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Differential regulation of NPR-B/GC-B by protein kinase c and calcium

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Abstract

C-type natriuretic peptide (CNP) activation of the guanylyl cyclase-linked natriuretic peptide receptor-B (NPR-B) stimulates vasorelaxation and bone growth. Hormones and phorbol esters (PMA) inhibit NPR-B in calcium and protein kinase c-dependent manners, respectively. Here, we characterize the kinetic properties of NPR-B in membranes from cells exposed to PMA, the calcium ionophore, ionomycin, or sphingosine-1-phosphate (S1P). PMA and ionomycin primarily increased the K_m and decreased the V_{max} of NPR-B for GTP, respectively, whereas S1P caused modest changes in both parameters. PMA and S1P treatment increased the EC_{50} for CNP activation by eight- and three-fold, whereas ionomycin was ineffective. All three agents caused NPR-B dephosphorylation, but the basis for the loss of phosphate differed between treatments. In vitro phosphorylation of NPR-B in membranes was markedly diminished by prior whole cell PMA or S1P exposure, whereas ionomycin pretreatment had no effect. The involvement of the known phosphorylated residues in each process was tested with a mutant receptor containing glutamates substituted for these sites. While the effect of PMA was lost on this receptor, the effects of S1P and ionomycin were only partially blocked. Our data suggest that the molecular bases for PMA- and calcium-dependent inhibition of NPR-B are unique. The former results from reduced phosphorylation of a known site and primarily affects the affinity of NPR-B for CNP and GTP. The latter is associated with reductions in maximal velocities by a mechanism that does not involve inhibition of NPR-B phosphorylation and requires a process in addition to the dephosphorylation of the known sites.

Keywords: ANF; NPR-B; Guanylyl cyclase; cGMP; PKG; GC-A

1. Introduction

C-type natriuretic peptide (CNP) is one of three structurally similar but genetically distinct hormones that constitute the mammalian natriuretic peptide family. It is most highly expressed in vascular endothelial cells [1], brain [2] and bone tissue [3–7]. Circulating factors, such as tumor necrosis factor-α [1] or transforming growth factor-β [1] stimulate vascular endothelial CNP synthesis and secretion, which induces relaxation of adjacent vascular smooth muscle cells and inhibits their proliferation. In chondrocytes, CNP stimulates long bone growth [7]. Homozygous loss of function mutations in the receptor for CNP causes severe dwarfism in mice and humans [8–10].

The signaling effects of CNP are elicited through the activation of natriuretic peptide receptor-B (NPR-B), also known as guanylyl cyclase B or GC-B [11]. NPR-B contains an extracellular ligand binding domain, a single membrane-spanning region and intracellular kinase homology, dimerization and guanylyl cyclase catalytic domains. Under basal conditions, NPR-B is phosphorylated on five known sites (Ser^{513}, Thr^{516}, Ser^{518}, Ser^{523}, and Ser^{526}) [12] within its kinase homology domain. The mutation of any known phosphorylation site to alanine decreases receptor activity and the mutation of all known sites to alanine yields a receptor that is unresponsive to ligand [12]. Chronic exposure of NPR-B to CNP in whole cells causes a time-

Abbreviations: ANP, atrial natriuretic peptide; BNP, b-type natriuretic peptide; CNP, C-type natriuretic peptide; KHD, kinase homology domain; AVP, arginine-vasopressin; NPR-A, natriuretic peptide receptor A; NPR-B, natriuretic peptide receptor B; S1P, sphingosine-1-phosphate; PMA, phorbol 12-myristate 13-acetate

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dependent decrease in activity (homologous desensitization) that correlates with receptor dephosphorylation [13].

NPR-B and NPR-A are dephosphorylated and inhibited pharmacologically by phorbol ester activation of protein kinase C (PKC) [14,15]. However, unlike the global dephosphorylation associated with homologous desensitization, phorbol ester-dependent inhibition is characterized by the specific dephosphorylation of a single residue, Ser^{523} within NPR-B [15]. Recent data indicate that physiologic signaling molecules, such as platelet-derived growth factor (PDGF) [16,17], arginine-vasopressin (AVP) [18], sphingosine-1-phosphate (S1P) [19,20], lysophosphatidic acid (LPA) [16], gonadotrophin hormone-releasing hormone [21] as well as fetal bovine serum [16,17] and hyperosmolarity [22], inhibit NPR-B in various cell types.

Because of the abundance of prior data demonstrating phorbol ester (PMA)-induced heterologous desensitization, it was initially speculated that the vasoactive agent-dependent inhibition of NPR-B results solely from the activation of PKC. Surprisingly, we discovered that AVP, S1P, and LPA-dependent desensitization does not require PKC isoforms that are activated and downregulated by PMA. Instead, these pathways involve increased calcium concentrations because a general calcium ionophore mimics and an intracellular calcium chelator blocks part (S1P) or all (AVP and LPA) of the inhibitory action of these agents, whereas PKC inhibition is without effect [16,18,20]. Hence, there are at least two independent mechanisms for the heterologous regulation of NPR-B activity, one that requires PKC activation and one that requires calcium elevations.

