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 peptide play an inhibitory regulation for smooth muscle motility in gastrointestinal tract, however, 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 0.01μmol/L, 0.1 μmol/L and 1 μmol/L, respectively, at +60mV. When the cells were pre-incubated with 0.1μmol/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 pre-incubated with 0.1μmol/L zaprinast, a cGMP-sensitive phosphoesterase inhibitor, the 0.01μmol/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 suggested 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

Introduction

Potassium currents represent the dominant repolarizing conductance within the physiological range of membrane potentials (-50mV to 0 mV) and are critical in determining the membrane potential Em of smooth muscle cells. There is generally 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 [$I_{(to)}$]. 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(Sulayma 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 is variable among different animals as well as among different organs. Wade *et al*(Wade et al. 1999) identified a $I_{K(V)}$ current and a $I_{K(ca)}$ current in esophagus smooth muscle cells of human and demonstrated that $I_{K(V)}$ current play a dominant role in regulating resting tension of esophageal muscle, whereas $I_{K(ca)}$ current largely limited contraction associated with excitation. Koh *et al*(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 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 et al. 2002). NPR-A and NPR-B include intracellular particulate guanylyl cyclase(pGC) domains. Much knowledge has 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 receptor distributed 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.

Materials and 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 ºC for 15 min. Then they were incubated at 36 ºC in 4 ml digestion medium (Ca^{2+}-free PSS) containing 0.1 % collagenase Type Ⅱ, 0.1 % dithioerythreitol (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 4ºC 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 ºC 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 MΩ) filled with pipette solution were used to make giga seal of 3-5 GΩ. 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).

Drugs and solutions All drugs were purchased from Sigma Chemical Co, USA. Tyrode's solution contained (mmol/L) NaCl 147, KCl 4, $CaCl_2 \cdot 2H_2O$ 2, $MgCl_2 \cdot 6H_2O$ 1.05, $NaH_2PO_4 \cdot 2H_2O$ 0.42, $Na_2HPO_4 \cdot 2H_2O$ 1.81 and glucose 5.5, pH was adjusted to 7.35 with NaOH. PSS contained (mmol/L) NaCl 134.8, KCl 4.5, $MgCl_2 \cdot 6H_2O$ 1.0, $CaCl_2 \cdot 2H_2O$ 2.0, glucose 5.0 and HEPES 10.0, and pH was adjusted to 7.4 by using Tris. In Ca-free PSS, $CaCl_2 \cdot 2H_2O$ 2.0 mmol/L was omitted from PSS. The pH of modified Kraft-Bruhe solution containing (mmol/L) egtazic acid 0.5, HEPES 10, $MgCl_2 \cdot 6H_2O$ 3, KCl 50, glucose 10, L-glutamata 50, Taurine 20 and KH_2PO_4 20, was adjusted to 7.40 with KOH. The pipette solution contained(mmol/L) potassium-aspartic acid 110, Mg-ATP 5, HEPES 5, $MgCl_2 \cdot 6H_2O$ 1.0, KCl 20, egtazic acid 10, di-*tris*-creatine phoshate 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 consubstantiality comparison. Control is the current before perfusion with CNP or other tool drugs. All values were expressed as mean ± SE. Statistical significance was evaluated by *t*-test.

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 -60mV, when the pipette solution contained egtazic acic 10 mmol/L, $I_{K(V)}$ was elicited by step voltage command pulse from -40 mV to +80mV for 440ms with a 20mV increment at 10 sec intervals. The mean peak current of $I_{K(V)}$ was 921.8±33.5pA（n=60）at +60mV. First $I_{K(V)}$ was elicited by a single depolarizing step pulse (depolarized to +60mV, 15 sec intervals) for 440ms, and observed the effect of CNP on $I_{K(V)}$. $I_{K(V)}$ was sharply suppressed by 1μmol/L CNP, and the inhibitory effect

started at 45 seconds 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, 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 1μmol/L CNP at every membrane potential from 0mV to +80mV 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 0.01μmol/L, 0.1μmol/L and 1μmol/L, respectively, at +60mV (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 inhibitory effect of CNP, and 1mmol/L 8-Br-cGMP suppressed $I_{K(V)}$ from 801.8± 224.8pA of control to 686.3± 193.5pA (Fig.3, n=6, P<0.05).

