

The Effect of C-Type Natriuretic Peptide on Delayed Rectifier Potassium Currents in Gastric Antral Circular Myocytes of the Guinea-Pig

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Summary

C-type natriuretic peptides (CNP) play an inhibitory role in smooth muscle motility of the gastrointestinal tract, but the effect of CNP on delayed rectifier potassium currents is still unclear. This study was designed to investigate the effect of CNP on delayed rectifier potassium currents and its mechanism by using conventional whole-cell patch-clamp technique in guinea-pig gastric myocytes isolated by collagenase. CNP significantly inhibited delayed rectifier potassium currents [$I_{K(V)}$] in dose-dependent manner, and CNP inhibited the peak current elicited by depolarized step pulse to 86.1±1.6 % (n=7, P<0.05), 78.4±2.6 % (n=10, P<0.01) and 67.7±2.3 % (n=14, P<0.01), at concentrations of 0.01 μmol/l, 0.1 μmol/l and 1 μmol/l, respectively, at +60 mV. When the cells were preincubated with 0.1 μmol/l LY83583, a guanylate cyclase inhibitor, the 1 μmol/l CNP-induced inhibition of $I_{K(V)}$ was significantly impaired but when the cells were preincubated with 0.1 μmol/l zaprinast, a cGMP-sensitive phosphodiesterase inhibitor, the 0.01 μmol/l CNP-induced inhibition of $I_{K(V)}$ was significantly potentiated. 8-Br-cGMP, a membrane permeable cGMP analogue mimicked inhibitory effect of CNP on $I_{K(V)}$. CNP-induced inhibition of $I_{K(V)}$ was completely blocked by KT5823, an inhibitor of cGMP-dependent protein kinase (PKG). The results suggest that CNP inhibites the delayed rectifier potassium currents *via* cGMP-PKG signal pathway in the gastric antral circular myocytes of the guinea-pig.

Key words

Delayed rectifier potassium currents • C-type natriuretic peptide • Gastric myocytes • Cyclic GMP

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Introduction

Potassium currents represent the dominant repolarizing conductance within the physiological range of membrane potentials (-50 mV to 0 mV) and are critical in determining the membrane potential E_m of smooth muscle cells. There is a general agreement that three kinds of outward potassium currents are in the smooth muscle cells (Kurgama *et al.* 1998), i.e. the delayed rectifier potassium currents [$I_{K(V)}$], the calcium-activated potassium currents [$I_{K(ca)}$] and the transient outward potassium currents [$I_{(to)}$]. In our previous study, $I_{(to)}$ was not observed in the gastric antral circular myocytes of the guinea-pig (Li *et al.* 2000, Piao *et al.* 2001). $I_{K(V)}$ has already been characterized in various types of smooth muscle cells, for example, coronary artery (Leblanc *et al.* 1994), trachea (Boyle *et al.* 1992, Fleischmann *et al.* 1993), portal vein (Miller *et al.* 1993), cerebral artery (Albarwani *et al.* 2003) and pulmonary artery smooth muscle cells (Post *et al.* 1992, Wade *et al.* 1999), however, only a little has been done on $I_{K(V)}$ in the gastrointestinal smooth muscle myocytes.

The functions of different types of potassium currents in smooth muscles are variable among different animals as well as among different organs. Wade *et al.* (1999) identified a $I_{K(V)}$ current and a $I_{K(ca)}$ current in smooth muscle cells of human esophagus and demonstrated that $I_{K(V)}$ current play a dominant role in regulation of resting tension of esophageal muscle, whereas $I_{K(ca)}$ current largely limited contraction associated with excitation. Koh *et al.* (1998, 1999)

observed the basal activation of ATP-sensitive potassium current (I_{KATP}) contributed to membrane potential in murine colonic smooth muscles and I_{KATP} could contribute to dual regulation of membrane conductance and generate either depolarization or hyperpolarization, depending on the open probability of I_{KATP} channels. However, $I_{(to)}$ contributed to the maintenance of negative resting membrane potentials in murine antral smooth muscle cells (Amberg *et al.* 2002).

