C-type natriuretic peptide (CNP) is the third member of the natriuretic peptide family that also includes atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). ANP and BNP are found primarily in cardiac myocytes of the atria and ventricle, respectively, and are secreted into the circulation in response to increased cardiac wall stretch. In contrast, only traces of CNP are found in the bloodstream. It is most highly expressed in vascular endothelial cells, chondrocytes, and the brain. Unlike ANP and BNP, which signal in a true endocrine fashion, CNP exerts its effects in a paracrine manner.

ANP and BNP decrease blood pressure by stimulating renal salt and water excretion, inhibiting the renin-angiotensin-aldosterone system and possibly causing vasodilation. In contrast, CNP functions as a local regulator in vascular walls and fibroblasts, where the endothelial cell production of CNP is transcriptionally regulated by paracrine factors like tumor necrosis factor-α, interleukin-1α/β, and transforming growth factor-β as well as by shear stress. Once released from endothelial cells, CNP relaxes and inhibits the proliferation of adjacent vascular smooth muscle cells and adventitial fibroblasts. The clinical relevance of this signaling pathway was demonstrated in studies where CNP infusions dramatically inhibited the intimal thickening and restenosis that accompanies balloon angioplasty.

The effects of natriuretic peptides are mediated through the activation of the particulate guanylyl cyclases, natriuretic peptide receptor-A (NPR-A) and natriuretic peptide receptor-B (NPR-B), enzymes that convert GTP into the intracellular second messenger, cyclic GMP. NPR-A is activated by both ANP and BNP, whereas NPR-B is activated by CNP. A third natriuretic peptide binding protein, natriuretic peptide receptor-C (NPR-C), is structurally related to NPR-A and NPR-B, but it does not possess any known enzymatic activity.

NPR-B activity is inhibited by hormones such as arginine-vasopressin or growth factors like platelet-derived growth factor (PDGF) that activate phospholipase C. These agents counter the actions of natriuretic peptides by stimulating vasoconstriction and cell proliferation. Fetal bovine serum elicits the most dramatic inhibitory effect on NPR-B activity, but the serum factors responsible for this effect are currently unknown. We recently reported that both PDGF and lysophosphatidic acid (LPA) inhibit NPR-B in NIH3T3 cells, although neither agent was as potent as fetal bovine serum, suggesting that another unidentified factor may be responsible for the inhibitory action of serum on NPR-B.

In this report, we demonstrate that sphingosine-1-phosphate (S1P), a novel phospholipid signaling molecule
that is released in micromolar concentrations from activated platelets, is an acute inhibitor of NPR-B in both fibroblasts and vascular smooth muscle cells. To our knowledge, S1P is the most potent NPR-B desensitizing factor identified to date.

Materials and Methods

Materials

Rat CNP-22, GF-109203X, and ionomycin were from Sigma-Aldrich. BAPTA-AM was purchased from Calbiochem. S1P, LPA and dihydro-sphingosine-1-phosphate (sphingoamine-1-phosphate) were purchased from Avanti Polar Lipids. These powders were suspended in ethanol or 95% ethanol (LPA) by warming to 55°C and briefly sonicating in a water bath. A10 cells (CRL-1476) and NIH3T3 cells (CRL-1658) were acquired from American Type Culture Collection.

Cell Culture and Preparation of Crude Membranes

Cells were maintained in Dulbecco modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (A10) or 10% calf serum (NIH3T3). Crude membranes were prepared as previously described.14,16

Whole Cell Stimulations

Cells plated in 12-well dishes were grown to 90% confluency and incubated overnight at 37°C in serum-free media. Cells were stimulated with various agents, and intracellular cGMP concentrations were determined as previously described.14,16

Guanylyl Cyclase Assays

Guanylyl cyclase assays were performed for 3 minutes in the presence of activator cocktails consisting of either 1 μmol/L CNP, 1 μmol/L ATP, and 5 μmol/L MgCl₂, or 1% Triton X-100 and 5 μmol/L MnCl₂ as previously described.14,16

