Biochemical and Biophysical Research Communications 452 (2014) 60–65

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

AGAP, a new recombinant neurotoxic polypeptide, targets the voltage-gated calcium channels in rat small diameter DRG neurons

Xifang Liu [a], Chunli Li [a,*], Jianzhao Chen [a], Jingnan Du [a], Jinghai Zhang [b], Guixia Li [a], Xiaoquan Jin [a], Chunfu Wu [a,*]

[a] Department of Pharmacology, School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, Liaoning 110016, PR China
[b] Department of Biochemistry, School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang, Liaoning 110016, PR China

ARTICLE INFO

Article history:
Received 21 July 2014
Available online 19 August 2014

Keywords:
Calcium channel
Scorpion toxin
AGAP
Whole-cell patch clamp
Dynamic function

ABSTRACT

A previous study showed that antitumor-analgesic peptide (AGAP), a novel recombinant polypeptide, which had been expressed in *Escherichia coli*, exhibits analgesic and antitumor effects in mice. In the present study, we investigated the underlying analgesic mechanism of AGAP. The effect of AGAP on voltage-gated calcium channels (VGCCs) was assessed in acutely isolated rat dorsal root ganglia (DRG) neurons using the whole-cell patch clamp technique. The results showed that AGAP potently inhibited VGCCs, especially high-voltage activated (HVA) calcium channels. AGAP inhibited HVA and T-type calcium currents in a dose-dependent manner, but had no significant effect on their dynamic functions in rat small-diameter DRG neurons. AGAP inhibited N- and L-type calcium currents at 78.2% and 57.3%, respectively. Thus, the present study demonstrates that AGAP affects calcium currents through the inhibition of N-, L- and T-type channels in DRG neurons, explaining the potential mechanisms of antinociception.

1. Introduction

Scorpion venoms are complex mixtures that contain a variety of peptide neurotoxins which can affect ion channels from mammals and insects [1]. Toxins that recognize sodium and calcium channels are usually from 60 to 76 amino acid residues, whereas those that recognize potassium and chloride channels are shorter, 29–41 amino acids residues [2]. Antitumor-analgesic peptide (AGAP) is a recombinant "long-chain" polypeptide with 66 amino acid residues, which exhibits analgesic activity in vivo [3,4]. However, it has not yet been reported whether AGAP acts on the sodium or calcium channels. The cDNA encoding mature AGAP was amplified by PCR from the total RNA of the scorpion venom gland, and then cloned into an expression plasmid pET28a. The researches about AGAP provide a new opportunity to elucidate structure–activity of the pharmacophore [5,6].

Voltage-gated calcium channels (VGCCs) play an important role in the regulation of membrane ion conductance and membrane hyperexcitability associated with chronic pain states [7,8]. As previously reviewed [9–12], many calcium channels are regarded as cellular targets for the development of new drugs. Some new polypeptides, which can inhibit calcium channels, have been shown to be potent pain relievers in various animal pain models [13–15]. Based on the pharmacological and biophysical properties, VGCCs can be grouped into two classes: high-voltage activated (HVA) and low-voltage activated (T-type) calcium channels [16], the HVA calcium channels can be further subdivided into L-, N-, P/Q-, and R-type calcium channels. N-type calcium channel is known to be strongly associated with the pathological processes of neuropathic pain [10]. Amlodipine, a selective L-type calcium channel blocker, can potentiate the analgesic effect of morphine [17]. T-type calcium channels are primarily found in scattered small and medium sized DRG neurons, whereas the extremely large neurons do not express these channels [18]. Recent studies indicate that T-type calcium channels play an important role in regulating cellular excitability [19,20]. Several pharmacological blockers and modulators of T-type calcium channels can ease neuropathic pain in a chronic constriction injury model [21]. Together, the findings of those studies indicate that both HVA and T-type calcium channels are correlated with pain. Therefore, in the present

* Corresponding authors. Fax: +86 24 23986339 (C. Li), +86 24 23986012 (C. Wu).
E-mail addresses: lichunli_2014@126.com (C. Li), Chunfuw@gmail.com (C. Wu).

http://dx.doi.org/10.1016/j.bbrc.2014.08.051
0006-291X/

study, we investigated the effects of AGAP on these channels to find the possible antinociceptive mechanisms.

2. Materials and methods

2.1. Regents

Recombinant AGAP was provided by Professor Zhang (Shenyang Pharmaceutical University, PR China) [3] and stored at −20 °C. Stock solutions of AGAP were prepared in physiological saline and diluted in assay buffer immediately before use. Nifedipine was purchased from Sigma–Aldrich. ω-Conotoxin GVIA, ω-Agatoxin IVA, and rSNX-482 were purchased from Alomone.

