Natriuretic peptides bind their cognate cell surface guanylyl cyclase receptors and elevate intracellular cGMP concentrations. In vascular smooth muscle cells, this results in the activation of the type I cGMP-dependent protein kinase and vasorelaxation. In contrast, pressor hormones like arginine-vasopressin, angiotensin II, and endothelin bind serpentine receptors that interact with Gq and activate phospholipase Cβ. The products of this enzyme, diacylglycerol and inositol trisphosphate, activate the conventional and novel forms of protein kinase C (PKC) and elevate intracellular calcium concentrations, respectively. The latter response results in vasoconstriction, which opposes the actions of natriuretic peptides. Previous reports have shown that pressor hormones inhibit natriuretic peptide receptors NPR-A or NPR-B in a variety of different cell types. Although the mechanism for this inhibition remains unknown, it has been universally accepted that PKC is an obligatory component of this pathway primarily because pharmacologic activators of PKC mimic the inhibitory effects of these hormones. Here, we show that in A10 vascular smooth muscle cells, neither chronic PKC down-regulation nor specific PKC inhibitors block the AVP-dependent desensitization of NPR-B even though both processes block PKC-dependent desensitization. In contrast, the cell-permeable calcium chelator, BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester), abrogates the AVP-dependent desensitization of NPR-B, and ionomycin, a calcium ionophore, mimics the AVP effect. These data show that the inositol trisphosphate/calcium arm of the phospholipase C pathway mediates the desensitization of a natriuretic peptide receptor in A10 cells. In addition, we report that CNP attenuates AVP-dependent elevations in intracellular calcium concentrations. Together, these data reveal a dominant role for intracellular calcium in the reciprocal regulation of these two important vasoactive signaling systems.

The natriuretic peptide family consists of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (1, 2). ANP and BNP are stored primarily in the cardiac atria and ventricles, respectively, and are released into the circulation upon an increase in cardiac wall stretch that usually results from increased blood pressure. CNP is found in reasonably high quantities in cytokine-treated vascular endothelial cells (3), porcine seminal plasma (4), the brain (5), and bone tissue (6–9). Unlike ANP, CNP is not stored in granules. Instead, it is regulated at the level of transcription by various signaling molecules, such as transforming growth factor-β and tumor necrosis factor-α (3, 10) as well as shear stress (11, 12).

The physiological responses elicited by natriuretic peptides are similar but not identical. In general, ANP and BNP counterbalance the renin-angiotensin-aldosterone system (1, 2). Acutely, they decrease blood pressure by increasing renal sodium and water excretion, stimulating vascular vasorelaxation, and inhibiting aldosterone and renin secretion. Similarly, CNP has been implicated in a "vascular natriuretic peptide system," but in this scenario it signals in an autocrine/paracrine manner (3). CNP binding to NPR-B relaxes phenylephrine-contracted rat aortic rings and, unlike ANP, is equally effective at relaxing veins and arteries (13). Furthermore, CNP inhibits the proliferation of vascular smooth muscle cells (14) and has been shown by many groups to inhibit balloon angioplasty-induced coronary artery restenosis (15–18).

CNP also regulates the growth of long bones (19). In mice, transgenic overexpression of BNP results in skeletal overgrowth (8), and CNP, but not ANP, increases the height of the proliferative and hypertrophic chondrocyte zones in cultured tibia preparations (9). Consistent with these findings, mice lacking NPR-C display increased natriuretic peptide half-lives and skeletal overgrowth (20), whereas mice lacking either CNP (6) or type II cGMP-dependent protein kinase (21) exhibit dwarfism.