Data Analysis and Statistics

The data were graphed, analyzed, and IC₅₀/EC₅₀ estimated with GraphPad Prism4 for the MacIntosh. In Figures 1B, 2B, and 3 the dose response curves were fitted using the equation: Y = Bottom + (Top − Bottom)/(1 + 10^(logEC₅₀− X)×Hill Slope)). The “top” is the best-fit highest value and “bottom” is the best-fit lowest value. In Figure 2 and Figure 3 this equation was used to determine the IC₅₀ from the program’s estimated logEC₅₀. The caveat to this method of estimation is the lowest value assumption when it is unclear whether S1P is able to further inhibit activity at higher concentrations. Unfortunately, we were unable to test concentrations of S1P higher than 10 μmol/L due to nonspecific effects of the buffer. Furthermore, because we were unable to fully dissolve the S1P and the lipid is known to bind to plastic surfaces, the actual concentration in the media may be less than reported. Thus, we may have overestimated the IC₅₀ for S1P-dependent inhibition of NPR-B. Statistical analysis for Figure 2 was performed with GraphPad Prism4 using 2-way ANOVA and Bonferroni post tests to determine P values for significant differences between −S1P and +S1P values. To determine the P values as indicated in Figure 7, the values were analyzed using 1-way ANOVA and Dunnett’s post test for multiple comparisons.

Results

S1P Potently Inhibits NPR-B in Fibroblasts

S1P is one of the most highly concentrated mitogens in serum and binds receptors in the same family as those activated by lysophosphatidic acid.17 Therefore, we asked whether S1P, like LPA, inhibits CNP signaling. To test this hypothesis, we treated serum-starved NIH3T3 fibroblasts that endogenously express NPR-B, but not NPR-A,16 with medium alone, 10% fetal bovine serum, 10 μmol/L LPA, or 5 μmol/L S1P before exposing them to media containing 20 nM CNP and measuring intracellular cGMP concentrations (Figure 1A). In cells incubated with medium alone, CNP increased intracellular cGMP levels from 0.01 pmol per 100 000 cells to 1.1 pmol per 100 000 cells, or 110-fold. However, prior incubation with 10% fetal bovine serum inhibited more than two-thirds of the CNP response, whereas LPA treatment blocked about half the effect (Figure 1A). Interestingly, S1P was a more robust inhibitor than LPA, blocking cGMP elevations to the same extent as fetal bovine serum.

The ability of S1P to inhibit NPR-B guanylyl cyclase activity in fibroblasts was tested by preparing crude membranes from cells incubated with increasing amounts of S1P for 30 minutes and then measuring guanylyl cyclase activity

Figure 1. S1P inhibits NPR-B in NIH3T3 fibroblasts. A, NIH3T3 cells were incubated with 10% fetal bovine serum (FBS), 10 μmol/L LPA, 5 μmol/L S1P, or medium alone for 30 minutes at 37°C and then were stimulated with 20 nM CNP for 5 minutes. Total cGMP levels were determined by radioimmunoassay. Wells contained 62 500±3708 cells and cell numbers were unaffected by the treatments. Values are the mean of 3 replicates (±SEM), and where error bars are not visible, they are contained within the column. B, Cells were treated with the indicated concentrations of S1P for 30 minutes and crude membranes were prepared and assayed for guanylyl cyclase activity in the presence of either CNP (1 μmol/L CNP, 1 μmol/L ATP, 5 μmol/L MgCl₂, circles) or detergent (1% Triton X-100, 5 μmol/L MnCl₂, squares). The control values for CNP- and detergent-dependent activities were 1.2 and 2.4 nmol/mg per 3 minutes, respectively. Values are the mean of duplicates (±range). Where error bars are not visible, they are contained within the data point. Figures are representative of 3 experiments.

ANOV and Bonferroni post tests to determine

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in the presence of 1 μmol/L CNP, 1 mmol/L ATP, and 5 mmol/L MgCl₂ (Figure 1B, circles) or 1% Triton X-100 and 5 mmol/L MnCl₂ (Figure 1B, squares). The former treatment measures hormone-dependent activity, whereas the detergent treatment maximally activates NPR-B independently of CNP and is an excellent indicator of the amount of NPR-B protein present in the membrane preparations. We found that S1P inhibits CNP-dependent NPR-B guanylyl cyclase activity in a dose-dependent manner (Figure 1B). The IC₅₀ for S1P was approximately 0.08 μmol/L and the response plateaued around 1 μmol/L (Figure 1B). To our knowledge, this is the most efficacious inhibitor of NPR-B thus far identified.

