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Reduced ability of C-type natriuretic peptide (CNP) to activate natriuretic peptide receptor B (NPR-B) causes dwarfism in *lbab*$^{-/-}$ mice

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Abstract

C-type natriuretic peptide (CNP) stimulates endochondrial ossification by activating the transmembrane guanylyl cyclase, natriuretic peptide receptor-B (NPR-B). Recently, a spontaneous autosomal recessive mutation that causes severe dwarfism in mice was identified. The mutant, called long bone abnormality (*lbab*), contains a single point mutation that converts an arginine to a glycine in a conserved coding region of the CNP gene, but how this mutation affects CNP activity has not been reported. Here, we determined that thirty to greater than one hundred-fold more CNP^{lbab} was required to activate NPR-B as compared to wild-type CNP in whole cell cGMP elevation and membrane guanylyl cyclase assays. The reduced ability of CNP^{lbab} to activate NPR-B was explained, at least in part, by decreased binding since ten-fold more CNP^{lbab} than wild-type CNP was required to compete with [^{125}I][Tyr^{0}]CNP for receptor binding. Molecular modeling suggested that the conserved arginine is critical for binding to an equally conserved acidic pocket in NPR-B. These results indicate that reduced binding to and activation of NPR-B causes dwarfism in *lbab*$^{-/-}$ mice.

Keywords

Endochondrial ossification; Cyclic-GMP; Guanylyl cyclase B; Type II cGMP dependant protein kinase; *Nppc*

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1. INTRODUCTION

Natriuretic peptides (NPs) are structurally related pleiotropic factors that regulate the cardiovascular, skeletal, and possibly other systems [25]. Atrial natriuretic peptide (ANP) reduces blood pressure by stimulating renal sodium and water excretion [11], reducing intravascular volume [8], and relaxing vascular smooth muscle [32]. Infusion of synthetic B-type natriuretic peptide (BNP) into animals or humans elicits similar responses as ANP, but gene deletion studies in mice indicate that BNP is not required for the maintenance of normal blood pressure [29]. C-type natriuretic peptide (CNP) is also a vasorelaxant [10], but its most obvious function is to stimulate endochondral ossification, resulting in the lengthening of long bones [19, 28].

Natriuretic peptides exert their functions via interaction with single membrane-spanning guanylyl cyclases called natriuretic peptide receptors. Peptide binding activates their catalytic domains resulting in elevated intracellular cGMP concentrations. Both ANP and BNP bind and activate natriuretic peptide receptor A (NPR-A/GC-A) [6, 27], whereas CNP selectively activates natriuretic peptide receptor B (NPR-B/GC-B) [16]. ANP, BNP, CNP, as well as osteocrin, also bind the natriuretic peptide clearance receptor (NPR-C), which controls local concentrations of these peptides through constitutive receptor-mediated internalization and degradation [18].

CNP is translated as a preprohormone that has multiple biologically active forms. Tissues primarily contain a 53-amino acid form of CNP, but an amino-terminal deleted 22-amino acid form of CNP is the predominant circulating species found in blood and cerebral spinal fluid (Fig. 1). Both the 53- and 22-residue forms have similar NPR-B activation and binding profiles [35].

CNP is postulated to regulate long bone growth through its induction of endochondral ossification [23, 34]. CNP-dependent activation of NPR-B produces cGMP, which activates the type II cGMP dependent protein kinase, PKGII. Functional inactivation of the genes encoding CNP [7], NPR-B[28, 30], or PKGII [5, 24] produce dwarfism. The relevant substrates for PKGII in the bone growth pathway have not been determined, but transgenic overexpression of CNP was shown to inhibit MAP kinase activity and partially compensate for fibroblast growth factor receptor-3-dependent dwarfism in a mouse achondroplasia model [17, 33].

In humans, familial homozygous loss-of-function mutations in the gene encoding NPR-B, *Npr2*, lead to a form of dwarfism known as acromesomelic dysplasia type Maroteaux (AMDM), which is characterized by disproportionately short arms and legs after birth [2]. Individuals that are heterozygous for mutations in NPR-B have normal limb proportions but are significantly shorter than comparable individuals without the mutation [22]. Recently, an AMDM patient was shown to have a novel mutation in NPR-B which allowed the receptor to bind CNP, but abolished cGMP signaling by the receptor in a dominant negative fashion [13].

In 1996, researchers at The Jackson Laboratory identified a spontaneous autosomal recessive mouse mutation characterized by overall smaller body size and proportional

dwarfing of all organs and long bones [31]. They called this mutation, *lbab*, for "long bone abnormality" and mapped the mutation to chromosome 1 of the mouse genome. Recently Jiao and colleagues further characterized this mutation and found that the *lbab* phenotype was associated with a single point mutation in the *Nppc* gene [15]. A C to G transversion was found in exon 2 of *Nppc* that results in the substitution of a glycine for an arginine at position 117 in proCNP. An absolutely conserved D-R-I sequence is present within the ring structure of all natriuretic peptides. The *lbab* mutation changes this sequence to D-G-I. After processing, the 22-amino acid peptide encoded by the *lbab* mutation differs from by a single amino acid in position 13 (R13G) and is referred to henceforth as CNP^{lbab} (Fig. 1). Jiao and colleagues speculated that this point mutation results in loss of function but provided no experimental data to support this hypothesis.

The purpose of this study was to determine if the peptide encoded by the *lbab* mutation is less biologically active than the wild-type peptide. This was evaluated by whole cell ligand binding as well as whole cell cGMP elevation and membrane guanylyl cyclase assays. We found that the single amino acid difference between the mutant and wild-type forms of CNP dramatically reduce its ability to bind and activate NPR-B.

2. MATERIALS AND METHODS

2.1. Reagents

CNP^{lbab} (GLSKGCFGLKLDGIGSMSGLGC, disulfide bridge: 6–22) was synthesized by AnaSpec, Inc (San Jose, CA). [^{125}I][Tyr^{0}]CNP (1–22) was purchased from Phoenix Pharmaceuticals (Phoenix, AZ). [α-^{32}P]GTP was purchased from Perkin Elmer (Waltham, MA).

