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Original Article

C-type atriuretic peptide causes relaxation of the internal anal sphincter through natriuretic peptide receptor B

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ABSTRACT

Objectives: The internal anal sphincter (IAS) plays an important role in maintaining continence. Atrial natriuretic peptide (ANP) could relax the IAS. The purpose of the present study was to investigate the roles of natriuretic peptide receptors (NPRs) in the IAS.
Materials and methods: Relaxation of isolated rat IAS strips caused by natriuretic peptides was measured using isometric transducers. Expression of NPRs was evaluated by reverse transcription–polymerase chain reaction (PCR), immunohistochemistry and real-time PCR.
Results: In the rat IAS, C-type natriuretic peptide (CNP) produced a marked and concentration-dependent relaxation while ANP caused a mild relaxation. By contrast, brain natriuretic peptide, dendroaspis natriuretic peptide, and des[Gln18, Ser19, Gly20, Leu21, Gly22]ANP(4–23) amide did not generate relaxation. CNP was much more effective than ANP and brain natriuretic peptide in stimulating the relaxation, indicating that NPR-B mediates IAS relaxation. The CNP-induced relaxation was inhibited by the protein kinase G inhibitor Rp-8CPT-cGMPS and potassium channel blocker charybdotoxin but not by protein kinase A inhibitor Rp-cAMPS. This suggests the involvement of cGMP and calcium-activated potassium channels in the CNP-induced IAS relaxation. Reverse transcription PCR and immunohistochemistry revealed the existence of NPR-B in the rat IAS. Furthermore, real-time PCR identified abundant expression of CNP and NPR-B in the rat IAS.
Conclusion: CNP causes relaxation of the IAS via NPR-B, cGMP, and potassium channel pathways in rats. CNP might regulate IAS motility. NPR-B is a potential therapeutic target in anorectal motility disorders.

1. Introduction

Natriuretic peptides are a family of peptides with natriuretic, diuretic, and vasorelaxant activities. They are involved in cardiovascular and renal homeostasis [1,2], and cardiovascular diseases as well as metabolic syndrome [3,4]. Recently, natriuretic peptides have been associated with a novel gut-heart interaction [5–7]. The major natriuretic peptides are atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and dendroaspis natriuretic peptide (DNP). ANP and BNP are synthesized in the heart and kidney, and act as cardiac hormones, whereas CNP is produced in vessels, the kidney and gastrointestinal tissues and acts as an autocrine or paracrine regulator. DNP is a potent natriuretic peptide isolated from snake venom. Three natriuretic peptide receptors (NPRs), NPR-A, NPR-B, and NPR-C, have been identified. NPRs are expressed in the kidney, lung, vessels, and brain [1,2]. NPR-A has a high affinity for ANP, BNP, and DNP but a low affinity for CNP. By contrast, NPR-B has a high affinity for CNP but a low affinity for ANP, BNP, and DNP. NPR-C has a high affinity for ANP, DNP, and des[Gln18, Ser19, Gly20, Leu21, Gly22] ANP(4–23) amide [cANF (4–23)], which is a selective NPR-C agonist [1,2,8,9].

In the gastrointestinal system, natriuretic peptides are involved in gastrointestinal motility and secretion [10,11]. Previous studies showed that natriuretic peptides relax the esophagus, stomach, colon, and gallbladder. CNP produces relaxation of the esophagus [12] and ANP, BNP, and DNP generate relaxation of the lower esophageal sphincter [13,14]. In addition, ANP and CNP inhibit

Conflict of interest: none.