Here, we asked whether the PKC- and calcium-dependent inhibitory pathways utilized the same or different mechanisms. Specifically, we measured the effects of PKC activation and calcium elevations on the kinetic properties and phosphorylation status of NPR-B. We used ionomycin and PMA to pharmacologically elevate intracellular calcium or PKC activity, respectively. We used S1P as an example of a physiologic agent that inhibits NPR-B [20]. We found that PMA and ionomycin primarily increased the K_m and decreased the V_{max} of NPR-B for GTP, respectively, whereas S1P affected both parameters. In addition, all three agents decrease the phosphate content of NPR-B. PMA and S1P treatments inhibit the ability of NPR-B to be phosphorylated, whereas ionomycin does not affect the rate of NPR-B phosphorylation. Finally, we directly tested the requirement of dephosphorylation in the desensitization process by using a NPR-B mutant that had all its known phosphorylation sites mutated to glutamate. In contrast to the effects of PMA, which are completely lost on this mutant receptor, we found that the S1P- and ionomycin-dependent inhibition is only partially blocked. These data indicate for the first time that PMA and S1P-, but not ionomycin, -dependent NPR-B dephosphorylation results from reduced receptor phosphorylation and provide the first evidence of a novel NPR-B regulatory mechanism.

2. Materials and methods

2.1. Materials

Rat CNP-22, GF-109203X, phobol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma–Aldrich (www.sigma-aldrich.com). Sphingosine-1-phosphate was purchased from Avanti Polar Lipids (www.avantilipids.-com).

2.2. Cell culture and preparation of crude membranes

293T-NPR-B cells were maintained as previously described [23]. Crude membranes were prepared and resuspended in phosphatase inhibitor buffer as previously described [18]. For the kinetic studies, membranes were frozen in a dry ice/ethanol bath and stored at −80 °C until used.

2.3. Guanylyl cyclase assays

Guanylyl cyclase assays were performed and cGMP was purified as previously described [18]. In the kinetic experiments, increasing concentrations of Mg-GTP were added to cocktails containing 1 mM ATP, 5 μM CNP and 5 mM $MgCl_2$. Experiments with increasing CNP contained 1 mM ATP, 1 mM GTP and 5 mM $MgCl_2$. Between 15 and 25 μg of crude membranes were assayed for 2 min at 37 °C. Reactions were linear for up to 2 min and were started by the addition of cocktails to crude membranes in a final volume of 100 μl.

2.4. Metabolic labeling

293T-NPRB cells were washed twice with phosphate and serum-free DMEM and then incubated overnight in 5 ml of the same media containing 1 mCi/ml [^{32}P]-orthophosphate. After exposure to the various treatments, NPR-B was immunoprecipitated, fractionated by SDS–PAGE and blotted to polyvinylidene diflouride (Immobilon P) membrane as previously described [24]. The membrane was then exposed to a phosphoimaging screen, and the ^{32}P-content was quantitated using ImageGauge software (Fuji).

2.5. Immunoblot analysis

NPR-B was purified, blotted to an Immobilon membrane and probed with rabbit polyclonal antiserum that recognizes the last 10 carboxyl-terminal amino acids of NPR-B as previously described [18].

2.6. In vitro kinase assay

Twenty microliter of each membrane preparation in phosphatase inhibitor buffer (2 mg/ml) was supplemented with 40 μl of kinase buffer (50 mM HEPES, pH 7.4;

100 mM NaCl; 20 mM NaH_2PO_4; 12.8 mM $MgCl_2$; 2 μM microcystin; 1X complete EDTA free protease inhibitor cocktail from Sigma), 20 μl of 2 mCi/ml [^{35}S]ATPγS and 20 μl of 0.5 mM ATP for a final ATP concentration of 0.1 mM. Samples were incubated at 37 °C for the times indicated. Reactions were terminated with 1 ml of ice-cold stop buffer (50 mM Hepes, pH 7.4; 10% glycerol; 100 mM NaCl; 5 mM EDTA; 1 μM microcystin and 1X EDTA free protease inhibitor cocktail). Tubes were centrifuged at 4 °C for 10 min at 20,000 × *g*, and the pellets were used for the receptor purification by immunoprecipitation and SDS–PAGE as described above. The radioactivity associated with NPR-B was measured with a Fuji FLA 5000 laser scanner (Fuji Photo Film Co. Ltd., Tokyo, Japan) and the ^{35}S-content was quantitated using ImageGauge software (Fuji Photo Film Co. Ltd., Tokyo, Japan).

2.7. Data analysis and statistics

The data were graphed and analyzed with GraphPad Prism 4 for the MacIntosh (http://www.graphpad.com/). K_m and V_{max} values were determined by nonlinear analysis to Michaelis–Menten kinetics using the equation $y = (V_{max}x)/(Km + x)$. CNP EC_{50}'s were estimated using variable dose-response nonlinear analysis. The data were normalized to the percent of the maximum experimental activity measured at 7 μM CNP and then fit to the equation:

$$y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{((\log \text{EC}_{50} - x)\text{HillSlope})}}$$

where "Bottom" is the best-fit basal activity and "Top" is the activity at 7 μM CNP. Statistical significance in all cases except Fig. 7 was determined by performing one-way ANOVA followed by Dunnett's multiple comparison analysis and was defined as a *p*-value less than 0.05 unless otherwise indicated. The data in Fig. 7 were first normalized to the percent of control values to correct for differences in receptor expression between experiments. These data were then analyzed by one-way ANOVA followed by Bonferroni's multiple comparison analysis.

