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Full Length Research Paper

Effects of astragaloside IV on L-type calcium channel currents in adult rat ventricular myocytes

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Astragaloside IV (AGS-IV), a traditional Chinese herb, has been found to have potent cardioprotective effects. However, the relevant mechanisms still remain unclear. In the present study, using standard whole-cell patch clamp recording technique, we identified a novel functional role of AGS-IV in regulating L-type calcium channel currents (L-currents) in adult rat ventricular myocytes. Our results showed that AGS-IV exerted inhibitory effects on L-currents of the single adult rat ventricular myocytes. This L-current inhibitory action of AGS-IV was concentration-dependent. The current density at +10 mV was reduced by about 48.7% after exposure of the cells to AGS-IV (1 µM), from the control value of 13.7±1.7 to 7.6±1.2 pA/pF. AGS-IV did not markedly affect the activation of L-currents but significantly shifted the inactivation curve to the left. Pretreatment of cells with H89, a protein kinase A (PKA) inhibitor, completely abolished AGS-IV-induced L-current inhibition. Similar results were obtained by another PKA inhibitor PKI 5-24. Taken together, these results suggested that AGS-IV inhibited L-currents via a PKA-dependent pathway in rat ventricular myocytes, which could contribute to its cardioprotective effects.

Key words: Astragaloside IV, L-type calcium channels, ventricular myocytes, patch clamp.

INTRODUCTION

Astragalus membranaceus, a traditional Chinese herb, has been reported to have a range of pharmacological effects. Astragaloside IV (AGS-IV; 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosyl-cycloastragenol, as the main active ingredient of Astragalus membranaceus, is a small molecular weight (MW 784) saponin. Current clinical and laboratory-based research has focused on the effects of AGS-IV with regards to anti-inflammation (Zhang et al., 2003), immunomodulation (Wang et al., 2002), ischemia injury protection (Zhang et al., 2006; Luo et al., 2004), and cardioprotection (Luo et al., 1995) etc.

In cardiovascular system, AGS-IV produces protection against cardiac arrhythmias in animal and clinical trials. It exerts the protective action against hypoxia, and reduces the level of lactate dehydrogenase (LDH) and superoxide dismutase (SOD) during myocardial infarction (Luo et al., 1995). However, the direct evidence for the action mechanism of AGS-IV on the cardiomyocytes is still lacking. It is well established that cardiac ischemia/reperfusion damage is associated with calcium overload ((Stamm and del Nido, 2004) therefore, these cardioprotective effects of AGS-IV suggest that it may reduce cardiac intracellular calcium, which is highly relevant to the state of L-type calcium channels in cardiovascular system. However, to date, the detailed mechanisms remain still unclear. More research on the cellular mechanisms of AGS-IV will not only contribute to the understanding of the efficacies of astragalus membranaceus for clinical treatment, but would also lead to the development of potential therapeutic strategies for the treatment of relative diseases.

In the present study, using the whole-cell patch clamp technique we first demonstrated that AGS-IV played a novel role in modulating L-type calcium channels in adult rat ventricular myocytes. Based on pharmacological

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manipulation of AGS-IV-induced L-current inhibition, we propose that this response is coupled to a PKA-dependent pathway and could contribute to the cardioprotective effects induced by AGS-IV.

MATERIALS AND METHODS

Reagents and solutions

All drugs were obtained from Sigma (USA), unless otherwise indicated. Astragaloside IV (JC Biotech, NJ) was prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO in the bath solution is expected to be less than 0.01%, and had no functional effects on L-type calcium channels. The composition of the external solution for L-type calcium channel current recording was composed of (in mM): TEA-Cl 140, $BaCl_2$ 5, $MgCl_2$ 0.5, glucose 5.5, CsCl 5, and HEPES 10, pH 7.35, with TEA-OH. The pipette solution contained (in mM): CsCl 110, EGTA 10, ATP-Mg 4, GTP-Na 0.3, HEPES 25, Tris-phosphocreatine 10, pH 7.3 with CsOH, 290 mOsm. The Tyrode's solution was composed of the following (in mM): NaCl 150, KCl 5.4, MgCl 2.0, HEPES 10, glucose 11. pH was adjusted with NaOH to 7.4. The KB solution was composed of the following (in mM): KOH 70, KCl 40, L-glutamic acid 50, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, HEPES 10, EGTA 0.5, glucose 10 (pH 7.35 to 7.40 with KOH).