2.2. Cell Lines

Human embryonic 293 cells lacking any known natriuretic peptide receptor were transfected with 10 μg of pRK5-NPR-B [26] and 1 μg of pWL-neo to confer neomycin resistance. An individual clone stably expressing NPR-B was selected with plastic cloning cylinders after 10–14 d of growth in medium containing 200 μg/ml neomycin. NIH3T3 cells were maintained as previously described [1].

2.3. Preparation of Crude Membranes

293-NPR-B cells were scraped off 10 cm plates with 0.75 ml of phosphatase inhibitor buffer (25 mM Hepes pH 7.4, 20% glycerol, 50mM NaCl, 50 mM NaF, 2 mM EDTA, 0.5 μM microcystin, EDTA-free protease inhibitors (Roche)), sonicated for 1 s with a Misonix Ultrasonic Processor at 4°C, and centrifuged at 20,000 × g for 15 min at 4°C. Membrane pellets were resuspended in phosphatase inhibitor buffer at 5–10 mg protein/ml.

2.4. Guanylyl Cyclase Assays

Guanylyl cyclase assays were performed at 37°C for 3 min in a buffer containing 25 mM Hepes pH 7.4, 50 mM NaCl, 0.1% BSA, 0.5 mM 1-methyl-3-isobutylxanthine, 1 mM GTP, 0.5 μM microcystin, 1 mM EDTA, 1–2 μCi of [α-^{32}P]GTP, and 5 mM $MgCl_2$ with or without CNP. Reactions were started by the addition of 80 μl of the above reagents to 50–

200 μg of crude membrane protein suspended in 20 μl of phosphatase inhibitor buffer. Reactions were stopped by the addition of 0.5 ml 110 mM ZnOAc and 0.5 ml 110 mM $NaCO_3$ on ice. Cyclic-cGMP accumulation was determined as described previously [4].

2.5. Whole Cell Stimulations

Cells plated in poly-D-lysine coated 48-well plates were incubated overnight in serum-free media. Medium was aspirated and 0.2 ml DMEM containing 1 mM 1-methyl-3-isobutylxanthine (IBMX) was added and incubated for 10 min. Medium was aspirated and cells were treated with DMEM containing 1 mM IBMX with or without natriuretic peptide for 1 to 5 min. Treatment medium was aspirated and the reaction was stopped with 0.2 ml ice-cold ethanol. An aliquot of the supernatant was dried in a centrifugal vacuum concentrator and analyzed for cGMP content using a [^{125}I]-radioimmunoassay kit from Perkin Elmer as per manufacturer's instructions.

2.6. Radioligand Binding Assays

Cells in 24-well plates were washed with DMEM and then incubated with 0.2% BSA in DMEM at 37°C for 1 h. Medium was aspirated and 150 μl of binding medium containing 75 pM [^{125}I][Try^0]CNP and 1% BSA, alone or with unlabeled ligand, was added to the well. The plate was incubated for 1 h at 4°C and then medium was aspirated and wells were washed with 0.5 ml ice-cold PBS. The PBS was aspirated and 0.5 ml 1 N NaOH was added to the well to remove cells. The supernatant was transferred to glass tubes and the amount of [^{125}I][Tyr^0]CNP present was assessed in a gamma counter. Nonspecific binding was determined by repeating the above assay in the presence of 1 μM unlabeled CNP. Specific binding was calculated by subtracting the nonspecific binding from the total binding.

2.7. Modeling of NPR-B With CNP

Modeller-9.3 was used to model the NPR-B/CNP structure based upon the crystal structure of NPR-A bound to ANP solved by Ogawa and colleagues (RCSB Protein Data Bank access code 1T34) [21]. Numbering of human NPR-B starts with initiation methionine as position 1. This model includes the entire extracellular domain, residues Arg23-Gly464. Spatial constraints were created from the sequence alignment between NPR-B and NPR-A. The structure was then randomized and minimized upon the constraints. Figures were drawn with the PyMol program [9].

3. RESULTS

3.1. Reduced potency of CNP^{lbab} in elevating whole cell cGMP concentrations

Cells overexpressing the rat NPR-B receptor (293-NPR-B, Fig. 2A) or cells endogenously expressing the mouse NPR-B receptor (NIH3T3, Fig. 2B) were stimulated with increasing concentrations of CNP or CNP^{lbab} and intracellular cGMP concentrations were estimated by radioimmunoassay. Although cells were incubated with as much as 1 μM and 10 μM concentrations of the wild-type and mutant forms of CNP, saturation was not achieved for either peptide, consistent with a previous report for wild-type CNP and human NPR-B [16]. Importantly, the concentration-response curve for CNP^{lbab} was significantly shifted to the right compared to wild-type CNP in 293 cells expressing rat NPR-B. The stimulation

observed with 10 μM CNP^{lbab} was equivalent to the level of activation observed with 115 nM CNP, an approximately 85-fold difference. Whole cell stimulations of NIH3T3 cells demonstrated an exaggerated trend with 10 μM CNP^{lbab}, yielding the same level of activation as about 30 nM concentrations of wild-type CNP, about a 300-fold difference. Thus, with respect to both the rat and the mouse NPR-B receptors, CNP^{lbab} has a markedly diminished ability to elevate intracellular cGMP concentrations compared to the wild-type molecule.

3.2. Reduced potency of CNP^{lbab} in stimulating NPR-B guanylyl cyclase activity

Crude membrane preparations from 293-NPR-B cells were used to access the ability of CNP and CNP^{lbab} to directly activate NPR-B in broken cell preparations (Fig. 2C). Concentration-response curves generated by CNP^{lbab} were shifted to the right of those generated with wild-type CNP. The activity seen with 10 μM CNP^{lbab} was equivalent to the activation seen with 275 nM CNP, amounting to a 36-fold difference. Hence, the decreased cGMP production observed in whole cells (Fig. 2A and 2B) is a direct result of the diminished ability of CNP^{lbab} to activate the NPR-B receptor.