Effect of LY83583, zaprinast 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 zaprinast, a cGMP-sensitive phosphoesterase inhibitor conditions CNP-induced inhibition of $I_{K(V)}$ was observed. 0.1μmol/L LY83583 itself had no effect on $I_{K(V)}$ (Fig.4A and B, n=7, P>0.05), but LY83583 significantly diminished the inhibitory effect of CNP on $I_{K(V)}$ in gastric antrial myocytes (Fig.4A and B, n=7, P<0.05). The inhibitory percentage of CNP on $I_{K(V)}$ was from 78.4±2.6% of control diminished to 89.5±4.5% by LY83583 at +60 mV (Fig.4C, n=7, P<0.05). However, zaprinast significantly potentiated CNP-induced inhibition of $I_{K(V)}$ (Fig.5A and B), and the inhibitory percentage of CNP on $I_{K(V)}$ was from 78.4±2.6% of control potentiated to 70.3±2.2% at +60mV (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 1μmol/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 guinea-pig caecum(Itaba et al. 2004). We demonstrated CNP inhibited spontaneous contractions of gastric smooth muscles in rat, guinea-pig and human (Guo et al. 2003b), and CNP hyperpolarized membrane potential in guinea-pig gastric smooth muscle and potentiated calcium-activated potassium currents [$I_{K(Ca)}$](Guo et al. 2003c). Many evidence indicated that $I_{K(Ca)}$ is involved in inhibitory effects of CNP on smooth muscle motility, for example, CNP mediated 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 increase in the outward potassium currents and relaxing smooth muscles, and CNP-induced relaxation is mainly mediated by $I_{K(Ca)}$. Many studies support our standpoint, for example, 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 regulating resting tension of human esophageal muscle, whereas $I_{K(Ca)}$ current largely limited contraction associated with excitation(Wade et al. 1999); Otsuka *et al* found that the apparent extent of BK_{Ca} channels contributed 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). Therefore, in our opinion, in the guinea pig gastric antral circular myocytes, $I_{K(V)}$ may play a dominant role in regulating resting membrane potential, 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 by 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 GC which produces cGMP(Winquist 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 phosphoesterase 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 $I_{K(V),}$ it is suggest that cGMP-PKG pathway was involved in this process. Similarly, Zhang *et al*(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.

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Footnote

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 $I_{K(V).}$

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.

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.

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 +60mV. $^{*}P<0.01$ *vs* control group, $^{a}P<0.05$ *vs* LY83583 group, $^{\#}P<0.05$ *vs* CNP group, respectively.

Fig 5. Effect of zaparinast 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.

Fig 6. Effect of KT5823 on CNP-induced inhibition of $I_{K(V)}$. A shows the raw current traces. B shows the I-V relationship and C 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