3. Results

3.1. Phorbol esters, ionomycin and S1P inhibit NPR-B in 293T-NPR-B Cells

To investigate the mechanisms of NPR-B desensitization by PKC activation and calcium elevations, we first validated the desensitization in a 293T cell line stably expressing NPR-B (293T-NPR-B cells). We tested the ability of PMA, S1P and ionomycin to inhibit NPR-B guanylyl cyclase activity in these cells. S1P is a hormone which elevates calcium in 293T cells (data not shown) and ionomycin is a pharmacological calcium ionophore in 293 [25] and other cell lines. We prepared crude membranes

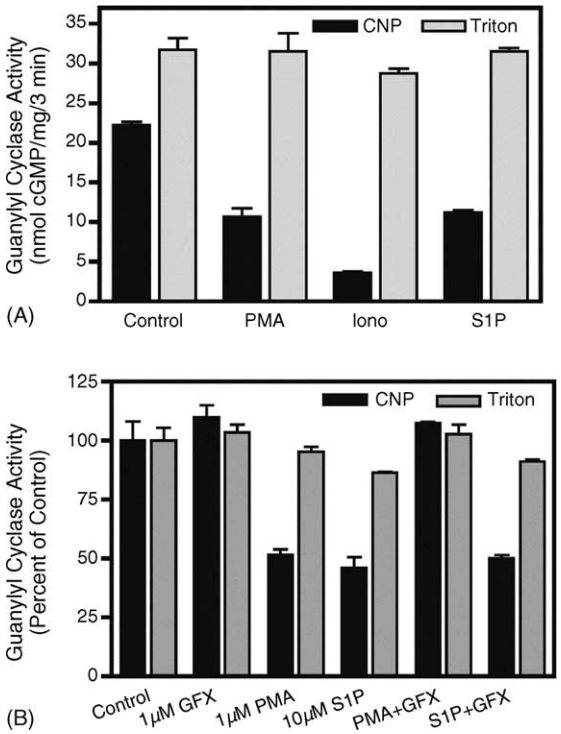

Fig. 1. Calcium and PKC-dependent NPR-B inhibitory pathways are intact in 293T cells. (A) 293T-NPR-B cells were treated in the presence or absence of 1 μM PMA, 1 μM ionomycin, and 10 μM S1P. Crude membranes were prepared and then assayed for CNP- (1 μM CNP, 1 mM ATP, 5 mM $MgCl_2$) or detergent- (1% Triton X-100, 5 mM $MnCl_2$) dependent guanylyl cyclase activity for 3 min. Each treatment was measured in triplicate. Bars within columns represent the S.E.M. (B) 293T-NPR-B cells were pretreated with or without 1 μM GFX for 1 h and then incubated in the presence or absence of 1 μM PMA or 10 μM S1P for an additional 30 min. Crude membranes were prepared and then assayed for CNP- (1 μM CNP, 1 mM ATP, 5 mM $MgCl_2$) or detergent- (1% Triton X-100, 5 mM $MnCl_2$) dependent guanylyl cyclase activity for 3 min. Error bars within each column indicated the range between two values. Control values are 36.5 nmol/mg/3 min for CNP-dependent activation and 67.44 nmol/mg/3 min for detergent-dependent activation. This experiment is representative of three.

from cells incubated with or without 10 μM S1P or 1 μM PMA for 30 min, or 1 μM ionomycin for 12 min (longer exposure to ionomycin caused cell detachment) and measured guanylyl cyclase activity in the presence of 1 μM CNP, 1 mM ATP and 5 mM $MgCl_2$ or 1% Triton X-100 and 5 mM $MnCl_2$ (Fig. 1). The former treatment measures hormone-dependent activity, whereas the detergent treatment maximally activates NPR-B independently of CNP and indicates the total amount of NPR-B protein present in the membrane preparations. Importantly, all treatments significantly inhibited CNP-dependent, but not detergent-dependent, guanylyl cyclase activity in membranes prepared from these cells (Fig. 1A), similar to their effects in A-10 [18,20] and NIH3T3 [16,22] cell lines. Furthermore, to test the involvement of PKC in the S1P-dependent desensitization of NPR-B, we preincubated 293T-NPR-B cells with or without the selective PKC inhibitor, GF-109203X, for 1 h before exposure to S1P or PMA. We found that while GF-109203X completely blocked the

