese but not magnesium is required to activate PP2Cα and CaMKIIPase appears to eliminate these phosphatases from consideration as well (43, 44). It is not clear which of the two remaining PP2C family members regulates NPR-A, but because an antibody that recognizes PP2Cα and some PP2Cβ alternative splicing forms detected significant amounts of PP2C in membranes from baby hamster kidney cells, these two isoforms are prime candidates.

One puzzling finding that we made during the course of these
studies is that high concentrations of NaF (50 mM) did not affect the ANP/ATP-dependent guanylyl cyclase activity of NPR-A in time course experiments such as those shown in Fig. 1. This led us to suspect that the microcystin-sensitive phosphatase was novel, because, to our knowledge, there are no known microcystin- or okadaic acid-sensitive phosphatases that are not also inhibited by NaF. However, when we subsequently tested the ability of NaF to inhibit the loss of NPR-A activity in the two-stage assay, we found that it completely blocked the temperature- and magnesium-dependent losses in cyclase activity. The reason for the discrepancy is not known at the moment, but it may be related to the fact the cyclase reaction mixture in the time course experiment contains compounds that might bind and decrease the amount of free fluoride available to inhibit the phosphatases. Alternatively, it may be that the receptor is rapidly rephosphorylated in the time course experiment due to the presence of ATP and magnesium. At the moment, a definitive explanation for these results is unavailable.

In two of the three systems tested, ANP binding markedly decreased subsequent ANP/ATP-dependent guanylyl cyclase activities, suggesting either that hormone binding stimulates receptor dephosphorylation or that a liganded receptor is a better substrate for dephosphorylation. The reason for diminished effect in the 293 cells is not known, but it may be related to the fact that NPR-A in these cells is only slightly inhibited by phorbol ester treatment.2 In our opinion, the mouse kidney membranes are the best model system to reproduce the regulation of NPR-A in vivo because they express physiologic amounts of the receptor and, unlike the transfected cells, express the necessary kinases and phosphatases in the appropriate stoichiometries. However, of the two established stable cell lines, it appears that the 3T3-NPR-A cell line is better suited for studies on the desensitization of NPR-A because it is regulated more like NPR-A in the mouse kidney membranes.

Finally, it will be interesting to determine the relative contribution of these phosphatases to the dephosphorylation of NPR-A that is initiated by homologous and heterologous stimuli. Recently, Joubert et al. (28) reported that ANP-dependent dephosphorylation of NPR-A in whole transfected 293 cells results primarily from the inactivation of the NPR-A kinase, with little contribution from increased phosphatase activity. These data are completely consistent with our findings in the 293-NPR-A model system. Whether this is a general phenomenon or restricted to 293 cells remains to be determined, but our data using membranes from mouse kidneys and NIH3T3 cells suggest that ANP binding may increase NPR-A phosphatase activity as well.

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2. L. R. Potter, unpublished observation.
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