The PKC Inhibitor Gö 6976 Blocks C-Type Natriuretic Peptide Activation of Guanylyl Cyclase B

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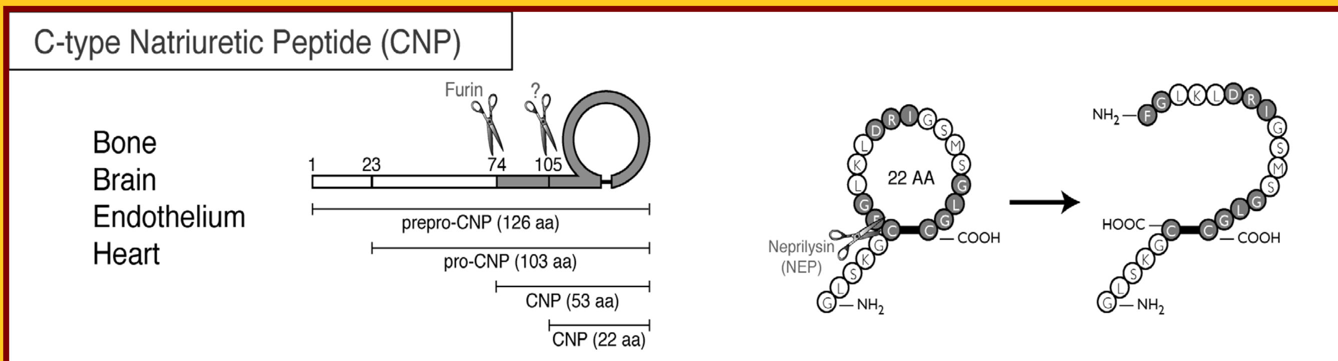
Introduction

C-type natriuretic peptide is one of three structurally related but genetically distinct mammalian hormone/paracrine factors that regulate long bone growth, vasorelaxation and axonal guidance. CNP is most highly expressed in chondrocytes, the brain, and vascular endothelial cells, and elevated concentrations of CNP can be found in patients with congestive heart failure (1).

FIG. 1. C-Type Natriuretic Peptide (CNP) processing and structure.



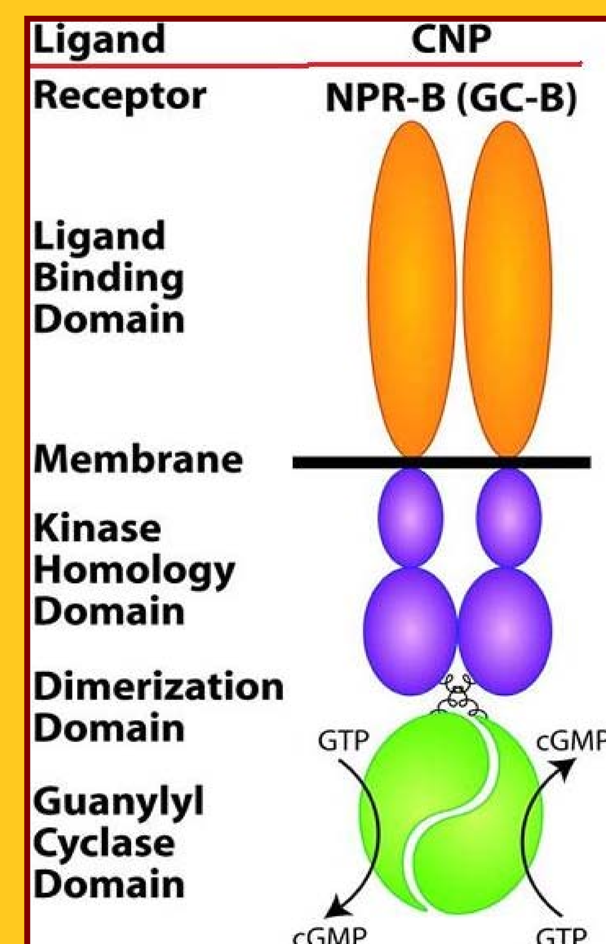
Potter, L.R., et al. Endocr. Rev. 2006:27:42-72

During processing, the serine endoprotease furin cleaves proCNP into a 53 amino acid carboxyl terminal biologically active peptide, CNP-53, the major active form of CNP at the tissue level. Additional cleavage by a yet unknown protease yields the 22 amino acid peptide, CNP-23, which is active in the systemic circulation.

FIG. 2. Model of NPR-B and its ligand, CNP.

CNP binding to the transmembrane guanylyl cyclase natriuretic peptide receptor-B (NPR-B/GC-B) stimulates the synthesis of the intracellular secondary messenger, cGMP, and activates phosphodiesterases, protein kinases and ion channels.