3.3. CNP^{lbab} has reduced affinity to NPR-B

Using a competitive binding assay, the affinity of CNP and CNP^{lbab} for rat NPR-B was examined using 293-NPR-B cells. Specific binding of 75 pM [^{125}I][Tyr^{0}]CNP was progressively inhibited when increasing amounts of CNP or CNP^{lbab} were included in the binding assay (Fig. 3). The concentrations needed for 50% inhibition of specific [^{125}I][Tyr^{0}]CNP binding to NPR-B (IC_{50}) were 370 pM and 3900 pM for CNP and CNP^{lbab}, respectively. Thus, CNP^{lbab} binds NPR-B at least ten-fold less avidly than wild-type CNP binds NPR-B.

4. DISCUSSION

In this study, the ability of CNP^{lbab} to activate and bind to NPR-B was examined. In our experiments, the capacity of CNP^{lbab} to activate both mouse and rat NPR-B in whole cells and in crude membrane guanylyl cyclase assays was markedly reduced. Similarly, whole cell binding assays indicated that ten-fold more CNP^{lbab} compared to the wild-type peptide was required to compete with [^{125}I][Tyr^{0}]-CNP for binding to NPR-B in whole cells. Hence, we conclude that the *lbab* mutation in *Nppc* causes dwarfism because it encodes an amino acid substitution that decreases the ability of CNP to bind and activate its cognate receptor, NPR-B.

One interesting aspect of our data is that we saw varying degrees of diminished responses to CNP^{lbab} in our assays. We saw a 10-fold decrease in binding affinity, but an 85-fold decrease in whole cell cGMP accumulation with CNP^{lbab}. One might expect to see similar fold shifts across all assays. However, that was not the case. Although there may be two separate effects of the single amino acid change seen in CNP^{lbab}—decreased binding affinity as well as decreased ability to activate NPR-B once bound—we cannot separate these effects due to the differences in assay conditions as previously noted by Garbers and colleagues [12].

In order to elucidate the basis for the decreased ability of CNP^{lbab} to bind and activate NPR-B, we sought to identify critical molecular interactions between CNP and NPR-B via molecular modeling. A previous molecular model of human NPR-B complexed to CNP was created by He and colleagues [14], which focused on interactions that provided ligand specificity. The mutation in CNP^{lbab} occurs in a highly conserved region, which was not discussed in the previous model. Unfortunately, the coordinates for this model were not deposited into the RSCB database. Hence, we created a new structural model of human NPR-B bound to either CNP or CNP^{lbab} based on the crystal structure of the rat ANP/NPR-A complex. Like NPR-A [21], NPR-B is thought to function as a head-to-head dimer, with two-fold symmetry in the ligand binding pocket. By examining the interactions of the R13G mutation of CNP^{lbab} compared to wild-type CNP, we have identified key differences in the molecular interaction of the ligands with the receptor. For wild-type CNP, Arg13 fits tightly into an acidic pocket, with its positively charged guanidinium group juxtaposed to two acidic residues, Asp176 and Glu77, of the receptor. An additional interaction between the planar ring of Tyr103 of the receptor and CNP further stabilizes the interaction (Fig. 4A). When CNP^{lbab} is bound to NPR-B, the hydrogen side chain of the glycine residue is neither long enough to fill the receptor pocket nor is it appropriately charged to ion pair with the acidic residues lining the base of the pocket (Fig. 4B). In solution, CNP does not have a well-defined structure but is forced into a particular conformation when it binds the receptor. Although Gly13 in CNP^{lbab} affords the peptide greater conformational flexibility, it does not allow for the favorable electrostatic interactions with Asp176 or Glu77 of NPR-B envisioned for wild-type CNP. Much like Arg13 is absolutely conserved in CNP across all species, the importance of Asp176, Glu 77, and Tyr103 in the receptor-ligand interaction is underscored by the fact that all three residues are absolutely conserved in all mammalian homologs of NPR-B whose primary amino acid sequence is deposited in the NCBI database (data not shown). The disruption of these critical points-of-contact are likely to reduce binding of CNP to NPR-B, but this remains to be quantified.

In conclusion, we have demonstrated a blunted cellular and *in vitro* response to the version of CNP encoded by the murine *lbab* allele. To our knowledge, this is the first report to characterize a functional consequence of a single point mutation in a ligand for any guanylyl cyclase. At physiologic concentrations, the mutated natriuretic peptide CNP^{lbab} is unlikely to cause significant elevations in cGMP, which would fail to trigger the down stream signal transduction pathways that normally ensue after CNP release in bone tissue. Recently, human chromosomal 2:7 translocations located close to the NPPC gene were identified that result in elevated CNP mRNA expression, increased CNP protein levels, and skeletal overgrowth [3, 20]. Whether mutations exist in the coding region of CNP that modify human skeletal growth has yet to be reported, but based on the *lbab*$^{-/-}$ mouse, we believe this scenario is likely.