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contraction in the stomach [10,15] whereas ANP, BNP, CNP, and DNP inhibit contraction in the colon and cecum [8,10,16–18]. In the biliary system, CNP elicits relaxation in the gallbladder [19]. NPRs have been identified in various gastrointestinal tissues. NPR-A and NPR-B are found in the colon whereas NPR-B and NPR-C are detected in the gastric muscles [10,16]. Natriuretic peptides stimulate relaxation of gastrointestinal smooth muscle through NPR-A, NPR-B, or NPR-C. NPR-A mediates relaxation of the lower esophageal sphincter [14]. NPR-B promotes relaxation of the esophagus and gallbladder, whereas NPR-C mediates relaxation of the stomach [12,16,19]. ANP has been reported to cause relaxation in the internal anal sphincter (IAS) [13]. However, the effects of other natriuretic peptides in the IAS are not clear. We hypothesized that natriuretic peptides alter IAS motility through interaction with NPR subtypes. The aim of the present study was to characterize NPRs mediating relaxant effects of natriuretic peptides in the IAS, which play an important role in maintaining continence.

2. Materials and methods

All procedures were performed in compliance with relevant laws and national guidelines. The Institutional Animal Care and Use Committee of E-Da Hospital approved the protocol for this study (IACUC-99013and IACUC-100009). Male Sprague–Dawley rats were obtained from BioLASCO Taiwan (Taipei, Taiwan). Buffer reagents and charybdotoxin were purchased from Sigma-Aldrich (St. Louis, MO, USA). ANP, BNP, CNP, and cANF (4–23) were obtained from American Peptide Company (Sunnyvale, CA, USA). DNP was purchased from Bachem (Bubendorf, Switzerland). Tetrodotoxin was obtained from Tocris Cookson Inc. (Avonmouth, Bristol, UK). Reverse transcription–polymerase chain reaction (RT-PCR) reagents were obtained from Invitrogen (Carlsbad, CA, USA) and RT-PCR primers for NPR-B were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Real-time PCR reagents and TaqMan primer/probe sets for rat NPR-A (Rn00561678_m1), NPR-B (Rn00587693_m1), NPR-C (Rn00563495_m1), ANP (Rn00664637_g1), BNP (Rn00676450_g1), CNP (Rn00587070_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Rn01775763_g1), hypoxanthine phosphoribosyltransferase 1 (HPRT1; Rn01527840_m1), and β-actin (Rn00667869_m1), were purchased from Life Technologies (Grand Island, NY, USA). Rp-8-(4-chlorophenylthio)-guanosine 3′,5′-cyclic monophosphorothioate (Rp-8CPT-cGMPS), Rp-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS), and rabbit polyclonal anti-NPR-B antibody (sc-25486) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). A polymeric horseradish peroxidase (HRP)-linker antibody conjugate system kit (Bond PolymerRefine Detection, DS9800) was obtained from Leica Biosystems Newcastle (Newcastle, Tyneside, UK). Normal rabbit immunoglobulin G (IgG) was purchased from Dako (Copenhagen, Denmark).

2.1. Measurements of relaxation of isolated rat IAS strips

Measurements of relaxation of muscle strips from the IAS were performed according to a procedure published previously with minor modifications [20–22]. Male rats, weighing 300–350 g, were euthanized with CO_2. The IAS muscle strips (~3 mm × 10 mm) were isolated and suspended in a 37°C organ bath containing Krebs–Henseleit solution (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 11.1 mM glucose, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, and 1.2 mM $MgSO_4$, pH 7.4), gassed with 95% O_2/5% CO_2. The IAS muscle strips were connected to isometric force transducers (FORT 10 g, World Precision Instruments Inc., Sarasota, FL, USA), which were connected to an amplifier and a computer recording system (BIOPAC Systems, Santa Barbara, CA, USA). The basal tension of the muscle strips was adjusted to 1.0 g. Natriuretic peptides were added in a noncumulative fashion, i.e., using single dose administration. The relaxation responses were represented as a percentage of the relaxation to 100μM papaverine (% papaverine). Only one single dose response was studied with each preparation. For studies using tetrodotoxin, the muscle strips were exposed to tetrodotoxin for 15 minutes and then to CNP. For studies using potassium channel or protein kinase inhibitors, the muscle strips were exposed to the indicated concentrations of these inhibitors for 30 minutes, and then to CNP.