Isolation of single cardiac myocytes

The animal use protocol was reviewed and approved by the Animal Care and Use Committee of Soochow University. Ventricle myocytes were dissociated enzymatically by a modified method described previously (Tao et al., 2004). Briefly, Sprague-Dawley rats (male, 200 to 300 g) were injected intraperitoneally with 1000 IU heparin and euthanized in a CO_2 chamber. The heart was quickly removed and transferred to an ice-cold Tyrode's solution. The aorta was cannulated and the heart was mounted on a Langendorff apparatus. The heart was perfused with prewarmed (at 37°C) and oxygenated Tyrode solution containing Protease Type XIV (Sigma) Collagenase Type I (Sigma C0130) for 10 min until the heart was flaccid. The ventricles were dissected out, cut into small pieces, and gently stirred in the Tyrode's solution. Isolated cells were filtered and maintained in an oxygenated KB solution on ice. Only the cells with a rod shape and clear cross striation were used for experiments.

The whole-cell patch clamp

Voltage-clamp experiments for recording cardiac myocytes were performed at room temperature (20 to 22ºC) as described previously (Tao et al., 2009; Zhang et al., 2010; Wang et al., 2011). Electrodes were pulled from borosilicate glass microcapillary tubes (World Precision Instruments). They had resistances from 2 to 3 MΩ when filled with internal solution. We made recordings using a MultiClamp 700B amplifier (Molecular Devices) and controlled voltage commands and digitization of membrane currents using a Digidata 1440A interfaced with Clampex 10.2 of the pClamp software package (Molecular Devices), running on a personal computer. Currents were low-pass filtered at 2 to 5 kHz. Series resistance (Rs) and capacitance (Cm) values were taken directly from readings of the amplifier after electronic subtraction of the capacitive transients. Series resistance was compensated to the maximum extent possible (at least 70%). Current traces were corrected for linear capacitive leak with online P/6 trace subtraction. The steady-state inactivation curves were obtained from experiments by stepping from a holding potential of − 70 mV to a 30-ms normalizing pulse to 0 mV followed by a family of 5-s-long prepulses from − 100 to 20 mV. A 100-ms test-pulse to 0 mV was recorded finally. Each test-pulse was normalized to the maximal current amplitude of the normalizing pulse.

Data analysis

All data are expressed as mean±S.E.M., and GraphPad Prism software was used for electrophysiological data plotting. Student's *t*-tests or one-way ANOVA were used to compare the different values, and were considered significant at $P<0.05$, $P<0.01$, or $P<0.001$. Concentration–response curves were fitted by sigmoidal Hill equation $I/I_{control} = 1/(1+10^{(\log IC50-X)n_h})$, where X is the decadic logarithm of the concentration used, IC_{50} is the concentration at which the half-maximum effect occurs, and n_H is the Hill coefficient. The I-V curves were fitted by $I_{Ba} = G_{max}(V-E\text{rev})/\{1+\exp((V-V_{1/2})/k_{I-V})\}$. Where I is the test-pulse current, Imax is the normalization peak current, Gmax = maximum conductance, E_{rev} = reversal potential, $V_{1/2}$ = voltage at 50% of Ba^{2+} current activation, and k_{I-V}=slope factor. Activation data were fitted by: $G/G_{max} = F_{low}/\{1+\exp((V_{1/2,low}-V)/k_{low})\} + (1-F_{low})/\{1+\exp((V_{1/2,high}-V)/k_{high})\}$, where $V_{1/2act}$ is the potential for half-activation calculated from dual Boltzmann functions when $G = 0.5G_{max}$. Steady-state inactivation data were fitted by a Boltzmann function of the form: $I/I_{max} = (A1-A2)/\{1+\exp((V-V_{1/2inact})/k_{inact})\}+A2$. The data for inactivation curves were fitted with a simple Boltzmann function: I/Imax = (A1−A2) / {1+exp[(V−V1/2inact)/k]}+A2, where I is the test-pulse current, Imax is the normalization current, A_1 is initial current amplitude, A_2 is final current amplitude, V is the membrane potential of the conditioning pulse, $V_{1/2}$ is the potential for half-inactivation, and *k* is the slope factor.

RESULTS

Identification of L-type calcium channel currents (L-currents) in adult rat ventricular myocytes

Voltage-gated calcium channels (VGCC) fall into two categories: High-voltage activated (HVA), including L-, N-, P/Q-, and R-type, and low-voltage activated (LVA) T-type. To detect the effect of AGS-IV on L-currents, we first isolated this subtype of VGCC currents in adult rat ventricular myocytes.

Whole-cell currents were recorded using 5 mM Ba^{2+} as charge carrier. Currents were elicited by a depolarization step from -70 to 0 mV (Figure 1A). Addition of $NiCl_2$ (100 µM), a specific T-type calcium channel blocker, did not affect the inward currents (Figure 1A), which suggested that there is no contamination of T-channels in the recorded currents. Application of nifedipine (10 µM), a specific L-type calcium channel blocker, completely abolished the HVA channel currents (Figure 1B), indicating that L-type HVA channels are functional in adult rat ventricular myocytes. As a complementary of our hypothesis, Bay K8644, a specific L-type calcium channel activator, significantly increased the current density. The high voltage-activated, slow inactivating, long steady-state component and Ni^{2+}-insensitive, but nifedipine-sensitive currents showed typical properties of the L-currents.

