The indolocarbazole, Gö6976, inhibits guanylyl cyclase-A and -B

Jerid W Robinson¹, Xiaoying Lou² and Lincoln R Potter¹,²

¹Department of Pharmacology, University of Minnesota – Twin Cities, Minneapolis, MN, USA, and ²Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota – Twin Cities, Minneapolis, MN, USA

BACKGROUND AND PURPOSE

Atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) decrease vascular volume and pressure by activating guanylyl cyclase-A (GC-A). C-type natriuretic peptide (CNP) activation of guanylyl cyclase-B (GC-B) stimulates long bone growth. This study investigated the effects of the indolocarbazole, Gö6976, on the guanylyl cyclase activity of GC-A and GC-B as a first step towards developing small molecule regulators of these enzymes.

EXPERIMENTAL APPROACH

Whole cell cGMP concentrations or ³²P-cGMP accumulation in membrane preparations measured the effects of indolocarbazoles on the enzymatic activity GC-A and GC-B from transfected 293T or endogenously expressing 3T3-L1 cells.

KEY RESULTS

Gö6976 blocked cellular CNP-dependent cGMP elevations in 293T-GC-B cells. The $t_{1/2}$ for Gö6976 inhibition was 7 s and IC₅₀ was 380 nM. Gö6976 increased the EC₅₀ for CNP 4.5-fold, but increasing the CNP concentration did not overcome the inhibition. Half of the inhibition was lost 1 h after removal of Gö6976 from the medium. Cellular exposure to Gö6976 reduced basal and natriuretic peptide-dependent, but not detergent-dependent, GC-A and GC-B activity. Inhibition was also observed when Gö6976 was added directly to the cyclase assay. A constitutively phosphorylated form of GC-B was similarly inhibited.

CONCLUSIONS AND IMPLICATIONS

These data demonstrate that Gö6976 potently, rapidly and reversibly inhibited GC-A and GC-B via a process that did not require intact cells, known phosphorylation sites or inactivation of all catalytic sites. This is the first report of an intracellular inhibitor of a transmembrane guanylyl cyclase and the first report of a non-kinase target for Gö6976.

Abbreviations

ANP, atrial natriuretic peptide; BNP, B-type (brain) natriuretic peptide; CNP, C-type natriuretic peptide; DMEM, Dulbecco’s modified Eagle’s medium; GC-A, guanylyl cyclase-A; GC-B, guanylyl cyclase-B; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate

Introduction

Natriuretic peptides are pleiotropic factors that regulate the cardiovascular and skeletal systems (Potter et al., 2009). Humans express three structurally related but genetically distinct natriuretic peptides known as atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Four natriuretic peptide receptors (NPR1-3 and GUCY2C; nomenclature follows Alexander et al., 2009) have been identified and two of these, guanylyl cyclase-A (GC-A; NPR1) and guanylyl cyclase-B (GC-B; NPR2) are composed of large extracellular ligand binding domains,
single membrane-spanning regions and intracellular kinase homology, dimerization, and carboxyl-terminal guanylyl cyclase catalytic domains (Potter et al., 2009; Potter, 2009; 2011). GC-A is activated by ANP and BNP, whereas GC-B is activated by CNP. Gene inactivation experiments in mice indicate that GC-A mediates the blood pressure and volume reducing effects of ANP and BNP, whereas GC-B mediates the effects of CNP on skeletal growth (Lopez et al., 1995; Tamura et al., 2004).

Homozygous inactivating mutations in GC-B cause a severe form of human dwarfism called acromesomelic dysplasia, type Maroteaux (AMDM). Human chromosomal translocations that increase CNP concentrations are associated with Marfanoid-like skeletal overgrowth (Bacciardi et al., 2007; Moncla et al., 2007). Recently, CNP infusions were shown to increase long bone growth in a murine model of fibroblast growth factor receptor-3-dependent dwarfism (Yasoda et al., 2009). Together, these data indicate that GC-B inactivation causes dwarfism, that GC-B overactivation causes Marfanoid-like skeletal overgrowth, and that CNP rescues long bone growth in a murine model of the most common form of human dwarfism. Thus, identifying small molecule inhibitors and activators of GC-B may lead to a new class of drugs for skeletal diseases.