SIP Potently Inhibits NPR-B in Vascular Smooth Muscle Cells

We then tested whether S1P also inhibits NPR-B in vascular smooth muscle cells, a relevant cardiovascular tissue that is exposed to serum under conditions such as vascular injury where the endothelial lining is compromised. As a model system, we used rat aortic smooth muscle A10 cells that we have previously shown to express NPR-B but not NPR-A. We incubated confluent, serum-starved A10 cells with or without 1 μmol/L S1P for 30 minutes and then stimulated cGMP synthesis with increasing concentrations of CNP (Figure 2). At each CNP concentration tested, S1P reduced the amount of intracellular cGMP detected. Stimulation for 3 minutes with 8 nM CNP elevated cGMP levels to approximately 4 pmol per well, whereas a 30 minute exposure to 1 μmol/L S1P decreased CNP-dependent cGMP formation to less than 0.75 pmol per well. The inhibition was statistically significant (P<0.01) for cells stimulated with CNP concentrations of 8 nM or greater (Figure 2). In contrast, S1P treatment had no significant effect on basal cGMP levels.

Next, we tested whether the reduced CNP-dependent cGMP levels were due to a suppression of hormone-dependent guanylyl cyclase activity similar to that observed in fibroblasts. A10 cells were treated with increasing concentrations of S1P for 30 minutes and then crude membranes were prepared and assayed for guanylyl cyclase activity in the presence of either CNP (circles) or detergent (squares). The control values for CNP- and detergent-dependent activities were 0.37 and 1.42 nmol/mg per 3 minutes, respectively. Values are the mean of 6 replicates (±SEM), and where error bars are not visible, they are contained within the data point.

SIP-Dependent Inhibition of NPR-B Is Rapid, Reversible, and Receptor Mediated

We then determined the time course of the S1P-dependent desensitization in vascular smooth muscle cells. Serum-starved A10 cells were incubated in the presence of 10 μmol/L S1P for increasing periods of time up to 1 hour and then crude membranes were prepared and assayed for guanylyl cyclase activities (Figure 4A). S1P rapidly inhibited hormone-dependent (Figure 4, circles), but not detergent-dependent (squares) NPR-B activity. The time required for half of the inhibition to occur (t½) was between 2.5 and 5
minutes. The inhibition was essentially complete by 10 minutes and was sustained for up to 1 hour. Hence, the S1P-dependent desensitization of NPR-B is rapid and not subject to acute (<1 hour) desensitization.

Next, we tested the effect of long-term (overnight) S1P incubation on NPR-B activity. A10 cells were incubated in media with or without 10 μmol/L S1P for either 1 hour, overnight (18 hours), or overnight followed by 1 additional hour incubation with a second fresh aliquot of S1P, such that the total S1P concentration was 20 μmol/L. Membranes were prepared from these cells and assayed for guanylyl cyclase activity as described above (Figure 4B). One hour of exposure suppressed CNP-dependent NPR-B activity similar to that shown in Figure 4A. However, hormone-dependent cyclase activity in membranes from cells exposed to S1P overnight (18 hours) was not different from activities obtained in membranes from untreated cells, indicating that the S1P-dependent inhibition is reversible (Figure 4B). The time-dependent loss of the S1P effect is due to the desensitization of the S1P pathway and not due to degradation of S1P itself because membranes from cells incubated overnight with S1P and then treated with a second aliquot of S1P were similarly unaffected. These data indicate that the S1P-dependent inhibition of NPR-B is maintained for at least 1 hour, but desensitizes with prolonged S1P exposure.

S1P binds and activates a subset of the endothelial differentiation gene (Edg) class of G protein-coupled receptors, but S1P also has been suggested to exert direct intracellular effects. To investigate whether the S1P-dependent desensitization of NPR-B requires a cell surface receptor, we utilized a known plasma membrane impermeable agonist of Edg receptors, dihydro-S1P. Because of its increased hydrophilic nature, this compound can bind and activate the S1P receptors, but it is unable to reach the cytoplasmic face of the plasma membrane inner leaflet. Thus, if S1P is mediating its effects directly on the intracellular guanylyl cyclase domain of NPR-B, the dihydro-S1P variant should not substitute. Alternatively, if S1P is inhibiting NPR-B via activation of a cell surface receptor, then the dihydro variant should be equally effective. When we treated A10 cells with 10 μmol/L S1P or dihydro-S1P for 30 minutes, prepared crude membranes, and assayed for guanylyl cyclase activity, we found that S1P and dihydro-S1P treatment resulted in equivalent inhibition of NPR-B (Figure 5A). We next tested whether the inhibition results from a direct interaction of S1P with NPR-B, by incubating A10 crude membranes with 10 μmol/L S1P, buffer, or MgCl2 as a positive control for in vitro desensitization.20 We found that MgCl2 inhibited CNP-dependent NPR-B cyclase activity, presumably by activating a metal-dependent protein phosphatase, but that S1P was ineffective (Figure 5B). Together, these results support a surface receptor-mediated, not a direct intracellular, mechanism of action.

