Effects of Dendroaspis Natriuretic Peptide on Calcium-Activated Potassium Current and Its Mechanism

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Abstract: In this study, we sought to investigate the effect of dendroaspis natriuretic peptide (DNP) on calcium-activated potassium current (IK(Ca)) and its mechanism in gastric antral circular smooth muscle cells (SMCs) using the whole-cell patch-clamp technique. DNP concentration-dependently increased macroscopic IK(Ca) and spontaneous transient outward currents (STOCs) in freshly isolated guinea pig gastric antral circular SMCs. The effects of DNP on IK(Ca) and/or STOCs were not blocked by applying calcium-free bath solution or the ryanodine receptor (RyR) antagonist ryanodine (10 µM), but they were inhibited by the inositol triphosphate receptor (IP3R) inhibitor heparin or the guanylate cyclase inhibitor LY83583. Moreover, a DNP-induced increase in STOCs was potentiated by the cyclic guanosine monophosphate (cGMP)–sensitive phosphoesterase inhibitor zaprinast. In conclusion, our results suggest that DNP increases IK(Ca) in gastric antral circular SMCs by increasing cGMP production and activating IP3Rs.

Key words: dendroaspis natriuretic peptide, calcium-activated potassium channels, Ca2+-induced Ca2+ release, inositol 1,4,5-trisphosphate receptors, ryanodine receptor.

Natriuretic peptides (NPs), including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), and dendroaspis natriuretic peptide (DNP), are the bioactive members of a polypeptide family [1]. These peptides are distributed throughout the body and exert numerous physiological functions in the control of urine excretion [2], vasorelaxation [3], blood pressure [4], and electrolyte homeostasis [5].

Dendroaspis natriuretic peptide (DNP), a 38-amino residue peptide that contains a disulfide ring structure and was originally isolated from the venom of Green Mamba [6], is a new member of the natriuretic peptide family with a structure and function similar to other members. Studies about its physiological functions are limited and mainly focus on the cardiovascular [7], urinary [8], and genital systems [9]. Moreover, little is known about the functional role of DNP in gastrointestinal cells. Kim et al. [10] have for the first time demonstrated that DNP is present in rat colon and can control colonic motility as a local regulator. We have found that NPs can also regulate gastric motility [11–14]. Our further study indicates that the regulatory effect of DNP on spontaneous contraction in gastric antral circular SMCs occurs via a cGMP-dependent pathway [15]. However, the underlying mechanisms are unknown. Until now, there have been few studies about the relationship between DNP-induced increase of IK(Ca) and IP3Rs. In our study [16], we found that SNP increased by cGMP via IP3Rs. NPs are similar to nitric oxide which is a cGMP generation system in the living body, and their physiological function exhibits very important in life science. Considering that calcium-activated potassium channels play an important role in the regulation of contractility in gastric SMCs, in this study we first intended to investigate whether DNP could affect IK(Ca) in gastric antral circular SMCs using the conventional whole-cell patch clamp technique, and then to determine the potential involvement of calcium mobilization in the effect of DNP. Moreover, the effect of cGMP on DNP-induced change of IK(Ca) was observed.

