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Effect of agmatine on L-type calcium current in rat ventricular myocytes

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KEY WORDS agmatine; patch-clamp techniques; myocardium; L-type calcium channels

ABSTRACT

AIM: To study the effect of agmatine (Agm) on L-type calcium current ($I_{Ca\text{-}L}$) in rat ventricular myocytes. **METHODS**: Whole-cell configuration of the patch-clamp technique was used to record $I_{Ca\text{-}L}$ in single rat ventricular myocytes which were dissociated by enzymatic dissociation method. **RESULTS**: (1) Agm (0.5, 1, 2 mmol/L) reduced the voltage-dependently activated peak amplitude of $I_{Ca\text{-}L}$ (pA) from 1451 ± 236 (control) to 937 ± 105 ($n = 8$, $P < 0.05$), 585 ± 74 ($n = 8$, $P < 0.01$), and to 301 ± 156 ($n = 8$, $P < 0.01$) in a concentration-dependent manner. (2) Agm (1 mmol/L) blocked $I_{Ca\text{-}L}$ in a use-dependent manner. The degree of use-dependent blocking effect was 53 % ± 12 % ($n = 8$, $P < 0.05$) at 1 Hz, and 69 % ± 11 % ($n = 8$, $P < 0.01$) at 3 Hz. (3) Agm upshifted the current-voltage (*I-V*) curve, but the characteristics of *I-V* relationship were not significantly altered by Agm, the maximal activation voltage of $I_{Ca\text{-}L}$ was not different from that of control. Steady-state activation of $I_{Ca\text{-}L}$ was not affected markedly. The half activation potential ($V_{0.5}$) and the slope factor (k) were not significantly different from those of the control. $V_{0.5}$ value was (−20.2 ± 2.5) mV in the control and (−20.5 ± 2.7) mV in the presence of Agm 1 mmol/L. The k value was (7.1 ± 0.4) mV and (7.5 ± 0.5) mV, respectively ($n = 8$, $P > 0.05$). (4) Agm 1 mmol/L markedly shifted the steady-state inactivation curve of $I_{Ca\text{-}L}$ to the left, and accelerated the voltage-dependent steady-state inactivation of calcium current. $V_{0.5}$ value was (−32 ± 6) mV in the control and (−40 ± 5) mV in the presence of Agm. The k value was (7.6 ± 0.9) mV and (12.5 ± 1.1) mV, respectively ($n = 8$, $P < 0.05$). (5) Agm 1 mmol/L markedly delayed half-recovery time of Ca^{2+} channel from inactivation (92 ± 28) ms to (249 ± 26) ms ($n = 8$, $P < 0.01$). **CONCLUSION**: Agm inhibited $I_{Ca\text{-}L}$ and mainly acted on the inactivated state of L-type calcium channel, manifested as acceleration of calcium channel inactivation and slowdown of recovery from inactivated state in rat ventricular myocytes.

INTRODUCTION

Agmatine (Agm) is a polycationic amine synthesized by the decarboxylation of *L*-arginine by the enzyme arginine decarboxylase and has been identified as an endogenous clonidine-displacing substance (CDS) in mammalian brain[1]. Our previous works demonstrated that Agm decreased action potential amplitude (APA), overshoot (OS), maximal rate of depolarization in phase 0 (V_{max}), duration of plateau phase, and action potential durations at 50 % and 90 % of repolarization ($APD_{50,90}$) in guinea pig papillary muscles[2], and reduced APA, OS, V_{max}, velocity of diastolic (phase 4) depolarization (VDD), and rate of pacemaker firing (RPF) in sinoatrial node pacemaker cells of rabbits[3] and human atrial fibers[4]. Moreover, Agm inhibited early afterdepolarization (EAD) and delayed afterdepolarization (DAD) induced by isoproterenol in guinea pig papillary muscles[5], and elevation of Ca^{2+} concentration antagonized the effects of Agm on sinoatrial node pacemaker cells of rabbits[3]. In our previous studies, we have suggested that Agm may exert its action by affecting the calcium influx. However, the direct evidence for the action mechanism of Agm on the cardiomyocytes is needed to be presented. The purpose of this study was to investigate the effect of Agm on L-type calcium current of ventricular myocytes.