In broken cell preparations, ATP increases the guanylyl cyclase activity of GC-A and GC-B when activated by natriuretic peptides but inhibits when activity is measured in the presence of manganese and non-ionic detergent (Kurose et al., 1987; Gazzano et al., 1991). Whether ATP is required for the initial activation of GC-A and GC-B by natriuretic peptide is controversial. We found that initial activation does not require ATP (Antos et al., 2005) but that activities measured at longer time periods are increased in the presence of ATP due to reduction of the Michaelis constant for GTP (Antos and Potter, 2007).

Both GC-A and GC-B are phosphorylated on multiple residues in resting cells (Yoder et al., 2010). Prolonged exposure to natriuretic peptide causes the dephosphorylation and desensitization of the receptors (Potter and Garbers, 1992; Potter, 1998). Conversion of known phosphorylation sites to alanine or glutamate yields hormonally unresponsive receptors or responsive receptors respectively (Potter and Hunter, 1999). Thus, phosphorylation is required for hormonal activation and dephosphorylation is a mechanism of desensitization.

Activation of protein kinase C (PKC) by phorbol 12-myristate, 13-acetate (PMA) inhibits hormone-dependent activation of GC-A and GC-B in a manner that correlates with site-specific receptor dephosphorylation (Potter and Garbers, 1994; Potter and Hunter, 2000; Potthast et al., 2004). Guanylyl cyclase inhibition and dephosphorylation by PMA is blocked by the general PKC inhibitor GF-109203X, which competes for ATP binding to the active site of PKC (Potter and Garbers, 1994; Abbey-Hosch et al., 2005). GF-109203X has no inhibitory effect on natriuretic peptide receptors in the absence of PKC activators. To identify the PKC isoform required for PMA-dependent inhibition of GC-B, cells were incubated with Gö6976, a compound that inhibits the conventional subgroup of the PKC family. However, an unexpected inhibitory effect of Gö6976 on GC-B activation was observed in the absence of PMA, which led to the studies described in this report. We found that Gö6976 potently, rapidly and reversibly inhibits GC-B by a mechanism that did not require changes in known phosphorylation sites, cellular architecture or complete disruption of the active site.

**Methods**

**Cells**

Human 293T cells stably expressing rat GC-A or GC-B were cultured as described (Potter and Hunter, 2000; Fan et al., 2005). Mouse 3T3-L1 cells were cultured as described (Student et al., 1980).

**Whole cell stimulations**

Cells were seeded on poly-D-lysine-coated 48-well plates and incubated >5 h in serum-free media upon reaching 70% confluence. The medium was aspirated and replaced with 0.25 mM Dulbecco’s modified Eagle’s medium (DMEM) containing 1 mM 1-methyl-3-isobutylxanthine (IBMX) and 25 mM HEPES pH 7.4 for 10 min. Following pretreatment, the medium was aspirated and cells were incubated with the new medium with or without 1 μM CNP for 1 min. The medium was aspirated and the reaction was stopped with 0.5 mL ice-cold 80% ethanol. The cGMP content of the extract was determined by radioimmunoassay as described (Abbey and Potter, 2002).

**Guanylyl cyclase assays**

Crude membranes were prepared in phosphatase inhibitor buffer as previously described (Bryan and Potter, 2002). Assays were performed at 37°C for 5 min in a buffer containing 25 mM HEPES pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM IBMX, 1 mM GTP, 10–30 μCi of α-32P-GTP, 1 mM EDTA, 0.5 mM microcystin, 1 mM ATP and 5 mM MgCl2 in the presence (stimulated) or absence (basal) of the indicated natriuretic peptide. In some assays, Mn2+ was substituted for Mg2+ and Triton X-100 was added to a final concentration of 1% to artificially activate the enzyme. Synthesized 32P-cGMP was purified and quantified as described (Bryan and Potter, 2002).

**Concentration response assays**

Cells in 48-well plates were exposed to different concentrations of Gö6976 or an equivalent volume of DMSO for 1 h at 37°C. The medium was aspirated and new medium with or without 1 μM CNP for was added. After 1 min, the medium was aspirated and the reaction was stopped with 0.5 mL ice-cold 80% ethanol and the cGMP content of the extract was determined.