2.2. Cell preparation

DRG neurons were dissected from 4 to 8-week-old SD rats as previously described [22]. Briefly, the rats were deeply anesthetized, and the $L_{4\text{-}6}$ lumbar DRG were removed quickly from the spinal cord, minced immediately in ice cold, oxygenated D-Hank's, and incubated in Dulbecco's Modified Eagle Medium/Ham's F-12 medium (DMEM/F12) containing 0.5 mg/ml trypsin, 0.5 mg/ml collagenase IA, and 30 Kunitz units/ml DNase I for 13 min at 37 °C. Then the ganglia were gently triturated with fire-polished glass pipettes. The suspension was dissociated in DMEM/F12 supplemented with 10% fetal bovine serum and 10% horse serum and plated on glass coverslips coated with poly-L-lysine. The neurons were cultured at 37 °C for 1–2 h before electrophysiology experiments, which were carried out within 10 h. DRG neurons with diameters of 15–25 μm were selected, because many of these neurons express many proteins involved in pain [10,11,23,24].

2.3. Electrophysiology

All recordings were conducted at room temperature (22–25 °C). Neurons were perfused with external recording solution for 3 min at a rate of 3 ml/min before recordings. The external recording solution contained (in mM): 130 choline chloride, 0.0025 TTX, 25 TEA-Cl, 3 KCl, 5 $BaCl_2$, 0.6 $MgCl_2$, 1 $NaHCO_3$, 10 HEPES, 4 glucose, and pH 7.4 adjusted with NaOH. Recording electrodes were filled with an internal solution containing (in mM): 140 CsCl, 10 EGTA, 0.1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 2 ATP, and pH 7.2 adjusted with Tris. Patch-clamp electrodes were pulled with a P-97 puller, and had a resistance of 3–5 MΩ [25]. Peak current amplitudes were measured before and after a 3–5-min incubation with AGAP. Data was acquired using an Axopatch 200B amplifier running pClamp 10.0 software (Molecular Devices). Currents were low pass-filtered at 1 kHz, and digitized at 20 kHz.

2.4. Statistical analysis

All data are presented as mean ± standard error of mean (SEM). The voltage dependence for the activation curves was fitted by a Boltzmann function according to the equation: $G/G_{max} = 1/[1 + \exp[(V - V_{1/2})/k]]$, where G/G_{max} is the normalized conductance, $V_{1/2}$ is the membrane potential of half-maximum channel activation, and k is equal to the slope factor. The voltage dependence of inactivation data was fitted by a Boltzmann equation: $I/I_{max} = 1/[1 + \exp[(V - V_{1/2})/k]]$. The curves for the concentration–response relationship were fitted to the Hill equation: $E = E_{max}/[1+(IC_{50}/C)^{nH}]$, where nH and IC_{50} represent the Hill coefficient and the concentration producing half-maximal inhibition, respectively. Statistical analyses were performed using one-way analysis of variance (ANOVA). The differences of values under control and test conditions were tested for significance using t-tests. A p-value < 0.05 was considered to indicate a significant difference.

3. Results

3.1. AGAP inhibits HVA calcium currents in DRG neurons

To investigate the effect of AGAP on state-dependent inhibition of HVA calcium channels, different voltages were used when applying AGAP to DRG neurons. Representative HVA calcium current traces under control conditions and in the presence of 1000 nM AGAP are shown in Fig. 1A. The bar diagram shows that AGAP attenuated the HVA calcium currents in a concentrations-dependent manner (Fig. 1B), and the maximum value of inhibition rate was 73.0%. The concentration-dependence of AGAP's effect conformed to the Hill equation with an estimated IC_{50} of 89.6 nM (Fig. 1C). 1000 nM AGAP decreased the current–voltage relationships (I–V curves) in a concentration-dependent manner (Fig. 1D). Representative time course for inhibition of HVA calcium peak current amplitudes by 10–1000 nM AGAP is shown in Fig. 1E. AGAP inhibited HVA calcium currents in a time-dependent manner for each concentration.

Isolation of components of HVA calcium currents were determined using nifedipine (4 μM) to block L-type current, ω-Conotoxin GVIA (1 μM) to block N-type current, ω-Agatoxin IVA (200 nM) to block P/Q-type current, and rSNX-482 (200 nM) to block R-type current. Our results showed a full mix of calcium current subtypes in small-diameter DRG neurons, with a dominance of N- and L-type currents, whereas P/Q- and R-type currents were present at lower levels, consistent with previous findings [26]. As shown in Fig. 1F, 1000 nM AGAP significantly reduced N-type current (78.2 ± 8.1%, n = 3) and L-type current (57.3 ± 0.8%, n = 3), administration of ω-Conotoxin GVIA (1 μM) and nifedipine (4 μM) inhibited the remaining currents, respectively.

3.2. Effects of AGAP on the dynamics of HVA calcium channels in DRG neurons

The activation kinetic of HVA calcium channels by voltage pulses to potentials ranging from −30 to +30 mV was analyzed. As shown in Fig. 2B, 1000 nM AGAP did not significantly change the activation curve of HVA calcium channels, and the $V_{1/2}$ values were −16.5 ± 0.1 mV and −15.0 ± 0.6 mV in the absence and presence of AGAP, respectively.

In addition, the effect of AGAP on the inactivation kinetic of HVA calcium channels was also examined. Steady-state inactivation of HVA calcium channels was studied with current traces obtained using a prepulse potential from −110 mV to +20 mV in the absence and presence of AGAP. Likewise, there was very little difference between the control and AGAP-treated condition (Fig. 2D). The values of the parameters of $V_{1/2}$ and k were −49.2 ± 1.6 mV and −14.4 ± 1.3 mV under control conditions and −47.0 ± 2.2 mV and −15.6 ± 1.7 mV in the presence of 1000 nM AGAP, respectively.