MATERIALS AND METHODS

Isolation of ventricular myocytes Single rat

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ventricular myocytes were obtained from the hearts of adult Sprague-Dawley rats provided by Experimental Animal Center of Hebei Province (n = 15, either sex, weighing 260 g ± 38 g, Grade Ⅱ, Certificate № 04064), using enzymatic dissociation technique similar to that previously described[6]. The ventricles were cut, and then minced, incubated for 10 min in Ca^{2+}-free Tyrode's solution containing 0.1 % bovine serum albumin. Myocytes were harvested after filtration through a 200 μm nylon mesh, and resuspended in Tyrode's solutions containing different concentrations of Ca^{2+}. The concentration of Ca^{2+} was gradually increased to 1 mmol/L. All experiments were performed within 36 h of isolation.

Measurement of Ca^{2+} current Isolated ventricular myocytes were placed in the experimental chamber (0.4 mL) mounted on the stage of an inverted microscope (CK2, Olympus). After settling to the bottom of chamber, cells were superfused with external solution for 10 min at a rate of 2 – 3 mL/min at 25 ℃. Transmembrane currents were recorded with an Axopatch amplifier (200B, Axon Instruments, Inc). Glass microelectrodes were made using a microelectrode puller (PB-7, Narishige, Japan) by two-stage pulling and had a resistance of 2.0 to 4.0 MΩ when filled with electrode internal solution. Only rod-shape cells with clear cross-striations were used for experiments. Liquid junction potential between the pipette solution and external solution was corrected after the pipette tip got into the external solution. After gigaseal formation, the membrane was ruptured with a gentle suction to obtain the whole cell voltage-clamp configuration. Membrane capacitance and series resistance were compensated after membrane rupture to minimize the duration of capacitive currents. The external solution was changed to the Na^+-free solution in which Na^+ was replaced by equimolar tetraethylammonium chloride (TEA-Cl). Na^+ current was also inactivated at the holding potential (E_h) of – 40 mV and blocked by tetrodotoxin (TTX). K^+ current were suppressed by substituting K^+ by Cs^+. Computer-generated voltage or current pulses were programmed using the pCLAMP 6.0 software (Axon Instruments, Inc). On-line acquired data were stored on a hard disk of the microcomputer.

Solutions and drugs Agmatine, collagenase type Ⅱ, bovine serum albumin (BSA), taurine, TEA-Cl, HEPES, egtazic acid, CsOH, CsCl, MgATP, nisoldipine, 3-(*N*-morpholino) propanesulfonic acid (MOPS), and TTX were purchased from Sigma Co.

The Ca^{2+}-free Tyrode's solution contained NaCl 100, KCl 10, $MgSO_4$ 5.0, NaH_2PO_4 1.2, glucose 20, taurine 10, MOPS 10 mmol/L, pH was adjusted to 7.4 with KOH. The electrode internal solution for whole-cell recording was composed of MgATP 3, CsCl 140, HEPES 10, egtazic acid 10 mmol/L, pH 7.2 adjusted with CsOH. The external solution[7] was composed of TEA-Cl 140, $MgCl_2$ 2.0, $CaCl_2$ 1.5, glucose 10, HEPES 10, TTX 0.002 mmol/L, gassed with 100 % O_2, pH was adjusted with TEAOH to 7.3 – 7.4. Agm was dissolved in external solution at the concentrations of 0.5, 1, and 2 mmol/L before experiment.

Statistics The values were expressed as $x \pm s$. Statistical analysis was performed using t-test.

RESULTS

Effect of Agm on L-type calcium current After a 5 min period of external solution perfusion for control measurement, the external solution was changed with external solution containing Agm (0.5, 1, or 2 mmol/L) and data were collected at 30 s, 1, 2, 3, 4, and 5 min, respectively. Cells were superfused again with drug-free solution for 5 min to determine the reversibility of drug action. L-type calcium current in rat ventricular myocytes was evoked by a depolarizing step pulse from E_h of – 40 mV to 0 mV at the frequency of 0.1 Hz. The pulse duration was 300 ms. Agm (0.5, 1, and 2 mmol/L) inhibited the peak amplitude of $I_{Ca\text{-}L}$ (pA) from 1451 ± 236 (control) to 937 ± 105 (n = 8 cells from 6 hearts, $P < 0.05$), 585 ± 74 (n = 8 cells from 6 hearts, $P < 0.01$), and to 301 ± 156 (n = 8 cells from 6 hearts, $P < 0.01$), respectively (Fig 1). The inhibitory effect was concentration-dependent and the peak amplitude of $I_{Ca\text{-}L}$ (pA) was to be recovered from 301 ± 156 to 1236 ± 156 during washout with drug-free solution for 5 min.