The signaling receptors for natriuretic peptides are cell surface guanylyl cyclases, which catalyze the synthesis of the intracellular messenger cGMP (22–24). Natriuretic peptide receptor A (NPR-A) is activated by both ANP and BNP, whereas the B-type natriuretic peptide receptor (NPR-B) is activated by CNP. Both NPR-A and -B are constitutively phosphorylated...
when expressed in tissue culture cells (25–28), and receptor phosphorylation is absolutely essential for hormonal activation (29, 30). The dephosphorylation of NPR-A and NPR-B in response to hormone binding has been shown to correlate with the declining activity of these receptors in whole cells (25, 27, 28), suggesting that receptor dephosphorylation mediates the homologous desensitization of these receptors. Consistent with this idea, a mutant version of NPR-A that cannot be dephosphorylated is resistant to ANP-dependent desensitization in whole cells and in membrane preparations (31, 32).

The pressor hormones arginine vasopressin, angiotensin II, and endothelin, which stimulate phospholipase C-β, oppose the actions of natriuretic peptides (33). Therefore, from a teleological point of view, it is reasonable that all three pressor peptides decrease natriuretic peptide-dependent cGMP elevations in cultured cell lines (34–39). Three primary observations have implicated PKC in this inhibitory response. First, all hormones that inhibit natriuretic peptide signaling activate PKC via phospholipase C. Second, direct pharmacologic activation of PKC with phorbol esters mimics the desensitizing effect of the hormones on natriuretic peptide receptors (36, 37, 39–47). Third, in a few instances relatively specific inhibitors of PKC, like H7, block all or part of the hormone-dependent desensitization (35, 36, 42). Hence, for more than 15 years it has been generally assumed that PKC is obligatory component in the heterologous desensitization of natriuretic peptide signaling. In this report, we provide evidence for a calcium-dependent desensitization pathway that appears to be distinct from the previously characterized PKC-dependent desensitization pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rat ANP, rat CNP, arginine-vasopressin, GF-109203X, phorbol 12-myristate 13-acetate, BAPTA-AM, ionomycin, and the aluminia resin used for cGMP purification were purchased from Sigma. [α-32P]GTP (NEG-006H) was from PerkinElmer Life Sciences. The horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody was purchased from Amersham Biosciences.

**Cell Culture and Preparation of Crude Membranes**—A10 rat aortic smooth muscle cells (CRL-1476) were purchased from American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (Mediatech, Inc.) in a humidified atmosphere (1% CO2) at 37 °C. Cells were cultured on cover slips coated with poly-L-lysine. When confluent, the cells were washed and incubated for at least 4 h with serum-free media. The medium was aspirated and replaced with 0.5 ml of DMEM containing 10% fetal bovine serum (Mediatech, Inc.) in a humidified atmosphere (1% CO2) at 37 °C. After a 30-min incubation, the medium was aspirated and replaced with 0.5 ml of DMEM containing 10% fetal bovine serum, 50 mM HEPES, 20 mM glycerol, 50 mM NaCl, 50 mM NaF, with phosphate-buffered saline, scraped into 0.5 ml of phosphatase inhibitor buffer (25 mM HEPES, 20% glycerol, 50 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM microcystin, 0.1% bovine serum albumin, 0.5 μM 1-methyl-3-isobutylxanthine, 1 mM EDTA, 5 mM creatine phosphate, and 0.1 mg/ml creatine kinase (as a nucleotide regeneration system), 1 μM microcystin, 0.1–0.2 μCi of [α-32P]GTP, and 0.1 or 1 mM GTP. Activator mixtures consisted of 1 mM ATP, 1 μM CNP, and 5 μM MgCl2 or 1% Triton X-100 and 5 μM MnCl2. Between 25 and 50 μg of crude membrane preparations were assayed for 3 min at 37 °C by the addition of a mixture containing the above reagents to a total volume of 100 μl. The reactions were initiated by the addition of a mixture containing the substrate and terminated with 500 μl of 110 mM zinc acetate. To purify the cGMP, 0.5 ml of sodium carbonate was added to the mixture, and the sample was vortexed and centrifuged at 3000 × g for 10 min at 2 °C. The supernatant was added to chromatography columns (Bio-Rad model 731-1550) containing ~0.5 g of dry neutral aluminia resin (Sigma, AnalaR). The columns were washed with 5 ml of 1X perchloric acid. The columns were then washed with 10 ml of 1X perchloric acid followed by 10 ml of water. The purified [α-32P]GTP was then eluted with 5 ml of freshly prepared 200 mM ammonium formate and quantitated using the Cerenkov method in a Beckman 3801 scintillation counter.