C-type natriuretic peptide (CNP), a member of the natriuretic peptides family, was first isolated in porcine brain. It is a peptide of 22 amino acid residues including 17-residue sequences flanked by two cysteine residues which is common to all the natriuretic peptides (Sudoh *et al.* 1990). It is widely distributed and has been found in the central nervous system, cardiovascular system, digestive system, reproductive system, pulmonary system and almost all over the body (Barr *et al.* 1996). Natriuretic peptides elicit their physiological effects by binding to specific cell surface receptors, which have been denoted natriuretic peptide type A, B and C receptors (NPR-A, NPR-B and NPR-C). NPR-A preferentially binds atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), whereas NPR-B is more selective for CNP (Misono 2002). NPR-A and NPR-B include intracellular particulate guanylyl cyclase (pGC) domains. Many facts have become available about the effect of CNP on $I_{K(ca)}$ and its mechanism (Hiromi *et al.* 1994). CNP causes relaxation of vascular smooth muscle through activation of large-conductance calcium-activated potassium channels and activation of particulate and soluble guanylate cyclase (Banks *et al.* 1996). Inhibition of Ca^{2+} -dependent potassium channels significantly attenuated human forearm vasodilation caused by CNP (Honing *et al.* 2001).

Our previous study demonstrated natriuretic peptide receptors were expressed in rat gastric smooth muscle (Guo *et al.* 2003a), and CNP induced relaxations of gastric smooth muscle in rat, guinea-pig and human (Guo *et al.* 2003b). The inhibitory effect of CNP on spontaneous contraction in guinea-pig was related to activation of large conductance calcium-activated potassium channels and activation of particulate guanylate cyclase (Guo *et al.* 2003c). However, the effect of CNP on $I_{K(v)}$ and its mechanism is still unclear in gastric smooth muscle. Therefore, in this study we have investigated the effect of CNP on $I_{K(v)}$ and the possibility of NPR-pGC-cGMP-PKG pathway involved in this process in guinea-pig gastric circular myocytes.

Methods

Preparation of cells

Single gastric myocytes were isolated enzymatically from the antrum of guinea pigs stomach, according to a protocol derived from the method as described previously (Xu *et al.* 1996). Briefly, EWG/B guinea pigs (obtained from the Experimental Animal Department of Jilin University College of Medicine, Certificate No. 10-6004) of either sex weighing 300-350 g were euthanized by lethal dose of pentobarbital sodium (50 mg/kg, i.p.). The antral part of the stomach was promptly excised and equilibrated in Ca^{2+} -free physiological salt solution (Ca^{2+} -free PSS) which was oxygenated. After cutting out the mucosal layer using fine scissors, the muscle layer was separated and dissected into small segments (1 mm × 4 mm). These segments were kept in modified Kraft-Bruhe (K-B) medium at 4 °C for 15 min. Then they were incubated at 36 °C in 4 ml digestion medium (Ca^{2+} -free PSS) containing 0.1 % collagenase Type II, 0.1 % dithioerythritol (DTT), 0.15 % trypsin inhibitor and 0.2 % bovine serum albumin (BSA) for 25-35 min. After digestion, the muscle segments were transferred into the modified K-B medium, and single myocytes were dispersed by gentle agitation with a wide-bored fire-polished glass pipette. Isolated gastric antral myocytes were kept in modified K-B medium at 4 °C until use.

Electrophysiological recordings

The isolated myocytes were transferred to a small chamber on the stage of an inverted microscope (IX-70 Olympus, Japan) for 10-15 min and well-attached to the bottom of the chamber, then continuously superfused with PSS (2-3 ml/min). An 8-channel perfusion system (L/M-sps-8, List Electronics, Germany) was used to change the perfusate. Experiments were performed at 20-25 °C and the whole-cell configuration of the patch-clamp technique was applied. The patch-clamp micropipettes were manufactured from borosilicate glass capillaries (GC) (150T-7.5, Clark Electromedical Instruments, UK) by a two-stage puller (PP-83, Narishige, Japan). Glass pipettes (2-5 M Ω) filled with pipette solution were used to make giga seal of 3-5 G Ω . Pipette and membrane capacitance and series resistance were electronically compensated and the whole-cell currents were recorded with a patch-clamp amplifier EPC-10 (HEKA Instruments, Germany).