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GLOSSARY

CNP	C-type natriuretic peptide
NPR-B/GC-B	Natriuretic peptide receptor B
NP	natriuretic peptide
ANP	atrial natriuretic peptide
BNP	B-type natriuretic peptide
NPR-A/GC-A	natriuretic peptide receptor A
NPR-C	natriuretic peptide clearance receptor
PKGII	type II cGMP dependant protein kinase

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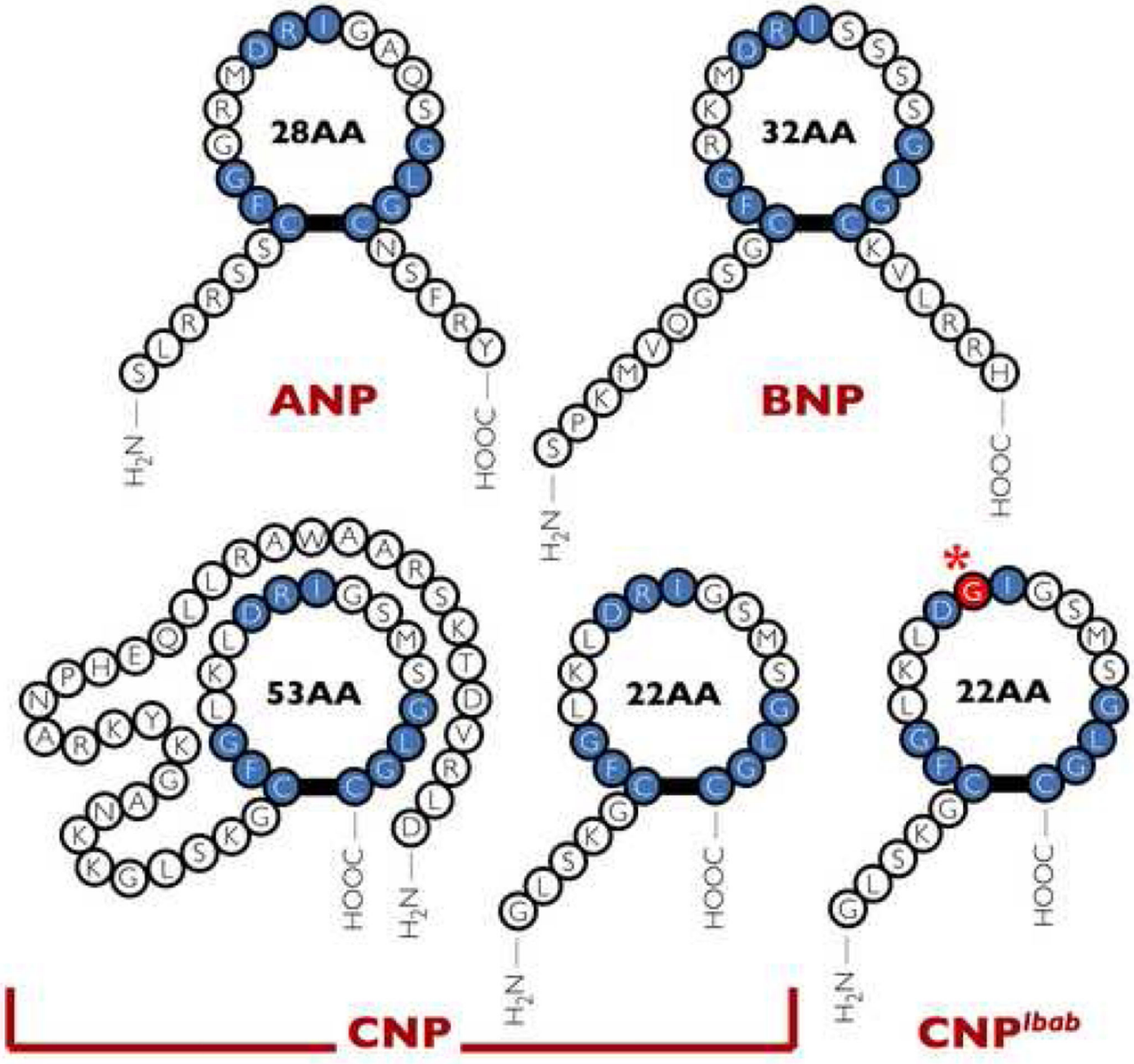
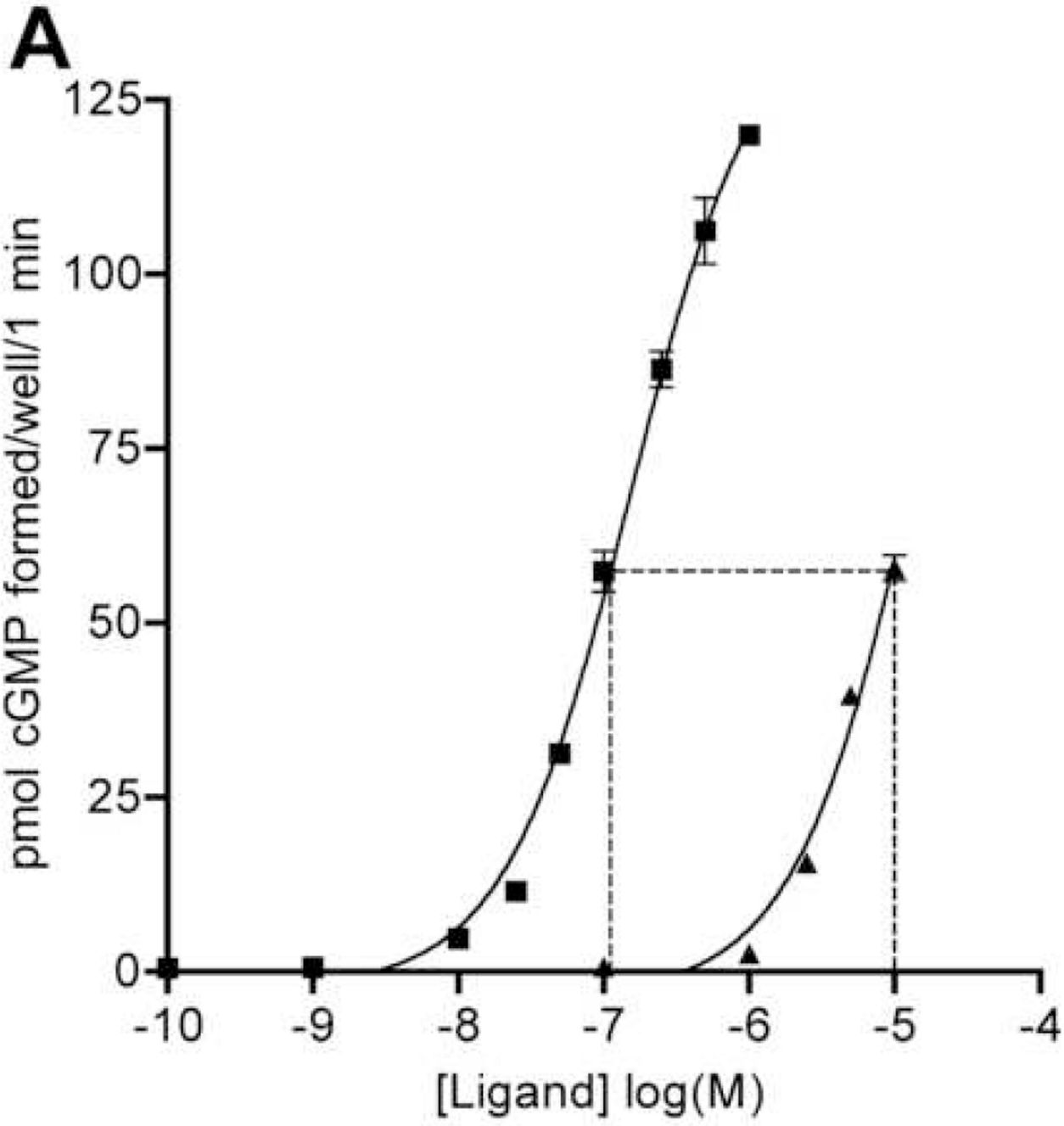
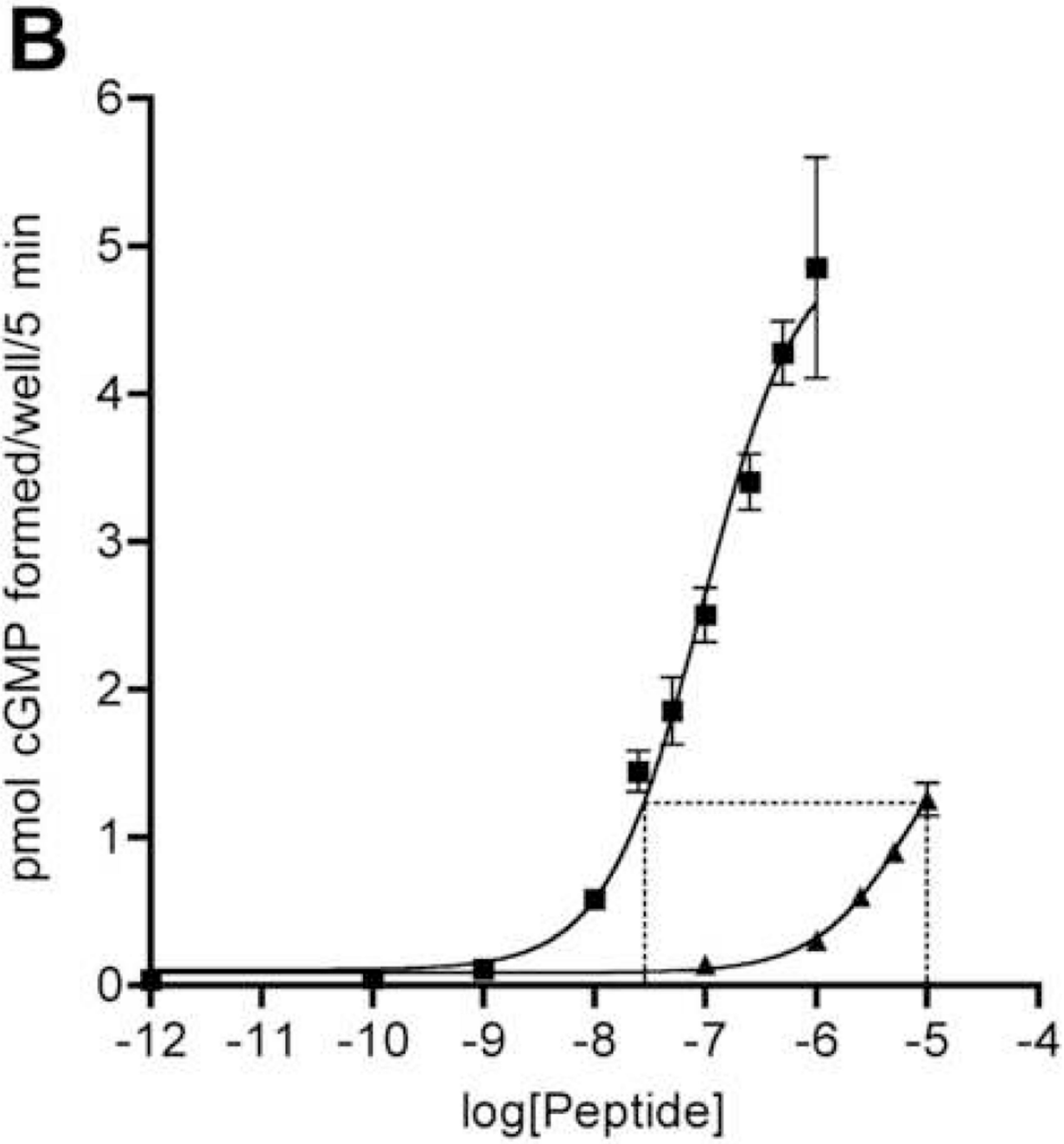