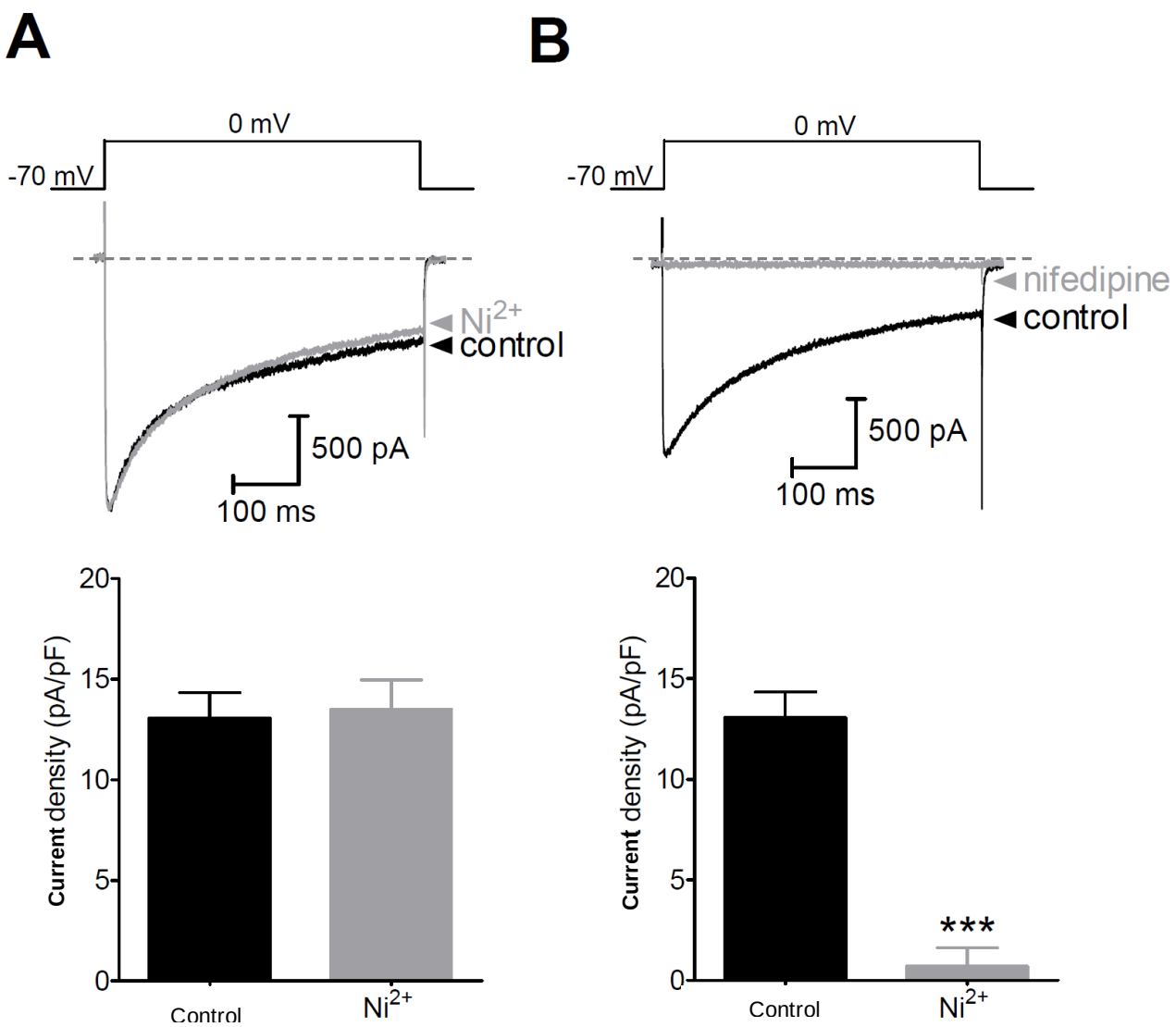

Figure 1. Characterization of L-type calcium channel currents in adult rat ventricular myocytes. A-B, Exemplary traces and pooled data showed the effects of Ni^{2+} (100 μM, n=7, (A) or nifedipine (10 μM, n=6, (B) on barium currents elicited by a depolarizing step pulse from the holding potential of -70 mV to 0 mV. Current with 5 mM barium as a charge carrier were elicited by a 500-ms long depolarization step pulse. ***p<0.001 *vs.* control.