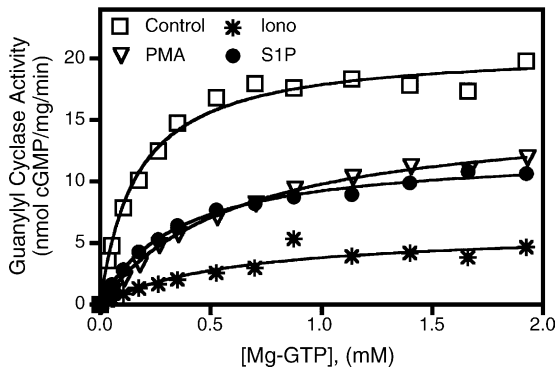

Fig. 2. Calcium- and PKC-dependent mechanisms of NPR-B inhibition are kinetically distinct. 293T-NPR-B cells were treated in the presence or absence of 10 μM S1P, 1 μM PMA, or 1 μM ionomycin for 30, 30 or 12 min, respectively. Crude membranes were prepared and assayed for CNP-dependent guanylyl cyclase activity (5 μM CNP, 1 mM ATP, 1 mM $MgCl_2$) with increasing concentrations of Mg-GTP for 2 min. Data are representative of four independent experiments.

PMA-dependent desensitization, it had no effect on the ability of S1P to inhibit NPR-B (Fig. 1B). This indicates that an alternative pathway is responsible for the S1P-dependent inhibition of NPR-B in the 293T-NPR-B cells. Thus, both the PKC-dependent and calcium-dependent NPR-B desensitization pathways occur in the NPR-B stably transfected 293T cell line.

3.2. Ionomycin and PMA inhibit NPR-B activity through kinetically distinct mechanisms

Next, we compared the kinetic properties of NPR-B desensitized by PKC and calcium-dependent pathways by measuring cyclase activity in membranes from 293T-NPR-B cells that had been treated in the presence or absence of 1 μM PMA, 1 μM ionomycin and 10 μM S1P as described above. To our knowledge, this is the first report to describe the kinetic properties of NPR-B. The Michaelis constants (K_m values) and maximal velocities (V_{max}) of NPR-B for Mg-GTP were determined for 2 min at 37 °C (Fig. 2). Average kinetic constants of four experiments are shown in Table 1. The average K_m of NPR-B in crude membranes isolated from resting cells was 180 μM. S1P and ionomycin modestly increased the K_m, 1.7- and 2-fold, respectively, whereas PMA treatment increased the K_m 3.3-fold, which was statistically significant with a p-value of <0.01 (Table 1 and Fig. 2). The average V_{max} of NPR-B in membranes isolated from resting cells was 23.5 nmol of cGMP/mg protein/min. Ionomycin and S1P exposure decreased the average V_{max} to 33 and 52% of velocity obtained from untreated cells, respectively. In contrast, the V_{max} measured in membranes from cells exposed to PMA was reduced to 77% of control values. This difference was not statistically significant. Interestingly, PMA, ionomycin or S1P treatments resulted in similar reductions in the apparent catalytic efficiency of NPR-B, reducing the V_{max}/K_m ratios to 23, 21 and 31% of values obtained in membranes from control cells (Table 1).

Table 1
Kinetic constants for NPR-B

	Control	PMA	Ionomycin	S1P
K_m (μM)	180 ± 6	593 ± 19**	363 ± 95	314 ± 11
V_{max} (nmol/mg/min)	23.5 ± 5	18.1 ± 3	7.7 ± 0.7**	12.2 ± 1.5*
V_{max}/K_m (μL/mg/min)	130 ± 25	30 ± 5	27 ± 7	40 ± 5
CNP EC_{50} (nM)	39 ± 8	305 ± 52**	87 ± 40	128 ± 23

Crude membranes prepared from 293T-NPR-B cells treated with or without 1 μM PMA, 1 μM ionomycin or 10 μM S1P were assayed for guanylyl cyclase activities to determine kinetic parameters as in Fig. 2. Catalytic efficiency was estimated by V_{max}/K_m. Data are four individual trials ± S.E.M.

* Statistical significance is indicated by p-value <0.05 as determined by one-way ANOVA analysis followed by Dunnett's multiple comparison to control values.

** Statistical significance is indicated by p-value <0.01 as determined by one-way ANOVA analysis followed by Dunnett's multiple comparison to control values.

We also assessed whether the PMA, ionomycin or S1P treatments alter the effective CNP concentration required to activate NPR-B to half its maximum level, a value commonly referred to as the EC_{50}. The same membranes used in the kinetic experiments were assayed for guanylyl cyclase activity with increasing concentrations of CNP (Fig. 3). We observed no statistically significant difference in the EC_{50} for CNP activation of NPR-B in membranes from control cells versus membranes from cells exposed to ionomycin (Fig. 3 and Table 1). In contrast, PMA treatment shifted the CNP EC_{50} from 39 ± 8 to 305 ± 52 nM or approximately eight-fold. S1P treatment resulted in a more modest three-fold increase in the EC_{50}. Thus, the kinetic and the CNP dose-response data are consistent with a model where the calcium and PKC-dependent pathways inhibit NPR-B through different mechanisms.