2.2. RT-PCR for detection of NPR-B mRNA in rat IAS

RT-PCR for detection of NPR-B mRNA in rat IAS was performed as described before with minor modifications [20,23]. Total RNA was isolated from the rat IAS using TRIzol reagent, treated with DNase I and reverse transcribed into cDNA with superscript II RNase H^- reverse transcriptase. PCR amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems/Life Technologies, Grand Island, NY, USA) with Taq polymerase for one cycle at 94°C for 10 minutes, followed by 35 cycles at 94°C for 30 seconds, 47°C for 1 minutes, 72°C for 1 minute, and, finally 72°C for 10 minutes. Similarly, PCR amplification for GAPDH was performed with Taq polymerase for one cycle at 94°C for 2 minutes, followed by 30 cycles at 94°C for 30 seconds, 48°C for 30 seconds, 72°C for 30 seconds, and, finally 72°C for 5 minutes. After amplification, the PCR products were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and analyzed under ultraviolet light. The following primers were used [23,24]: NPR-B: F: 5′-ACGGGCTGGCTAGCTTCCGA-3′; R: 5′-GCCTTCTGCATGCCCGTGGT-3′; GAPDH: F: 5′-GACCCCTTCATTGACCTCAACT-3′; and R: 5′-CTCAGTGTAGCCCAGGATGCC-3′.

2.3. Immunohistochemistry for detection of NPR-B in rat IAS

To localize NPR-B in the rat IAS, immunohistochemistry was performed as described previously with modifications [25]. Briefly, immunohistochemical staining was performed using an automated staining system (BOND-MAX; Leica Microsystems, Nussloch, Germany). Deparaffinized IAS tissue sections (4 μm) were subjected to heat-induced antigen retrieval with citrate buffer, pH 6.0. The staining procedure involved peroxidase blocking with 3% hydrogen peroxide for 5 minutes, and sequential applications of the primary rabbit anti-NPR-B antibody (0.4 μg/mL) for 30 minutes and an anti-rabbit IgG HRP polymer for 8 minutes at room temperature. Subsequently, the tissue sections were treated with a chromogen, 3,3′-diaminobenzidine, for 10 minutes and counterstained with hematoxylin for 5 minutes at room temperature. Normal rabbit IgG (0.4 μg/mL) was used as the negative control.

2.4. Real-time PCR for detection of mRNA of NPRs and natriuretic peptides in rat IAS

Real-time PCR for detection of mRNA of NPRs and natriuretic peptides was performed using the TaqMan Gene Expression Assay as described previously with minor modifications [26]. In brief, total RNA was isolated from the rat IAS using TRIzol reagent and treated with DNase I. The RT reaction was performed using a recombinant moloney murine leukemia virus reverse transcriptase (MultiScribe reverse transcriptase). Real-time PCR was performed using an ABI 7500 Detection System (Applied Biosystems/Life Technologies). Three housekeeping genes, GAPDH, HPRT1, and β-actin, were used as endogenous controls to standardize the amount of cDNA. Thermal cycling conditions were one cycle of 2 minutes at 50°C and one cycle of 10 minutes at 95°C, followed by 40 cycles of amplification for 15 s at 95°C and 1 minute at 60°C. Cycle threshold

values were obtained and quantification was performed by the relative expression method using the geometric mean of the housekeeping genes [27]. Negative controls had no reverse transcriptase added.

2.5. Analysis of data

Results were expressed as mean ± standard error of the mean. Statistical evaluation was performed using unpaired Student *t* test for two samples or one-way analysis of variance (ANOVA) with Dunnett's procedure for more than two samples. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of natriuretic peptides in rat IAS

CNP provoked a marked and concentration-dependent relaxation (Figs. 1 and 2) of the IAS strips. Specifically, CNP stimulated detectable IAS relaxation at 30nM and maximal relaxation at 300nM, which generated a 55 ± 7% papaverine (100μM)-induced relaxation. The highest tested concentration (1μM) of CNP produced a 52 ± 7% relaxation of the rat IAS. ANP at 0.1μM did not trigger any relaxation whereas at 1μM, it caused a mild, 13 ± 5%, relaxation (Figs. 1 and 2). BNP, DNP, and cANF (4–23), up to 1μM, did not produce any detectable relaxation of the IAS strips (Fig. 2). The CNP-induced relaxation was not significantly affected by tetrodotoxin. Specifically, in the presence of 1μM tetrodotoxin, CNP (300nM) produced a 70 ± 4% relaxation ($p = 0.15$, compared with CNP 300nM alone).