**Time course assays**

Cells in 48-well plates were exposed to either 10 μM Gö6976 or an equivalent volume of DMSO for the periods of time indicated. The medium was then aspirated and replaced with new medium with or without 1 μM CNP. After 1 min, the medium was aspirated and the reaction was stopped with 0.5 mL ice-cold 80% ethanol and cGMP concentrations were determined.
Wash out experiments
293T GC-B cells were incubated with medium containing 1 μM Gö6976, 10 μM Gö6976 or DMSO for 5 min. After 5 min the cells were washed twice with medium and then new medium containing 1 μM CNP was added for 1 min at the indicated post-wash time points. The medium was aspirated and the reaction was stopped with 0.5 mL ice-cold 80% ethanol and cellular cGMP concentrations of cell extracts determined.

Statistical analysis
All curves were fitted using GraphPad Prism 5.0 software. All P-values were obtained using a paired Student’s t-test in Microsoft Excel. Error bars represent the standard error of the mean and when not visible, are contained within the symbol.

Materials
Cyclic GMP radioimmunoassay kits and 32P-α GTP were from Perkin Elmer (Waltham, MA). Gö6976 was acquired from Sigma-Aldrich (Saint Louis, MO), GF-109203X, also known as Gö6850, was purchased from EMD chemicals (Gibbstown, NJ).

Results
Gö6976 inhibits CNP-dependent elevation of intracellular cGMP
Initial studies investigated the ability of PMA, Gö6976 and GF-109203X to modulate CNP-dependent cGMP elevations in 293T-GC-B cells. The structural similarity between Gö6976 and GF-109203X is shown in Figure 1A. CNP (1 μM) elevated intracellular cGMP concentrations 48-fold, but prior PMA exposure reduced these increases by more than 60% (Figure 1B). Exposure to GF-109203X for 60 min prior to CNP stimulation yielded intracellular cGMP concentrations that were elevated 55-fold higher than those from control cells. PMA did not significantly reduce CNP-dependent cGMP concentrations in cells exposed to GF-109203X, consistent with PMA inhibiting GC-B through a PKC-dependent mechanism. Surprisingly, Gö6976 alone completely blocked CNP-dependent cGMP concentrations, and exposure to both Gö6976 and PMA reduced CNP-dependent intracellular cGMP concentrations to levels that were below basal values.

Gö6976 inhibits the guanylyl cyclase activity of GC-B
To directly measure the effect of Gö6976 on the enzymic activity of GC-B, intact 293T-GC-B cells were incubated with 10 μM Gö6976 for 1 h and guanylyl cyclase activities in crude membranes were determined under basal, CNP-stimulated or detergent-stimulated conditions (Figure 2A). Exposure to Gö6976 (10 μM) reduced basal and CNP-dependent guanylyl cyclase activities by 51 and 49% respectively, but failed to significantly reduce activity measured in the presence of Triton X-100 and Mn²⁺GTP.

Gö6976 inhibits GC-A
Intact 293T cells stably expressing GC-A (293T-GC-A) were also incubated with 10 μM Gö6976 for 1 h and guanylyl cyclase activities in crude membranes were determined as described above except that ANP was substituted for CNP (Figure 2B). Incubation with Gö6976 (10 μM) reduced basal and ANP-dependent guanylyl cyclase activities by 53 and 40% respectively, but failed to significantly reduce activity measured in the presence of Triton X-100 and Mn²⁺GTP.

Gö6976 is a potent inhibitor of GC-B
293T-GC-B cells were incubated with increasing concentrations of Gö6976 and then CNP-dependent increases in cGMP were determined (Figure 3A). The concentration required to
inhibit half of the maximal CNP-dependent cGMP response (IC$_{50}$) was approximately 380 nM.

**Gö6976 is a rapid inhibitor of GC-B**

The time required for Gö6976 to reduce CNP-dependent concentrations to half of control values (t$_{1/2}$) was also determined. 293T-GC-B cells were incubated with 10 μM Gö6976 for the periods shown and then the cells were incubated with 1 μM CNP for 1 min and intracellular cGMP concentrations were determined. The t$_{1/2}$ was estimated to be 7 s (Figure 3B).