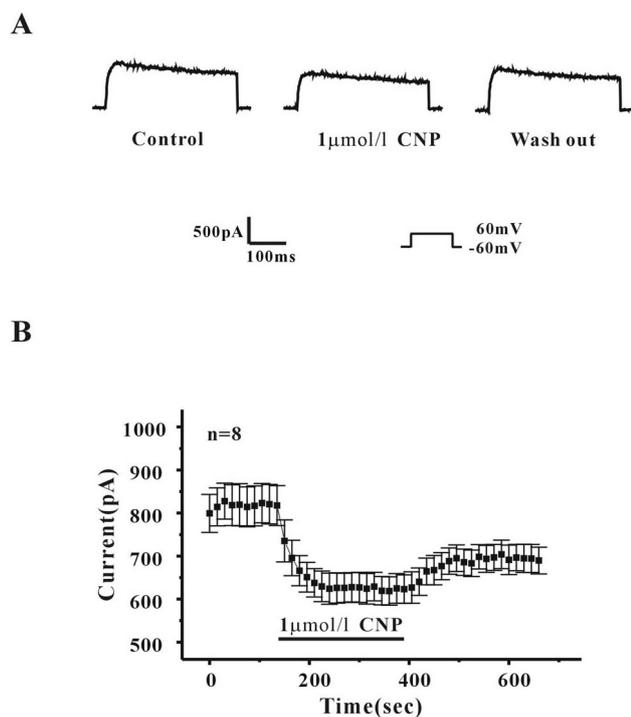


Fig. 1. Effect of CNP on $I_{K(V)}$ in the gastric antral circular myocytes. **A** shows raw current traces elicited by single depolarized step pulse. **B** shows the time-effect relationship of CNP on $I_{K(V)}$.

Drugs and solutions

All drugs were purchased from Sigma Chemical Co, USA. Tyrode's solution contained (mmol/l) NaCl 147, KCl 4, CaCl₂ 2, MgCl₂ 1.05, NaH₂PO₄ 0.42, Na₂HPO₄ 1.81 and glucose 5.5, pH was adjusted to 7.35 with NaOH. PSS contained (mmol/l) NaCl 134.8, KCl 4.5, MgCl₂ 1.0, CaCl₂ 2.0, glucose 5.0 and HEPES 10.0, and pH was adjusted to 7.4 by using Tris. In Ca-free PSS, CaCl₂ 2.0 mmol/l was omitted from PSS. The pH of modified Kraft-Bruhe solution containing (mmol/l) egtazic acid 0.5, HEPES 10, MgCl₂ 3, KCl 50, glucose 10, L-glutamate 50, taurine 20 and KH₂PO₄ 20, was adjusted to 7.40 with KOH. The pipette solution contained (mmol/l) potassium-aspartic acid 110, Mg-ATP 5, HEPES 5, MgCl₂ 1.0, KCl 20, egtazic acid 10, di-tris-creatine phosphate 2.5 and disodium-creatine phosphate 2.5, pH was adjusted to 7.30 with KOH. CNP, LY83583, zaprinast and KT5823 were prepared as 1 mmol/l stock solution respectively.

Data analysis

This experiment is substantiality for comparison. Control is the current before perfusion with CNP or other tool drugs. All values were expressed as mean \pm S.E.M. Statistical significance was evaluated by the *t*-test.