**Guanylyl Cyclase Assays**—Guanylyl cyclase assays were performed in the presence of 25 mM HEPES, 50 mM NaCl, 0.1% bovine serum albumin, 0.5 μM 1-methyl-3-isobutylxanthine, 1 mM EDTA, 5 mM creatine phosphate, and 0.1 mg/ml creatine kinase (as a nucleotide regeneration system). 1 μM microcystin, 0.1–0.2 μCi of [α-32P]GTP, and 0.1 or 1 mM GTP. Activator mixtures consisted of 1 mM ATP, 1 μM CNP, and 5 μM MgCl2 or 1% Triton X-100 and 5 μM MnCl2. Between 25 and 50 μg of crude membrane preparations were assayed for 3 min at 37 °C by the addition of a mixture containing the above reagents to a total volume of 100 μl. The reactions were initiated by the addition of a mixture containing the substrate and terminated with 500 μl of 110 mM zinc acetate. To purify the cGMP, 0.5 ml of sodium carbonate was added to the mixture, and the sample was vortexed and centrifuged at 3000 × g for 10 min at 2 °C. The supernatant was added to chromatography columns (Bio-Rad model 731-1550) containing ~0.5 g of dry neutral aluminia resin (Sigma, AnalaR). The columns were washed with 5 ml of 1X perchloric acid. The columns were then washed with 10 ml of 1X perchloric acid followed by 10 ml of water. The purified [α-32P]GTP was then eluted with 5 ml of freshly prepared 200 mM ammonium formate and quantitated using the Cerenkov method in a Beckman 3801 scintillation counter.

**Immunoblot Analysis**—NPR-B present in crude membranes was fractionated on an 8% SDS-polyacrylamide gel and blotted to polyvinylidene difluoride (Immobilon P) membrane using a BioRad Trans-Blot semidyv transfer cell. The membrane was then incubated for 1 h in TBST (20 mM tris(hydroxymethyl)aminomethane, 500 mM NaCl, and 0.05% polyoxylene sorbitan monolaurate, pH 7.5) containing 3% bovine serum albumin followed by two 5-min washes with TBST. The primary antisera was diluted 1:2,500 in TBST and incubated with the membrane for 2 h at 4 °C. The secondary and the four washes followed for 30 min. The specific antisera were raised against synthetic peptides corresponding to the last 17 or 10 carboxyl-terminal amino acids of NPR-A (antiserum 6326) or NPR-B (antiserum 6328), respectively, which conjugated to keyhole limpet hemocyanin. These antisera are specific for each receptor and do not cross-react (see Fig. 1, bottom panel). The membrane was then incubated with donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) diluted 1:10,000 in TBST for 45 min. After four washes for 5 min with TBST, the NPR-B antibody complex was visualized by chemiluminescence using the ECL Western blot detection system (Amersham Biosciences).

**Calcium Imaging**—A10 cells were plated on 15-mm glass coverslips and grown until they formed a monolayer. The cells were washed with HBS and resuspended solute salt solution (HBS) and incubated at 37 °C for 30 min with 5 μM fura-2-acetoxymethyl ester (a ratiometric fluorescent Ca2+ indicator). The coverslip was then washed with HBSS and placed on a 150-μl open slide chamber (RC-25F, Warner Instruments) mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was perfused at ~2.5 μl/min at room temperature with HBSS. In experiments using ionomycin or CNP, the reagents were added directly to the chamber with constant perfusion. Fura-2-loaded cells were alternately excited at 340 and 380 nm with a digitally controlled filter wheel (DG-4, Sutter Instrument Co.). The fluorescence emissions at 510 nm were collected with a cooled CCD 12-bit digital camera (Princeton Scientific Instruments). The digital camera output was then analyzed by a digital computer (Universal Imaging). Fluorescent signals were determined from regions of interest and the images corrected for system background, 80% shading errors, and the very low autofluorescence of the unloaded cells. In each experiment, fura-2-loaded cells were exposed to 1 μM 4-bromo-A23187 and 10 mM EGTA to obtain maximum and minimum F340/F380 ratios, respectively. Calcium concentrations were calculated using an in vitro calibration method as previously described (48).