Fig 1.

Cartoon schematic of the primary amino acid structure of atrial natriuretic peptide (ANP), B-type-natriuretic peptide (BNP), the 53- and 22- amino acid forms of C-type natriuretic peptide (CNP), and the peptide encoded by the *lbab* mutation in *Nppc* (CNP^{lbab}). The shading indicates residues conserved among family members, the dark bars indicate disulfide bridges, and the asterisk indicates the arginine-to-glycine substitution present in CNP^{lbab}.





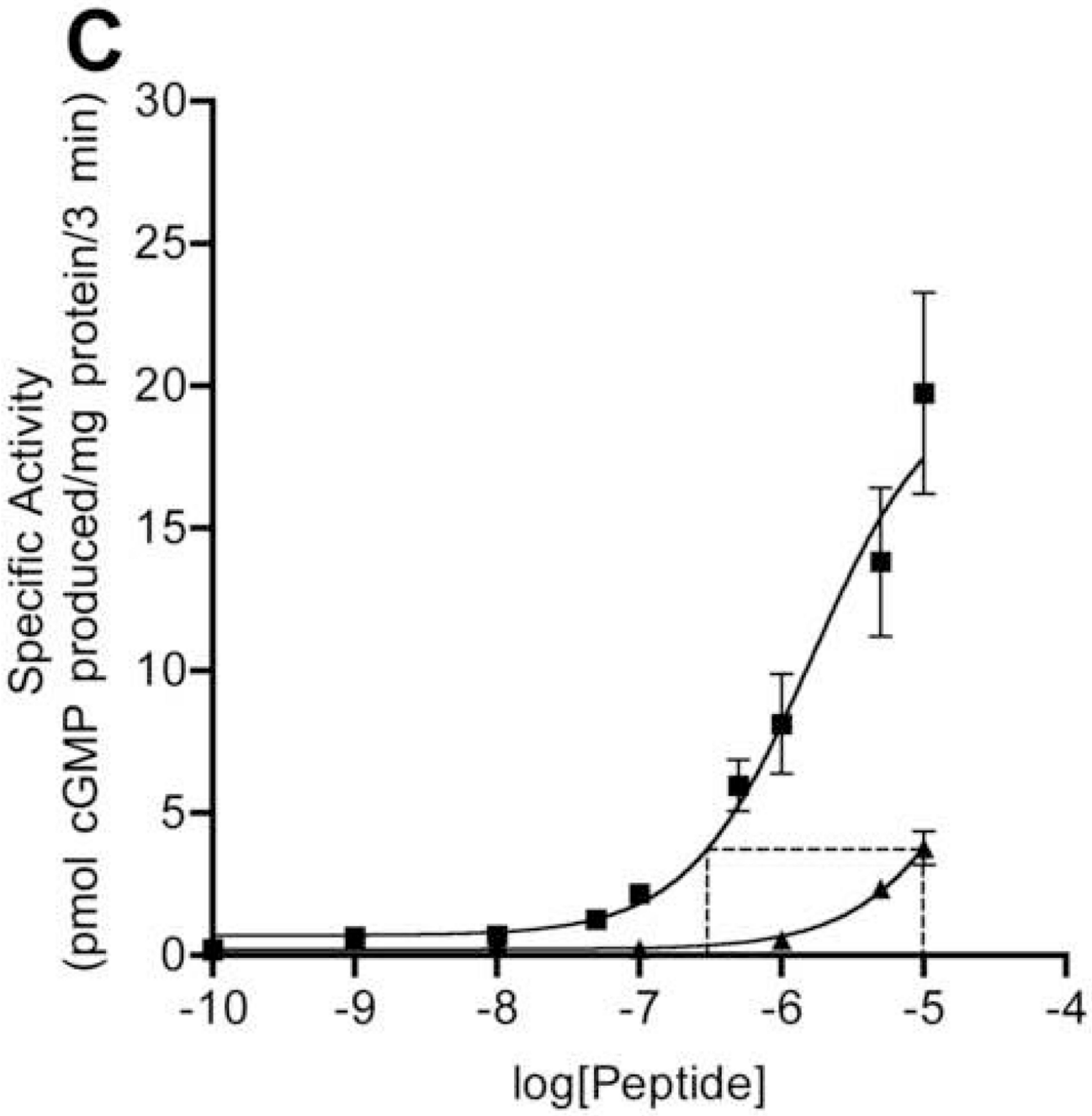


Fig 2.

Effect of increasing concentrations of CNP (■) or CNP^{lbab} (▲) on cGMP generation in intact cells and membrane preparations. (A) One-minute stimulations of 293-NPR-B cells stably overexpressing rat NPR-B. Results are expressed as the mean of triplicate determinations ± SEM. This data is representative of three separate trials. (B) Five-minute stimulations of mouse NIH3T3 cells endogenously expressing NPR-B. Results are expressed as the mean of triplicate determinations ± SEM. This data is representative of two separate trials. (C) Three-minute stimulation of membrane preparations from 293-NPR-B cells.

Results are expressed as the mean of three separate experiments ± SEM. Each individual experiment contained duplicate data points.