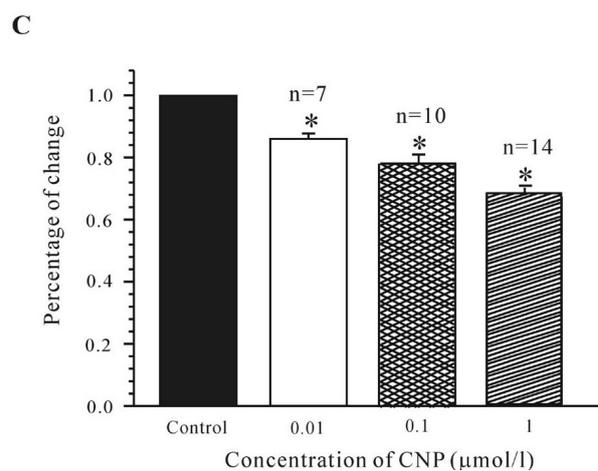
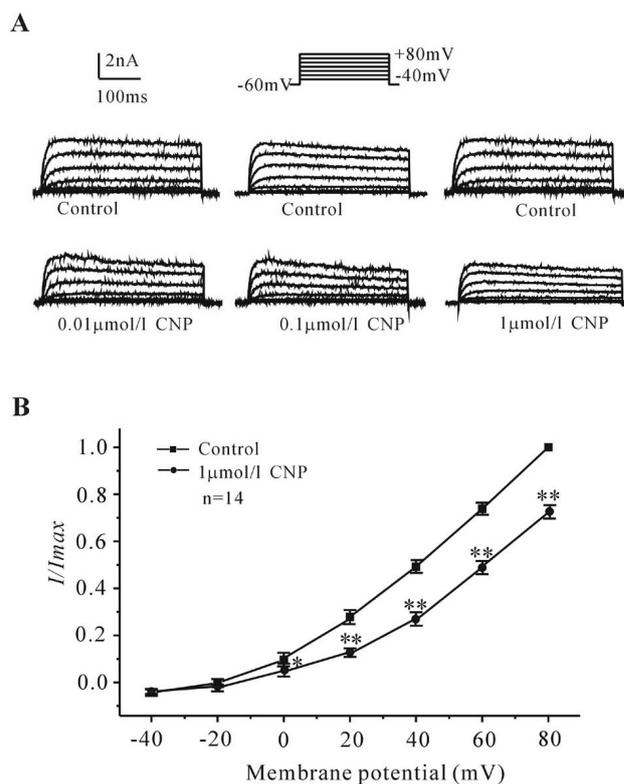


Fig. 2. Effect of different concentrations of CNP on $I_{K(V)}$ in the gastric antral circular myocytes. **A** shows the raw current traces elicited by depolarizing step pulse. **B** shows I-V relation curve of 1mmol/L CNP on $I_{K(V)}$. **C** shows the dose-dependent inhibition of CNP on $I_{K(V)}$ at +60mV. * P <0.05, ** P <0.01 vs control group, respectively.

Results

Effect of CNP on $I_{K(V)}$

Delayed rectifier potassium currents were recorded from gastric antral myocytes by using conventional whole-cell patch-clamp technique in guinea pigs. Under the whole cell configuration, the membrane potential was clamped at -60 mV, when the pipette solution contained egtazic acid 10 mmol/l, $I_{K(V)}$ was