Effect of Agm on use-dependent of $I_{Ca\text{-}L}$ Use-dependent characteristics were examined by eliciting the $I_{Ca\text{-}L}$ with 10 repetitive clamp steps from – 40 mV to 0 mV for 200 ms at 1 and 3 Hz pacing rates. $I_{Ca\text{-}L}$ was decreased as the stimulating frequency increased. The reduction of $I_{Ca\text{-}L}$ at the first pulse after Agm was defined as the tonic block. The use-dependent block was determined by the reduction of $I_{Ca\text{-}L}$ at the end of the 10th pulse, where the reduction of $I_{Ca\text{-}L}$ reached steady state. The tonic block of Agm 1 mmol/L at 1 and 3 Hz was

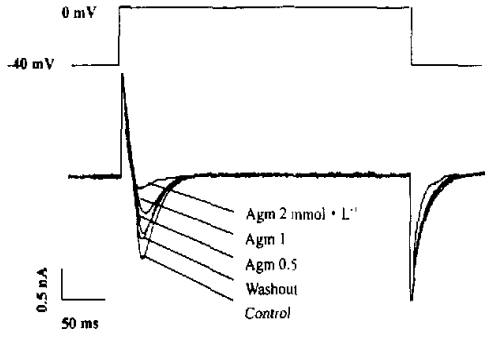

Fig 1. Effect of Agm on $I_{Ca\text{-}L}$ in isolated rat ventricular myocytes. Currents were recorded during 300 ms depolarizations from a holding potential of − 40 mV to 0 mV.

49 % ± 8 % and 52 % ± 11 % (n = 8 cells from 6 hearts), respectively. After exposure to Agm 1 mmol/L, the degree of use-dependent block was 53 % ± 12 % (P < 0.05) at 1 Hz, and 69 % ± 11 % (P < 0.01) at 3 Hz. The blocking effect at 3 Hz was stronger than that at 1 Hz (P < 0.05) (Fig 2A). To further elaborate the use-dependenence, the average beat-to-beat reduction of $I_{Ca\text{-}L}$ (I/I_{1st}) during the 10-pulse train is shown in Fig 2B. In presence of Agm 1 mmol/L, the reduction at the 10th pulse was greater at 3 Hz than that at 1 Hz (n = 8 cells from 6 hearts, P < 0.05), while the reduction at the 10th pulse on drug-free condition showed no difference between at 1 Hz and 3 Hz (P > 0.05).

Effects of Agm on current-voltage relationship and activation kinetics of $I_{Ca\text{-}L}$ Current-voltage (I-V) curves of L-type calcium current was obtained by 300 ms depolarizing pulses from the E_h of − 50 mV to various test potentials ranging from − 40 mV to + 50 mV in 10-mV increments. The pulse frequency was 0.1 Hz. $I_{Ca\text{-}L}$ was activated at − 30 mV and the peak amplitude occurred at the potential of 0 mV. Agm (0.5, 1, and 2 mmol/L) inhibited the peak amplitude of $I_{Ca\text{-}L}$ by 35 % ± 8 %, 60 % ± 12 %, and 79 % ± 17 % without altering the shape of the I-V (Fig 3). Although Agm upshifted the I-V curve, the maximal activation of $I_{Ca\text{-}L}$ appeared at 0 mV in the presence of Agm, which was not different from the control. The activation curves were fitted according to the Boltzmann equation: $I/I_{max} = 1/\{1 + EXP[-(V\text{-}V_{0.5})/k]\}$. Agm (1 mmol/L) did not markedly influence the activation kinetics with half activation potential ($V_{0.5}$) from (− 20.2 ± 2.5) mV to (− 20.5 ± 2.7) mV, and slope parameter (k) from (3.2 ± 0.4) mV to (3.0 ± 0.5) mV (n = 8 cells from 6 hearts, P > 0.05) (Fig 4A).