3.3. Ionomycin, PMA and S1P stimulate NPR-B dephosphorylation

To determine whether physiologic or pharmacologic elevations of intracellular calcium by S1P or ionomycin, respectively, decrease the phosphorylation state of NPR-B, we metabolically labeled NPR-B with ^{32}P-orthophosphate overnight. The cells were then treated with 1 μM PMA, 10 μM S1P, or 1.5 μM ionomycin for 25, 25, or 10 min, respectively. NPR-B was then purified from cell lysates by immunoprecipitation, fractionation by SDS–PAGE, and blotting to an Immobilon membrane. The amount of ^{32}P associated with the receptor was visualized by autoradiography (Fig. 4, top panel). All three treatments reduced the ^{32}P-content of NPR-B, but the most dramatic loss was associated with the ionomycin treatment, consistent with ionomycin having the greatest inhibitory effect on guanylyl cyclase activity (Fig. 1A). The decreased ^{32}P-content was not due to receptor degradation because immunoblot analysis performed on the

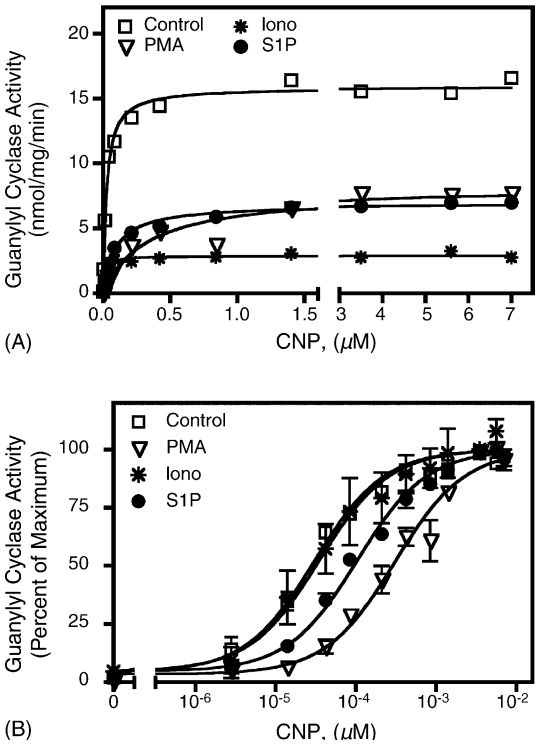

Fig. 3. PMA, but not ionomycin, treatment increases the EC_{50} for CNP activation of NPR-B. 293T-NPR-B cells were treated in the presence or absence of 10 μM S1P, 1 μM PMA, or 1 μM ionomycin as described in Fig. 2. Crude membranes were prepared and assayed for CNP-dependent guanylyl cyclase activity (1 mM GTP, 1 mM ATP, 1 mM $MgCl_2$) with increasing concentrations of CNP. Data are representative of three individual experiments. A. Absolute values are plotted. Note the change in units on the broken *x*-axis. (B) Data are normalized to the percent of maximum activity for each treatment to indicate differences in the EC_{50} for CNP.

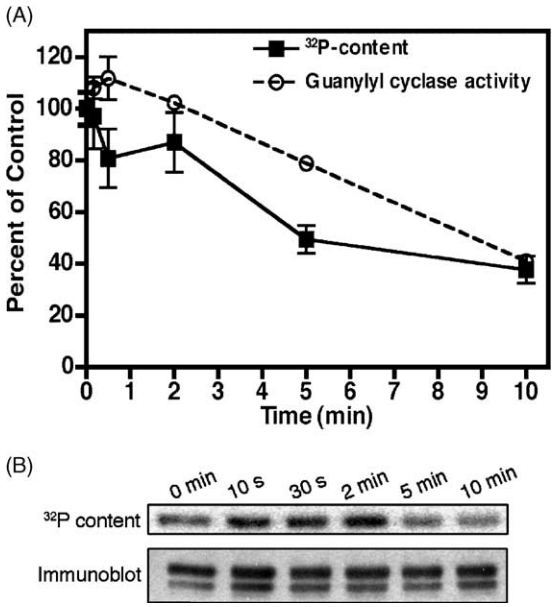

Fig. 5. S1P treatment causes time-dependent decreases in NPR-B $^{32}PO_4$-content and guanylyl cyclase activity. (A) $^{32}PO_4$-labeled or unlabelled 293T-NPR-B cells were treated with 10 μM S1P for the indicated periods of time. Whole cell lysates were prepared from the labeled cells, and NPR-B was immunoprecipitated, separated by SDS-PAGE, blotted to membrane and visualized by autoradiography. Quantitation of the ^{32}P associated with NPR-B was performed by phosphoimaging analysis and represents the average of six independent determinations ± S.E.M. (squares). Crude membranes were prepared from the unlabelled cells and assayed for CNP-dependent guanylyl cyclase activity for three min. The average control value for guanylyl cyclase activity is 43.7 ± 10.8 nmol cGMP/mg/3 min. Values are the mean of six independent replicates ± S.E.M. (circles). (B) A representative autoradiograph (top) and its corresponding immunoblot (bottom) of $^{32}PO_4$- labeled samples described in (A). These data are representative of six replicates.

same membrane indicated similar amounts of receptor were present in each sample (Fig. 4, bottom panel).