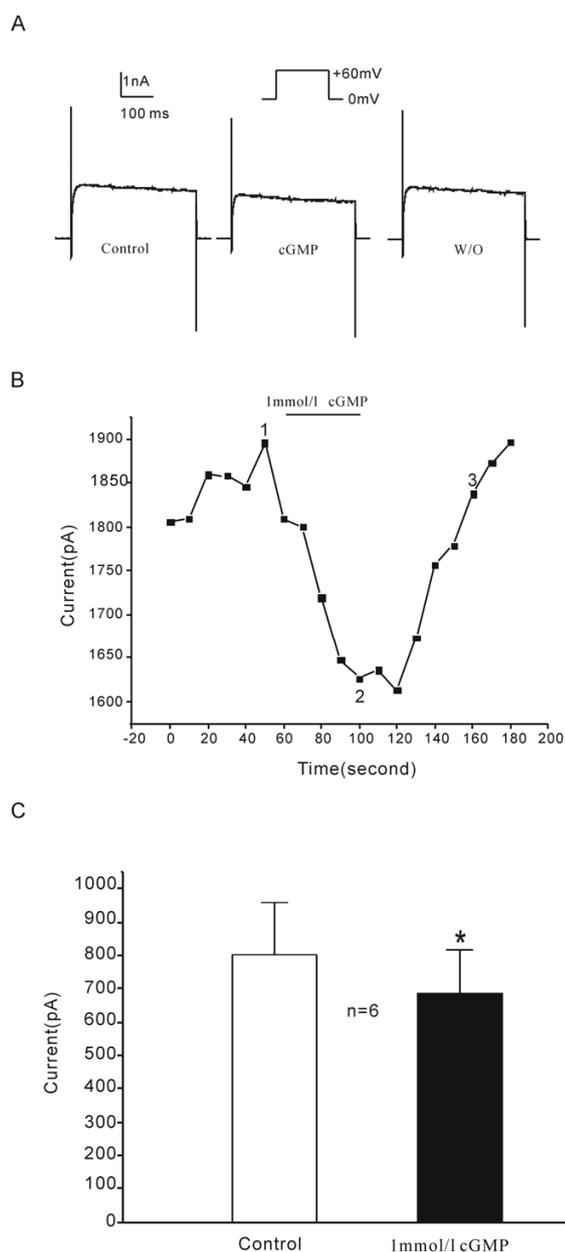


Fig.3. Effect of 8-Br-cGMP on $I_{K(V)}$. **A** shows raw current traces elicited by single depolarized step pulse. **B** shows the time-effect relationship of 8-Br-cGMP on $I_{K(V)}$, and **C** shows inhibitory effect of 8-Br-cGMP on $I_{K(V)}$ which summarized six cells.

elicited by step voltage command pulse from -40 mV to $+80$ mV for 440 ms with a 20 mV increment at 10 sec intervals. The mean peak current of $I_{K(V)}$ was 921.8 ± 33.5 pA $n=60$ at $+60$ mV. First $I_{K(V)}$ was elicited by a single depolarizing step pulse (depolarized to $+60$ mV, 15 sec intervals) for 440 ms, and observed the effect of CNP on $I_{K(V)}$. $I_{K(V)}$ was sharply suppressed by $1 \mu\text{mol/l}$ CNP, and the inhibitory effect started at 45 sec after cells were exposed to CNP (Fig. 1). The inhibitory effect of CNP on $I_{K(V)}$ was partially recovered after CNP was washed out (Fig. 1B). Using the same pulse protocol,