AGS-IV inhibits L-currents in ventricular myocytes

Bath application of 1 μM AGS-IV inhibited the amplitude of basal L-currents in ventricular myocytes by 48.7±2.37% (Figure 2A). Upon washout of AGS-IV, the amplitude of L-currents partially returned within 3 min (Figure 2A), which indicated that the effect of AGS-IV on L-currents was not due to the rundown. Using the magnitude of the effect that AGS-IV has on currents elicited by depolarization to +10 mV, it is clear that AGS-IV inhibited L-currents in a concentration-dependent manner (Figure 2D). The relationship between the concentration of AGS-IV used and the degree of inhibition observed is described by a logistic equation where the concentration of AGS-IV producing half-maximal inhibition (IC_{50}) is 0.41 μM, the apparent Hill coefficient is 0.87, and the maximal inhibitory effect is 62.5±9.7% (Figure 2D).

Effects of AGS-IV on current-voltage relationship of $I_{Ca,L}$

The current-voltage (I-V) relations were obtained from a holding potential of -70 mV, 500 ms depolarizing pulses to different membrane potentials (10 mV increments from -40 to +60 mV). As demonstrated in Figure 3, AGS-IV significantly up-shifted the I-V curve. The current density at +10 mV was declined from 13.7±1.7 to 7.6±1.2 pA/pF in

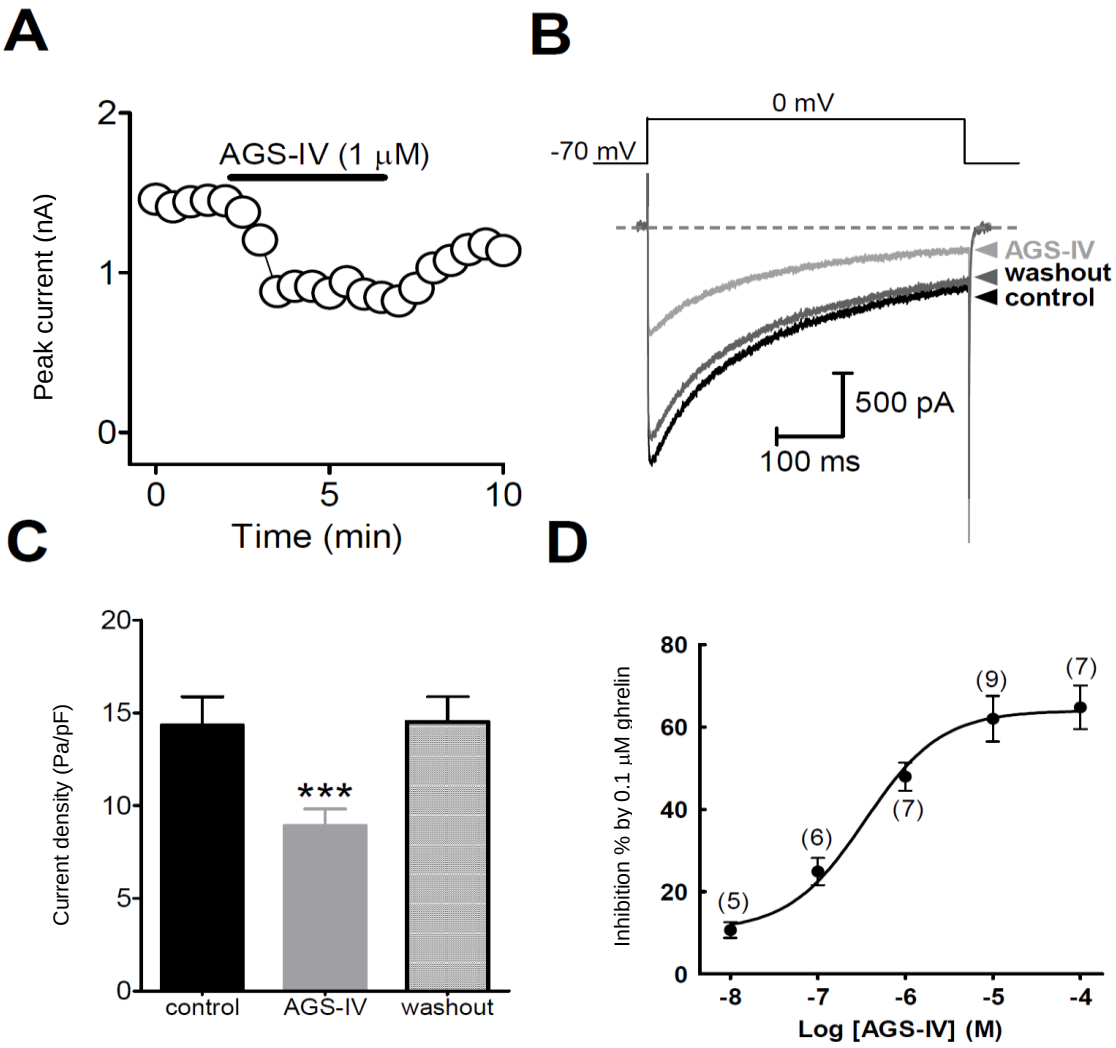

Figure 2. AGS-IV dose dependently inhibited L-currents. A-C, Time course (A), exemplary traces (B) and pooled data (C) of L-type calcium channel currents (L-currents) recorded under the control conditions, during exposure to 1 μM AGS-IV, and washout. D, Dose-response curve for the inhibitory effects of AGS-IV on L-currents. The line represents the best fit of the data points to the sigmoidal Hill equation (see *Methods and Materials*). Number of cells tested at each concentration of AGS-IV is indicated in brackets.

the presence of AGS-IV at 1 μM. These results suggested that AGS-IV did not affect the voltage-dependent activation.

