Mechanism underlying blockade of voltage-gated calcium channels by agmatine in cultured rat hippocampal neurons[1]

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KEY WORDS agmatine; patch-clamp techniques; calcium channels; imidazoline receptors; α adrenergic receptors

ABSTRACT

AIM: To investigate whether agmatine could selectively block a given type of the voltage-gated calcium channels (VGCC) and whether related receptors are involved in the blocking effect of agmatine on VGCC. **METHODS:** The whole-cell patch recording technique was performed to record VGCC currents in the cultured neonatal rat hippocampal neurons. **RESULTS:** Verapamil (100 μmol/L), a selective blocker of L-type calcium channel, significantly inhibited VGCC current by 80 %± 7 %. Agmatine (100 μmol/L) could further depress the remained currents by 25 %±6 %. The α_2-adrenoceptor antagonist yohimbine (10 μmol/L) and the I_2 imidazoline receptor antagonist idazoxon (10 and 40 μmol/L) had no significant effect on VGCC currents when used respectively. When the mixture of yohimbine and agmatine was applied, VGCC currents were still depressed remarkably. However, the blocking effect of agmatine was decreased by 29 %± 8 % in the presence of idazoxon (10 μmol/L). The effect of idazoxon did not increase at a higher concentration (40 μmol/L). **CONCLUSION:** Agmatine could block the L- and other types of VGCC currents in the cultured rat hippocampal neurons. Blocking effect of agmatine on VGCC was partially related to I_2 imidazoline receptor and had no relationship with α_2-adrenoceptors.

INTRODUCTION

Agmatine [4-(aminobutyl) guanidine] 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]. Now, accumulating evidence has revealed that agmatine meets most criteria for a central neurotrans-mitter. It is synthesized in brain, stored in synaptic vesicles in heterogeneously distributed neurons, inactivated by reuptake, degraded by a specific enzyme, agmatinase, released from axon terminals by depolarization, and binds with high affinity to α_2-adrenoceptors and imidazoline receptors[3,4].

Pharmacologically, agmatine seems to possess a lot of evident therapeutic and neuroprotective potential, even though the mechanism remains to be uncovered thoroughly[5-7]. It was found that agmatine could regulate the release of vasopressin from neurohypophysial nerve terminals by inhibiting voltage-gated calcium channels (VGCC)[8]. Our former studies also demonstrated that agmatine blocked VGCC in rat hippocampal neurons with higher efficacy[9]. The evidence suggested that agmatine might perform its physiological and pharmacological effects, at least partially, by blocking the calcium channel.

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In the present experiment, we tried further to figure out that whether agmatine had selective effect on the different types of VGCC. As agmatine is accepted as a central neurotransmitter and has a higher affinity to imidazoline and α_2-adrenoceptors, we also wanted to determine whether these receptors were involved in its blocking effect on VGCC currents.

MATERIALS AND METHODS

Cell culture Hippocampal neurons were isolated from fetal Wistar rat's 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[10]. Briefly, the hippocampi were cut into small pieces and digested at 36.5 ºC for 30 min with 0.25 % trypsin in Hanks' balanced salt solution. 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 ºC.

Electrophysiology Whole-cell recordings (Axopatch-1D, Axon Instruments, USA) were performed at room temperature (21-25 ºC). All kinds of hippocampal pyramidal neurons were used for the experiment after 7-15 d culture. Patch electrodes, pulled from boroscilicate glass tubing, had a resistance in the range of 1-5 MΩ. For the calcium currents recording, the electrode was filled with the solution containing (mmol/L): CsCl 140, edetic acid 10, Na_2ATP 2, and HEPES 10, pH 7.25-7.40. The extracellular solution contained (mmol/L): NaCl 140, KCl 5, $CaCl_2$ 3, HEPES 10, $MgCl_2$ 1, and glucose10, tetraethyl ammonium (TEA) 10, 4-aminopyridine (4-AP) 1, tetrodotoxin (TTX) 0.5, pH 7.25-7.4. The remarkable run-down phenomenon of VGCC currents was found in about 3 min after the whole-cell recording configuration was formed. To eliminate the influence of the run-down problem, we have added ATP 2 mmol/L in intracellular solution, and finished the observation of the effect of agmatine on VGCC currents as quickly as possible, usually in 1-2 min.