3.4. S1P-dependent inhibition of NPR-B activity correlates with receptor dephosphorylation

Next, we asked whether the calcium-dependent NPR-B inhibition is temporally correlated with receptor dephosphorylation. We chose to continue these studies with S1P as opposed to ionomycin for two reasons. First, unlike ionomycin, S1P is a physiological calcium elevator. Secondly, ionomycin treatment made the cells float, which severely compromised the accuracy of experiments involving multiple time points.

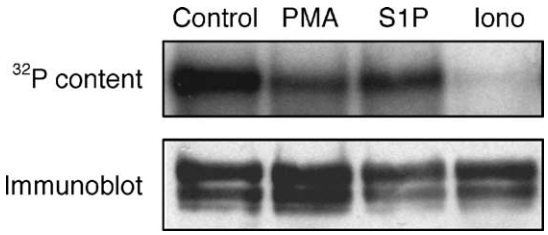

Fig. 4. Ionomycin, S1P and PMA treatment decreases the $^{32}PO_4$ content of NPR-B. $^{32}PO_4$-labeled 293T-NPR-B cells were treated with or without 5 μM PMA (25 min), 10 μM S1P (25 min) or 1.5 μM ionomycin (12 min). Whole cell lysates were prepared from the labeled cells, and NPR-B was immunoprecipitated, separated by SDS-PAGE, blotted to membrane and visualized by autoradiography (^{32}P-content). The same membrane was probed with a specific antibody against NPR-B (immunoblot). Data are representative of three individual experiments.

Cells were metabolically labeled as above and treated with 10 μM S1P for the indicated periods of time. The amount of ^{32}P associated with purified NPR-B was visualized by autoradiography and quantitated by phosphoimaging analysis (Fig. 5). Although a general dephosphorylation trend was apparent upon short S1P exposures, no statistically significant changes in the ^{32}P-content of NPR-B were observed after 10 s, 30 s, or 2 min S1P exposures compared to NPR-B isolated from untreated cells (Fig. 5A). The time-dependent dephosphorylation roughly paralleled the reductions in CNP-dependent guanylyl cyclase activity observed in membranes prepared from cells treated in the same manner. The decreases in activity were statistically significant after 5 min (p-value <0.01) and 10 min (p-value <0.01). In fact, after a 10 min S1P incubation, the reductions in NPR-B ^{32}P-content and CNP-dependent guanylyl cyclase activities were equivalent. The decreased ^{32}P-content and enzymatic activity were

not due to receptor degradation because there was no change in the amount of receptor in each sample as indicated by immunoblot (Fig. 5B). This experiment demonstrates that a physiologic calcium elevating agent causes temporally correlated dephosphorylation and desensitization of NPR-B.

3.5. PMA and S1P inhibit the in vitro phosphorylation of NPR-B

Since de Lean and colleagues have previously reported that ANP-dependent dephosphorylation of NPR-A results primarily from reduced phosphorylation [26], not increased dephosphorylation of NPR-A (possibly due to decreased kinase activity, not increased phosphatase activity); we investigated whether reduced phosphorylation of NPR-B explains its dephosphorylation in response to PMA, ionomycin and S1P exposure. Crude membranes were isolated from cells exposed to the desensitizing agents or media alone as described in Fig. 1. The membranes were then resuspended in kinase buffer containing ^{35}S-γ-ATP and incubated at 37 °C for 1, 3 or 5 min. ^{35}S-γ-ATP was used as the kinase substrate in an effort to reduce NPR-B dephosphorylation during the kinase assay because thiophosphorylated proteins are dephosphorylated less efficiently than non thiophosphorylated proteins. Importantly, because PMA, S1P and ionomycin exposure results in dephosphorylated receptors at the time of membrane purification (Fig. 4), thiophosphate incorporation is a reasonable measure of the ability of NPR-B to be phosphorylated. The reactions were terminated by the addition of a buffer containing EDTA, and NPR-B was purified by sequential immunoprecipitation and fractionation by SDS–PAGE. The amount of ^{35}S-phosphate associated with NPR-B was visualized by autoradiography and quantitated on a phosphorimager (Fig. 6).

Phosphorylation of NPR-B in all membrane preparations was linear during the assay period. Interestingly, the rate of phosphorylation was markedly diminished in the membranes from cells treated with PMA, and to a lesser extent, in membranes from cells exposed to S1P. In contrast, the phosphorylation of NPR-B from ionomycin treated cells was unaffected, even though ionomycin treatment resulted in the most dramatic dephosphorylation of NPR-B in whole cells (Fig. 4A). The reduced ^{35}S-signal was not due to decreased protein levels because western blot analysis indicated that the same amount of NPR-B was present in each lane (data not shown). These data indicate that the reduced phosphorylation state of NPR-B observed in cells exposed to PMA and S1P, but not from ionomycin, results at least in part, from a decreased ability of the receptor to be phosphorylated.