AGS-IV leftward shifted steady-state inactivation curve

We further investigated whether the electrophysiological properties of L-type calcium channels were affected by AGS-IV. Steady-state activation and inactivation potentials of L-type calcium channels were then investigated (Figure 4A and B). We did not observe a significant shift in the hyperpolarized direction of the activation potential ($V_{1/2}$ from -11.9±0.7 to -12.1±1.2 mV, and *k* value from 6.5±0.8 to 6.3±0.7) (Figure 4A). However, AGS-IV at 1 μM leftward shifted the steady-state inactivation potentials of L-type Ca^{2+} channels by -11 mV ($V_{1/2}$ from -21.7±1.9 to -32.5±1.7 mV, and *k* value from -9.3±0.3 to -11.6±0.8) (Figure 4B). These results suggested that the reduced L-currents observed upon application of AGS-IV could be due to more channels remaining in the inactivated state after activation.

Protein kinase A is involved in the AGS-IV-induced L-current inhibition

We next investigated the detailed mechanism underlying

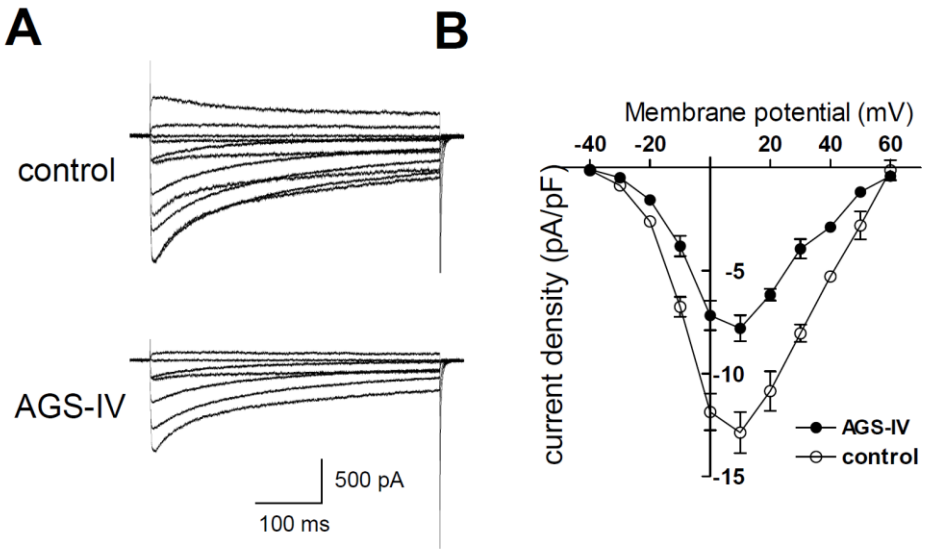

Figure 3. Effects of AGS-IV on current-voltage (I-V) curve. A-B, Exemplary traces (A) and pooled data (B) showed the effects of 1 μM AGS-IV on I-V curve. I-V curves were obtained from a holding potential of -70 mV, 500 ms depolarizing pulses to different membrane potentials (10 mV increments from -40 mV to +60 mV).

