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Agmatine blocked voltage-gated calcium channel in cultured rat hippocampal neurons

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KEY WORDS agmatine; patch-clamp techniques; calcium channels; hippocampus; neurons

ABSTRACT

AIM: To investigate the mechanism of agmatine by observing the effect of agmatine on the voltage-gated channels in rat hippocampal neurons. **METHODS:** The whole-cell patch recording technique was performed to record the voltage-gated potassium, sodium, and calcium currents in cultured rat hippocampus. Agmatine was applied directly to the single neuron using a pressure injector with microtubules. **RESULTS:** Agmatine (500 μmol/L) had no significant effect on the voltage-gated potassium and sodium channels. Agmatine reversibly blocked the voltage-gated calcium channel and the blockade was enhanced with the increasing concentration of agmatine. The inhibitory rates were 21 %±4 %, 35 %±6 %, 49 %±6 %, 67 %±4 %, 69 %±6 %, 86 %±8 %, and 87 %± 9 %, at the concentration of 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100 μmol/L, respectively. IC_{50} was (1.2±0.4) μmol/L. Two-way ANOVA revealed that change of membrane potential displayed a significant interaction with the blockade by agmatine. **CONCLUSION:** Agmatine reversibly blocked the voltage-gated calcium channel in rat hippocampal neurons in a concentration- and voltage-dependent way. Agmatine might perform its physiological and pharmacological effects partially by blocking the calcium channel.

INTRODUCTION

Agmatine [4-(aminobutyl) guanidine] is synthesized by decarboxylation of *L*-arginine with arginine decarboxylase (ADC) and hydrolyzed to putrescine and urea by agmatinase. Agmatine was first identified in 1910 by Kossel in herring sperm and was known as an intermediate in the polyamine metabolism of various bacteria, fungi, parasites and marine fanna[1,2].

Until the mid-1990s, agmatine was not believed to express in mammals. However, in 1994, it was discovered that agmatine, ADC, and agmatinase were also expressed and stored in mammalian brains[3] and many other organs[4]. Evidences have revealed that agmatine meets most criteria for a central neurotransmitter. It is synthesized in brain, stored in synaptic vesicles in heterogeneously distributed neurons, inactivated by reuptake, degraded enzymatically by a specific enzyme, agmatinase, released from axon terminals by depolarization, and binds with high affinity to α2-adrenoceptors and imidazoline receptors[5,6]. More and more publications have shown that agmatine might possess a lot of functions rather than that of a metabolic intermediate for polyamines. In the central nervous system, agmatine could produce release of several hormones and neurotramsmitters. It could enhance morphine analgesia and block morphine tolerance and withdrawal[7,8]. It could selectively block the NMDA subclass of glutamate receptor channels[9] and inhibit all

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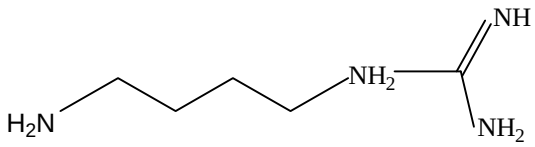
isoforms of NOS. Pharmacologically, agmatine seems to possess a lot of evident therapeutic and neuroprotective potential, even though the mechanism remains to be uncovered.

As an organic cation, agmatine could block NMDA receptor channel[9] and other ligand-gated cationic channels, including nicotinic acetylcholine and 5-HT_3 receptors[10]. However, there is no evidence to show whether agmatine interacts with the voltage-gated channels. As the voltage-gated channels underlie many important biological functions, such as cell excitability, neurotransmitters and hormone secretion, activity of enzymes and gene expression, we hypothesized that agmatine might present its physiological and pharmacological functions partially by modulating some kinds of these channels. Therefore, the effects of agmatine on several kinds of voltage-gated channels were observed in the present experiment.



Agmatine

MATERIALS AND METHODS

Cell culture Hippocampal neurons were isolated from fetal Wistar rat brain of either sex provided by Medical Experiment Animal Center of our institute. The dissociating and culture method was followed as described in our previous work[11]. Briefly, the hippocampi were cut into small pieces and digested at 36.5 °C with 0.25 % trypsin in Hanks' balanced salt solution for 30 min. Neurons were fed with Dulbecco's modified Eagle's medium plus 10 % horse serum and maintained in a CO_2 (9.6 %) incubator at 36.5 °C.