To investigate the role of the known phosphorylation sites in the desensitization process, we tested whether a version of NPR-B containing glutamate substitutions at all five of its known phosphorylation sites [15] could be

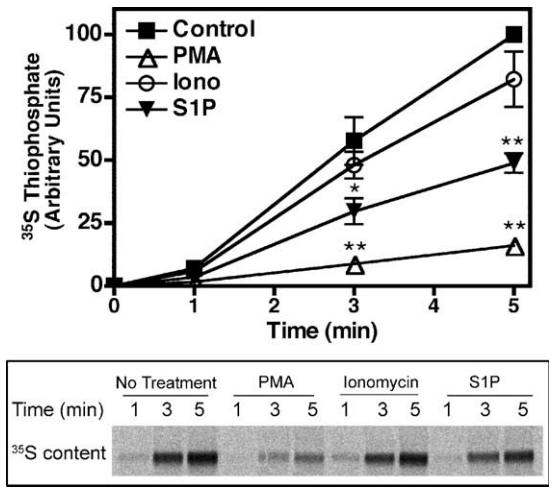

Fig. 6. PMA and S1P, but not ionomycin, exposure decreases the ability of NPR-B to be phosphorylated in crude membranes. 293T-NPR-B cells were treated with medium alone, 1 μM PMA, 1 μM ionomycin or 10 μM S1P for 30, 12 and 30 min, respectively. Crude membranes were suspended in kinase buffer containing ^{35}S-γ-ATP and incubated at 37 °C for the indicated periods of time. NPR-B was purified by immunoprecipitation and fractionation by SDS-PAGE. The amount of ^{35}S-phosphate associated with the receptor was visualized by autoradiography and quantitated on a phosphorimager. Upper panel. Values are the mean ± S.E.M. (N = 4). * and ** indicate statistical significance with a p-value <0.05 and <0.01, respectively. The bottom panel depicts a representative autoradiograph from a single experiment.

inhibited by S1P and ionomycin. 293T cells were transiently transfected with wild type NPR-B or NPR-B-5E. Two days later, they were incubated with or without 1 μM PMA, 10 μM S1P or 1 μM ionomycin for 30, 30 or 12 min, respectively. PMA, S1P and ionomycin reduced cyclase activity in membranes from cells expressing the wild type receptor to 52 ± 3, 35 ± 3, and 42 ± 4% of control activity, respectively (Fig. 7). In contrast, exposure of cells transfected with NPR-B-5E to PMA increased

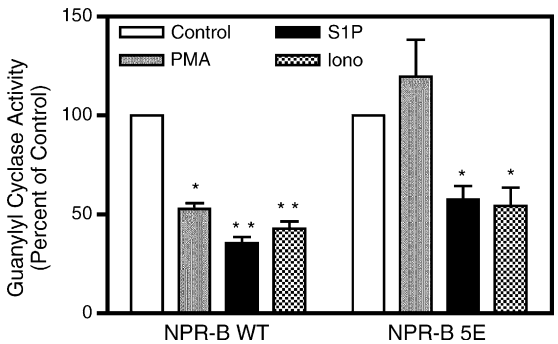

Fig. 7. NPR-B-5E inhibition in response to S1P and ionomycin is blunted. 293T cells were transiently transfected with wild type NPR-B or NPR-B-5E. Between 24 and 48 h post transfection, serum-starved cells were treated in the presence or absence of PMA (30 min), S1P (30 min) or ionomycin (10 min) as described in Fig. 2. Crude membranes were prepared and then assayed for CNP-dependent guanylyl cyclase activities for 3 min. The average control values were 4.5 ± 1.2 (WT) and 0.93 ± 2 (5E) nmol/mg/3 min. Values are the mean of four–six replicates (±S.E.M.). * and ** indicate statistical significance when compared to their respective control values with a p-value <0.01 and <0.001, respectively.

cyclase activity in membranes from these cells. S1P and ionomycin treatments decreased NPR-B-5E activity to 57 ± 6 and $55 \pm 9\%$ of the control, which is a smaller reduction than was observed for the wild type receptor, but still statistically significant at a p-value of less than 0.01 (Fig. 7). Because the wild type NPR-B is more affected by S1P and ionomycin exposure than the mutant, it suggests dephosphorylation of one or more of the known five sites plays a significant role in the desensitization of NPR-B in response to calcium. However, since the mutant receptor is still inhibited by these agents, it suggests that an additional mechanism that does not require the dephosphorylation of the known NPR-B phosphorylation sites also is involved in this process.

4. Discussion

In this report, we describe mechanistic differences in both the kinetic constants and phosphorylation status of NPR-B from cells exposed to PKC activators or intracellular calcium elevating agents. We determined that PMA and ionomycin treatment primarily increased the K_m and decreased the V_{max} of NPR-B, respectively, while S1P treatment resulted in kinetic effects associated with moderate activation of both pathways. Similarly, PMA increased the EC_{50} for CNP activation of NPR-B by eight-fold, whereas, S1P increased it by three-fold and ionomycin was ineffective. We also determined that PMA and S1P decreased the ability of NPR-B to be phosphorylated in vitro, whereas ionomycin has no effect on this process. Finally, we demonstrated that about half of the S1P and ionomycin-dependent desensitization, but none of the PMA effect, was still observed in cells expressing a mutant form of NPR-B containing glutamate substitutions at all five known phosphorylation sites.