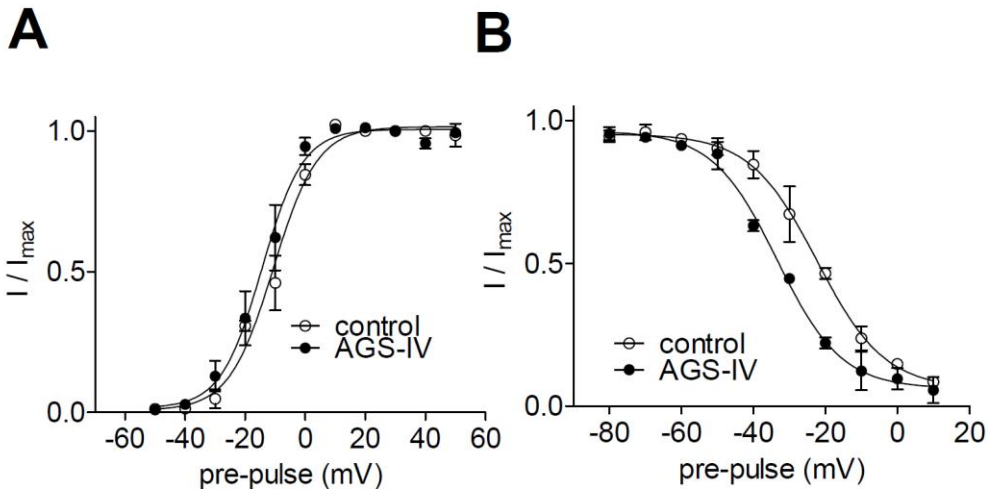

Figure 4. AGS-IV hyperpolarized shifted steady-state inactivation curve. A, The steady-state activation of L-type calcium channels is not altered by 1 μM AGS-IV application. Tail currents were elicited by repolarization to -70 mV after 200 ms test pulses from -70 to 0 mV in increments of 10 mV. B, AGS-IV shifted steady-state inactivation curve of L-type calcium channels to the hyperpolarizing direction. The steady-state inactivation curves were obtained from experiments by stepping from a holding potential of − 70 mV to a 30-ms normalizing pulse to 0 mV followed by a family of 5-s-long prepulses from − 100 to 20 mV. A 100-ms test-pulse to 0 mV was recorded finally. Each test-pulse was normalized to the maximal current amplitude of the normalizing pulse.

AGS-IV-induced L-current inhibition. To determine whether protein kinase A (PKA) was involved in AGS-IV-mediated L-current inhibition, we pre-incubated the cells with a PKA inhibitor, H89 (1 μM). Our results showed that pretreatment of cells with H89 abolished the AGS-IV-induced L-current inhibition in adult rat ventricular myocytes (inhibition%=2.7±1.1, Figure 5A and B). In contrast, H85 (1 μM), a structurally related but inactive analogue, had no effect on AGS-IV-induced L-current effects (not shown). To further confirm the PKA-mediated L-current inhibition, we dialyzed the cells with a pipette solution containing another PKA inhibitor, PKI 5-24. Our