### S1P-Dependent Inhibition of NPR-B Requires Calcium Elevations but not Protein Kinase C Activation

Edg receptors couple to several members of the G-protein family, including Gs. The subsequent activation of phospholipase CB, production of inositol trisphosphate, and increases in intracellular calcium occur through both pertussis toxin-sensitive and -insensitive pathways.21 This is of particular importance because both the synthetic protein kinase C (PKC) activator phorbol myristate 13 acetate (PMA)22 and pharmacological increases in intracellular calcium have been shown to inhibit NPR-B.14 Therefore, we tested the requirement for phorbol ester-sensitive PKC isozymes in the S1P-dependent desensitization of NPR-B (Figure 6). Pretreatment of A10 cells with 100 nM PMA for 30 minutes resulted in crude membranes containing approximately 40% of the CNP-dependent guanylyl cyclase activity obtained in membranes from untreated cells. Likewise, hormone-dependent
activity, assayed in membranes isolated from cells treated with 10 μmol/L S1P, was reduced to similar levels. Neither treatment affected detergent-dependent activities, again indicating that the activity losses were not due to receptor degradation. To block the activation of PKC, the cells were incubated for 1 hour with 1 μmol/L GF-109203X, a cell permeable inhibitor of the “classic” (α1, β1, β2, δ, ε, and γ) PKC isoforms, prior to the addition of S1P or PMA. As previously reported, pretreatment with the PKC inhibitor completely blocked the PMA-dependent desensitization. In contrast, the PKC inhibitor failed to prevent the majority of S1P-dependent desensitization of NPR-B, indicating that the GF-109203X sensitive forms of PKC are not involved in this process (Figure 6).

To test the involvement of the IP3/calcium arm of the PLC pathway, we used the general calcium ionophore ionomycin to nonspecifically elevate intracellular calcium concentrations and the membrane permeable calcium chelator BAPTA-AM to sequester free intracellular calcium. In this experiment, A10 cells were treated in the presence or absence of 75 μmol/L BAPTA-AM prior to incubation with or without 10 μmol/L S1P or 1 μmol/L ionomycin, then crude membranes were prepared and assayed for guanylyl cyclase activity. Exposure of cells to BAPTA alone slightly, but reproducibly increased NPR-B activity, whereas ionomycin decreased NPR-B activity (Figure 7). As expected, pretreatment...
ment of cells with BAPTA before ionomycin exposure completely blocked the ionomycin-dependent desensitization of NPR-B, verifying the ability of BAPTA to block a calcium-mediated inhibition of NPR-B. Interestingly, although BAPTA-AM completely blocked the ionomycin-dependent inhibition, it only blocked slightly more than half (56%) of the S1P-dependent effect (Figure 7). The remaining S1P-dependent decrease in activity in the presence of BAPTA-AM was statistically significant with \( P<0.01 \) when compared with control levels (Figure 7). These data are consistent with the notion of calcium elevations playing an important role in the S1P-dependent desensitization, but suggest that complete inhibition requires additional components.

**Discussion**

In this report, we have demonstrated that S1P antagonizes CNP signaling in both fibroblasts and vascular smooth muscle cells. We found that (1) S1P stimulates reductions in whole cell CNP-dependent cGMP concentrations, (2) the reductions result from decreased NPR-B guanyllyl cyclase activity in a process that does not require receptor degradation or activation of protein kinase C, (3) the inhibition has an IC\(_{50}\) of 0.08 \( \mu \text{mol/L} \) in NIH3T3 cells and 0.3 \( \mu \text{mol/L} \) in A10 cells, (4) the inhibition is a cell surface receptor mediated process, (5) the inhibition is rapid, having a \( t_{1/2} \) of between 2.5 and 5 minutes, (6) the process is reversible, and (7) the full inhibitory effect of S1P requires elevated intracellular calcium concentrations.