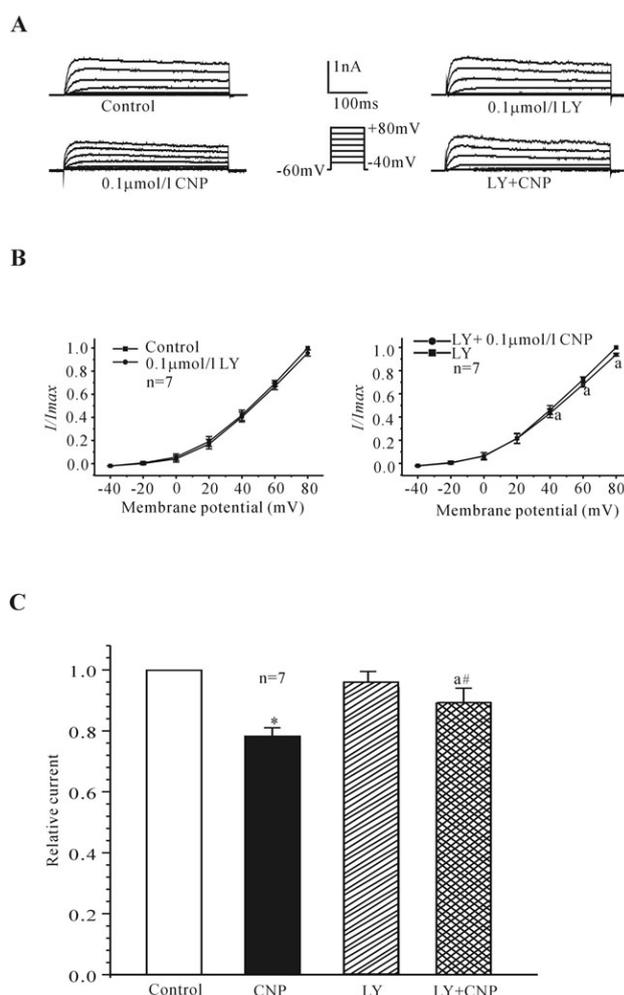


Fig.4. Effect of LY83583 on CNP-induced inhibition of $I_{K(V)}$. **A** shows the raw current traces elicited by depolarizing step pulse. **B** shows the I-V relationships and **C** shows the effect of LY83583 on CNP-induced inhibition of $I_{K(V)}$ at $+60$ mV. * $P < 0.01$ vs control group, ^a $P < 0.05$ vs LY83583 group, # $P < 0.05$ vs CNP group, respectively.

the effect of different concentrations of CNP on $I_{K(V)}$ was observed. CNP significantly inhibited $I_{K(V)}$ in a dose-dependent manner (Fig. 2A), and $I_{K(V)}$ values was significantly decreased by $1 \mu\text{mol/l}$ CNP at every membrane potential from 0 mV to $+80$ mV in I-V curve (Fig. 2B, $n=14$, $P < 0.01$). CNP-induced inhibitions of $I_{K(V)}$ from 100 % of control suppressed to 86.1 ± 1.6 % ($n=7$, $P < 0.05$), 78.4 ± 2.6 % ($n=10$, $P < 0.01$) and 67.7 ± 2.3 % ($n=14$, $P < 0.01$) at concentrations of 0.01 $\mu\text{mol/l}$, 0.1 $\mu\text{mol/l}$ and $1 \mu\text{mol/l}$, respectively, at $+60$ mV (Fig. 2C).

Effect of 8-Br-cGMP on $I_{K(V)}$

Since CNP can bind with NPR-B and activate pGC we investigated whether the inhibitory effect of CNP on $I_{K(V)}$ is mediated by cGMP. The result showed that cell membrane permeable 8-Br-cGMP mimicked the