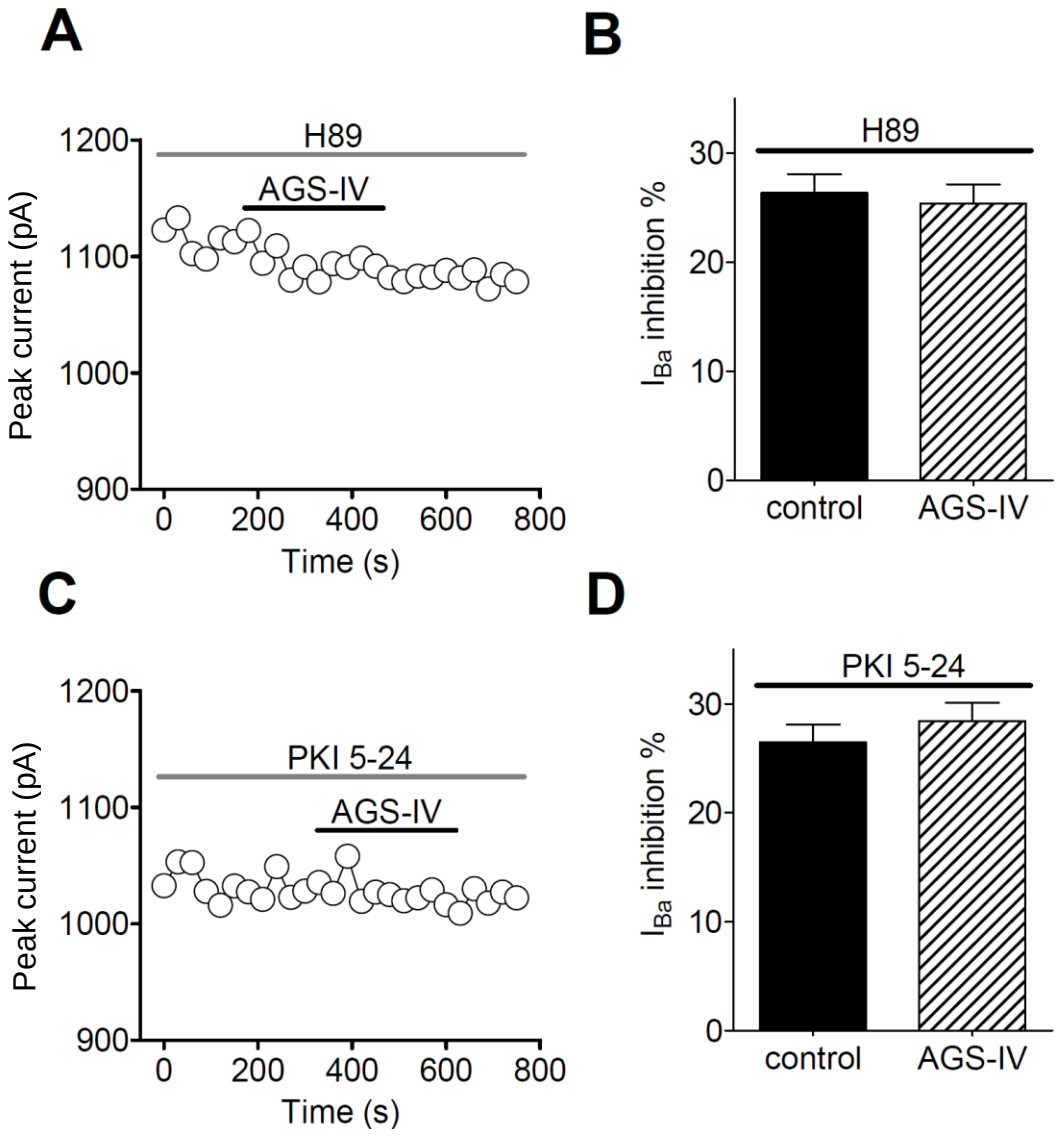

Figure 5. PKA was involved in AGS-IV induced L-current inhibition. A-D, Exemplary current traces and pooled data showed the effects of AGS-IV (1 μM) on L-currents in the presence of H89 (1 μM, A and B) or PKI 5-24 (1 μM, C and D).

results showed that intracellular application of PKI 5 to 24 (1 μM) to the recording pipette solution blocked AGS-IV-induced L-current inhibition (Figure 5C and D).

DISCUSSION

AGS-IV, the main functional ingredient of the Chinese herb Astragalus membranaceus, has many pharmacological functions. Although AGS-IV has a cardioprotective effect in the model of ischemic injury (Luo et al., 1995), there is almost no immediate evidence to demonstrate the cellular mechanism of AGS-IV on the protective effect of cardiomyocytes. In the present study, we first demonstrated that AGS-IV played a novel role in modulating L-type calcium channels in adult rat ventricular myocytes. Based on pharmacological manipulation of AGS-IV-induced L-current inhibition, we propose that this response is coupled to a PKA-dependent pathway and could contribute to the cardiac protective effects.

To elicit calcium currents, 500-ms-long pulses were applied from a holding potential of -70 to 0 mV under conditions where calcium was the only charge carrier for inward current (Heubach et al., 2000). This calcium current was completely blocked by 1 μM nifedipine, a specific L-type calcium channel blocker, indicating that it was not contaminated by K^+, Na^+, and T-type calcium currents (Tao et al., 2004). Furthermore, Na^+ free solution

was used to eliminate the possibility of contamination by Na^+ current. This strategy has the additional virtue of disabling Na^+-Ca^{2+} exchange. Rundown of ionic currents is always a concern in whole-cell voltage-clamp recording (Tao et al., 2004). We minimized time-dependent changes in L-currents by using high resistance pipettes filled with Mg-ATP 4 µM and beginning the experiments within 5 min after membrane rupture (Belles et al.,1998).

AGS-IV exerted some influence on the inactivation kinetics of L-type calcium channels. It shifted the inactivation curve of calcium currents to the left. These data strongly suggested that AGS-IV exerted its inhibitory effect via stabilizing the inactivation state of L-type calcium channels. Binding to the inactivated state is an important feature of the AGS-IV block, since strong inactivation at physiological resting potentials of L-type calcium channels could contribute to tissue selectivity. For example, dihydropyridines (DHP) that bind preferentially to the inactivated state of L-type calcium channels are useful as anti-hypertensive drugs by acting on vascular smooth muscle while having little effect on the heart (Triggle, 1992).

There are several examples of L-current modulation by protein kinase C (PKC) activation. In mouse hippocampal neurons, activation of PKC inhibits L-currents while an inactive analogue has no effect (Zhang et al., 2010). By contrast, PKC-induced L-current increase was also described in rat and in chick ventricular myocytes (Bray and Mynlieff, 2011). In the present study, we found that the inhibitory effects of AGS-IV on L-currents were PKA, but not PKC-dependent. Our data showed that pretreatment of ventricular myocytes with H89 could completely abolish the inhibition of AGS-IV on L-currents. Our present results were supported by previous studies that L-current inhibition by CB1 cannabinoid receptor activation in GT1-7 hypothalamic neurons was prevented by application of PKA inhibitors (Hoddah et al., 2009). Similarly, Cav1.2 L-current inhibition by integrin receptor activation was blocked by addition of PKA inhibitor H89 (Gui et al., 2006). In contrast, some previous workers have reported that T-type Ca^{2+} currents were mildly affected by PKA (Collis et al., 2007). On the contrary, L-currents recorded from isolated cardiomyocytes from adult canine left ventricles, were shown to be increased by the cAMP-PKA pathway (Xiao et al., 2011). Together, these results suggest that the PKA regulatory effects on L-currents can be variable in different tissues expressing different L-type calcium channel subtypes (Cav1.2 or Cav1.3).