Under basal conditions, NPR-B is phosphorylated on five known serine and threonine sites in its kinase homology domain (2). Substitution of individual sites with glutamic acid or alanine mimics the effects of a phosphorylated or dephosphorylated residue, respectively. Chronic exposure of NPR-B to ligand results in a time-dependent decrease in activity that is temporally correlated with receptor dephosphorylation.



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NPR-B is inhibited by phorbol 12-myristate 13-acetate (PMA) activation of protein kinase C (3). In preliminary experiments, the indolocarbazole Gö 6976 inhibited CNP mediated NPR-B activity in the absence of PMA.
This study characterizes the effects of the widely used protein kinase C inhibitor, Gö 6976, on NPR-B guanylyl cyclase activity as a means to identify its inhibitory mechanisms.

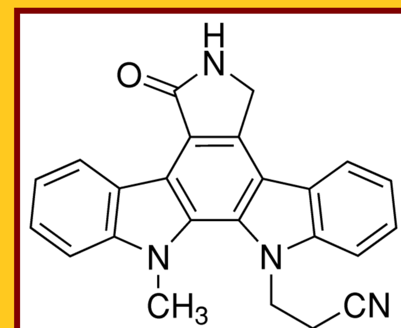


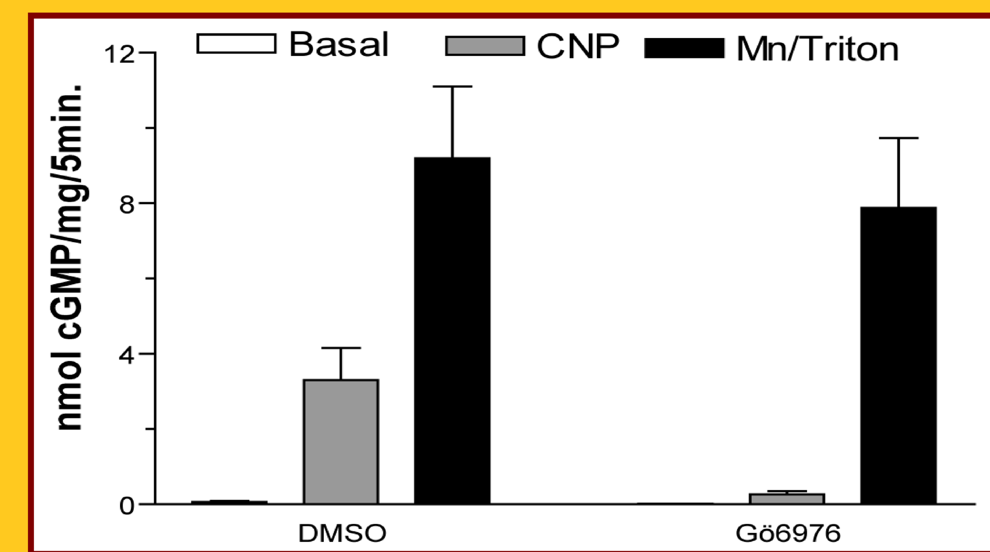
FIG. 3. Chemical structure of Gö 6976.
Gö 6976 selectively inhibits the Ca^{2+}-dependent, conventional PKC isoforms, α and β1, but exerts no effect on the kinase activity of the Ca^{2+}-independent PKC subtypes, δ, ε, and ζ (4).

Methods and Results

Serum starved cells were treated in the presence or absence of Gö 6976 for 1-h. Crude membranes were prepared and then assayed for CNP-dependent guanylyl cyclase activities for 5 min at 37°C in the presence of [α-^{32}P]GTP. Activator mixtures contained 1 mM ATP, 1 µM CNP, and 5 mM $MgCl_2$ or 1% Triton X-100 and 5 mM $MnCl_2$.
Purification of [α-^{32}P]GTP was achieved with chromatography columns containing alumina resin, and samples were quantified using a Beckman 3801 scintillation counter. Protein concentrations were estimated using Pierce Coomassie Plus Protein Assays, and GraphPad Prism4 was used for data analysis and significance testing (5).

FIG. 4. Gö 6976 inhibits >80% CNP mediated NPR-B activity in HEK 293PMA cells expressing NPR-B.

HEK 293PMA cells were transiently transfected with a plasmid encoding the wild type (WT) NPR-B receptor. Confluent, serum-starved cells were pre-incubated with DMSO solvent or 1 µM Gö 6976 for 1 h at 37°C. Crude membranes were prepared, and guanylyl cyclase activities were determined in the presence of CNP or nonionic detergent (Mn/Triton), which maximally activates NPR-B in a ligand independent, non physiologic manner. Values represent the average of two duplicate plate treatments assayed in duplicate (± S.E.). This experiment was performed at least three times with similar results.