Electrophysiology Whole-cell recordings (Axopatch-1D, Axon Instruments, USA) were performed at room temperature (21-25 °C) from cultured hippocampus neurons 7-15 d after plating. Patch electrodes, pulled from boroscilicate glass tubing, had a resistance in the range of 1-5 MΩ. When the calcium currents were recorded, the electrode filling solution contained (μmol/L): CsCl 140, edetic acid 10, Na_2ATP 2, and HEPES 10, pH 7.25-7.4. The extracellular solution contained (μmol/L): NaCl 140, KCl 5, $CaCl_2$ 3, HEPES 10, $MgCl_2$ 1, and glucose 10, tetraethylammonium (TEA) 10, 4-aminopyridine (4-AP) 1, tetrodotoxin (TTX) 0.5, pH 7.25-7.4. The electrode filling solution for the recording of sodium and potassium currents contained (μmol/L): KCl 140, $CaCl_2$ 1, HEPES 10, $MgCl_2$ 1, Na_2ATP 2, and edetic acid 10, pH 7.4. The extracellular solution contained (μmol/L): NaCl 125, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 1, $NaHCO_3$ 26, NaH_2PO_4 1.25, and glucose 25, pH 7.4.

Application of drugs Agmatine was purchased from Sigma Chemical Co. Agmatine was dissolved in the extracellular solution and filled in a micro-manifold consisting of 3 microtubules, each of them had a diameter of 5-10 μm. The drugs were applied directly to the single neuron using a pressure injector (BH-2 Medical Systems Corp).The microtubule was placed approximately 20-30 μm from the cell and the puff pressure of N_2 (30-50 kPa) was adjusted to achieve rapid drug application while avoiding any mechanical disturbance in the recording of the electronic signal. One of the microtubules was filled with extracellular solution as control and others with different concentrations of agmatine as test groups.

Calculation Data acquisition and analysis were controlled by pCLAMP 7.0 software (Axon instruments). All the data were expressed as mean ± SD. The dose-response curve was fitted by Sigmaplot 2000 software (SPSS Inc, USA) with Hill equation. The software of Origin (MicroCal software) was used for the *I-V* graphic display and the SAS 6.12 software (SAS Inc, USA) was used to conduct the two-way analysis of variance (ANOVA). $P<0.05$ was considered as statistically significant.

RESULTS

No effect of agmatine on the potassium and sodium currents The voltage-gated whole-cell currents were evoked by depolarizing the membrane potential from -90 mV to +30 mV in 10 mV increments (Fig 1A). The inward currents were TTX-sensitive sodium currents (I_{Na}). The outward currents consisted of rapidly inactive, 4-AP-sensitive potassium currents (I_A) and delayed rectified, TEA-sensitive potassium currents (I_K). The drugs were applied for 1 s while the currents were induced. Agmatine (500 μmol/L) did not present any visible effect on the outward potassium currents and inward sodium currents (Fig 1).

Agmatine depressed calcium currents concen-

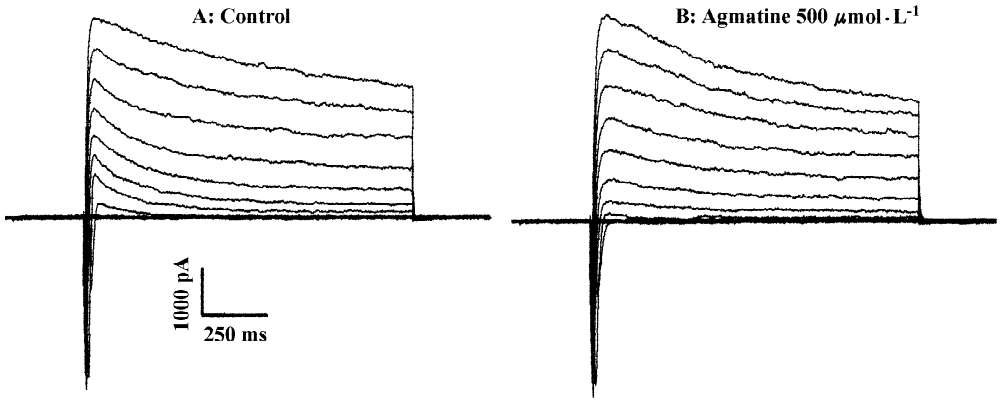


Fig 1. Effect of agmatine on the voltage-gated currents in hippocampal neurons. There was no effect found on the voltage-gated currents of I_A, I_K, and I_{Na} (P>0.05, n=6) while agmatine 500 mmol/L was applied.

tration-dependently To record the much smaller voltage-gated inward calcium currents (I_{Ca}), TTX, TEA, and 4-AP were extracellularly used to block I_{Na}, I_A, and I_K currents, respectively. Furthermore, intracellular K^+ was replaced by Cs^+ to depress the outward potassium currents. Adenosine triphosphate (Na_2ATP) was added in the pipette to retard the "run-down" of calcium currents. After the whole cell recording configuration was formed, the voltage-dependent calcium currents were elicited by the pulse stepped from -80 mV to 20 mV in a 10 mV increment. Calcium currents were depressed significantly with the increasing concentration of agmatine (Fig 2B-D). After 2 or 3 min, the calcium currents recovered partially (Fig 2E).