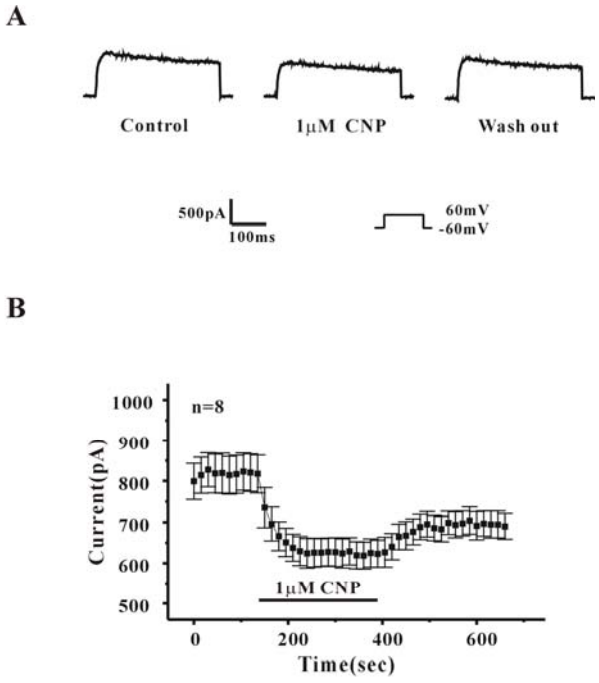

A
Control
1μM CNP
Wash out
500pA
100ms
60mV
-60mV
B
n=8
Current(pA)
1000
900
800
700
600
500
1μM CNP
0
200