3.3. AGAP inhibits T-type calcium currents in DRG neurons

A typical example of the T-type calcium channel under the control conditions and in the presence of 1000 nM AGAP is shown in Fig. 3A. Neurons were depolarized by a 500-ms voltage pulse from −90 to −40 mV in 10-mV increments from a holding potential of −100 mV. A test pulse to −40 mV produced a maximum T-type calcium current without activating the HVA channels. As shown in Fig. 3B, the action of AGAP was tested over a broad range of concentrations (3–3000 nM). AGAP inhibited T-type calcium channels in a dose-dependent manner, with an estimated IC_{50} of

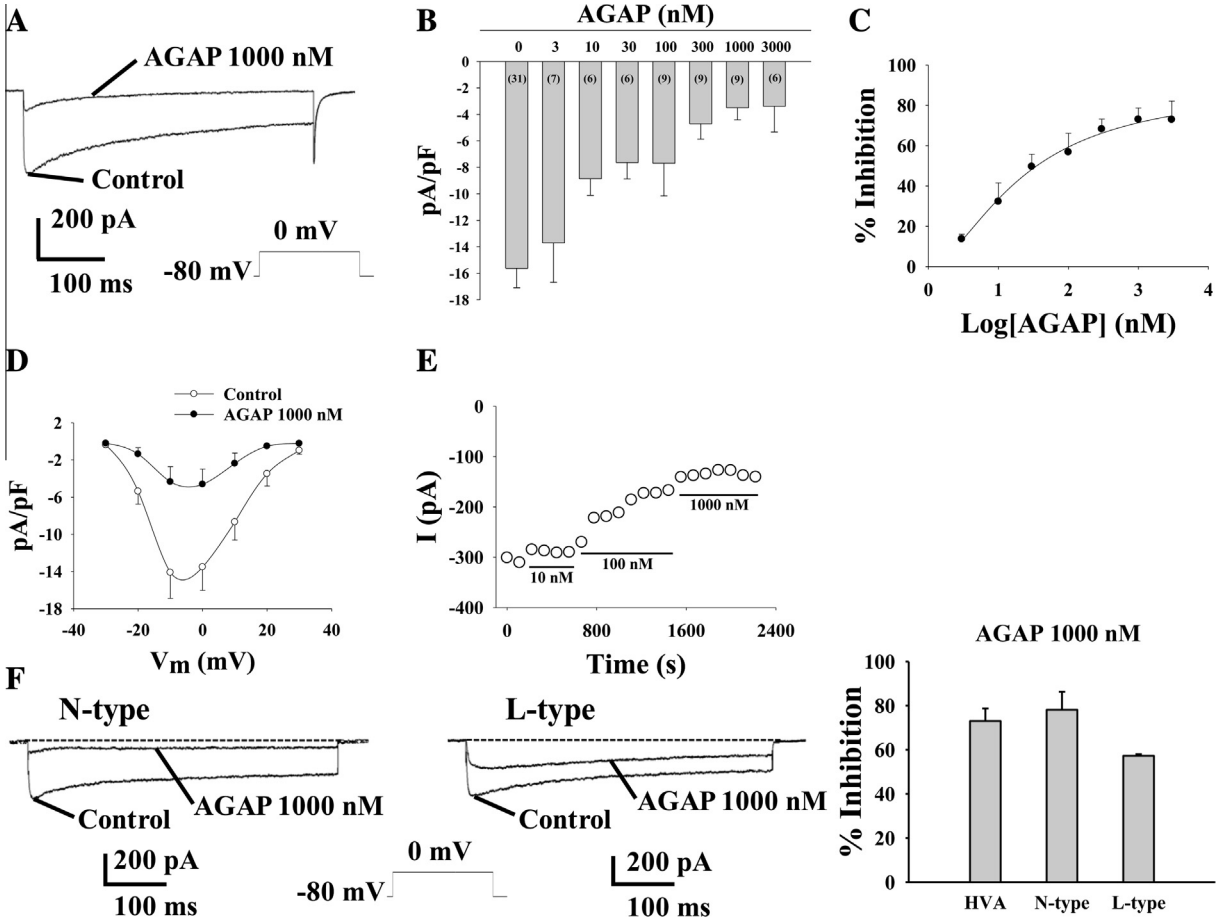

Fig. 1. AGAP inhibits HVA calcium currents in small-diameter DRG neurons. (A) Examples of HVA calcium current traces elicited by 10-mV progressive steps between −30 and +30 mV from a holding potential of −80 mV under control condition and after the application of 1000 nM AGAP. (B) Histogram showing the mean amplitude of the peak currents of HVA calcium channels under control conditions and the application of 3–3000 nM AGAP. The maximum inhibition value was 73.0 ± 5.8% (n = 6–9). (C) A fit with the Hill equation provided the IC_{50} values (nH = 1.7 ± 0.2, IC_{50} = 89.6 ± 7.6 nM). (D) I–V relationship obtained by plotting current density as a function of test potential under control conditions and after the application of 1000 nM AGAP (n = 10). (E) AGAP inhibited HVA calcium currents in a time- and concentration-dependent manner in the time-course experiment. (F) Histogram showing 1000 nM AGAP inhibited N- and L-type calcium currents at 78.2 ± 8.1% (n = 3) and 57.3 ± 0.8% (n = 3), respectively.