**Data Analysis and Statistics**—The data were graphed and IC50 values estimated with GraphPad Prism for the MacIntosh. In Fig. 2, the dose-response curve was fit using the equation: \( Y = \text{Bottom} + \ (\text{Top} - \text{Bottom})/ (1 + 10^{(EC_{50} - x)}) \). The “Top” is the best-fit highest value, which the program determined to be 4.990. The “Bottom” is the best-fit lowest value, given by 0.5670. With these two values, the logEC50 was estimated at ~10.5.

**RESULTS**

One of the primary goals of this study was to examine the heterologous regulation of a natriuretic peptide receptor in a physiological setting. Therefore, we chose rat vascular smooth A10 cells because vascular smooth muscle is a known target for natriuretic peptides (13, 49). However, because of discrepancies in the literature regarding whether A10 cells express NPR-A (34, 41, 50) or NPR-B (51–53), we first determined the expression profile of natriuretic peptide receptors in these cells. Initially, we examined the sensitivity of whole A10 cells to CNP or ANP by measuring cGMP elevations in response to increasing concentrations of ANP by measuring cGMP elevations in response to increasing concentrations of ANP.
Concentrations of each peptide. Cells treated with CNP responded with dose-dependent increases in intracellular cGMP (Fig. 1, top panel, squares). Statistically significant elevations in cGMP concentrations were first detected at 1 nM CNP, and the maximum dose (1 mM), resulted in a 230-fold increase in cGMP concentrations above basal levels. In contrast, 100 nM concentrations of ANP were required to detect an increase in cGMP levels, and 1 mM ANP only stimulated cGMP concentrations 34-fold above basal levels (Fig. 1, top panel, circles). The ANP-dependent dose response in the A10 cells is similar to that observed in cell lines only expressing NPR-B (54, 55). Therefore, it most likely results from ANP cross-activation of NPR-B.

In complete agreement with these data, Western blots on membranes prepared from A10 cells or 293 cells stably expressing NPR-A (293-NPR-A) or NPR-B (293-NPR-B) indicated that A10 cells express NPR-B but no detectable NPR-A (Fig. 1, bottom panel). Together, these data indicate that NPR-B is the primary and probably the only natriuretic peptide receptor expressed in A10 cells.

Arginine-vasopressin (AVP) has previously been shown to decrease ANP-dependent cGMP elevations in A10 cells (34, 50). However, based on the observation that micromolar concentrations of ANP were not able to saturate the cGMP response in these cells (34, 50) combined with the expression data shown in Fig. 1, it is likely that previous investigators were actually studying the regulation of NPR-B and not NPR-A as they suggested. This was a reasonable oversight because CNP and NPR-B had not yet been identified at the time their studies were conducted. Consistent with this hypothesis, we found that AVP reduced CNP-dependent cGMP elevations in these cells. Incubation of A10 cells with 1 mM AVP for 30 min reduced cGMP concentrations at every CNP dose tested (Fig. 2A). Cells stimulated with the highest concentration of CNP tested (5 mM) produced 12.5 pmol cGMP/well, whereas the same CNP concentration resulted in only 3.0 pmol cGMP/well after treatment with AVP, which equals 24% of the control values. AVP exposure had no effect on basal cGMP concentrations, which suggests that the reduced cGMP concentrations are not mediated through increased cGMP degradation. The ability of AVP to decrease cGMP elevations was also dose-dependent (Fig. 2B).

Diminished CNP-dependent cGMP elevations were first apparent at picomolar concentrations of AVP, and the maximum desensitization was reached around 10 nM AVP. The IC50 for the response was estimated to be 0.03 nM AVP.