Application of drugs Agmatine, verapamil, yohimbine and idazoxon were purchased from Sigma Chemical Co. Agmatine, verapamil, yohimbine, idazoxon and their mixtures were dissolved in the extracellular solution and filled into 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 the test groups.

Data analysis Data acquisition and analysis were controlled by pCLAMP 7.0 software (Axon instruments). All the data were presented as mean±SD. The SAS 6.12 software (SAS Inc, USA) was used to conduct the analysis of variance (ANOVA). $P<0.05$ was considered as statistically significant.

RESULTS

Agmatine inhibited the remained calcium currents after applying verapamil To record the much smaller voltage-gated inward calcium currents, TTX, TEA, and 4-AP were extracellularly used to block sodium and potassium 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 VGCC currents were elicited by a depolarization pulse stepped from -70 mV to -10 mV. To the same single neuron, the VGCC currents were recorded after applying verapamil, respectively. The drugs were applied for 1 s while the currents were induced. Verapamil 100 μmol/L significantly inhibited the calcium current and the inhibitory rate was 80 %±7 % ($P<0.05$, $n=6$, Fig 1B). Agmatine 100 μmol/L decreased remained currents after applying verapamil by 25 %±6 % ($P<0.05$, $n=6$, Fig 1C).

Both yohimbine and idazoxon had no effect on the calcium currents The VGCC currents were elicited by the pulse stepped from -70 mV to 20 mV in a 10 mV increment. The drugs were also applied for 1 s while the currents were induced. To exclude the possible direct interaction, we observed the effects of yohimbine and idazoxon on the VGCC by applying them onto the neurons. It was found that yohimbine (10 μmol/L) and idazoxon (40 μmol/L) did not present any visible effect on the inward calcium currents ($P>0.05$, $n=5$, Fig 2).

Yohimbine did not affect blockade of VGCC by agmatine The VGCC currents were elicited by depolarizing the membrane potential from -70 mV to -10 mV. The effects of agmatine and mixture of agmatine

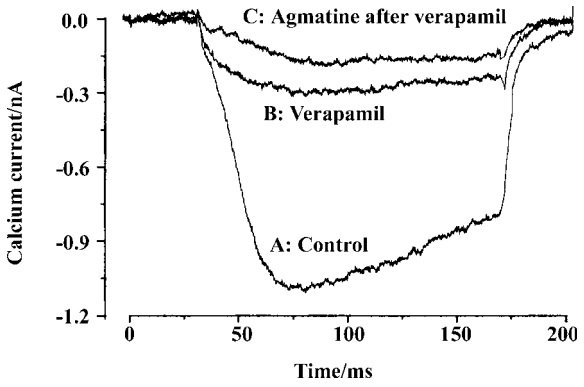

Fig 1. Effect of verapamil and agmatine on VGCC currents in hippocampal neurons. Currents were activated by stepping membrane potential from -70 to -10 mV. A) Control. B) Verapamil 100 μmol/L. C) Agmatine 100 μmol/L used after verapamil. *n*=6.

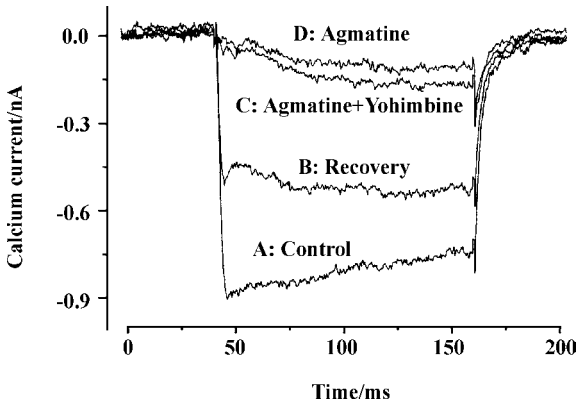

Fig 3. Effect of yohimbine on blockade of VGCC currents by agmatine. The VGCC currents were elicited by the pulse stepped from -70 mV to -10 mV. The VGCC currents were recorded from the same neuron. The inhibitory rates for agmatine plus yohimbine and agmatine alone were 78 %± 10 % and 86 %±8 %, respectively. There was no significant difference between the inhibitory rates (*P*>0.05, *n*=5).

with yohimbine on the VGCC currents were observed. Agmatine 100 μmol/L depressed the VGCC currents by 86 %± 8 %. The mixture blocked the VGCC currents with an inhibitory rate of 78 %±10 %. The difference of inhibitory rates between agmatine and the mixture had no statistical significance (*P*>0.05, *n*=5, Fig 3).