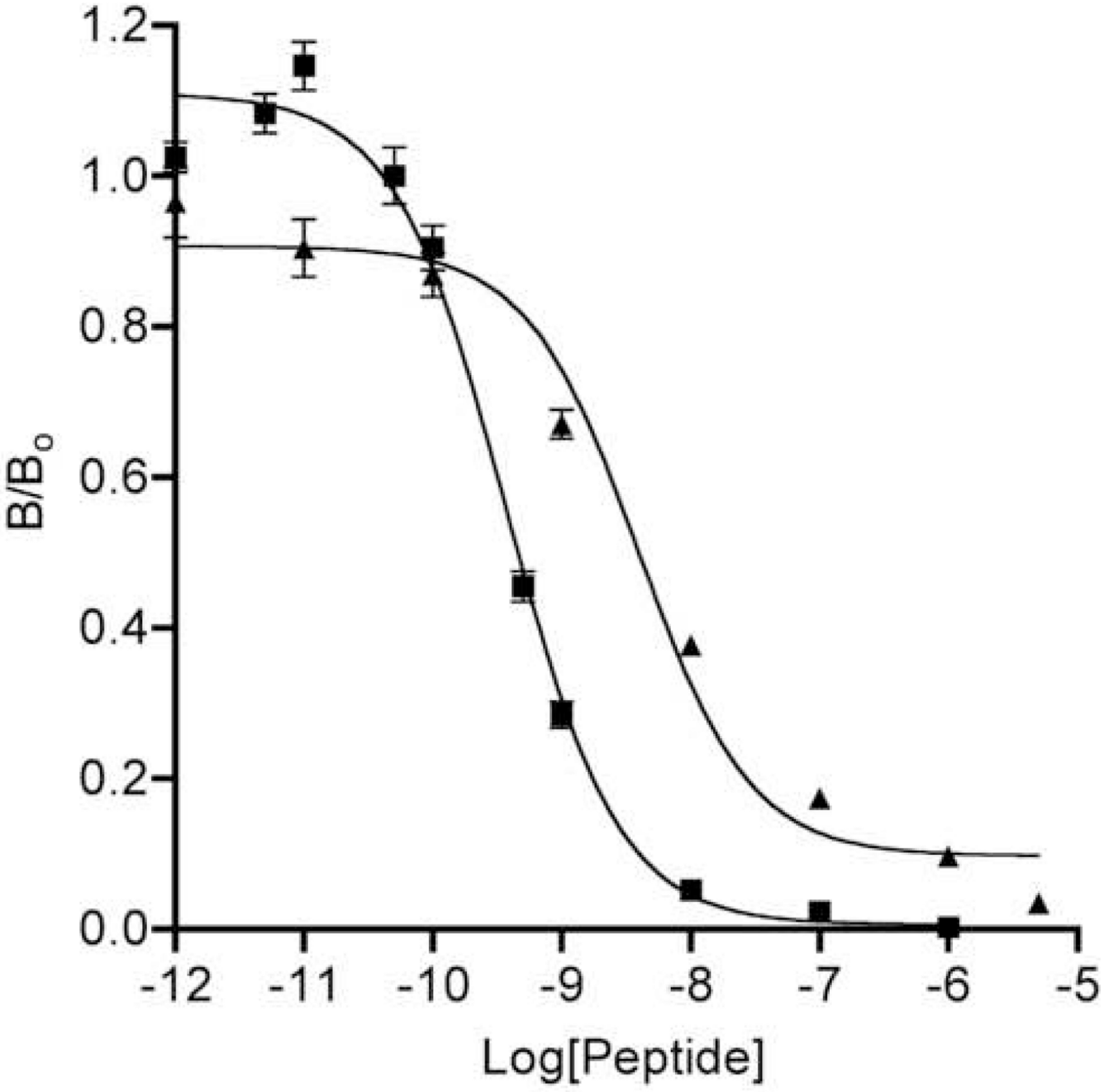
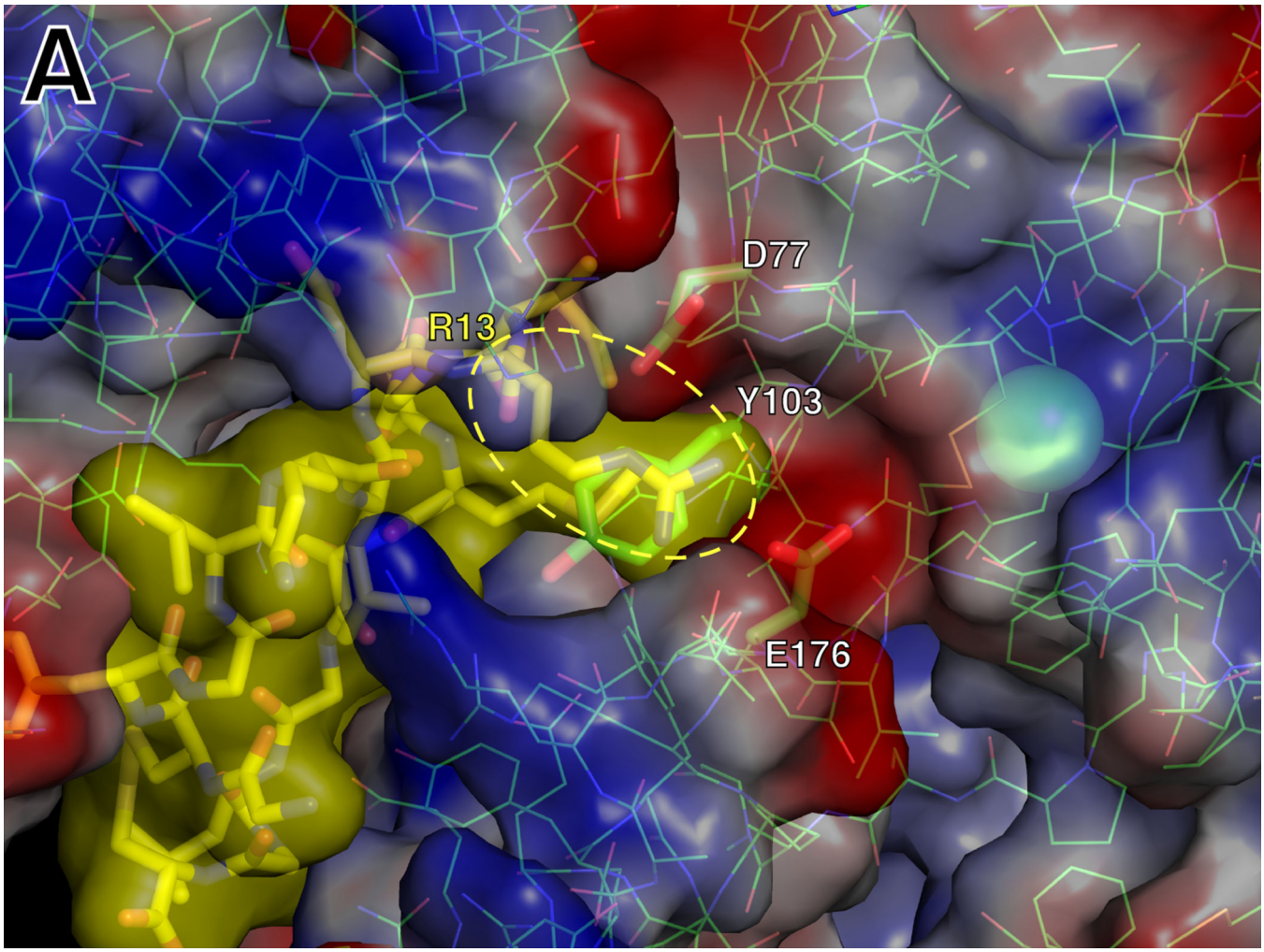


Fig 3.