To distinguish between the possibilities that the reduced cGMP concentrations could have resulted from increased phosphodiesterase activity or from decreased guanylyl cyclase activity, we performed guanylyl cyclase assays. Crude membranes prepared from A10 cells treated in the presence (circles) or absence (squares) of 100 nM AVP for 30 min were assayed for CNP-dependent (1 mM CNP, 5 mM MgCl2 and 1 mM ATP) guanylyl cyclase activity for 5 and 10 min (Fig. 3). Consistent with the whole cell stimulation data, AVP substantially reduced NPR-B activity, decreasing CNP-dependent cGMP for-
The data are representative of one of at least three similar experiments. The effect of AVP was rapid, an excellent indicator of the total amount of NPR-B present in any maximally activates NPR-B independently of CNP and is an
ments with similar results. Where error bars are not visible, they are contained within the data point. The data represent one of at least three experi-
ments that contained only

To characterize the time course of AVP-dependent inhibition of NPR-B activity, A10 cells were serum-starved for 4 h and then treated with AVP for 0, 2, 5, 10, 20, or 40 min. Crude membranes were prepared from these cells and assayed for guanylyl cyclase activity in the presence of CNP (1 mM ATP, 1 mM CNP, and 5 mM MgCl₂) guanylyl cyclase activity for 5 and 10 min. Values are the mean of two experiments assayed in duplicate (± range). This experiment was performed at least three times with similar results. Where error bars are not visible, they are contained within the data point. The data represent one of at least three experiments with similar results.

Calcium-dependent Desensitization of NPR-B

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To further investigate the potential role of PKC in the AVP-dependent desensitization of NPR-B, we performed a second set of experiments in which A10 cells were incubated for 24 h in serum-deficient medium containing vehicle (MeSO) or 1 μM PMA. The latter treatment is widely used to down-regulate phorbol ester-sensitive PKC isoforms (57). Following the incubation, the cells were treated in the absence or presence of 100 nM PMA or 100 nM AVP for 30 min. Crude membranes were then isolated and assayed for CNP-dependent guanylyl cyclase activity. In membranes isolated from control cells exposed to AVP or PMA for 30 min, NPR-B activity was decreased to less than 50% of control values (black bars). As anticipated, chronic PMA exposure (24 h) completely abolished subsequent PMA-induced decreases in CNP-dependent cGMP production, confirming that PKC was down-regulated (Fig. 5B). In contrast, AVP-induced inhibition of NPR-B was preserved despite the inactivation of PKC. Together, these two experiments indicate that AVP-induced desensitization of NPR-B does not require GF-109203X-sensitive or phorbol ester down-regulatable protein kinase C isoforms.

Because PKC was not required for the AVP response, we investigated the role of the inositol trisphosphate/calcium arm of the phospholipase C pathway in this process. As a first step, we determined the ability of AVP to elevate intracellular calcium concentrations in our A10 cells. We studied the response of a population of A10 cells that had been grown on glass coverslips and loaded with 5 μM fura-2-AM for 30 min. In this representative experiment, basal intracellular calcium concentrations in these cells ranged from 150 to 350 nM, and exposure to 1 μM AVP resulted in elevated calcium concentrations that ranged between 1.5 μM and 5 μM (Fig. 6A), which is similar to previously reported AVP-dependent calcium elevations in A10 cells (58). To further explore the involvement of intracellular calcium elevations in NPR-B regulation, we validated that the common calcium ionophore ionomycin could elevate intracellular calcium concentrations and that the cell-permeable calcium chelator, BAPTA-AM, could inhibit free calcium elevations. Treatment of A10 cells with 1 μM ionomycin mimicked the effect of AVP on calcium elevations, increasing the average net (maximum peak to average basal) intracellular Ca^{2+} concentration to above 3000 nM (Fig. 6B). In contrast, preincubation with 50 μM BAPTA-AM completely blocked the AVP-dependent intracellular calcium elevations. These experiments authenticated the use of these compounds in modulating intracellular calcium concentrations in A10 cells and suggested that they could be effective in assessing the requirement of calcium in AVP-dependent inhibition of NPR-B.

