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

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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

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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

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