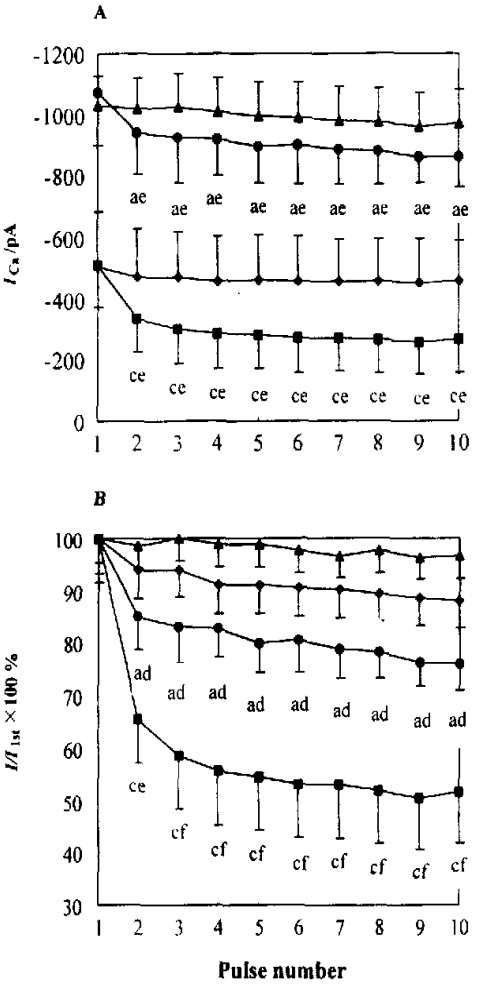

Fig 2. Use-dependent blocking curves of I_{Ca}. A) $I_{Ca\text{-}L}$ current at different frequency. B) The average beat-to-beat reduction of $I_{Ca\text{-}L}$ (I/I_{1st}) during the 10-pulse train. (▲) at 1 Hz, (●) 3 Hz before drug and (◆) at 1 Hz, (■) 3 Hz after Agm 1 mmol/L. n = 8 cells from 6 hearts. $x \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* 1 Hz before Agm. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* 1 Hz after Agm.

Effect of Agm on inactivation kinetics of $I_{Ca\text{-}L}$ Steady-state inactivation of the L-type channel was measured using a double protocol[8]. Membrane potential is first stepped from − 80 mV to various potentials for 400 ms and then to + 10 mV for 100 ms (test pulse) and finally clamped back to the holding potential of − 80 mV at the pulse frequency of 0.1 Hz. The inactivation curves were fitted according to the Boltzmann equation. Agm 1 mmol/L shifted half inactivation potential ($V_{0.5}$) from (− 32 ± 6) mV to

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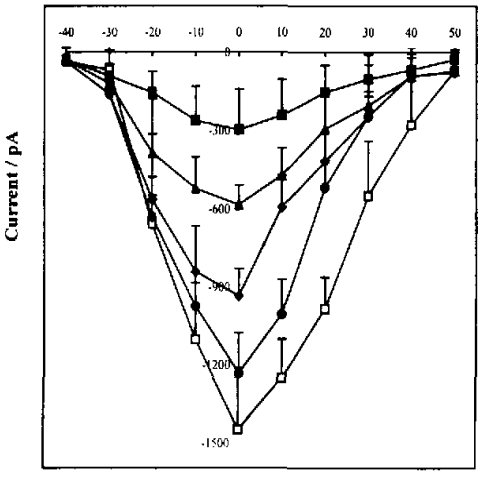

Fig 3. Effect of Agm on *I-V* curve of I_{Ca-L} in isolated rat ventricular myocytes. (□) Control, (◆, ▲, ■) Agm 0.5, 1, and 2 mmol/L, respectively. (●) Wash-out of Agm 2 mmol/L. n = 8 cells from 6 hearts. $\bar{x} \pm s$.

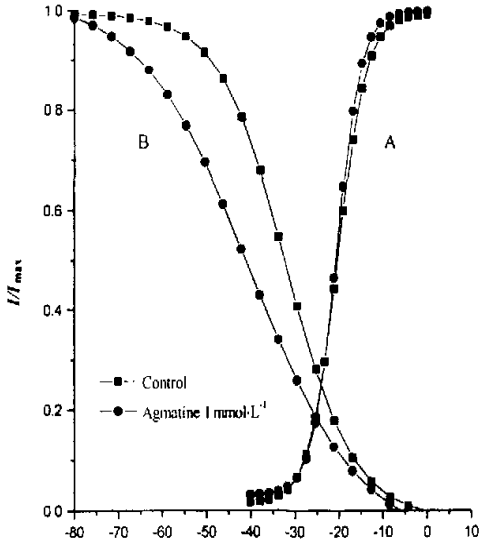

Fig 4. Effects of Agm on steady-state activation and inactivation kinetics of *L*-type Ca^{2+} current in myocytes. A) activation kinetics of I_{Ca-L}. B) inactivation kinetics of I_{Ca-L}.