In conclusion, this is the first report to electrophysiologically detail the inhibitory effects of AGS-IV on L-type calcium currents in adult rat ventricular myocytes. Our results provide ionic evidence of a possible link between the cardiacprotective effect of AGS-IV and calcium channels. Since L-type calcium channel inhibitors possess a high therapeutic potential in treating conditions like myocardial ischemia and hypertension (Tao et al., 2004), AGS-IV may also be a potential therapeutic traditional Chinese Medicine agent under some pathological conditions, such as ischemia, hypoxia and myocardial infarction, in which calcium-overload plays an important role.

ACKNOWLEDGEMENT

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REFERENCES

Belles B, Malecot CO, Hescheler J, Trautwein W (1998). “Run-down” of the Ca^{2+} current during long whole-cell recording in guinea pig hear cells: role of phosphorylation and intracellular calcium. Pflugers Arch., 411: 353-360.

Bray JG, Mynlieff M (2011). Involvement of protein kinase C and protein kinase A in the enhancement of L-type calcium current by GABAB receptor activation in neonatal hippocampus. Neurosci., 179: 62-72.

Collis LP, Srivastava S, Coetzee WA, Artman M (2007). beta2-Adrenergic receptor agonists stimulate L-type calcium current independent of PKA in newborn rabbit ventricular myocytes. Am. J. Physiol. Heart Circ. Physiol., 293: 2826-2835.

Gui P, Wu X, Ling S, Stotz SC, Winkfein RJ, Wilson E, Davis GE, Braun AP, Zamponi GW, Davis MJ (2006). Integrin receptor activation triggers converging regulation of Cav1.2 calcium channels by c-Src and protein kinase A pathways. J. Biol. Chem., 281: 14015-14025.

Hoddah H, Marcantoni A, Comunanza V, Carabelli V, Carbone E (2009). L-type channel inhibition by CB1 cannabinoid receptors is mediated by PTX-sensitive G proteins and cAMP/PKA in GT1-7 hypothalamic neurons. Cell Calcium, 46: 303-312.

Luo HM, Dai RH, Li Y (1995). Nuclear cardiology study on effective ingredients of Astragalus membranaceus in treating heart failure Chinese. Zhongguo Zhong Xi Yi Jie He Za Zhi, 15: 707-709.

Luo Y, Qin Z, Hong Z, Zhang X, Ding D, Fu JH (2004). Astragaloside IV protects against ischemic brain injury in a murine model of transient focal ischemia. Neurosci. Lett., 363: 218–223.

Stamm C, del Nido PJ (2004). Protein kinase C and myocardial calcium handling during ischemia and reperfusion: lessons learned using Rhod-2 spectrofluorometry. Thorac. Cardiovasc. Surg., 52: 127-134.

Tao J, Xu H, Yang C, Liu CN, Li S (2004). Effect of urocortin on L-type calcium currents in adult rat ventricular myocytes. Pharmacol. Res., 50: 471-476.

Tao J, Zhang Y, Huang H, Jiang X (2009). Activation of corticotropin-releasing factor 2 receptor inhibits Purkinje neuron P-type calcium currents via G (o)alpha-dependent PKC epsilon pathway. Cell. Signal., 21(9): 1436-1443.

Triggle DJ (1992). Calcium-channel antagonists: mechanisms of action, vascular selectivities, and clinical relevance. Cleveland Clin. J. Med., 59: 617-627.

Wang F, Zhang Y, Jiang X, Zhang Y, Zhang L, Gong S, Liu C, Zhou L, Tao J (2011). Neuromedin U inhibits T-type Ca^{2+} channel currents and decreases membrane excitability in small dorsal root ganglia neurons in mice. Cell Calcium, 49(1): 12-22.

Wang YP, Li XY, Song CQ, Hu ZB (2002). Effect of astragaloside IV on T, B lymphocyte proliferation and peritoneal macrophage function in mice. Acta Pharmacol. Sin., 23: 263-266.

Xiao YF, Nikolskaya A, Jaye DA, Sigg DC (2011). Glucagon-like peptide-1 enhances cardiac L-type Ca^{2+} currents via activation of the cAMP-dependent protein kinase A pathway. Cardiovasc. Diabetol., 20: 10-16.

Zhang WD, Chen H, Zhang C, Liu RH, Li HL, Chen HZ (2006). Astragaloside IV from Astragalus membranaceus shows cardioprotection during myocardial ischemia *in vivo* and *in vitro*. Planta Med., 72: 4-8.

Zhang WJ, Hufnagl P, Binder BR, Wojta J (2003). Antiinflammatory activity of astragaloside IV is mediated by inhibition of NFkappaB activation and adhesion molecule expression. Thromb. Haemost., 90: 904-914.

Zhang Y, Jiang D, Zhang J, Wang F, Jiang X, Tao J (2010). Activation of neuromedin U type 1 receptor inhibits L-type Ca^{2+} channel currents via phosphatidylinositol 3-kinase-dependent protein kinase C epsilon pathway in mouse hippocampal neurons. Cell. Signal, 22: 1660-1668.