At concentrations of 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100 μmol/L, agmatine inhibited I_{Ca} remarkably with the inhibitory rate 21 %±4 %, 35 %±6 %, 49 %±6 %, 67 %±4 %, 69 %±6 %, 86 %±8 %, and 87 %±9 %, respectively, at the -20 mV of membrane potential. The concentration-response curve (Fig 3) was fitted with Hill equation. The IC_{50} was (1.2±0.4) μmol/L with 95 % confidence limits of 0.79-1.57 μmol/L. Hill coefficient was 0.52±0.20 (n=6).

Voltage-dependent blockade by agmatine From the *I-V* curve (Fig 4), it could be found that I_{Ca} had a threshold about -60 mV and the top currents appeared at -20 mV. Agmatine inhibited the calcium current, but did not change the threshold and the membrane potential of top currents. To figure out whether the membrane potential had any effect on the inhibition

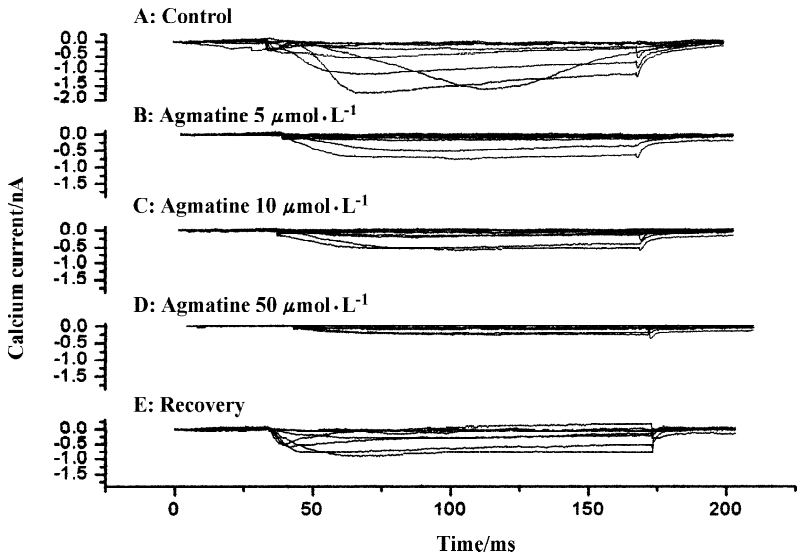


Fig 2. Effect of agmatine on voltage-gated calcium channel. A, B, C, D, and E were recorded from the same neuron. I_{Ca} was elicited by the pulse stepped from -70 mV to +20 mV in a 10 mV increments.

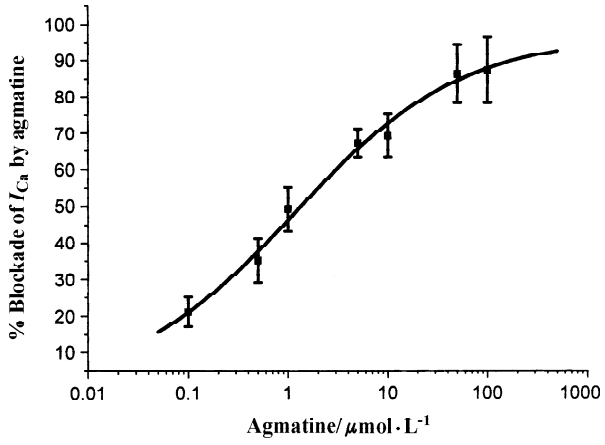


Fig 3. Concentration-response relationship of blockade of I_{Ca} by agmatine. The data were fitted with Hill equation. IC_{50} was (1.2±0.4) mmol/L and the Hill coefficient was 0.52±0.20 (n=6).

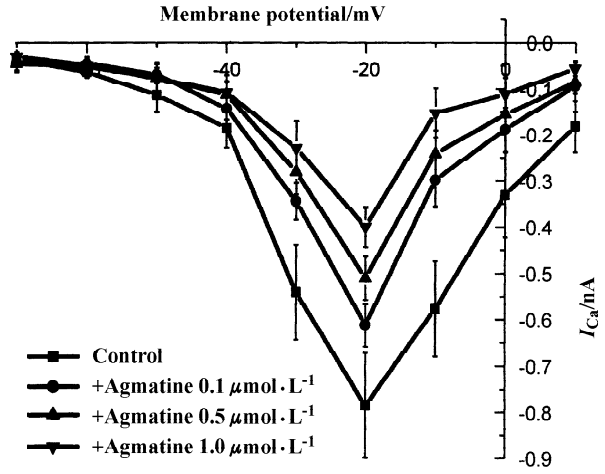


Fig 4. The *I-V* relationships of I_{Ca} obtained under the same conditions with different concentration of agmatine (n=6 cells). The holding potential was –90 mV and test potentials ranged from –70 to +20 mV in 10 mV increments.

by agmatine (Tab 1), two-way ANOVA was used. The analytic results revealed that the change of membrane potentials had a significant interaction with the inhibitory effect of agmatine (F=4.55, P<0.05).