To determine whether increases in intracellular calcium were sufficient for NPR-B desensitization, A10 cells were treated with 1 μM ionomycin for increasing periods of time. Crude membranes prepared from the treated cells were then assayed for guanylyl cyclase activity in the presence of CNP (Fig. 7, black bars) or Triton X-100 (gray bars). Membranes isolated from cells treated with ionomycin for 5 or 10 min contained 68 or 48%, respectively, of the CNP-dependent guanylyl cyclase activity measured in membranes from untreated cells (Fig. 7A). Similar to AVP-treated cells, ionomycin did not reduce the amount of NPR-B protein as evidenced by guanylyl cyclase measurements obtained in the presence of detergent. To verify that the ionomycin-induced inhibition of NPR-B was due to elevated intracellular calcium concentrations, cells were preincubated with 50 μM BAPTA-AM, a cell-permeable calcium chelator (Fig. 7B). In the presence of APTA-AM, ionomycin was a completely ineffective desensitizing agent, which suggest that elevated intracellular calcium concentrations are required and sufficient for the heterologous desensitization of NPR-B.
Calcium-dependent Desensitization of NPR-B

In this study we have shown that: 1) A10 cells express NPR-B and not NPR-A; 2) AVP decreases CNP-dependent but not basal cGMP levels in a time- and concentration-dependent manner; 3) the reduced cGMP concentrations are a result of decreased NPR-B guanylyl cyclase activity; 4) the NPR-B inhibition requires elevated intracellular calcium concentrations but not GF-109203X-sensitive forms of PKC or NPR-B degradation; and 5) CNP inhibits AVP-dependent intracellular calcium elevations. Together, these results reveal a dominant role for modulations of intracellular calcium in the reciprocal regulation of these two important vasoactive signaling pathways.

One surprising finding of this study was that of the two arms of the phospholipase C pathway, diacylglycerol/PKC or inositol trisphosphate/calcium, only the latter appears to be required for the AVP-dependent inhibition of NPR-B in A10 cells. This finding was unexpected because at least 15 published reports have suggested that PKC mediates the heterologous desensitization of natriuretic peptide receptors, whereas the role of calcium in this process has remained relatively unexplored (35–37, 40–47, 59–62). Nonetheless, because our experiments utilized two independent approaches, we believe that the notion of PKC being required for the heterologous desensitization

dependent guanylyl cyclase activity found in membranes isolated from untreated cells (Fig. 8). However, we observed maximum desensitization (13% of the control values) when cells were treated simultaneously with both agents, which suggests that intracellular calcium elevations and PKC activation are modulating different pathways. Interestingly, when cells were treated with AVP and PMA together, the desensitization was

Fig. 7. AVP-dependent desensitization of NPR-B requires intracellular calcium elevations. A, ionomycin inhibits CNP-dependent guanylyl cyclase activity. Confluent, serum-starved A10 cells were treated with 1 μM ionomycin for the indicated amounts of time or with 1 μM AVP for 15 min. Crude membranes were then prepared and assayed for CNP (1 mM CNP, 1 mM ATP, and 5 mM MgCl₂) or detergent-dependent (1% Triton X-100, 5 mM MnCl₂) guanylyl cyclase activity for 3 min. Approximate control values for CNP and detergent-dependent activities are 3.0 and 7.5 nmol cGMP/mg/3 min, respectively. B, BAPTA-AM blocks ionomycin-dependent NPR-B desensitization. A10 cells were grown to confluency, serum-starved, and pretreated with or without 50 μM BAPTA-AM for 30 min. Cells were then incubated in the presence or absence of 1 μM ionomycin for 10 min. Crude membranes were prepared and assayed as described in A. Average control values for CNP- and detergent-dependent activities are 0.4 and 2.0 nmol cGMP/mg/3 min, respectively. C, BAPTA-AM blocks AVP-dependent inhibition of NPR-B. Confluent, serum-starved A10 cells were treated with the indicated concentrations of BAPTA-AM for 30 min and then treated with or without 100 nM AVP for an additional 30 min. A guanylyl cyclase assay was then performed on the crude membranes as described in A. Control values for CNP-dependent and detergent-dependent activities are 0.5 and 1.7 nmol cGMP/mg/3 min, respectively. For each experiment, values represent the average of two separate experiments assayed in duplicate (± S.E.). They were each performed at least three times with similar results.