**Gö6976 modestly increases the EC$_{50}$ but inhibition is maintained at saturating CNP concentrations**

The ability of Gö6976 to competitively antagonize CNP activation of GC-B was investigated. Whole 293T-GC-B cells were incubated with or without 10 μM Gö6976 for 30 min and then exposed to various concentrations of CNP for 1 min and intracellular cGMP concentrations were determined (Figure 4A). Absolute cGMP concentrations are shown in Figure 4A and the data are replotted with two Y-axes, as shown in Figure 4B. The calculated EC$_{50}$ for control (DMSO) and Gö6976-treated cells were 40 nM and 180 nM respectively. A slight but significant 4.5-fold rightward shift in the dose–response curve in Gö6976-treated cells was observed (Figure 4B). However, the inhibitory effect was not overcome at saturating CNP concentrations.

**Gö6976 inhibition of GC-B is concentration-dependent and rapidly reversible**

293T-GC-B cells were incubated with 10 μM Gö6976 for 5 min, washed twice with DMEM, and incubated for the indicated periods of time before exposing the cells to CNP for 1 min to elevate intracellular cGMP concentrations (Figure 5A). Approximately half of the activity was recovered 1 h after removal of Gö6976. When the concentration of Gö6976 was reduced 10-fold to 1 μM, recovery was more rapid (Figure 5B). The apparent half-time for recovery was 9 min and complete recovery was observed by 30 min in cells exposed to 1 μM Gö6976.

**Gö6976 inhibition does not require changes in known GC-B phosphorylation sites**

Since Gö6976 inhibits several protein kinases and phosphorylation of GC-B is required for CNP-dependent activation (Potter, 1998; Potter and Hunter, 1998), we examined if Gö6976 reduces CNP-dependent activation by blocking GC-B phosphorylation (Figure 6). A cell line stably expressing a version of GC-B containing glutamate substitutions for all six known GC-B phosphorylation sites (293-GC-B-6E) was used in these experiments (Yoder _et al_. , 2010). Gö6976 inhibited GC-B-6E similarly to the wild-type receptor, consistent with a mechanism that is independent of changes in the phosphorylation of known sites.
Gö6976 inhibits GC-B in broken cell preparations

In all previous experiments, measuring cGMP concentrations in whole cells or cyclase activity in crude membranes assessed the effect of incubating whole cells with Gö6976. Here, the ability of Gö6976 to inhibit GC-B activity when directly added to membranes was examined. As a positive control for Gö6976 inhibition, whole 293T-GC-B cells were incubated in the presence or absence of Gö6976 for 1 h and then crude membranes were prepared and assayed for basal, CNP or detergent-dependent guanylyl cyclase activity (Figure 7A, Whole Cell). To test for direct inhibitory effects, crude membranes from 293T-GC-B cells that were not previously exposed to Gö6976 were incubated with Gö6976 or vehicle during the cyclase assay (Figure 7B, Membrane). Addition of Gö6976 to whole cells or crude membranes inhibited CNP-dependent GC-B activity to a similar extent, consistent with Gö6976 acting in a manner that does not require intact intracellular architecture or cellular incubation. We also investigated whether Gö6976 inhibited GC-B in a separate cell line. Gö6976 (10 μM) decreased CNP-dependent guanylyl cyclase activities at 5 and 10 min by 68 and 71% respectively, in membranes from mouse 3T3-L1 cells. We have previously shown that 3T3 cells express GC-B, but not GC-A (Abbey and Potter, 2003).

Discussion

Little is known about the inhibition of GC-A and GC-B. HS-142-1, a microbial polysaccharide, was shown to block CNP-dependent bone growth (Yasoda et al., 1998) and to inhibit CNP-dependent cGMP elevations in podocytes (Lewko et al., 2004), but no direct studies on GC-B guanylyl cyclase activities have been reported. Studies by Lewko and colleagues indicated an IC₅₀ of HS-142-1 between 0.25 and 2.5 μM, which is similar to the IC₅₀ of 380 nM determined for Gö6976. Both known GC-A antagonists, HS-142-1 and A71915, block ANP binding to the extracellular domain of
Gö6976 inhibition does not involve known GC-B phosphorylation sites. 293T-GC-B or 293T-GC-B 6E cells were incubated with DMSO or 10 μM Gö6976 for 1 h at 37°C. Crude membranes were prepared and guanylyl cyclase assays were determined under basal or stimulated conditions. **P < 0.01; ***P < 0.0005; n = 6 from three experiments.