71.9 nM (Fig. 3C). 1000 nM AGAP decreased the I–V curve of T-type calcium current in a concentration-dependent manner (Fig. 3D). Representative time course under control conditions and in the presence of 10–1000 nM AGAP is shown in Fig. 3E. AGAP inhibited T-type calcium currents in a time-dependent manner for each concentration. These results revealed that AGAP could remarkably affect the T-type calcium channels.

3.4. Effects of AGAP on the dynamics of T-type calcium channels in DRG neurons

The steady-state activation and inactivation curves of T-type calcium channels under control conditions and after exposure to 1000 nM AGAP are shown in Fig. 4. Neither the $V_{1/2}$ nor the k of the activation kinetics of T-type calcium channels was significantly different in control conditions (−51.5 ± 1.1 mV and 5.4 ± 0.8 mV) and with 1000 nM AGAP (−51.2 ± 2.0 mV and 6.3 ± 1.2 mV), respectively (Fig. 4B). Steady-state inactivation of T-type calcium currents was studied with current traces obtained using a prepulse potential from −100 mV to −40 mV in the absence and presence of AGAP (Fig. 4D). The application of 1000 nM AGAP produced only a slight shift toward negative potentials. The values of the parameters $V_{1/2}$ and k were −60.3 ± 1.8 mV and −5.4 ± 1.6 mV under the control conditions and −62.7 ± 3.8 mV and −6.1 ± 3.1 mV in the presence of 1000 nM AGAP, respectively.

4. Discussion

As previously reviewed [27], many peptides are potent and highly selective blockers or modulators of calcium channel function. They are commonly isolated from cone shells and spiders and also found in scorpions and insects [9]. A large number of blockers or modulators of potassium and sodium channels have been obtained from scorpion venoms, but a few polypeptides targeting calcium channels have been described including kurtoxin, which affects T-type calcium channels [28], and ryanotoxin [29] and imperatoxin I [30], which affect ryanodine-sensitive calcium channels in skeletal muscle.

Most of the long-chain scorpion peptides have been shown be sodium and calcium channel modulators [2,31,32]. Recombinant AGAP has 66 amino acid residues and belongs to the long-chain scorpion polypeptides. However, it has not yet been reported whether AGAP acts on the calcium channels. Previous results showed that AGAP has strong analgesic activities in the hot-plate, writhing and formalin tests in mice [3,4], but the mechanisms remain unclear. The present results first demonstrated that AGAP is a potent state-dependent inhibitor of calcium channels, including HVA and T-type calcium channels. AGAP strongly depressed calcium channels in a dose-, voltage- and time-dependence manner. A common view is that inhibition of VGCCs on sensory nerves attenuates membrane hyperexcitability and neurotransmitter release associated with chronic pain states [33]. Therefore, the