similar to that observed with AVP alone. We do not have a definitive explanation for these results, but because PMA inhibits AVP-dependent intracellular calcium elevations (data not shown), one possibility is that PMA is partially blocking the AVP-dependent activation of phospholipase C.

Finally, because AVP inhibited NPR-B in a calcium-dependent manner, we asked whether CNP inhibited AVP-dependent calcium concentrations in A10 cells. To this end, we plated A10 cells on glass coverslips, loaded them with fura-2-AM, and incubated the cells with 1 μM CNP for 5 min before calcium imaging. CNP treatment had a minimal effect on basal calcium concentrations, decreasing levels from 345 nM ± 8 to 300 ± 7 nM. However, when the CNP-treated cells were stimulated with 1 μM AVP, their calcium elevations were markedly blunted compared with elevations observed in cells not exposed to CNP (Fig. 9). CNP decreased average AVP-stimulated calcium elevations from 2129 to 449 nM, a reduction of 79%. These data provide direct evidence for reciprocal antagonism between the CNP and AVP signaling pathways in vascular smooth muscle cells.

DISCUSSION

Fig. 8. Effects of PMA and ionomycin on NPR-B desensitization are additive. Confluent, serum-starved A10 cells were treated with 1 μM PMA or 1 μM AVP for 30 min, 1 μM ionomycin for 15 min, or a combination of the treatments as indicated. Crude membranes were then prepared and assayed for CNP-dependent (1 mM ATP, 1 μM CNP, and 5 mM MgCl₂) or detergent-dependent (1% Triton X-100, 5 mM MnCl₂) guanylyl cyclase activity for 3 min. Approximate control values for CNP- and detergent-dependent activities are 0.5 and 1.7 nmol cGMP/mg/3 min, respectively. Values are the average of two duplicate plate treatments assayed in duplicate (± S.E.). This experiment was performed at least three times with similar results.
of natriuretic peptide signaling has been severely weakened. On the other hand, we cannot rule out the possibility that a form of PKC that is not inhibited by GF-109203X or that is down-regulated by chronic PMA exposure participates in this process. Nonetheless, our results indicate for the first time that elevated intracellular calcium concentrations are sufficient to inhibit NPR-B activity, revealing an alternative pathway by which NPR-B can be regulated. Hence, with this report both products of the phospholipase C catalyzed reaction have now been shown to inhibit NPR-B. It is of interest that the effects of AVP on NPR-B activity are greater than that of ionomycin, even though ionomycin exposure results in greater calcium elevations. This suggests that maximum desensitization results from something in addition to increased calcium concentrations. One obvious possibility is that diacylglycerol-dependent activation of PKC is required. Using PKC inhibitors and PMA-dependent down-regulation of phorbol ester-sensitive PKC isoforms, we have been unable to document a measurable contribution of PKC to this process. Therefore, it is currently unclear whether PKC activation or another unknown signaling event is required for the maximum desensitization of NPR-B.

With respect to previous studies, our data are not consistent with a report showing that the protein kinase C inhibitor H7 could block angiotensin II-dependent reductions in ANP-dependent guanylyl cyclase activity in primary glomerular mesangial cells (35). Similarly, Jaiswal used H7 to block the ability of endothelin to reduce ANP-dependent cGMP elevations in primary vascular smooth muscle cells (36). We do not know why our results differ from these previous studies, although one obvious reason is that NPR-A is regulated differently than NPR-B. This is clearly a possibility, but despite numerous attempts by our group as well as others, significant regulatory differences between these two receptors have not been identified. This is not completely unexpected given that the intracellular portions of these two receptors are 78% identical at the amino acid level. An alternative explanation may be related to the different cell systems employed in each study.