3.2. Effects of protein kinase and potassium channel inhibitors on CNP-induced relaxation in rat IAS

The protein kinase G inhibitor Rp-8CPT-cGMPS, protein kinase A inhibitor Rp-cAMPS, and potassium channel inhibitor charybdotoxin alone caused minimal or no alterations in the basal tone of the IAS. The relaxation produced by CNP in the IAS strips was attenuated by Rp-8CPT-cGMPS and charybdotoxin, but not Rp-cAMPS. Specifically, CNP (100nM) alone caused a 50 ± 6% papaverine-induced relaxation, while in the presence of Rp-8CPT-cGMPS (1μM) and charybdotoxin (0.1μM), it produced 26 ± 4% and 22 ± 8% relaxation, respectively ($p = 0.047$ and 0.015, respectively, compared with CNP alone; Fig. 3). By contrast, in the presence of Rp-cAMPS (1 μM), CNP (100 μM) caused 62 ± 9% relaxation ($p > 0.05$, compared with CNP alone).

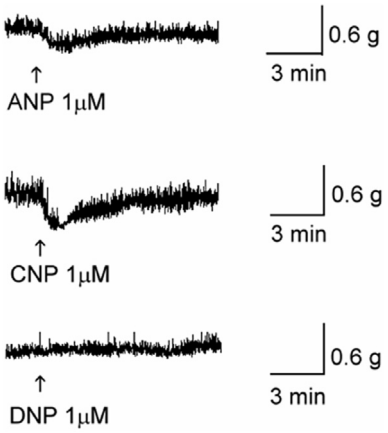

Fig. 1. Typical tracings showing relaxation of the rat IAS caused by atrial natriuretic peptide (ANP), 1μM and C-type natriuretic peptide (CNP), 1μM. Dendroaspis natriuretic peptide (DNP), 1μM, did not generate relaxation.

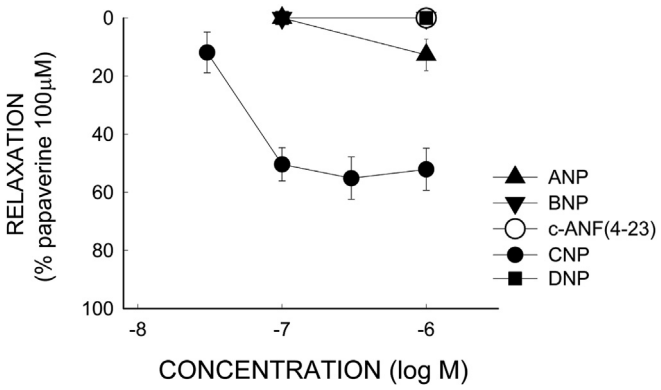

Fig. 2. The ability of natriuretic peptides to cause relaxation of the rat internal anal sphincter. The values are expressed as percent of papaverine (100 μM)-induced relaxation. The results given are from at least three experiments. The vertical bars represent standard error of the mean.

3.3. RT-PCR and immunohistochemical analysis of NPR-B expression in rat IAS

RT-PCR was used to examine mRNA expression of NPR-B in the rat IAS. As shown in Fig. 4, amplification of the cDNA yielded 127 base-pair and 732 base-pair products for NPR-B and GAPDH, respectively, as predicted [23,24]. In immunohistochemical studies, NPR-B receptor protein was detected in the rat IAS muscle (Fig. 5A). Incubation of IAS muscle with nonimmune rabbit IgG followed by the secondary antibody complex resulted in a complete lack of staining (Fig. 5B).