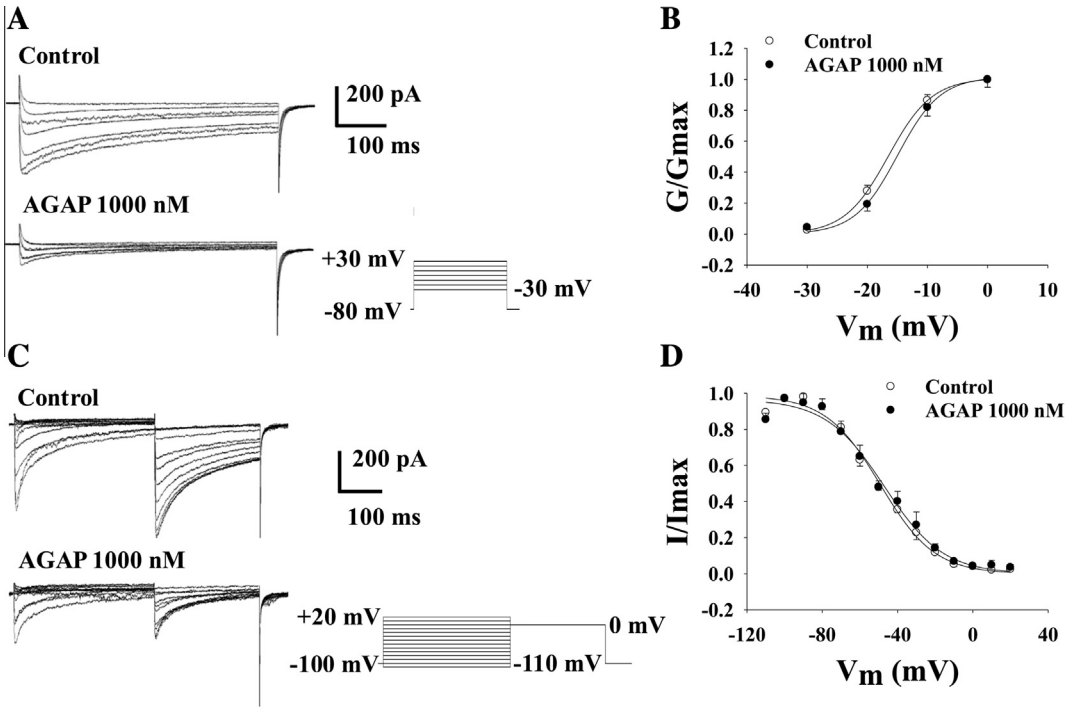

Fig. 2. AGAP has no significant effect on the dynamic function of HVA calcium channels in small-diameter DRG neurons. (A) Representative steady-state activation of HVA calcium channels under the control conditions and in the presence of 1000 nM AGAP. (B) Normalized activation kinetics determined before and after application of AGAP (n = 10). The values of the parameters of $V_{1/2}$ and k were −16.5 ± 0.1 mV and 3.6 ± 0.04 mV under control conditions and −15.0 ± 0.6 mV and 3.6 ± 0.4 mV in the presence of 1000 nM AGAP, respectively. (C) Representative steady-state inactivation of HVA calcium channels under the control conditions and in the presence of 1000 nM AGAP. (D) Effect of AGAP on steady-state inactivation kinetics of HVA calcium currents (n = 7). The values of the parameters of $V_{1/2}$ and k were −49.2 ± 1.6 mV and −14.4 ± 1.3 mV under control conditions and −47.0 ± 2.2 mV and −15.6 ± 1.7 mV in the presence of 1000 nM AGAP, respectively.

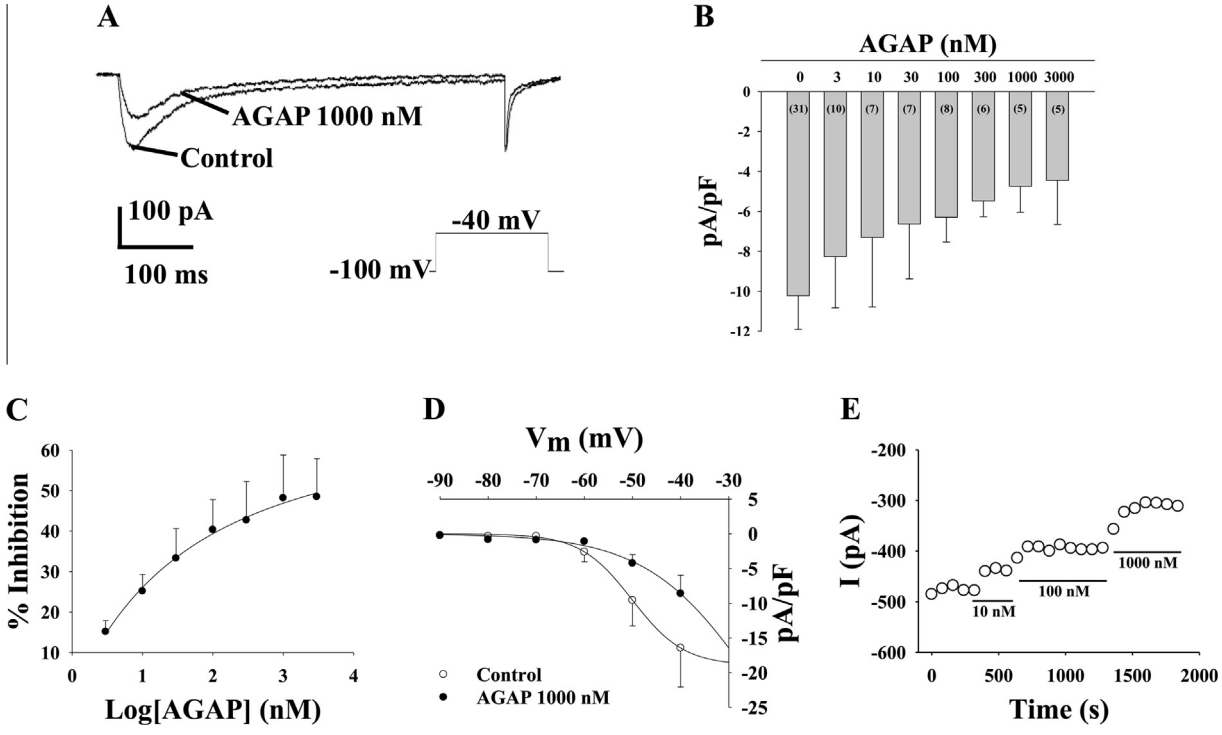

Fig. 3. AGAP inhibits T-type calcium currents in small-diameter DRG neurons. (A) Representative traces show the T-type calcium channels under control conditions and in the presence of 1000 nM AGAP. (B) Histogram showing the mean amplitude of the T-type calcium currents under control conditions and the application of 3–3000 nM AGAP. The maximum inhibition value was 48.5 ± 9.3% (n = 5–10). (C) A fit with the Hill equation provided the IC_{50} values (nH = 1.1 ± 0.2, IC_{50} = 71.9 ± 10.6 nM). (D) I–V relationships of T-type calcium channels were studied by eliciting voltage steps from −90 to −40 mV, under control conditions and in the presence of 1000 nM AGAP (n = 7). (E) AGAP inhibited T-type calcium channels in a time- and concentration-dependent manner in the time-course experiment.