In terms of NPR-B regulation, the protein kinase C inhibitor Ro 31-8220 blocked only 63% of the ability of endothelin-3 to inhibit CNP-dependent cGMP elevations in C6 glioma cells, which is consistent with the existence of both PKC-dependent and independent inhibitory pathways (39). Unfortunately, the role of intracellular calcium elevation in the endothelin-dependent desensitization of NPR-B was not described in this report. In contrast, gonadotropin-releasing hormone was shown to inhibit CNP-dependent cGMP elevations in pituitary T3-1 cells in a manner that is mimicked by phorbol esters but not by the Ca^{2+} ionophore A23187, which is completely opposite of our results (62).

Elevated intracellular calcium concentrations have been shown to decrease intracellular cGMP concentrations in several cell types (35, 63–65). Regardless of whether the calcium concentrations were elevated by hormones or ionophores, in most cases the decreased nucleotide concentrations resulted from increased phosphodiesterase activity, not reduced guanylyl cyclase activity. However, in mouse Leydig cells, Mukhopadhyay and colleagues (63) demonstrated that ionomycin decreased ANP-dependent, but not basal, cGMP concentrations in the presence of high concentrations of the general phosphodiesterase inhibitor isobutylmethylxanthine, suggesting that increased nucleotide degradation was not required for the diminished cGMP concentrations in these cells. On the other hand, these investigators were unable to detect any direct effect of calcium on ANP-dependent guanylyl cyclase activity in membranes from these cells, and the results of cyclase assays conducted on membranes isolated from cells treated in the presence or absence of ionomycin were not reported (63).

Although this study (63) suggests that PKC is not required for the desensitization of NPR-B in A10 cells, others and we have shown that PKC activation desensitizes NPR-B (39, 42, 44, 59, 62). Hence, it appears that both arms of the phospholipase pathway can lead to the inhibition of CNP-dependent guanylyl cyclase activity. The mechanism for this PKC-dependent loss of activity appears to involve the dephosphorylation of NPR-B at Ser-523, because the mutation of this residue to glutamate abrogates the effect (44). However, it is important to point out that this mechanism has not been shown to be required for any hormonal desensitization of NPR-B; it has been observed only upon pharmacologic activation of PKC by phorbol esters. In contrast, the mechanism for the calcium-dependent process is completely unexplored. We are currently investigating the role of NPR-B dephosphorylation in this process. However, the low concentration of NPR-B endogenously expressed in A10 cells combined with the low transfection efficiency of these cells has made this endeavor extremely difficult.

Finally, it is worth noting that the inhibitory effect of calcium on NPR-B is similar to the effect calcium has on the retinal guanylyl cyclases (RetGC-1 and RetGC-2/GC-E and GC-F) (66). These receptor cyclases have a predicted structural topology similar to NPR-A and NPR-B, but no specific extracellular activator of these receptors has been identified. It is known, however, that retinal cyclases are regulated by small intracellular calcium-binding molecules called guanylyl cyclase-activating proteins (GCAPs). Under low intracellular calcium conditions, GCAPs stimulate these receptors, whereas under elevated calcium concentrations the GCAPs inhibit them, presumably by causing a conformational change in the GCAP that is unfavorable to cyclase activation. Recently, visin-like protein-1 (VILIP-1), a member of the intracellular neuronal calcium sensor family that also includes GCAPs, was found to colocalize with NPR-B in cerebellar cell cultures. Unfortunately, the effect of altering intracellular calcium concentrations on this process was not investigated (67). It is tantalizing to speculate that VILIP-1 or perhaps a natriuretic peptide receptor-specific calcium-binding protein mediates the calcium-dependent desensitization of NPR-B; however, this remains to be determined.

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