400
600
Time(sec)

Fig.1

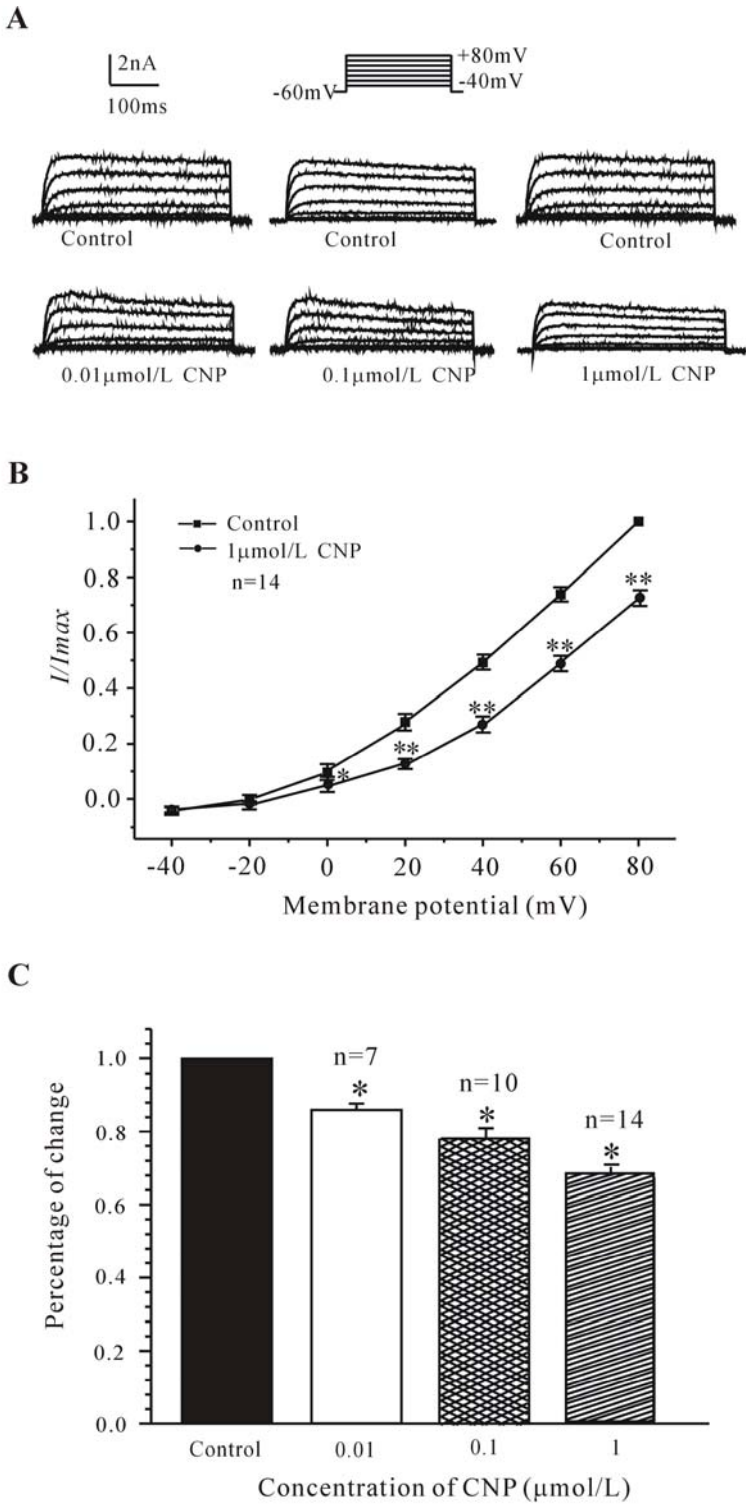
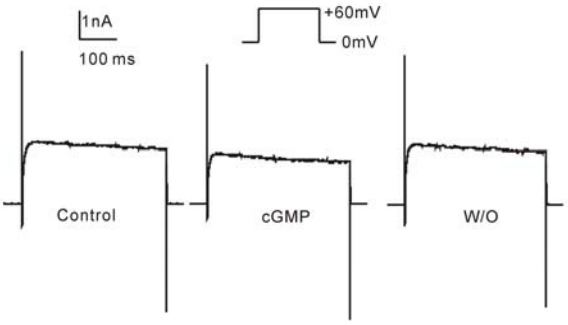
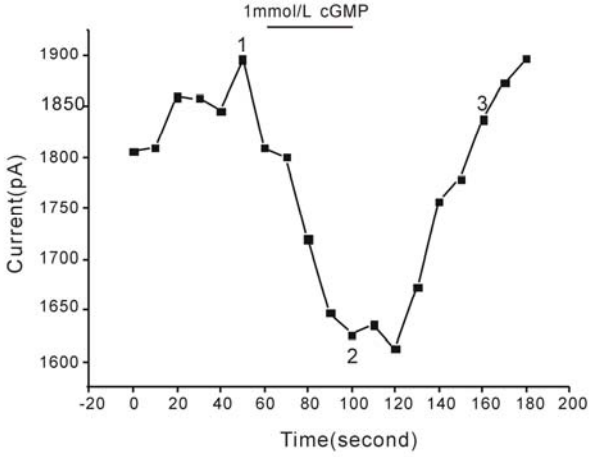