(-40 ± 5) mV (n = 8 cells from 6 hearts), and k from (7.6 ± 0.9) mV to (12.5 ± 1.1) mV (n = 8 cells from 6 hearts, $P > 0.05$) (Fig 4B).

Effect of Agm on recovery of I_{Ca-L} from inactivation The recovery of I_{Ca-L} from inactivation was studied using double-pulse protocol consisting of a 200 ms prepulse to + 10 mV (P1) followed by a 1000-ms test pulse to + 10 mV (P2) after a variable P1-P2 coupling interval from 0 to 2500 ms at the holding potential of − 40 mV. Double-pulse stimulation was repeated every 6 s. Agm 1 mmol/L markedly delayed the half-recovery time of Ca^{2+} channel from inactivation from (92 ± 28) ms to (249 ± 26) ms (n = 6 cells from 6 hearts, $P > 0.01$) (Fig 5).

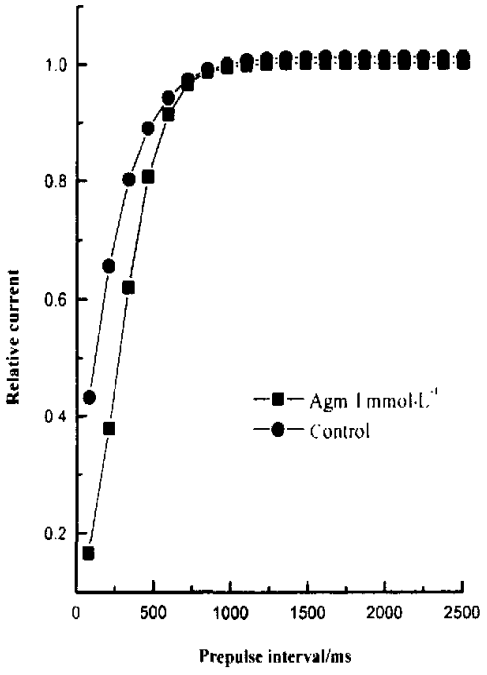

Fig 5. Time-dependent recovery of I_{Ca-L} from the steady-state inactivation. Agm 1 mmol/L markedly slowed the recovery time course of I_{Ca-L} from inactivation. n = 6 cells from 6 hearts.

DISCUSSION

In the present study, the slowly inactivated inward current recorded was *L*-type calcium current. Rundown of calcium current is always a concern in whole-cell patch-clamp recording. In this study, the rundown of I_{Ca} was minimized by adding MgATP (3 mmol/L) and egtazic acid (10 mmol/L) in pipette solution[9]. In the normal control group (n = 8), the I_{Ca-L} reduced only by 3 %, 6 %, and 10 %, respectively after 5, 10, and 15 min. Comparatively, in the experimental group, Agm 1 mmol/L reduced I_{Ca-L} by 53.2 % after 5 min. In addi-

tion, the currents were partially recovered after washout of Agm for 5 min. These results indicated that the reduction of $I_{Ca\text{-}L}$ was the action of Agm, but not the consequence of rundown of $I_{Ca\text{-}L}$. To eliminate the possibility of contamination by Na^+ current, Na^+ free external solution was used in our experiments. This strategy has the additional virtue of disabling the electrogenic process of Na^+-Ca^{2+} exchange. Although the beneficial effects of Agm on cardiovascular system have been extensively investigated, the study on $I_{Ca\text{-}L}$ in ventricular myocytes is not reported. In the present study, we found that Agm could concentration-dependently decrease the $I_{Ca\text{-}L}$. The inhibitory effect on $I_{Ca\text{-}L}$ was use-dependent. The *I-V* curve shifted upward by Agm, but the maximal activation of $I_{Ca\text{-}L}$ was not changed. Such effects indicated that Agm could block L-type calcium channel in rat ventricular myocytes, and had no marked effect on characteristics of voltage-dependence and activation kinetics of $I_{Ca\text{-}L}$. The inactivation kinetics of $I_{Ca\text{-}L}$ were changed by Agm. It shifted steady-state inactivation curve of calcium current to the left, and accelerated the voltage-dependent steady-state inactivation of calcium channel. It suggested that the blocking effect of Agm on inactivated state was stronger than that on activated state of L-type calcium channel. The recovery time of $I_{Ca\text{-}L}$ from inactivation was delayed, indicating that Agm could slow down the recovery of $I_{Ca\text{-}L}$ from inactivation. Therefore, from these findings, the myocardial electrophysiological effects of Agm observed in our previous studies[2-5] might be attributed to its action on $I_{Ca\text{-}L}$.