S1P is a novel phospholipid signaling molecule that has been recently implicated in the regulation of diverse cellular processes in a wide range of cell types, but appears to be especially important in smooth muscle.\(^{24}\) S1P is produced and released mainly by activated platelets and is found in serum at concentrations around 0.5 \( \mu \text{mol/L} \).\(^{25}\) Many of its biological effects are elicited through a subset of the Edg family of serpentine receptors that are coupled to a variety of G-proteins. However, some effects appear to be mediated directly by S1P in a receptor-independent manner.\(^{21}\) Our observation that a version of S1P that cannot cross the plasma membrane desensitizes NPR-B to the same level as authentic S1P is consistent with a receptor-mediated process. Furthermore, in experiments where S1P was directly added to membrane preparations, no inhibition was observed. These results suggest intact cell architecture is required for S1P to exert its effect and are not consistent with a direct intracellular mode of action.

It is interesting that elevated intracellular calcium concentrations do not account for all of the S1P-dependent inhibition because we have previously shown that BAPTA-AM completely blocks the ability of arginine-vasopressin to inhibit NPR-B in these same cells.\(^{14}\) There are 2 observations suggesting calcium alone is not sufficient to explain all of the S1P effect. First, as previously stated, BAPTA-AM was only able to block about half of the S1P-dependent inhibition (Figure 7). Secondly, although ionomycin elicits higher calcium elevations than S1P (data not shown), it only inhibited NPR-B to about half the level observed with S1P. Hence, these data suggest that an additional factor or process is required for the complete inhibitory response. One obvious class of molecules that is activated by S1P, and that has been shown to inhibit NPR-B, is the classic forms of protein kinase C. However, we saw very little effect of a protein kinase C blocker on the S1P-dependent inhibition. Importantly, we are sure that the inhibitor was working because it completely blocked the phorbol ester-dependent inhibition of NPR-B. Thus, it appears the classic GF-102390X-inhibitable protein kinase C isozymes are not playing a major role in this response.

**Perspectives**

To our knowledge, this is the first study describing cross-talk between S1P and cGMP. Recently, pathological roles for S1P in the cardiovascular system were identified. S1P was found to stimulate calcium elevations, vasoconstriction, as well as vascular smooth muscle cell proliferation and migration.\(^{24,26}\) S1P has also been implicated in atherosclerosis.\(^{27}\) The ablative of the primary receptor for S1P, Edg1/S1P\(_{1}\), in mice results in vascular maturation and vessel formation defects\(^{28}\) and was recently shown to be induced in neointima restenosis,\(^{29}\) a process where vascular blockage occurs at a site of earlier reconstruction that is caused by the migration of medial smooth muscle cells. Importantly, this process is inhibited by CNP.\(^{30}\) Thus, it is now exciting to speculate on the potential importance of the cross-talk between the CNP and S1P pathways with regard to pathological conditions such as wound healing, restenosis, atherosclerosis, and hypertension. Since activated platelets release S1P, the exposure of vascular smooth muscle cells to S1P on vascular injury may stimulate both their migration and proliferation, thereby leading to restenosis. On the other hand, CNP-dependent cGMP elevations antagonize these processes and inhibit restenosis. Similarly, S1P is present in high density and low-density lipoproteins that accumulate in atherosclerotic lesions,\(^{31}\) whereas CNP inhibits oxidized LDL-dependent coronary artery smooth muscle migration.\(^{32}\)

The time course of the interaction between S1P and CNP signaling pathways markedly affects the response. In the short term (Figure 4, minutes), S1P inhibits NPR-B (Figure 4A). However, after longer exposures (hours to days), the effect is markedly blunted or even absent (Figure 4B). This is completely consistent with a report by Brown and colleagues who showed that balloon angioplasty-induced CNP expression is temporally correlated with the subsiding of vascular smooth muscle cell proliferation despite the absence of endothelium.\(^{9}\) Together, these data suggest that acute exposure to S1P inhibits the repressive effect of the CNP/NPR-B system on vascular smooth muscle proliferation, but with time CNP expression is increased and NPR-B becomes refractory to S1P, allowing the restoration of the antiproliferative response. We hypothesize that the interplay between these two systems may determine the degree of intimal thickening and overall vascular injury. Further investigation into the cross-talk between natriuretic peptide and Edg/S1P receptor mediated signaling pathways may yield novel insights into the development, treatment, and prevention of vascular and other diseases.
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