Burczynska et al., 2007), but whether these sites are conserved in GC-B and are required for ATP-dependent activation of GC-A is not known. An alternative hypothesis is that ATP binds one of the two putative nucleotide-binding sites in the catalytic domain of GC-B. Structural and functional data on a guanylyl cyclase isolated from cyanobacteria indicates two nucleotide-binding sites that have similar affinities for GTP and ATP (Rauh et al., 2008). When manganese is used as the divalent metal, GC-A, GC-B and the cyanobacteria guanylyl cyclase exhibit positive cooperative kinetics with respect to increasing GTP concentrations, consistent with two catalytic sites. However, under physiological conditions where magnesium is the divalent cofactor, linear kinetics are observed for GC-A and GC-B, consistent with a single catalytic site. We found that ATP decreases the Michaelis constant for Mg\(^{2+}\)GTP, which is in agreement with a model where ATP increases the affinity of the catalytic site for GTP (Antos and Potter, 2007).

Thus, it is possible that Gö6976 inhibits GC-A and GC-B by blocking binding of ATP to a regulatory site in the catalytic domain. The fact that Gö6976 does not inhibit detergent-dependent activity suggests that it does not directly inhibit the catalytic site, but we cannot rule out the possibility that the catalytic sites differ in the detergent-activated and natriuretic peptide-activated forms of the enzymes.

Recently Duda et al. (2010) reported that staurosporine activates GC-A similarly to AMP-PNP in a broken cell assay, which suggests that staurosporine mimics the ability of ATP to activate the receptor. However, we failed to observe activation of GC-B or GC-A by any of the indolocarbazoles that we tested in this report, including staurosporine (unpublished work; Potter et al.). Thus, in our hands, indolocarbazoles do not mimic the ability of ATP to activate receptor guanylyl cyclases.
In addition to the classic forms of PKC (Martiny-Baron et al., 1993), Gö6976 has been shown to inhibit the tyrosine kinases associated with the neurotrophin receptors, trk A and trk B, and the JAK2 and FLT3 tyrosine kinases (Behrens et al., 1999; Grandage et al., 2006). However, GC-A and GC-B are the first reported non-kinase enzymic targets of Gö6976. The IC50 of Gö6976 for whole cell inhibition of GC-B was similar to that reported for inhibition of JAK3 but higher than that reported for inhibition of the trk A and trk B receptors (Behrens et al., 1999; Grandage et al., 2006). Our data indicate that inhibition of the natriuretic peptide receptor guanylyl cyclases should be considered when interpreting the effects of Gö6976 on cell function.

In conclusion, we have shown for the first time that Gö6976 is a rapid, potent and reversible inhibitor of GC-A and GC-B that does not require changes in phosphorylation sites or modification of the active site. Future studies will investigate the mechanism of action of Gö6976 on GC-A and GC-B.

Figure 7
Gö6976 inhibits C-type natriuretic peptide (CNP)-dependent activity in broken cell preparations. A. Intact 293T-GC-B cells were incubated with 10 μM Gö6976 or DMSO for 1 h at 37°C and then membranes were prepared and assayed for guanylyl activity (Whole Cell). Alternatively, 10 μM Gö6976 or DMSO was directly added to membranes prepared from naïve 293T-GC-B cells and guanylyl cyclase activity measured under stimulated conditions (Membrane). *P < 0.05; **P < 0.004, n = 11 from six experiments (Whole Cell) or n = 4 from two experiments (Membrane). B. 10 μM Gö6976 or DMSO was directly added to membranes prepared from 3T3-L1 cells and then guanylyl cyclase activity was measured in the presence of 1 μM CNP for the periods of time indicated. n = 10 from two experiments.

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Conflicts of interest
The authors declare no conflicts of interest.

References