3.4. Real-time PCR analysis of expression of NPRs and natriuretic peptides in rat IAS

The relative mRNA expression of NPRs and natriuretic peptides are shown in Figs. 6 and 7, respectively. Real-time PCR

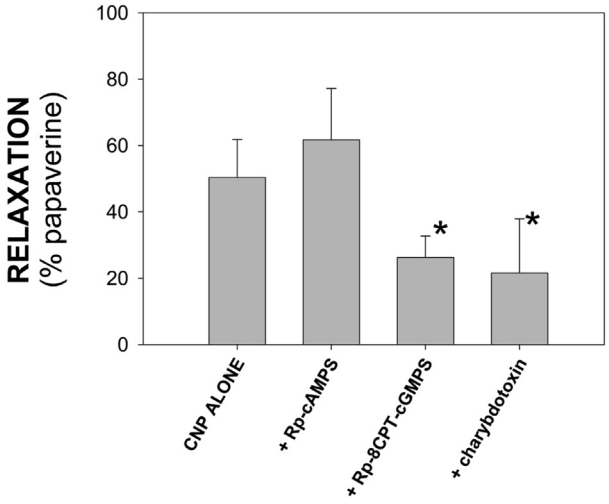

Fig. 3. The ability of C-type natriuretic peptide (CNP), in the presence or absence of the protein kinase A inhibitor Rp-cAMPS (1 μM), protein kinase G inhibitor Rp-8CPT-cGMPS (1 μM) and potassium channel blocker charybdotoxin (0.1 μM), to cause relaxation of the rat internal anal sphincter. The values are expressed as percent of papaverine (100 μM)-induced relaxation. The results given are from at least three experiments. The vertical bars represent standard error of the mean. * Represents significant difference from CNP (100 nM) alone ($p < 0.05$).

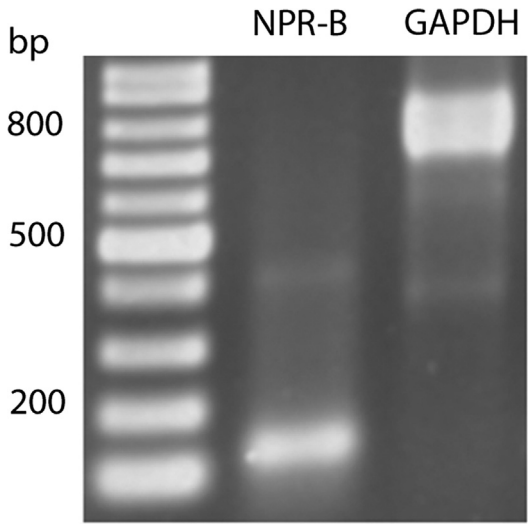

Fig. 4. Reverse transcription polymerase chain reaction analysis of the expression of natriuretic peptide receptor (NPR)-B mRNA in the rat internal anal sphincter. Total RNA was reverse transcribed and amplified using NPR-B and glyceraldehyde-3-phosphate dehydrogenase-specific primers. The amplified products were electrophoresed on an agarose gel, stained with ethidium bromide and analyzed under ultraviolet light. The results presented are representative of three experiments.

revealed that relative expression levels of NPRs in the rat IAS were $2.0 \pm 0.4 \times 10^{-3}$, $38 \pm 10 \times 10^{-3}$, and $35 \pm 10 \times 10^{-3}$ for NPR-A, NPR-B, and NPR-C, respectively (Fig. 6). Significantly higher levels of NPR-B ($p = 0.011$) and NPR-C ($p = 0.019$) were observed compared with NPR-A. In addition, real-time PCR showed that the relative expression levels of ANP, BNP, and CNP in the rat IAS were $1.3 \pm 0.1 \times 10^{-3}$, $0.13 \pm 0.06 \times 10^{-3}$, and $5.4 \pm 2.1 \times 10^{-3}$, respectively (Fig. 7). A significantly higher level of CNP was detected compared with ANP ($p = 0.038$) and BNP ($p = 0.013$).