DISCUSSION

The present results showed that 500 μmol/L of agmatine did not present any visible effect on the voltage-gated sodium and potassium currents. As high concentration of agmatine displayed a toxic effect on cultivated neurons (LD_{50}: 700 μmol/L[12]), we did not observe the effect of higher concentration of agmatine on the sodium and potassium currents.

Tab 1. Interaction of membrane potential with inhibition of I_{Ca} by agmatine. Mean±SD.

Membrane potential /mV	I_{Ca} current/nA 0 μmol·L^{-1}	0.1 μmol·L^{-1}	0.5 μmol·L^{-1}	1.0 μmol·L^{-1}
-40	-0.18±0.10	-0.14±0.08	-0.12±0.08	-0.10±0.06
-30	-0.54±0.27	-0.34±0.16	-0.28±0.12	-0.23±0.13
-20	-0.78±0.27	-0.61±0.10	-0.51±0.11	-0.40±0.10
-10	-0.58±0.22	-0. 28±0.12	-0.24±0.11	-0.16±0.10
0	-0.33±0.31	-0.17±0.06	-0.16±0.06	-0.10±0.07
10	-0.18±0.24	-0.10±0.06	-0.09±0.05	-0.06±0.04

Two-way ANOVA revealed that the membrane potential remarkably changed the amplitudes of I_{Ca} *vs* control group (n=6, F=64.54, P<0.05), and agmatine depressed the I_{Ca} significantly with increasing concentration (F= 69.24, P<0.05). The change of membrane potentials had a significant interaction with the inhibition by agmatine (F=4.55, P<0.05).

However, it was a little surprised to find that agmatine depressed the voltage-gated calcium currents with a higher potency [IC_{50}=(1.2±0.4) μmol/L]. The *I-V* relationship curve revealed that agmatine did not remarkably change the threshold of the voltage-gated calcium channel and the membrane potential level of maximum I_{Ca} (Fig 3). As the I_{Ca} could recover quickly upon the washing-off of agmatine (Fig 2E), it was suggested that the blockade was reversible.

Agmatine is charged with positive cation in the physical solution. It is possible for agmatine to block the calcium channel by plugging the open channels. To determine the mechanism of agmatine's action on the voltage-gated calcium channel, we observed the relationship between the membrane potential and the effect of agmatine. It was found that the interaction between the membrane potential and the inhibitory effect was significant (Tab 1), which meant that agmatine blocked the calcium channel in a voltage-dependent manner. The result suggested that agmatine interacted directly with the calcium channel pore by binding at a site partway across the membrane electric field. However, more evidences are needed to determine the action site of agmatine on the voltage-gated calcium channels.

In the cultured rat hippocampal neurons, L-, T-, and N-type calcium channels were found. Among these channels, L-type was expressed predominantly[13]. As 100 μmol/L of agmatine depressed 87 %±9 % of the calcium currents, we speculated that agmatine mainly

blocked the L-type calcium channel. In the future research, we are going to find out whether agmatine has any selective effect on the different subclasses of the voltage-gated calcium channels, such as L-, N-, or other types of calcium channels.

The estimated mean concentration of agmatine in the whole rat brain ranged from 0.1 to 6 μmol/L[9,14], and agmatine was not uniformly distributed within neurons; rather it was associated with subcellular structures such as clear synaptic vesicles[10] and dense-core vesicles[14] as demonstrated by electron microscopy. Therefore, it was reasonable to extrapolate that agmatine might modulate the voltage-gated calcium channels in the physiological condition, not mentioned that the higher concentration of agmatine administered exogenously.

Numerous results have demonstrated that agmatine possesses a lot of evident therapeutic and neuroprotective potential[5-10]. It is also well known that Ca^{2+} plays a much important role in a lot of physiological and pathological processes. Based on this experi-ment, we considered that agmatine might perform its biological and pharmacological functions, at least partially, by blocking the voltage-gated calcium channels. However, more evidences are needed to confirm the hypotheses.

In summary, agmatine reversibly blocked the voltage-gated calcium channel in cultured rat hippocampal neurons in a concentration- and voltage-dependent manner. The result might be helpful to explicate the functioning mechanism of agmatine in the physiological and pharmacological conditions.

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