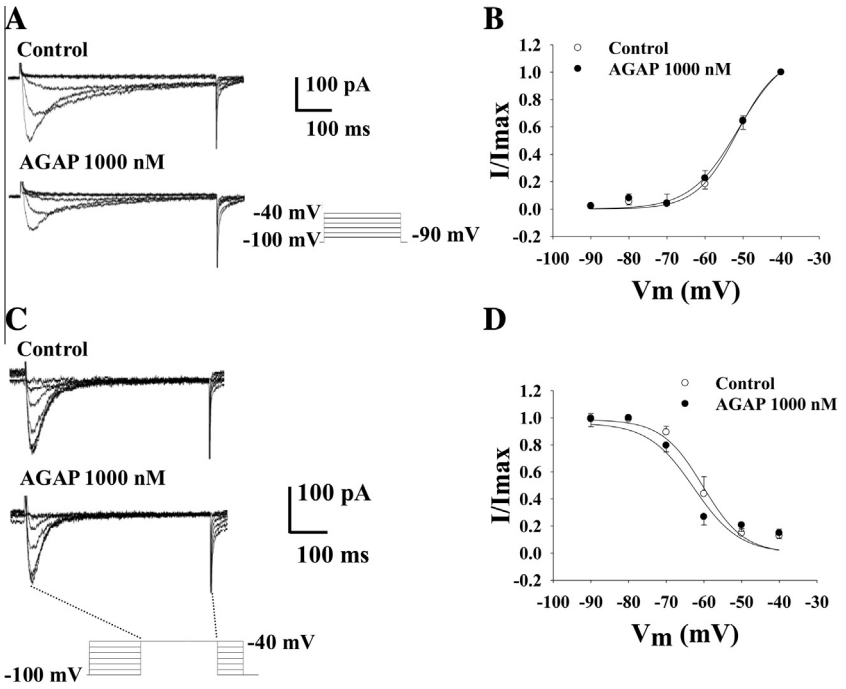

Fig. 4. AGAP has no significant effect on the dynamic function of T-type calcium channels in small-diameter DRG neurons. (A) Representative steady-state activation of T-type calcium channels under the control conditions and in the presence of 1000 nM AGAP. (B) Normalized activation kinetics determined before and after application of 1000 nM AGAP (n = 6). The values of the parameters $V_{1/2}$ and k were −51.5 ± 1.1 mV and 5.4 ± 0.8 mV under the control conditions and −51.2 ± 2.0 mV and 6.3 ± 1.2 mV in the presence of 1000 nM AGAP, respectively. (C) Representative steady-state inactivation of T-type calcium channels under the control conditions and in the presence of 1000 nM AGAP. (D) Effect of AGAP on steady-state inactivation kinetics of T-type calcium currents (n = 5). The values of the parameters $V_{1/2}$ and k were −60.3 ± 1.8 mV and −5.4 ± 1.6 mV under the control conditions and −62.7 ± 3.8 mV and −6.1 ± 3.1 mV in the presence of 1000 nM AGAP, respectively.

antinociceptive activity of AGAP may result from modulation of calcium channels.

With 1000 nM AGAP, both the half-maximal activation voltage and inactivation voltage of HVA calcium channels were shifted to become more positive. In addition, AGAP (1000 nM) modified T-type calcium currents by promoting slower activation and faster inactivation and by reducing steady-state currents. In general, peptides may inhibit channel function by either of two mechanisms: inhibition of ion permeation or alteration of channel gating. A pore-blocking polypeptide directly interacts with the binding site of permeant ions. Peptide affinity may be weakened by increased concentrations of permeant ions. Gating modifier peptides depress channel currents by making the channel less responsive to voltage, so that a physiological voltage stimulus sufficient to open the peptide-free channel is too weak or too short to open a peptide-bound channel. The biophysical signature of a gating modifier peptide is voltage-dependent inhibition, such that peptide-bound channels open only with strong depolarization and with much slower kinetics [34]. In the presence of AGAP, HVA calcium channels were activated more slowly and deactivated more quickly. All these effects of AGAP can be interpreted simply as a slowing of channel activation contributing to inhibition of the calcium currents. AGAP reduction of the HVA calcium currents in a voltage-dependent manner was accompanied by slowing of activation in DRG neurons. AGAP reduces membrane hyperexcitability because of the prolonged open time of HVA calcium channels. AGAP inhibition of T-type calcium channels might similarly be explained by the stabilization of closed states [35]. To our knowledge, this is the first experimental evidence demonstrating that AGAP is a gating modifier that has many different actions on a variety of neuronal channels including HVA and T-type calcium channels.

Our study also first demonstrated that AGAP has a potent inhibition on N-, L-, and T-type calcium channels in small-diameter DRG neurons. N- and L-type calcium currents contribute substantially to total HVA calcium currents in small-diameter DRG neurons, and T-type calcium currents are most evident in small-, and medium-diameter DRG neurons. N-, L-, and T-type calcium channels are essential for development of pain, and provide attractive candidates for the development of antihyperalgesic drugs [36]. The inhibition on most of N-type calcium currents, and large part of L- and T-type calcium currents by AGAP may contribute to its strong antinociception in mice.