4. Discussion

Although previous studies found that ANP could relax the IAS, the NPR subtypes mediating IAS relaxation are not clear [13]. The present study demonstrated that ANP and CNP could cause relaxation of the rat IAS, in agreement with a previous study showing that ANP relaxes the opossum IAS [13]. In addition, this study provided novel findings that CNP is the most effective natriuretic peptide stimulating IAS relaxation and NPR-B mediates rat IAS relaxation.

CNP was much more effective than ANP in causing relaxation in rat IAS strips, whereas BNP and cANF (4–23), the selective NPR-C agonist, did not trigger relaxation. This indicates that NPR-B mediates IAS relaxation. Thus, similar to that in the esophagus, colon and gallbladder, IAS relaxation is mediated by NPR-B. Furthermore, the ability of CNP to produce IAS relaxation was not affected by tetrodotoxin, suggesting that natriuretic peptides probably interacted directly with receptors in the smooth muscle of the IAS to cause relaxation. NPR-B expression in the IAS was detected by RT-PCR and immunohistochemistry. Real-time PCR analysis revealed that NPR-B and NPR-C were the predominant NPRs and CNP was the most abundant natriuretic peptide in the IAS. This implies that CNP, as a major natriuretic peptide, mediates relaxation via NPR-B in the rat IAS. The selective NPR-C agonist cANF (4–23) did not cause detectable relaxation, suggesting that the relaxant effect of CNP was through NPR-B and not NPR-C.

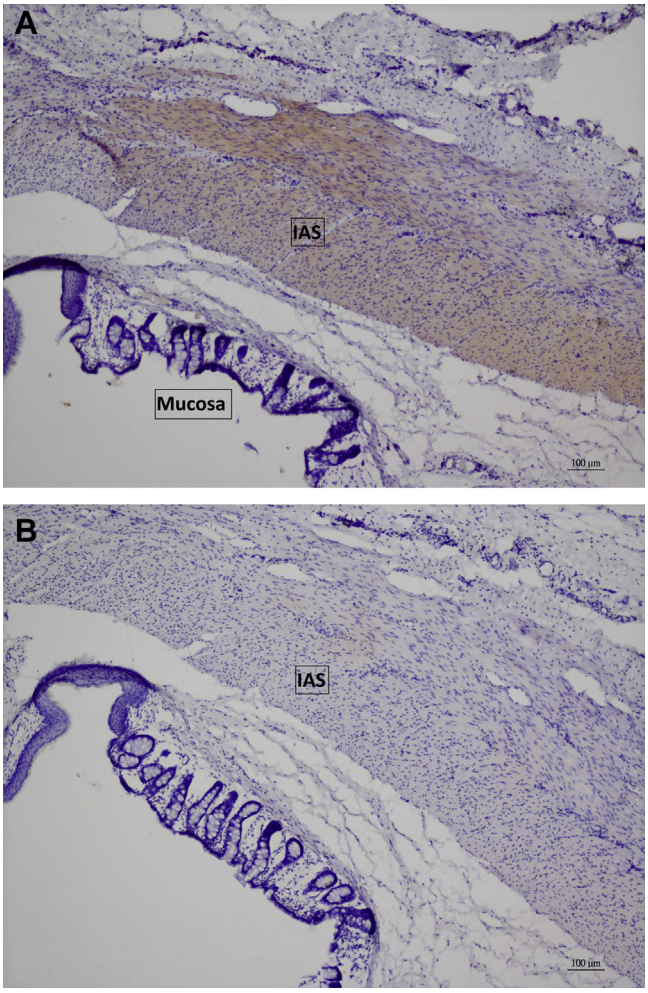

Fig. 5. Immunohistochemical analysis of the rat internal anal sphincter (IAS) stained with the natriuretic peptide receptor-B specific antibody. (A) Natriuretic peptide receptor-B immunostaining was observed in the IAS muscle (magnification 100×). (B) No immunostaining was observed in the negative control with normal rabbit immunoglobulin G (magnification 100×). The results presented are representative of three experiments.