As the concentrations of Agm used in this study seemed to be high, it was likely that the observed effects of Agm might be nonspecific. However, when the ventricular myocytes were pretreated with idazoxan, an antagonist at imidazoline receptors (IR) and alpha-2 adrenergic receptors (α_2-AR), the inhibitory effects of Agm on L-type calcium current were completely blocked in our pilot experiment (data have not shown), thus implying that the effects of Agm were not nonspecific but exclusively mediated by IR and/or α_2-AR. The results that the higher concentrations of agmatine were required for exerting its effects on the L-type calcium current of ventricular myocytes might be due to the low affinity of the relevant myocardial receptors to Agm. The results were supported by our standard microelectrode studies[4] showing that Agm 1 mmol/L had no effect on maximum diastolic potential (MDP), V_{max}, APD_{50}, and APD_{90} in human right atrial fibers, while at 5, 10 mmol/L induced a marked decrease in V_{max}, APD_{50}, APD_{90}, and APA in a concentration-dependent manner. Idazoxan (0.1 mmol/L) could completely block the above-mentioned effects induced by Agm (5 mmol/L).

In conclusion, Agm inhibits $I_{Ca\text{-}L}$ in rat ventricular myocytes, and the effect of it is mainly determined by accelerating the inactivation of calcium channel and slowing down the recovery of calcium channel from inactivation.

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胍丁胺对大鼠心室肌细胞 L-钙通道电流的影响

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关键词 胍丁胺；膜片箝技术；心肌；L-型钙通道

目的：观察胍丁胺(Agm)对大鼠心室肌细胞 L-型钙通道电流($I_{Ca\text{-}L}$)的影响．**方法**：以酶解法制备单个心室肌细胞．应用全细胞膜片箝技术记录大鼠单个心室肌细胞钙通道电流．**结果**：(1) Agm (0.5，1，2 mmol/L)可浓度依赖性地降低电压依赖性激活 $I_{Ca\text{-}L}$ (pA)峰值，其值从 1451 ± 236 (对照组)到 937 ± 105 ($n=8$，$P<0.05$)，585 ± 74 ($n=8$，$P<0.01$)，和 301 ± 156 ($n=8$，$P<0.01$)．(2) Agm 1 mmol/L 使用依赖性地阻滞 $I_{Ca\text{-}L}$．1 Hz 时抑制率为 53 % ± 12 % ($P<0.05$)，3 Hz 时为 69 % ± 11 % ($P<0.01$)．(3) Agm 使 I-V 曲线上移，但对 $I_{Ca\text{-}L}$的电压依赖特征、最大激活电压以及 $I_{Ca\text{-}L}$稳态激活无明显影响．在 Agm 1 mmol/L 作用下，半数激活电压($V_{0.5}$)和斜率参数(k)与对照组相比均无显著性差异．$V_{0.5}$分别为(−20.2 ± 2.5) mV 和(−20.5 ± 2.7) mV，k 分别为(3.2 ± 0.4) mV 和(3.0 ± 0.5) mV．(4) Agm 1 mmol/L 可明显使钙电流稳态失活曲线左移，加速钙通道电压依赖性稳态失活．$V_{0.5}$分别为(−32 ± 6) mV 和(−40 ± 5) mV，k 分别为(7.6 ± 0.9) mV 和(12.5 ± 1.1) mV ($P<0.05$)．(5) Agm 1 mmol/L 还使 I_{Ca}从失活状态下恢复明显减慢．**结论**：Agm 抑制 $I_{Ca\text{-}L}$，并主要作用于 L-型钙通道的失活状态，表现为钙通道失活加速和从失活状态下恢复减慢．

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