MATERIALS AND METHODS

Preparation of cells. Guinea pigs of either sex, weighing 250–350 g, were purchased from the Experimental Animal Center, Dalian Medical University. The guinea pigs were housed in plastic cages containing corn-chip bedding and had free access to food and water for 1 day after their purchase; thereafter they were used for experiments. All the guinea pigs in this study were used strictly in accordance with the National Institutions of Health...
The guinea pigs for our experiment were euthanized by a lethal intravenous injection of pentobarbital sodium (50 mg/kg). The antral part of the stomach was rapidly removed; the muscle layers were separated with the mucosal layer; and the longitudinal layer of muscle was dissected from the other muscle layers and cut into small segments (1 mm × 4 mm). These segments were kept in the modified Kraft-Bruhe (K-B) medium at 4°C for 15 min, and then in a digestion medium (Ca-free PSS) containing 0.1% collagenase II, 0.1% dithioerythreitol, 0.15% trypsin inhibitor, and 0.2% bovine serum albumin at 36°C for 25–35 min. The digested muscle segments were transferred into the enzyme-free K-B medium. Single isolated cells were dispersed by gentle trituration with a fire-polished, widebore glass pipette and kept in a modified K-B medium at 4°C for daily use. Electrophysiological recording isolated cells were transferred to a 0.1-ml chamber on the stage of an inverted microscope (IX-70, Olympus, Japan) and allowed to settle for 10–15 min. The cells were then continuously perfused with a physiologic salt solution at a rate of 0.9–1.0 ml/min, controlled by an 8-channel perfusion system (L/M-sps-8, List Electronics, Germany). Membrane currents were recorded using the conventional whole-cell patch-clamp technique. Patch-clamp pipettes were manufactured from borosilicate glass capillaries (GC 150T-7.5, Clark Electromedical Instruments, UK) using a two-stage puller (PP-83, Narishige, Japan). The resistance of a patch pipette was 3–5 MΩ when filled with pipette solution. Junction potentials were compensated prior to the seal formation. Whole-cell \( I_{K(Ca)} \) was recorded with an Axopatch 1-D patch-clamp amplifier (Axon Instrument, USA) and filtration at 1 kHz. Command pulses and data acquisition were generated using an IBM-compatible 486-grade computer with pCLAMP 6.02 software. Spontaneous transient outward currents (STOCs) were simultaneously recorded by using polygraph (RM6200, Nihon Kohden, Tokyo, Japan). All experiments were performed at room temperature (20º–25°C).

**Drugs and solutions.** Tyrode solution contained (mM) NaCl 147, KCl 4, MgCl\(_2\)-6H\(_2\)O 1.05, CaCl\(_2\)-2H\(_2\)O 0.42, Na\(_2\)PO\(_4\)-2H\(_2\)O 1.81, and 5.5 glucose. Ca\(^{2+}\)-free PSS contained (mM) NaCl 147, KCl 4, MgCl\(_2\)-6H\(_2\)O 1.05, CaCl\(_2\)-2H\(_2\)O 0.42, Na\(_2\)PO\(_4\)-2H\(_2\)O 1.81, and 5.5 glucose. Ca\(^{2+}\)-free PSS contained (mM) NaCl 134.8, KCl 4.5, glucose 5, HEPES 10, and egtazic acid 0.5; pH was adjusted to 7.40 with KOH. PSS contained (mM) NaCl 134.8, KCl 4.5, MgCl\(_2\)-6H\(_2\)O 1, CaCl\(_2\)-2H\(_2\)O 2, glucose 5, HEPES 10, and sucrose 110; pH was adjusted to 7.4 with Tris. In Ca\(^{2+}\)-free PSS, CaCl\(_2\)-2H\(_2\)O 2.0 mmol/l was omitted from PSS. The pipette solution contained (mM) potassium-aspartic acid 110, Mg\(_2\)-ATP 5, HEPES 5, MgCl\(_2\)-6H\(_2\)O 1.0, KCl 20, egtazic acid 0.1, di-tris-cresphosphate 2.5, and disodiumcreatine phosphate 2.5; pH was adjusted to 7.3 with KOH.

To eliminate delayed rectifier potassium currents \( I_{K(V)} \), the selective \( I_{K(V)} \) inhibitor 4-aminopyridine (4-AP, 10 mmol/l) was used in bath solution. Ryanodine was purchased from Vago Chemicals Ltd. (USA), and tetroethylammonium (TEA), dendraospatiatriuretic peptide, LY83583, and Zaparinast from Sigma (USA). All stock solutions were kept at 4°C and diluted in PSS before experiments.

**Data analysis.** Data were expressed as mean ± SE, and \( n \) indicates the number of guinea pigs. A Student’s \( t \)-test was used for statistical analysis. A value of \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**\( I_{K(Ca)} \) and STOCs in guinea pig gastric antral circular myocytes**

Membrane currents were recorded using the conventional whole-cell patch-clamp technique. \( I_{K(Ca)} \) was elicited by step voltage pulse from a holding potential of –60 to 100 mV for 400 ms with a 20-mV increment at a 10-s interval and confirmed with TEA (a nonselective potassium channel blocker [17]) and ChTX (a selective calcium-activated potassium channel blocker [18]), as we described previously [19]. STOCs, which can be activated by extracellular calcium influx and intracellular calcium release, were recorded at –20 mV using the same patch-clamp mode. As shown in Fig. 1, the currents were sensitive to TEA (4 mM) and ChTX (200 nm) (\( n = 11 \)).