In conclusion, our results demonstrated that AGAP could dose-dependently inhibit VGCCs in DRG neurons. Thus, AGAP may be used as a pharmacological tool to understand pain mechanisms related to calcium channels and as a potential drug to treat clinical pain syndrome.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (No: 81073081), the Excellent Talents Plan in Liaoning Province (No: LJQ2013105), the Shenyang City Science and Technology Project (No: F12-277-1-07) and Key Laboratory of Bio-pharmaceutical-engineering (Harbin Medical University), Ministry of Education.

References

[1] R.C. Rodríguez De La Vega, L.D. Possani, Overview of scorpion toxins specific for Na^+ channels and related peptides: biodiversity, structure-function relationships and evolution, Toxicon 46 (2005) 831–844.
[2] L.D. Possani, E. Merino, M. Corona, F. Bolivar, B. Becerril, Peptides and genes coding for scorpion toxins that affect ion-channels, Biochimie 82 (2000) 861–868.
[3] Y.F. Liu, R.L. Ma, S.L. Wang, Z.Y. Duan, J.H. Zhang, L.J. Wu, C.F. Wu, Expression of an antitumor-analgesic peptide from the venom of Chinese scorpion *Buthus martensii* Karsch in *Escherichia coli*, Protein Expr. Purif. 27 (2003) 253–258.
[4] Q.H. Mao, J.P. Ruan, X.T. Cai, W.G. Lu, J. Ye, J. Yang, Y. Yang, X.Y. Sun, J.L. Cao, P. Cao, Antinociceptive effects of analgesic-antitumor peptide (AGAP), a