Experiment performed by Jerid W. Robinson.

FIG. 5. In HEK 293T cells, Gö 6976 inhibits 50-60% of CNP mediated NPR-B 6E activity.

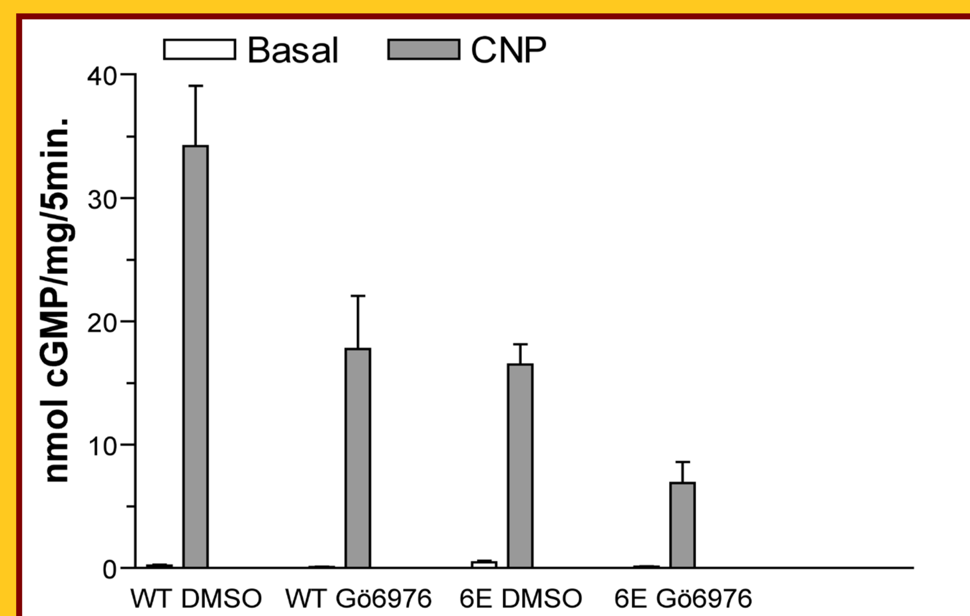


Figure 5 compares the inhibitory effect of Gö 6976 in HEK 293T cells stably expressing the WT NPR-B receptor versus in HEK 293T cells expressing the 6E mutant version of the NPR-B receptor, with all 5 known Ser/Thr sites (Ser^{513}, Thr^{516}, Ser^{518}, Ser^{523}, Ser^{526}) + the putative Thr^{529} site mutated to glutamic acid. Confluent, serum-starved cells were pre-incubated with DMSO solvent or 10 µM Gö 6976 for 1 h at 37°C and subjected to the treatments indicated. Values represent the average of two duplicate plate treatments assayed in duplicate (± S.E.). No difference was observed in levels of Mn/Triton activated guanylyl cyclase activity for either the WT NPR-B receptor or the NPR-B 6E receptor. This experiment was performed at least three times with similar results.

FIG. 6. Gö 6976 inhibits ≈50% of CNP mediated NPR-B activity when directly added to cell membranes.

Figure 6 compares the inhibitory effect of Gö 6976 when added to whole cells (WC) versus when added directly to crude cell membranes (Mem.). Confluent, serum-starved HEK 293T cells stably expressing the WT NPR-B receptor were pre-incubated with DMSO solvent or 10 µM Gö 6976 for 1 h at 37°C (WC) or for 15 min at RT (Mem.), and subjected to the treatments indicated. Values represent the average of two duplicate plate treatments assayed in duplicate (± S.E.). This experiment was performed at least three times with similar results.