**Effects of DNP on \( I_{K(Ca)} \) in guinea pig gastric myocytes**

DNP caused a significant increase in the amplitude of \( I_{K(Ca)} \) at 60 mM was respectively increased by 16.7 ± 1.12%, 26.5 ± 2.3%, 46.2 ± 3.3%, and 58.3 ± 3.7% following applications of DNP at 0.1, 1, 10, and 100 mM (Fig. 2A, \( n = 8 \)). DNP (10 mM) also significantly increased the
Effects of extracellular Ca\(^{2+}\) influx on DNP-induced increase of \(I_{K(Ca)}\)

It is well known that \(I_{K(Ca)}\) is activated by extracellular calcium influx. To test whether extracellular Ca\(^{2+}\) influx was involved in the effect of DNP, we examined the effect of DNP on \(I_{K(Ca)}\) in the absence of extracellular Ca\(^{2+}\). When Ca\(^{2+}\)-free solution (containing 10 µM egtaic acid) was used to replace normal Ca\(^{2+}\) solution, DNP (10 nM) still significantly increased \(I_{K(Ca)}\); the mean amplitude was increased by 47.1 ± 2.7% (Fig. 3, \(n = 8\)). Since L-type calcium channels provide a major route for calcium influx, we also examined whether the L-type calcium channel blocker nicardipine could affect a DNP-induced increase in \(I_{K(Ca)}\) and found that it (5 µM) markedly inhibited \(I_{K(Ca)}\), but did not prevent a DNP-induced increase in \(I_{K(Ca)}\) (Fig. 4, \(n = 8\)). These results suggest that extracellular Ca\(^{2+}\) was not involved in a DNP-induced increase of \(I_{K(Ca)}\).

Effects of intracellular Ca\(^{2+}\) release on DNP-induced increase in \(I_{K(Ca)}\)

Ca\(^{2+}\)-activated K\(^+\) channels can also be activated by intracellular Ca\(^{2+}\) release that is exclusively controlled by IP\(_3\)Rs and RyRs. Thus we sought to determine the potential role of IP\(_3\)-R- and RyR-mediate Ca\(^{2+}\) release in the effect of DNP on STOCs. An application of heparin (3 g/l) to selectively inhibit IP\(_3\)Rs markedly decreased the frequency and amplitude of STOCs. In the presence of heparin, the frequency and amplitude of STOCs were significantly decreased (Fig. 2B, \(n = 6\)).
arin, DNP (10 nM) no longer could increase STOCs (Fig. 5, n = 6). The RyR antagonist ryanodine (10 µM) initially (within about 4 min) increased and then inhibited the activity of STOCs. The inhibitory effect of ryanodine was not restored by the RyR agonist caffeine (1 mM). After the ryanodine-sensitive Ca^{2+} store was sufficiently depleted by ryanodine, DNP 10 nM still markedly increased the frequency and amplitude of STOCs (Fig. 6, n = 6).

**Effect of LY83583 and Zaparinast on a DNP-induced increase in STOCs**

To determine whether a DNP-induced increase of STOCs was mediated by cGMP, the effect of the guanylate cyclase LY83583 and the phosphoesterase inhibitor Zaprinast on a DNP-induced increase in STOCs was investigated. DNP 10 nM could not increase STOCs after treatment with LY83583 (10 nM) (Fig. 7, n = 6), and the
The effect of DNP was potentiated by Zaprinast (100 nM) (Fig. 8, n = 6).

**DISCUSSION**

In the present study, we have found that DNP increased $I_{K(Ca)}$ and STOCs in guinea pig gastric antral circular myocytes. A DNP-induced increase of $I_{K(Ca)}$ was not inhibited by removing external Ca$^{2+}$ and nicardipine. The effect of DNP on STOCs was markedly inhibited by heparin, but not by ryanodine. Moreover, LY83583 inhibited a DNP-induced increase in STOCs, but Zaprinast potentiated it.