Idazoxon retarded the inhibitory effect of agmatine on VGCC currents When agmatine 100 μmol/L alone was applied to neurons, 85 %±2 % of VGCC currents was depressed (Fig 4Ic). However, the inhibitory rate decreased to 60 %±8 % when the mixture of idazoxon 10 μmol/L and agmatine 100 μmol/L was applied (Fig 4Ib). The blocking effect of agmatine was declined by 29 %±8 % (*P*<0.05, *n*=5). When the con-

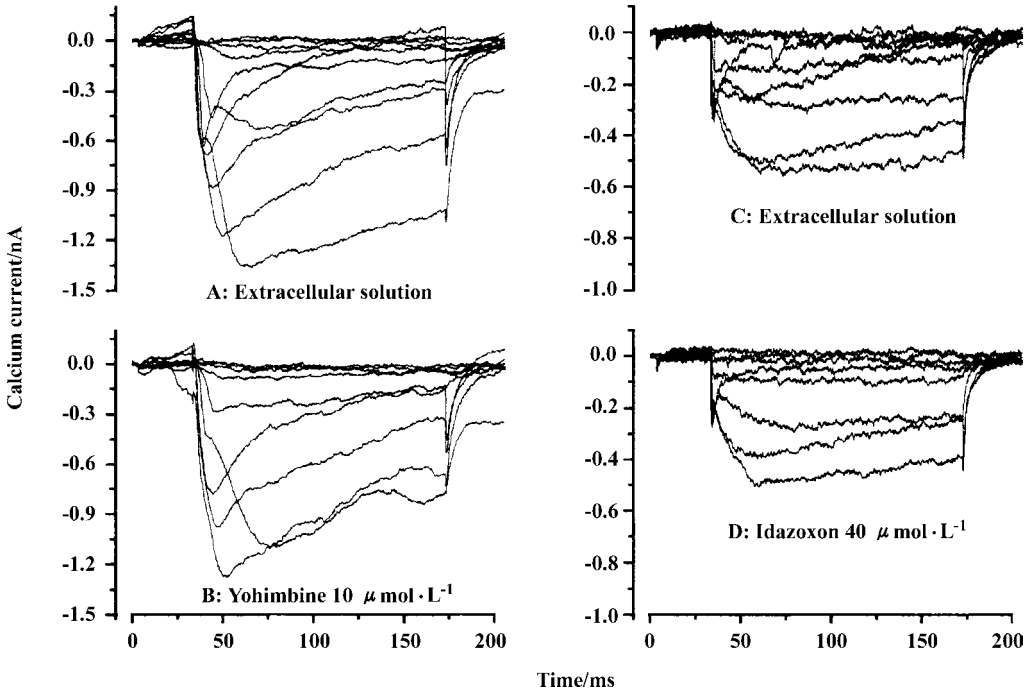

Fig 2. The effect of yohimbine and idazoxon on VGCC currents. The currents were elicited by depolarizing the membrane potential from -70 mV to 20 mV in a 10 mV increment. The currents in A and B were recorded from the same neuron and that in C and D from another one. Yohimbine and idazoxon did not present any visible effect on the VGCC currents (*P*>0.05, *n*=5).