A
2nA
100ms
-60mV
+80mV
-40mV
Control
Control
Control
0.01μmol/L CNP
0.1μmol/L CNP
1μmol/L CNP
B
Control
1μmol/L CNP
n=14
I/Imax
1.0
0.8
0.6
0.4
0.2
0.0
-40
-20
0
20
40
60
80
Membrane potential (mV)
C
Percentage of change
1.0
0.8
0.6
0.4
0.2
0.0
n=7
n=10
n=14
Control
0.01
0.1
1
Concentration of CNP (μmol/L)

Fig.2

A


1nA
100 ms
+60mV
0mV
Control
cGMP
W/O

B


1mmol/L cGMP
1
2
3
Current(pA)
Time(second)

C

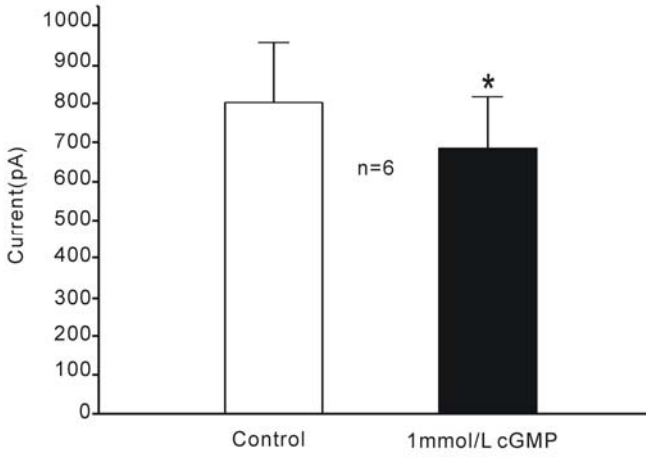

Current(pA)
n=6
*
Control
1mmol/L cGMP

Fig.3

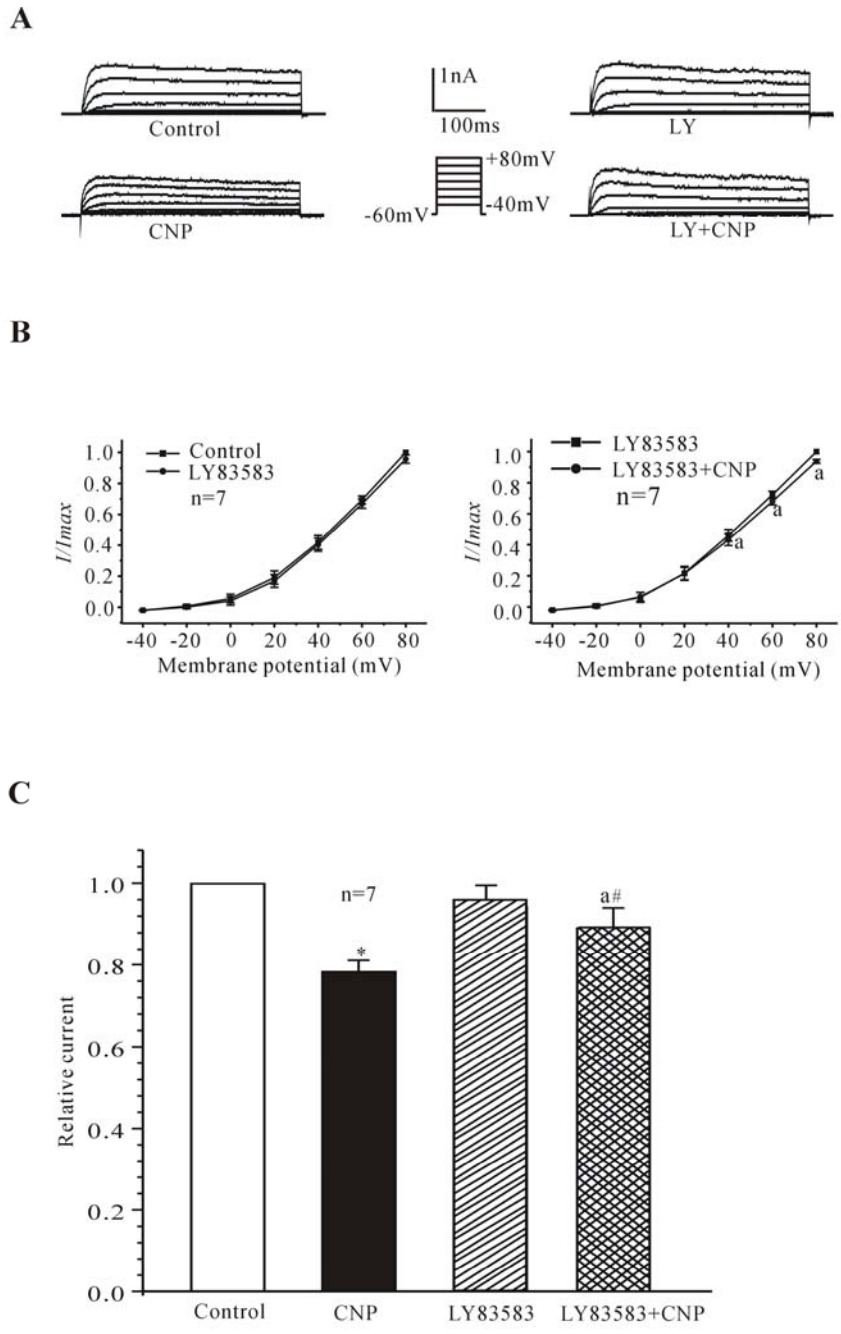
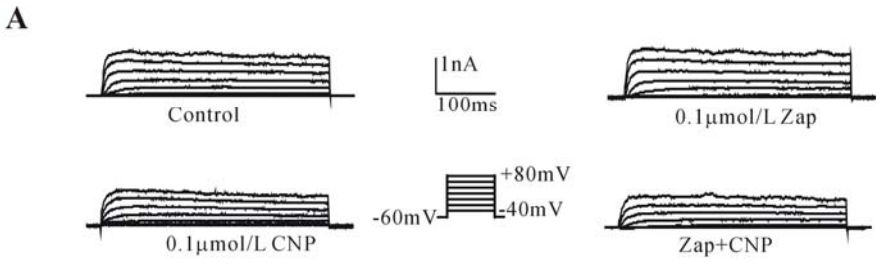
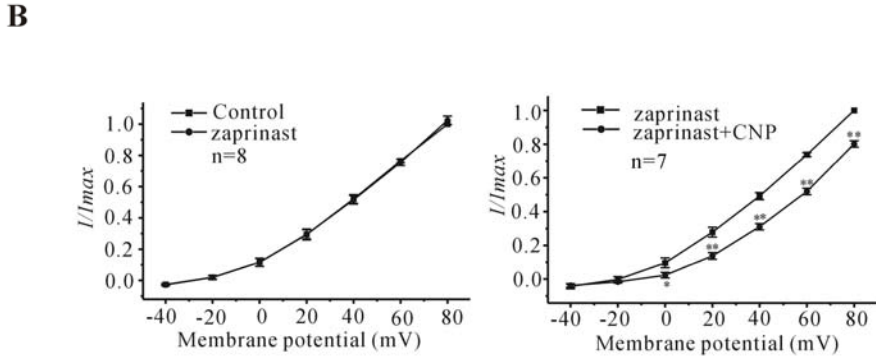