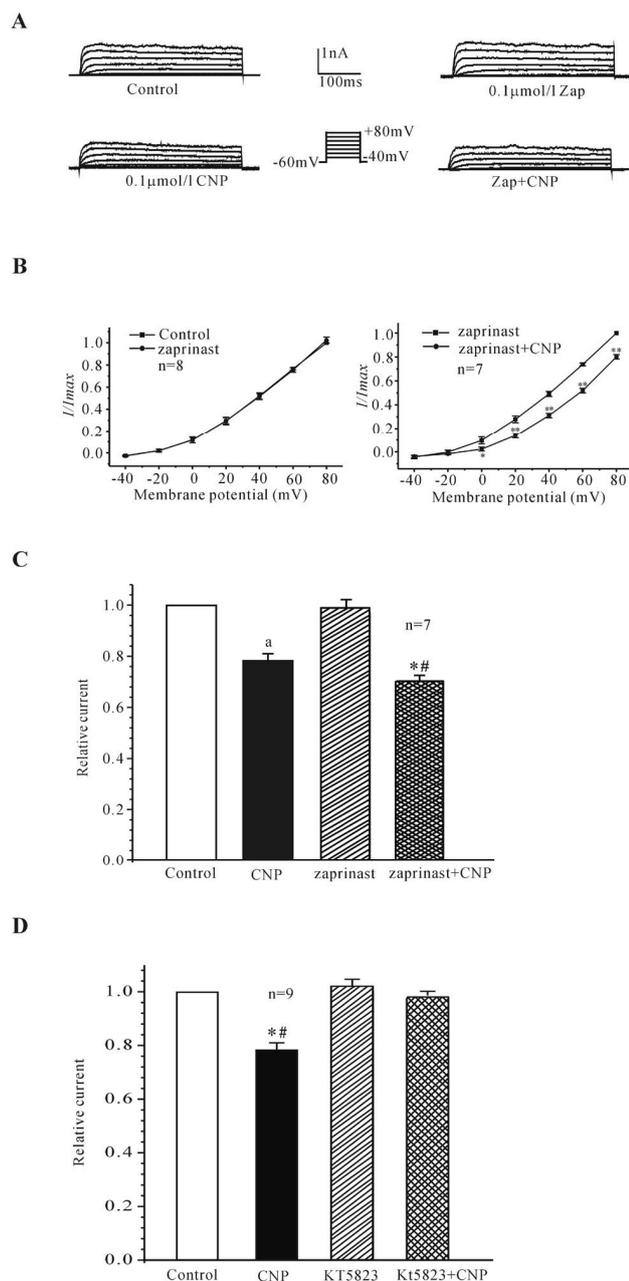


Fig 5. Effects of zaparinast and KT5823 on CNP-induced inhibition of $I_{K(V)}$. A shows the raw current traces elicited by depolarizing step pulse. B shows the I-V relationships and C shows the effect of zaparinast on CNP-induced inhibition of $I_{K(V)}$ at +60mV (^a $P < 0.01$ vs Control group; * $P < 0.05$, ** $P < 0.01$ vs zaparinast group; # $P < 0.01$ vs CNP group respectively). D shows the effect of KT5823 on CNP-induced inhibition of $I_{K(V)}$ at +60mV (* $P < 0.01$ vs Control group, # $P < 0.01$ vs CNP group respectively).

inhibitory effect of CNP, and 1 mmol/l 8-Br-cGMP suppressed $I_{K(V)}$ from 801.8 ± 224.8 pA of control to 686.3 ± 193.5 pA (Fig. 3, $n=6$, $P < 0.05$).

Effect of LY83583, zaparinast on CNP-induced inhibition of $I_{K(V)}$

To further investigate the relationship between

cGMP and inhibitory effect of CNP on $I_{K(V)}$, in the presence of LY83583, a guanylate cyclase inhibitor or zaparinast, a cGMP-sensitive phosphoesterase inhibitor conditions CNP-induced inhibition of $I_{K(V)}$ was observed. LY83583 itself ($0.1 \mu\text{mol/l}$) had no effect on $I_{K(V)}$ (Figs 4A and 4B, $n=7$, $P > 0.05$), but LY83583 significantly diminished the inhibitory effect of CNP on $I_{K(V)}$ in gastric antral myocytes (Figs 4A and 4B, $n=7$, $P < 0.05$). The inhibitory percentage of CNP on $I_{K(V)}$ was diminished from 78.4 ± 2.6 % of control to 89.5 ± 4.5 % by LY83583 at +60 mV (Fig. 4C, $n=7$, $P < 0.05$). However, zaparinast significantly potentiated CNP-induced inhibition of $I_{K(V)}$ (Figs 5A and 5B), and the inhibitory percentage of CNP on $I_{K(V)}$ was potentiated from 78.4 ± 2.6 % of control to 70.3 ± 2.2 % at +60 mV (Fig. 5C, $n=7$, $P < 0.05$).

Effect of PKG inhibitor on CNP-induced inhibition of $I_{K(V)}$

Since the inhibitory effect of CNP on $I_{K(V)}$ was related to cGMP, we further determined whether this process was mediated by cGMP-dependent protein kinase (PKG). The CNP-induced inhibition of $I_{K(V)}$ was completely blocked by $1 \mu\text{mol/l}$ KT5823, an inhibitor of PKG (Fig. 6, $n=9$, $P < 0.01$).

Discussion

The effects of CNP on the gastrointestinal motility have been described by only some reports – relaxant effect on chick rectum muscle strip (Sudoh *et al.* 1990), inhibitory effect on rat tenia coli (Kim *et al.* 2001), and relaxant effect on guinea-pig caecum (Itaba *et al.* 2004). We demonstrated that CNP inhibited spontaneous contractions of gastric smooth muscles in rat, guinea-pig and human (Guo *et al.* 2003b), and hyperpolarized membrane potential in guinea-pig gastric smooth muscle and potentiated calcium-activated potassium currents [$I_{K(Ca)}$] (Guo *et al.* 2003c). Much evidence indicated that $I_{K(Ca)}$ is involved in inhibitory effects of CNP on smooth muscle motility. For example, CNP mediated the relaxation of canine femoral veins through activation of large conductance calcium-activated potassium channels (BK_{Ca}) and activation of particulate and soluble guanylate cyclase (Banks *et al.* 1996). Inhibition of Ca^{2+} -dependent potassium channels significantly attenuated human forearm vasodilation caused by CNP (Honing *et al.* 2001).