Reduced binding of CNP^{lbab} to NPR-B. Specific binding of [^{125}I][Tyr^{0}]CNP to rat NPR-B stably expressed in 293 cells was competed with wild-type CNP (■) or CNP^{lbab} (▲). Cells were incubated with DMEM containing 1% BSA and 75 pM [^{125}I][Tyr^{0}]CNP, alone or in the presence of the indicated concentrations of nonradiolabeled CNP or nonradiolabeled CNP^{lbab} at 4°C for 1 h. Results are expressed as the counts per minute specifically bound at each peptide concentration (B) divided by counts per minute specifically bound in the absence of displacing peptide (B_0). Results are the mean of four trials that were assayed in triplicate. The vertical bar within each symbol represents the standard error of the mean.



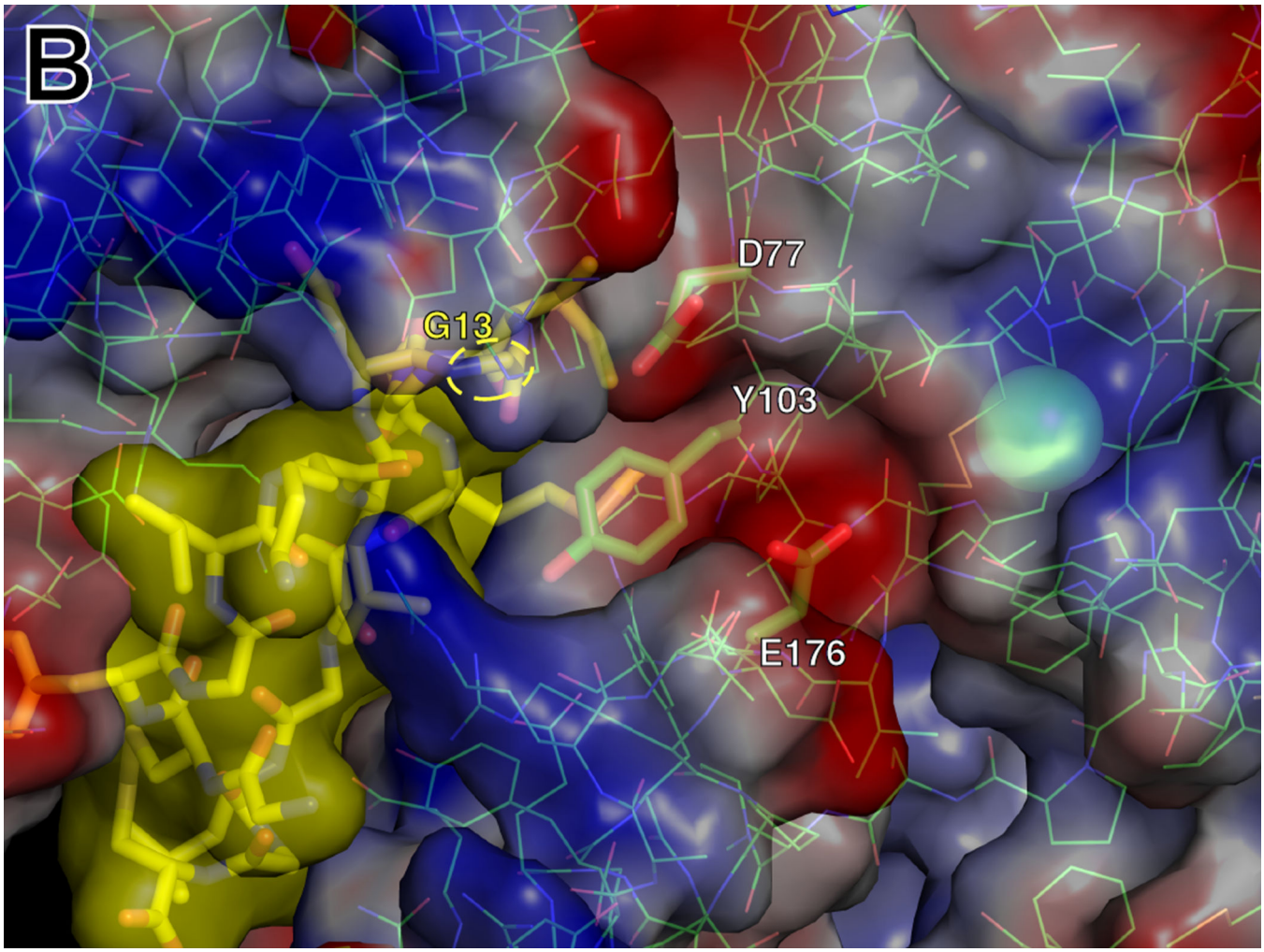


Fig 4.

Theoretical modeling of CNP and CNP^{lbab} binding to human NPR-B. Van der Waals surfaces are portrayed around all residues, with CNP depicted as thick yellow sticks and NPR-B colored according to electrostatic potential, with red indicating acidic regions and blue indicating basic regions. A bound chloride ion is shown as a light blue sphere. (A) Wild-type CNP (with arginine at position 13) is shown modeled with human NPR-B. Human NPR-B residues that have the potential to interact with this arginine are displayed as thick sticks and labeled in white. (B) CNP^{lbab} (with glycine at position 13) is shown modeled with human NPR-B.