A
Control
1nA
100ms
LY
+80mV
-60mV
-40mV
CNP
LY+CNP
B
Control
LY83583
n=7
I/Imax
Membrane potential (mV)
LY83583
LY83583+CNP
n=7
a
C
Relative current
n=7
*
a#
Control
CNP
LY83583
LY83583+CNP

Fig.4


A
1nA
100ms
Control
0.1μmol/L Zap
+80mV
-60mV
-40mV
0.1μmol/L CNP
Zap+CNP


B
Control
zaprinast
n=8
I/Imax
Membrane potential (mV)
zaprinast
zaprinast+CNP
n=7
I/Imax
Membrane potential (mV)

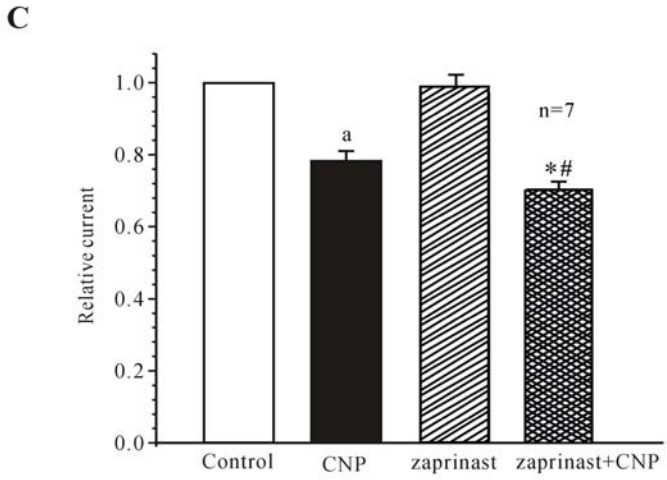

C
Relative current
a
n=7
*#
Control
CNP
zaprinast
zaprinast+CNP

Fig.5

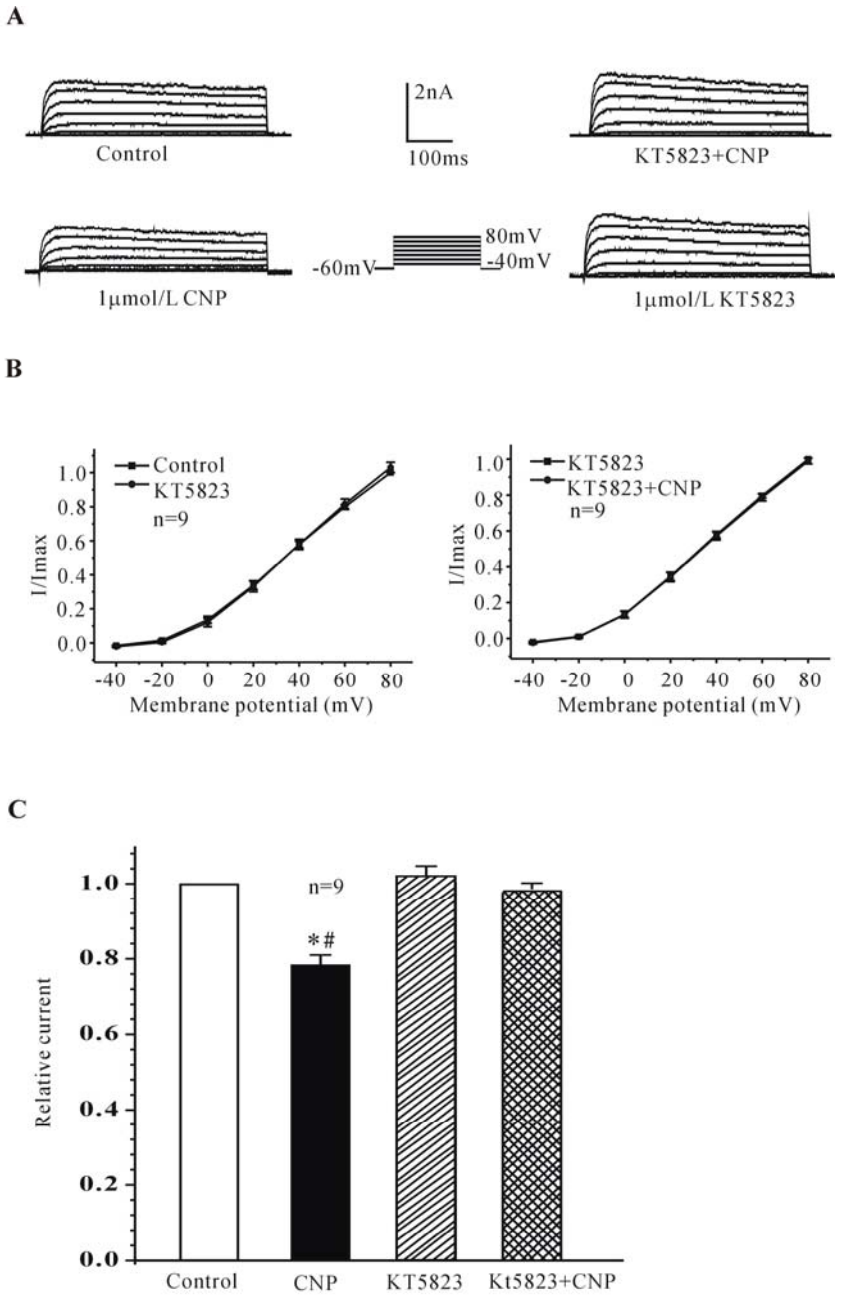

A
Control
2nA
100ms
KT5823+CNP
1μmol/L CNP
-60mV
80mV
-40mV
1μmol/L KT5823
B
Control
KT5823
n=9
I/Imax
Membrane potential (mV)
KT5823
KT5823+CNP
n=9
I/Imax
Membrane potential (mV)
C
n=9
*#
Relative current
Control
CNP
KT5823
Kt5823+CNP

Fig.6