There are three kinds of calcium-activated potassium channels in smooth muscle: large conductance (BKCa; approximately 200 pS) channels and intermediate conductance (IKCa; approximately 39 pS) blocked by 2 mM external TEA and 200 nM charybdotoxin); and small conductance (SKCa; approximately 10 pS) channels that were not blocked by 5 mM external TEA, but were sensitive to extracellular apamin (0.5 µM). Furthermore, KATP is blocked by apamin. In the present study, calcium-activated potassium current is blocked by 4 mM TEA and 200 nM CHTX [20].

Three kinds of potassium channels were activated by depolarization pulses in smooth muscle: calcium-activated potassium channel (BKCa); delayed rectified potassium channel (K(V)); and outward potassium channel (K(To)) [21]. In gastric antral circular myocytes of guinea pigs, depolarization pulses activated two types of K$^+$ currents, $I_{K(Ca)}$ and $I_{K(V)}$ [22]. To eliminate $I_{K(V)}$, we used three approaches in this study: (1) the pipette solution contained egtazic acid (0.1 mM); (2) the bath contained 4-AP 10 mM; and (3) STOCs were significantly blocked by TEA and CHTX (Fig. 1, A and B).

Calcium-activated potassium channels are regulated by intracellular Ca$^{2+}$ release and extracellular Ca$^{2+}$ influx. To determine the potential involvement of these calcium mobilizations in the effect of DNP on calcium-activated potassium channels, we examined the effects of DNP in the absence of extracellular Ca$^{2+}$. As shown in Figs. 3 and 4, DNP was also able to significantly increase $I_{K(Ca)}$ under this condition. The inhibition of L-type calcium channels by nicardipine markedly inhibited $I_{K(Ca)}$, but it did not block the DNP-induced increase of $I_{K(Ca)}$. Collectively, these results suggest that extracellular Ca$^{2+}$ influx was not involved in the DNP-induced increase of $I_{K(Ca)}$.

Intracellular calcium release is exclusively controlled by IP$_3$Rs and RyRs in SMCs [26]. Yu et al. [16] have shown that SNP increases $I_{K(Ca)}$ and causes relaxation in gastric antral circular smooth muscle by triggering IP$_3$-mediated Ca$^{2+}$ release. Kudoh et al. [27] have revealed that calcineurin- and calmodulin-dependent kinase II are both activated by calcium influx and subsequent RyRs, playing an important role in stretch-induced BNP gene expression during the development of cardiac hypertrophy. These studies indicate an intimate relationship between the relaxation of smooth muscle and intracellular calcium release. Thus we studied which calcium-release channels were involved in the DNP-induced activation of $I_{K(Ca)}$. Heparin completely blocked the DNP-induced increase of $I_{K(Ca)}$ (Fig. 5), but ryanodine had no effect (Fig. 6), dem-
onstrating that DNP may increase $I_{K(Ca)}$ by causing IP$_3$-mediated Ca$^{2+}$ release in gastric antral circular myocytes of the guinea pig.

DNP plays many physiological functions by activating the cGMP-dependent pathway. Best et al. [28] have reported that DNP relaxes human arteries and veins by increasing the generation of cGMP. Ha et al. [29] have described that DNP induces the apoptosis in H9c2 cardiomyocytes via cGMP production. Here we have found that the application of the guanylate cyclase inhibitor LY83583 to reduce cGMP production abolishes a DNP-induced increase in STOCs (Fig. 7), whereas the phosphoesterase inhibitor zaprinast to reduce cGMP-mediated IP$_3$R-mediated Ca$^{2+}$ release and increase cGMP production, causing an increase in $I_{K(Ca)}$ in gastrointestinal SMCs.

In summary, DNP increases $I_{K(Ca)}$ in a dose-dependent manner in gastric antral circular myocytes. The effects of DNP may be attributed to the increase in cGMP production and the activation of IP$_3$Rs.

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