In the present study, however, it was found that

CNP significantly inhibited $I_{K(V)}$ in a dose-dependent manner. Since CNP hyperpolarized membrane potential of gastric myocytes, it is suggested that the total effect of CNP on gastric smooth muscle is the increase of the outward potassium currents and relaxing smooth muscles, and CNP-induced relaxation is mainly mediated by $I_{K(Ca)}$. Many studies support our standpoint, because the dual regulation by Ca^{2+} and voltage allowed BK_{Ca} channels to play a more dynamic role in the regulation of cellular excitability than is possible with strictly voltage-gated K^+ channel homologues (Lingle *et al.* 2002), $I_{K(V)}$ current appeared to play a dominant role in regulation of resting tension of human esophageal muscle, whereas $I_{K(Ca)}$ current largely limited contraction associated with excitation (Wade *et al.* 1999), Otsuka *et al.* (2002) found that the apparent extent of BK_{Ca} channels contributing to the total CNP-induced relaxant response was $\approx 60\%$, supporting the substantial role of BK_{Ca} channels in the CNP-induced vascular relaxations (Otsuka *et al.* 2002). According to previous study and our results, $I_{K(V)}$ might play a dominant role in regulation of resting membrane potential in gastric antral circular myocytes of guinea-pig, whereas $I_{K(Ca)}$ may regulate the relaxation in response to the changes of $[Ca^{2+}]_i$ caused by inner or outer stimulations.

Many studies demonstrated that CNP exerted a physiological function through cGMP pathway. It was found that cGMP increased twofold within 10-60 s after the addition of CNP (Banks *et al.* 1996). ANP, BNP and CNP induce the relaxation of the vascular smooth muscle *via* particulate guanylate cyclase (GC) which produces cGMP (Winqvist *et al.* 1984, Protter *et al.* 1996, Akiho *et al.* 1995). Basal release of cGMP was increased up to 4-fold by CNP and this increase was reduced (-68%) in

presence of the natriuretic peptide receptor (NPR) antagonist HS-142-1 (Brunner *et al.* 2001). We previously reported that CNP significantly enhanced cGMP generation in rat gastric smooth muscle (Guo *et al.* 2003a). In the present study, 8-Br-cGMP mimicked inhibitory effect of CNP on $I_{K(V)}$ and LY83583, a kind of inhibitor of guanylate cyclase, markedly diminished the inhibitory effect of CNP on $I_{K(V)}$, but zaprinast, a cGMP-sensitive phosphodiesterase inhibitor, significantly potentiated the CNP-induced inhibition of $I_{K(V)}$. These data suggested that the CNP-induced inhibition of $I_{K(V)}$ was mediated by CNP-NPR-pGC-cGMP pathway. Meanwhile, KT5823, an inhibitor of cGMP-dependent protein kinase (PKG), completely blocked the CNP-induced inhibition of $I_{K(V)}$. This suggests that cGMP-PKG pathway was involved in this process. Similarly, Zhang *et al.* (2003) found that cGMP inhibited $I_{K(V)}$ in both hypoxic and normal rat pulmonary artery smooth muscle cells, and this inhibition was blocked by H-8, an inhibitor of PKG.

In summary, the major findings from this investigation were that CNP inhibited $I_{K(V)}$ in a dose-dependant manner and the cGMP-PKG pathway was involved in this process in guinea-pig gastric antral circular myocytes. $I_{K(V)}$ may play a dominant role in regulating resting membrane potential in the gastric antral circular myocytes of guinea-pigs.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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