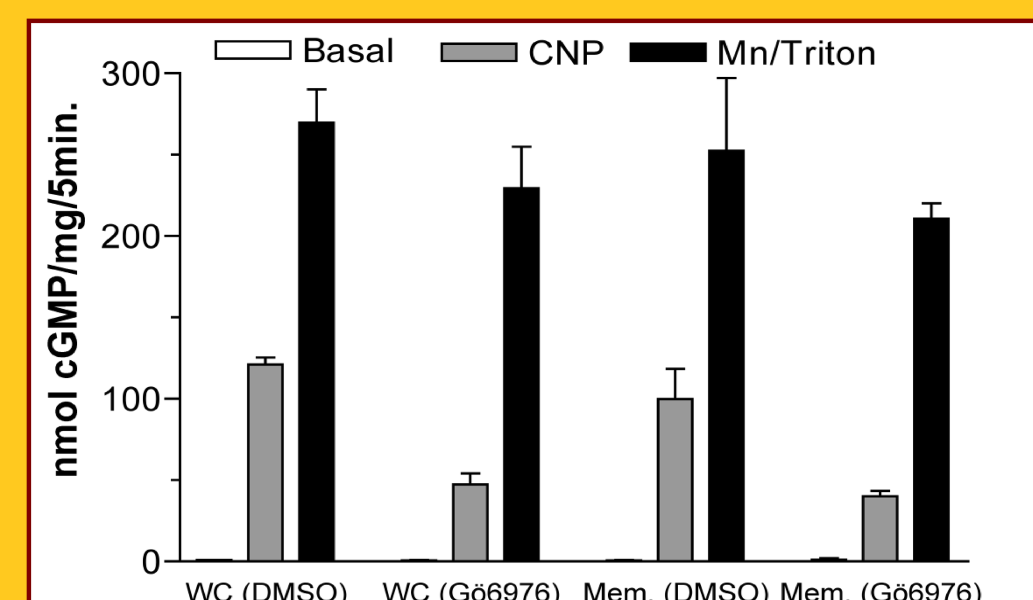
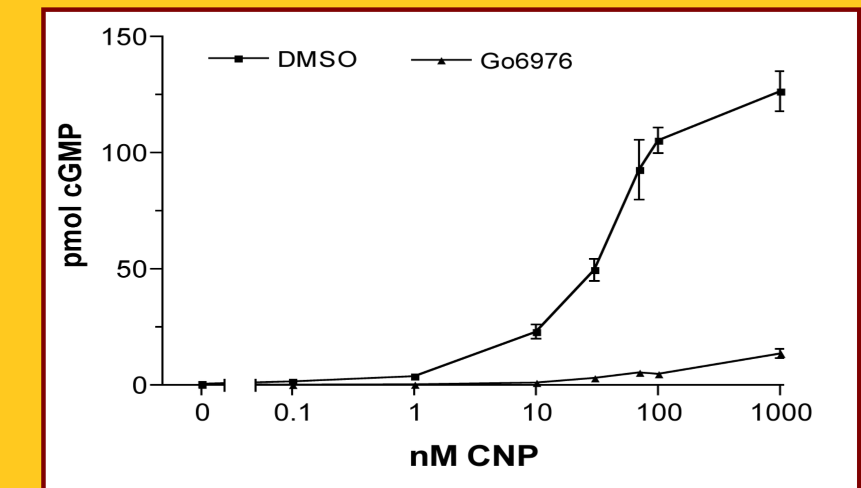


FIG. 7. Concentration-dependent dosage response for Gö 6976.



Experiment performed by Jerid W. Robinson.

HEK 293PMA cells were transiently transfected with a plasmid expressing the WT NPR-B receptor. Whole cells were incubated with 1 µM Gö 6976 or DMSO solvent for 1 h and then pre-incubated with increasing concentrations of CNP for 3 min. The cells were lysed and cGMP concentrations in the cells and bathing medium were determined by radioimmunoassay. One µM Gö 6976 dramatically inhibited cGMP elevations observed at all CNP concentrations.

Conclusion

- In **Figures 4**, **5**, and **6**, Gö 6976 inhibited CNP-dependent guanylyl cyclase activity, but had no effect on Mn/Triton dependent guanylyl cyclase activities, suggesting that the effect of Gö 6976 is on the CNP-dependent activation process and not on the formation of the catalytic site.
- Although 1 µM of Gö 6976 markedly inhibited NPR-B activity in HEK 293PMA cells (**Figure 4**), 10 times this concentration of Gö 6976 was necessary to produce a less pronounced effect on NPR-B activity in HEK 293T cells (**Figure 5**). The differential inhibition of NPR-B activity in these cell lines suggests that cellular environment plays a critical role in the mechanism of Gö 6976 dependent inhibition of NPR-B.
- Gö 6976 inhibited NPR-B 6E activity (**Figure 5**), consistent with an inhibitory process that does not require changes in receptor phosphorylation status.
- Addition of Gö 6976 to crude HEK 293T cell membranes reduced CNP-dependent guanylyl cyclase activity (**Figure 6**), indicating that the preservation of cellular machinery is not required for the inhibitory process and that Gö 6976 may exert its effects by acting directly on membrane components.

Future experiments will further refine regulatory pathways that mediate NPR-B activation and deactivation. The discovery of small, cell permeable molecules that either activate or inhibit NPR-B will facilitate a better understanding of the natriuretic peptide receptor activation mechanism and may prove to be clinically beneficial.

Because NPR-B is the major natriuretic peptide receptor active in the failed heart (6), these studies are the basis for the development of potential drug targets and therapeutics for the treatment of cardiovascular diseases.

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