The PMA treated samples display mixed inhibition kinetics with a greater effect on the K_m than the V_{max}. Importantly, because cellular GTP concentrations are in the 100–200 μM range, changes in K_m values due to receptor desensitization may affect intracellular cGMP levels and subsequent downstream signaling. Although PMA treatment also slightly decreased NPR-B's V_{max}, maximal velocities consistently approached control levels at higher Mg-GTP concentrations, whereas they were substantially below control levels at lower substrate concentrations (Fig. 2). These data indicate that PMA-desensitized NPR-B requires more GTP to achieve maximum velocity, suggesting a decrease in NPR-B's affinity for GTP. In contrast, ionomycin exposure had the greatest inhibitory effect on maximal velocities while only moderately increasing the K_m. S1P-dependent inhibition was mixed, showing intermediate effects on both V_{max} and K_m (Fig. 2). Interestingly, although their kinetic effects are unique, all three treatments yield receptors with approximately equivalent catalytic efficiencies as estimated by V_{max}/K_m ratios (Table 1).

The differences between treatments also extend to their effects on CNP activation of NPR-B. The most significant change in the EC_{50} for CNP occurred in response to PMA treatment (Fig. 3 and Table 1), whereas S1P was less effective and ionomycin had no effect. The almost order of magnitude shift in the EC_{50} observed in membranes from PMA treated cells suggests that PKC activation decreases the affinity of CNP for NPR-B, although this remains to be experimentally determined. One model of PMA-dependent desensitization that is consistent with these data is that the dephosphorylation of Ser^{523} causes a conformational change that not only alters the conformation of the catalytic domain, but also affects extracellular ligand binding.

The data presented in Figs. 4 and 5 clearly indicate the correlation between calcium-dependent dephosphorylation and NPR-B desensitization, which is similar to the effects of PMA treatment. However, the rates of NPR-B phosphorylation are less in membrane preparations from cells treated with PMA and S1P than those from cells treated with vehicle or ionomycin (Fig. 6). One explanation for these data is that PMA-treatment decreases the ability of NPR-B to be phosphorylated, without affecting its ability to be dephosphorylated. In contrast, ionomycin treatment appears to have the converse effect of enhancing NPR-B dephosphorylation without affecting its ability to be phosphorylated. Similar to the kinetic results, S1P-treatment yields receptors that are phosphorylated at a rate slower than receptors isolated after ionomycin-treatment, but faster than those from PMA-treated cells.

The simplest explanation for the ''mixed'' effects of S1P is that the S1P-dependent mechanism utilizes both PKC activation and calcium-dependent processes. However, the PKC inhibitor GF109203X had no effect on the S1P-dependent inhibition, while it completely blocked the PMA-dependent process (Fig. 1). Interestingly, in A10 cells BAPTA-AM only blocked about half of the S1P effect while it completely blocked the ionomycin-dependent inhibition. Thus, in these cells something in addition to calcium elevations is required for the maximal S1P effect. Unfortunately, BAPTA-AM does not appear to inhibit calcium-dependent processes in 293 cells. This may be a general feature of these cells because we have been unable to find any reports where BAPTA-AM was successfully used to inhibit a calcium-dependent process in 293 cells. Therefore, we are currently unable to quantify the relative contribution of calcium elevations to the S1P-induced inhibition in these cells. It is possible that a separate pathway is responsible for changes in the K_m and EC_{50} associated with the S1P-dependent desensitization. Although these effects are similar to those observed after PMA treatment, based on the insensitivity of this process to GF-109203X, PKC does not appear to be involved. Alternatively, it is possible that a GF-109203X insensitive form of PKC may contribute to the S1P-dependent inhibition or that

PKC is upstream of the calcium independent pathway activated by S1P.

Finally our data suggest a difference between the role of the known NPR-B phosphorylation sites in PMA and calcium-dependent desensitization of NPR-B. The NPR-B-5E data are consistent with the hypothesis that the dephosphorylation of the known phosphorylated residues does not account for the entire mechanism of desensitization by calcium elevations because both S1P and ionomycin effectively inhibit NPR-B-5E activity. Thus, it is possible that additional calcium regulated phosphorylation sites have yet to be identified that remain functional in NPR-B-5E. Alternatively, a portion of the calcium-dependent inhibition could be dephosphorylation-independent.

One assumption that we make in the interpretation of these data is that the mutant receptor is activated and deactivated similarly to the wild type receptor. In fact, we know that the mutant activity contains between 15–30% of the CNP-dependent activity of the wild-type receptor. Thus, it is possible that we are overestimating the contribution of the effect that is independent from the known phosphorylation sites on the activity of NPR-B. However, the absolute guanylyl cyclase activity that is lost in the mutant after S1P or ionomycin treatment is significant, being equivalent to approximately 10% of wild-type control activity. Unfortunately, we have no way of knowing whether the S1P and ionomycin-dependent activity loss observed in cells expressing the mutant receptor is proportional to the S1P and ionomycin-dependent desensitization measured in cells expressing the wild type receptor. Further studies are required to distinguish between these possibilities.

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