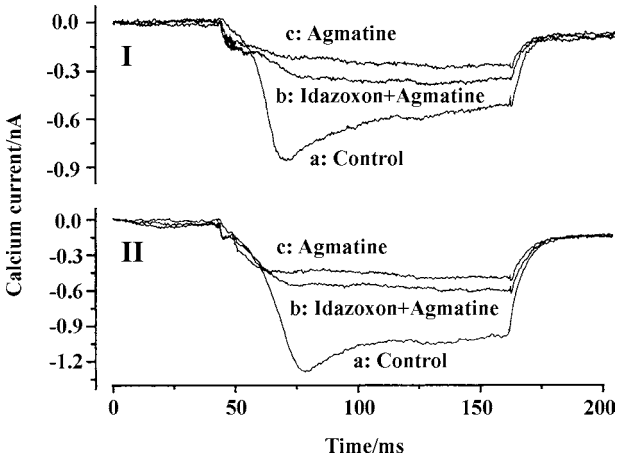

Fig 4. Effect of idazoxon on blockade of VGCC currents by agmatine. The currents were elicited by the pulse stepped from -70 mV to -10 mV. Ib: agmatine 100 μmol/L+idazoxon 10 μmol/L. The inhibitory rate of agmatine declined by 29 %±8 % (P<0.05, n=5); IIb agmatine 100 μmol/L+idazoxon 40 μmol/L. The inhibitory rate of agmatine decreased by 29 %± 10 % (P<0.05, n=5; Ic and IIc: agmatine 100 μmol/L).

centration of idazoxon was increased to 40 μmol/L, the blocking effect of agmatine was declined by 29 %± 10 % (Fig 4II). The retarding effect of idazoxon had not changed statistically at higher concentration (P>0.05, n=5).

DISCUSSION

Verapamil is a high selective L-type VGCC blocker and irreversibly blocks all the L-type calcium channel current at a concentration of 100 μmol/L[11]. In our study, 100 μmol/L verapamil was used to block L-type VGCC completely. However, only 80 %±7 % of the VGCC currents was depressed. It reconfirmed that L-type calcium channels were mainly expressed channels and other types of VGCC were also expressed in the cultured rat hippocampal neurons[12]. In our precious work, it was revealed that agmatine100 μmol/L decreased the calcium current by 87 %± 9 % by itself[9]. In the present experiment, we revealed an identical blocking effect of agmatine on VGCC currents (Fig 3, 4). It was suggested that agmatine, like verapamil, could block the L-type VGCC currents predominantly. The blocking effect of agmatine on VGCC currents was higher than verapamil, which implied that agmatine might block other types of VGCC besides the L-type calcium channel. In fact, we did find that agmatine still declined the remained currents by 25 %±6 %, after the L type VGCC currents were completely and irreversibly blocked by verapamil (Fig 1). However, more evidences are needed to demonstrate and identify whether agmatine blocks N-, T-, or other types of calcium channels.

Radioligand binding studies have revealed that K_d of agmatine to α_2-adrenoceptors, I_1 and I_2 imidazoline receptors was 4, 0.7, and 1 μmol/L, respectively. And a number of studies showed that many biological functions of agmatine were related to these receptors[3,13]. It reminded us that the inhibitory effect of agmatine on the VGCC currents might have some relationship to the receptors. In the present experiment, yohimbine (α_2-adrenoceptors antagonist) and idazoxon (I_2 imidazoline receptor antagonist) were used to explore whether α_2-adrenoceptors and/or imidazoline receptor were involved in blockade of VGCC by agmatine. It was found that yohimbine and idazoxon had no direct effect on VGCC currents (Fig 2). The result that yohimbine did not change the blocking effect of agmatine on the VGCC currents suggested that α_2-adrenoceptors were not involved in blocking effects of agmatine. However, in the presence of idazoxon 10 μmol/L, the inhibitory rate of agmatine on the VGCC current decreased by 29 %± 8 % (Fig 4). It implied that I_2 imidazoline receptor was related to the blocking effect of agmatine.

As the effect of agmatine was not blocked completely by high concentration of idazoxon, other mechanisms might be involved. It was known that there were two types of imidazoline receptors. Whether I_1 imidazoline receptor was involved in the agmatine block still remains unclear. We revealed that agmatine blocked the VGCC currents in a voltage-dependent way[9], which meant that agmatine could block the calcium channel by plugging the open channels directly. Our previous and present experiments only explained the blocking effect of agmatine from different profiles. To understand the mechanism underlying blockade of VGCC by agmatine completely, more researches are needed.

In summary, we revealed that agmatine could block the L- and other types of VGCC currents in the cultured rat hippocampal neurons and its inhibitory effect was partially related to I_2 imidazoline receptor. The result might be helpful to explicate the functioning mechanism of agmatine, an endogenous neurotransmitter, in the physiological and pharmacological conditions.

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