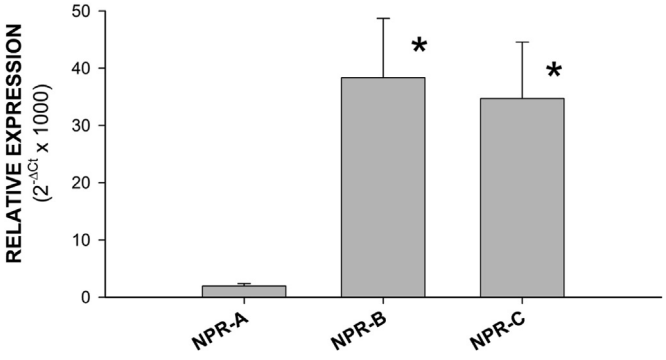

Fig. 6. Real-time polymerase chain reaction analysis of natriuretic peptide receptors (NPR)-A, -B, and -C mRNA expression in the rat internal anal sphincter. The expression levels were calculated using the comparative cycle threshold method and normalized against the geometric mean of three housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyltransferase 1, and β-actin. Data are expressed as means ± standard error of the mean ($n = 4$). * Represents significant difference from NPR-A ($p < 0.05$).

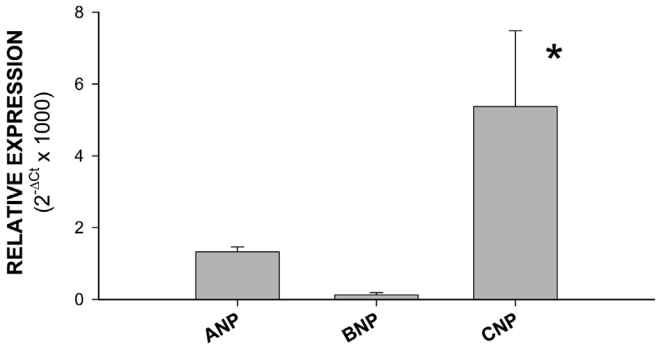

Fig. 7. Real-time polymerase chain reaction analysis of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) mRNA expression in the rat internal anal sphincter. The expression levels were calculated using the comparative cycle threshold method and normalized against the geometric mean of three housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyltransferase 1, and β-actin. Data are expressed as means ± standard error of the mean ($n = 4$). * Represents significant difference from ANP and BNP ($p < 0.05$).

NPR-A and NPR-B are membrane-bound guanylyl cyclases [1,2]. They produce cGMP, which activates protein kinase G and subsequently, membrane calcium-activated K+ channels, to trigger muscle relaxation [28]. The present study showed that the CNP-induced IAS relaxant response was inhibited by the protein kinase G inhibitor Rp-8-CPT-cGMPS and the calcium-activated potassium channel blocker charybdotoxin, but not by the protein kinase A inhibitor Rp-cAMPS. This suggests that the CNP-induced relaxation in the rat IAS involves cGMP and potassium channels but probably not cAMP, in agreement with previous studies of CNP signaling pathways [1,2].

The IAS tone is altered by neural and humoral signals through various membrane receptors [20,29]. The present study showed that NPR-B mediated relaxation in the IAS, which plays an important role in maintaining continence. Further studies are warranted to clarify the involvement of NPR-B in anorectal motility disorders. Currently, nitroglycerin and calcium channel blockers such as nifedipine are used for topical treatment of chronic anal fissure, a common disorder associated with increased tone of the IAS. However, there is a moderate incidence of side effects and the response is inadequate in some patients [30]. A recent study proposed that topical application of captopril, an angiotensin-converting enzyme inhibitor, might be a potential therapeutic option in chronic anal fissure [31]. The present study demonstrates that CNP can relax the IAS effectively. Therefore, NPR-B agonists might be of potential therapeutic value in anorectal motility disorders such as chronic anal fissure or anal pain after hemorrhoidectomy [31,32].

Taken together, these results demonstrate that CNP causes relaxation of the rat IAS through NPR-B, cGMP, and potassium channel pathways. CNP might regulate IAS motility. NPR-B is a potential therapeutic target in anorectal motility disorders.

Acknowledgments

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