neurotoxin from the scorpion *Buthus martensii* Karsch, on formalin-induced inflammatory pain through a mitogen-activated protein kinases-dependent mechanism in mice, PLoS One 8 (2013) e78239.
[5] R. Ma, Y. Cui, Y. Zhou, Y.M. Bao, W.Y. Yang, Y.F. Liu, C.F. Wu, J.H. Zhang, Location of the analgesic domain in scorpion toxin BmK AGAP by mutagenesis of disulfide bridges, Biochem. Biophys. Res. Commun. 394 (2010) 330–334.
[6] Y. Cui, G.L. Guo, L. Ma, N. Hu, Y.B. Song, Y.F. Liu, C.F. Wu, J.H. Zhang, Structure and function relationship of toxin from Chinese scorpion *Buthus martensii* Karsch (BmKAGAP): gaining insight into related sites of analgesic activity, Peptides 31 (2010) 995–1000.
[7] Q.H. Hogan, Role of decreased sensory neuron membrane calcium currents in the genesis of neuropathic pain, Croat. Med. J. 48 (2007) 9–21.
[8] P.J. Birch, L.V. Dekker, I.F. James, A. Southan, D. Cronk, Strategies to identify ion channel modulators: current and novel approaches to target neuropathic pain, Drug Discov. Today 9 (2004) 410–418.
[9] R.S. Norton, S.I. McDonough, Peptides targeting voltage-gated calcium channels, Curr. Pharm. Des. 14 (2008) 2480–2491.
[10] T. Yamamoto, A. Takahara, Recent updates of N-type calcium channel blockers with therapeutic potential for neuropathic pain and stroke, Curr. Top. Med. Chem. 9 (2009) 377–395.
[11] V. Jevtovic-Todorovic, S.M. Todorovic, The role of peripheral T-type calcium channels in pain transmission, Cell Calcium 40 (2006) 197–203.
[12] A. Dray, Neuropathic pain: emerging treatments, Br. J. Anaesth. 101 (2008) 48–58.
[13] H. Cuny, A. de Faoite, T.G. Huynh, T. Yasuda, G. Berecki, D.J. Adams, γ-Aminobutyric acid type B (GABAB) receptor expression is needed for inhibition of N-type (Cav2.2) calcium channels by analgesic α-conotoxins, J. Biol. Chem. 287 (2012) 23948–23957.
[14] H. Klimis, D.J. Adams, B. Callaghan, S. Nevin, P.F. Alewood, C.W. Vaughan, C.A. Mozar, M.J. Christie, A novel mechanism of inhibition of high-voltage activated calcium channels by α-conotoxins contributes to relief of nerve injury-induced neuropathic pain, Pain 152 (2011) 259–266.
[15] C.S. Dale, C. Altier, N. Cenac, R. Giorgi, M.A. Juliano, L. Juliano, G.W. Zamponi, N. Vergnolle, M.A.J.L. Renata Giorgid, N.V. Gerald, W. Zamponic, Analgesic properties of S100A9 C-terminal domain: a mechanism dependent on calcium channel inhibition, Fundam. Clin. Pharmacol. 23 (2009) 427–438.
[16] W.A. Catterall, Structure and regulation of voltage-gated Ca^{2+} channels, Annu. Rev. Cell Dev. Biol. 16 (2000) 521–555.
[17] A. Doğrul, Ö. Yeşilyurt, A. Işımer, M.E. Güzeldemir, L-type and T-type calcium channel blockade potentiate the analgesic effects of morphine and selective μ opioid agonist, but not to selective δ and κ agonist at the level of the spinal cord in mice, Pain 93 (2001) 61–68.
[18] E.M. Talley, L.L. Cribbs, J.H. Lee, A. Daud, E. Perez-Reyes, D.A. Bayliss, Differential distribution of three members of a gene family encoding low voltage-activated (T-type) calcium channels, J. Neurosci. 19 (1999) 1895–1911.
[19] M.T. Nelson, P.M. Joksovic, E. Perez-Reyes, S.M. Todorovic, The endogenous redox agent L-cysteine induces T-type Ca^{2+} channel-dependent sensitization of a novel subpopulation of rat peripheral nociceptors, J. Neurosci. 25 (2005) 8766–8775.
[20] S.M. Cain, T.P. Snutch, Contributions of T-type calcium channel isoforms to neuronal firing, Channels 4 (2010) 475–482.
[21] M.M. Jagodic, S. Pathirathna, P.M. Joksovic, W.Y. Lee, M.T. Nelson, A.K. Naik, P. Su, V. Jevtovic-Todorovic, S.M. Todorovic, Upregulation of the T-type calcium current in small rat sensory neurons after chronic constrictive injury of the sciatic nerve, J. Neurophysiol. 99 (2008) 3151–3156.
[22] Y.Q. Yu, F. Zhao, S.M. Guan, J. Chen, Antisense-mediated knockdown of $Na_v1.8$, but not $Na_v1.9$, generates inhibitory effects on complete Freund's adjuvant-induced inflammatory pain in rat, PLoS One 6 (2011) e19865.
[23] G. Berecki, L. Motin, A. Haythornthwaite, P.B.S. Vink, R. Drinkwater, C.I. Wang, M. Moretta, R.J. Lewis, P.F. Alewood, M.J. Christie, D.J. Adams, Analgesic ω-conotoxins CVIE and CVIF selectively and voltage-dependently block recombinant and native N-Type calcium channels, Mol. Pharmacol. 77 (2010) 139–148.
[24] M.J. Caterina, D. Julius, The vanilloid receptor: a molecular gateway to the pain pathway, Annu. Rev. Neurosci. 24 (2001) 487–517.
[25] C.L. Li, B.F. Yang, J.H. Zhang, J.D. Jiao, B.X. Li, C.F. Wu, Effect of ANEPIII, a novel recombinant neurotoxic polypeptide, on sodium channels in primary cultured rat hippocampal and cortical neurons, Regul. Pept. 164 (2010) 105–112.
[26] R.S. Scroggs, A.P. Fox, Calcium current variation between acutely isolated adult rat dorsal root ganglion neurons of different size, J. Physiol. 445 (1992) 639–658.
[27] T.S. Han, R.W. Teichert, B.M. Olivera, G. Bulaj, Conus venoms – a rich source of peptide-based therapeutics, Curr. Pharm. Des. 14 (2008) 2462–2479.
[28] R.S.I. Chuang, H. Jaffe, L. Cribbs, E. Perez-Reyes, K.J. Swartz, Inhibition of T-type voltage-gated calcium channels by a new scorpion toxin, Nat. Neurosci. 1 (1998) 668–674.
[29] J. Morrissette, M. Beurg, M. Sukhareva, R. Coronado, Purification and characterization of ryanotoxin, a peptide with actions similar to those of ryanodine, Biophys. J. 71 (1996) 707–721.
[30] F.Z. Zamudio, R. Conde, C. Arévalo, B. Becerril, B.M. Martin, H.H. Valdivia, L.D. Possani, The mechanism of inhibition of ryanodine receptor channels by imperatoxin I, a heterodimeric protein from the scorpion *Pandinus imperator*, J. Biol. Chem. 272 (1997) 11886–11894.
[31] H.H. Valdivia, M.S. Kirby, W.J. Lederer, R. Coronado, Scorpion toxins targeted against the sarcoplasmic reticulum Ca^{2+}-release channel of skeletal and cardiac muscle, Proc. Natl. Acad. Sci. 89 (1992) 12185–12189.
[32] N.X. Zhang, G. Wu, H.M. Wu, M.J. Chalmers, S.J. Gaskell, Purification, characterization and sequence determination of BmKK4, a novel potassium channel blocker from Chinese scorpion *Buthus martensii* Karsch, Peptides 25 (2004) 951–957.
[33] V.E. Scott, T.A. Vortherms, W. Niforatos, A.M. Swensen, T. Neelands, I. Milicic, P.N. Banfor, A. King, C. Zhong, G. Simler, A-1048400 is a novel, orally active, state-dependent neuronal calcium channel blocker that produces dose-dependent antinociception without altering hemodynamic function in rats, Biochem. Pharmacol. 83 (2012) 406–418.
[34] S.I. McDonough, Gating modifier toxins of voltage-gated calcium channels, Toxicon 49 (2007) 202–212.
[35] S.S. Sidach, I.M. Mintz, Kurtoxin, a gating modifier of neuronal high-and low-threshold Ca channels, J. Neurosci. 22 (2002) 2023–2034.
[36] H. Vanegas, H. Schaible, Effects of antagonists to high-threshold calcium channels upon spinal mechanisms of pain, hyperalgesia and allodynia, Pain 85 (2000) 9–18.