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Mechanisms of AMPA Receptor Inhibition by Diminazene

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Diminazene is an anti-infection agent for animals and is a member of the diarylamidine group. This study reports the first detection of its inhibitory effect on AMPA-type ionotropic glutamate receptors. Experiments were carried out on isolated Wistar rat neurons: striatal giant cholinergic interneurons were used to study calcium-permeable AMPA receptors and hippocampal field CA1 pyramidal neurons were used to study calcium-impermeable AMPA receptors. Cells were isolated by vibrodissociation and currents were recorded by voltage clamping in the whole cell configuration. Diminazene produced concentration-dependent inhibition of currents evoked by application of kainate in both neuron types. IC_{50} values for calcium-permeable and calcium-impermeable AMPA receptors were 60 ± 11 and 160 ± 30 μM, respectively. Of note is that the inhibitory action of diminazene increased with increases in agonist concentration. The plot of the voltage dependence of inhibition at a fixed diminazene concentration for calcium-permeable AMPA receptors was biphasic: minimal inhibition was seen at positive potentials and maximum at –40 to –60 mV, while further hyperpolarization produced a gradual decrease in blockade efficacy. All these properties provide evidence that diminazene blocks AMPA receptor channels, perhaps with penetration through channels into cells.

Keywords: AMPA receptors, diminazene, inhibition mechanisms, patch clamp.

Diminazene is an anti-infection agent for animals and is a member of the diarylamidine group [1]. It was developed more than 60 years ago and is used in the treatment of trypanosomiasis and various other diseases in animals caused by protozoa [2]. Studies in the last 20 years have also found other targets for diminazene. In particular, it is able to activate angiotensin-converting enzyme 2 [3] and has immunomodulatory properties [3, 4]. In addition, diminazene is a blocker of proton-controlled ion channels of the ASIC family [5, 6]. New pathways for the use of diminazene are under quite intense investigation at the current time, including in the light of the COVID-19 epidemic [7].

We have recently shown that diminazene can inhibit NMDA-type ionotropic glutamate receptors at micromolar concentrations [8]. AMPA receptors are another major type of ionotropic glutamate receptors [9]. They mediate rapid excitatory synaptic transmission in the CNS of vertebrates. These are conventionally divided into two main subtypes: calcium-permeable and calcium-impermeable, which have different pharmacological properties and physiological roles [9]. In particular, calcium-permeable AMPA receptors have greater channel permeability [10] and higher sensitivity to cationic channel blockers [11, 12]. Conversely, calcium-impermeable and calcium-permeable AMPA receptors have identical sensitivities to negative allosteric antagonists, such as the anticonvulsant perampanel [13, 14]. From the applied point of view, the search for novel AMPA receptor antagonists among drugs is relevant because of the relatively recent introduction of perampanel into clinical practice for the treatment of epilepsy [15, 16]. Considering the actions of diminazene on NMDA-type ionotropic glutamate receptors, it was interesting to determine whether it also inhibits AMPA-type receptors. It should be noted that the overall elongated shape of the molecule and the positive charge at physiological pH make diminazene reminiscent of classical AMPA receptor channel blockers.

Methods. All animal manipulation were carried out in compliance with the principles of the Basel Declaration and the guidelines of the Committee for the Humane Treatment of Animals of the Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences. Wistar rats (aged 14–19 days) were decapitated under urethane anesthesia. Brains were rapidly extracted and cooled to 2–4°C.

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A model 7000 smz2 vibratome (Campden Instruments, UK) was then used to cut transverse slices of the hippocampus and striatum of thickness 250 μm, which were kept in solution containing 124 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 2.0 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.24 mM NaH_2PO_4, and 10 mM D-glucose. The solution was aerated with carbogen (95% O_2, 5% CO_2) and had pH 7.4–7.5 at room temperature. Neurons were isolated from slices by vibrodissociation [17]. Studies with calcium-impermeable AMPA receptors used hippocampal field CA1 pyramidal neurons and studies of calcium-impermeable AMPA receptors used striatal giant cholinergic interneurons [18, 19]. Cell types were identified not only in terms of morphological, but also pharmacological criteria – sensitivity to the selective calcium-permeable AMPA receptor blocker IEM-1925 [20]. Correlations between AMPA receptor calcium permeability and sensitivity to dicationic channel blockers have been demonstrated in previous work [19].

Transmembrane currents were recorded by voltage clamping in the whole cell configuration. The extracellular solution contained 143 mM NaCl, 5 mM KCl, 2.0 mM $MgCl_2$, 2.5 mM $CaCl_2$, 18 mM D-glucose, and 10 mM HEPES; pH was adjusted to 7.3 by addition of HCl. A micropipette was filled with solution containing 100 mM CSF, 40 mM CsCl, 5 mM NaCl, 0.5 mM $CaCl_2$, 5 mM EGTA, and 10 mM HEPES; pH was adjusted to 7.2 by addition of CsOH. Substances were delivered using an RSC-200 eight-channel fast solution exchange system with electromagnetic valves (BioLogic Science Instruments, France). Solution exchange time was 50–60 msec. currents were recorded using an EPC8 amplifier (HEKA Elektronik, Germany). signals were filtered in the frequency band 0–5 kHz and digitized with a sampling frequency of 1 kHz. The holding membrane potential was monitored, the application system was controlled, and data were recorded and analyzed using a personal computer. Diminazene (diminazene aceturate, D7770) was purchased from Sigma Aldrich and other reagents were from Sigma Aldrich or Tocris Bioscience.

Data on the actions of diminazene are presented as mean ± standard deviation using at least five experiments. Statistically significant effects were identified using the paired *t* test.

Results. Application of kainate (100 μM) induced influx currents in hippocampal pyramidal neurons and striatal giant interneurons at a membrane potential of –80 mV. Figure 1, *a*, *b* shows representative examples of the inhibition of these currents by the selective calcium-permeable AMPA receptor blocker IEM-1925 (10 μM) and diminazene (100 μM). IEM-1925 effectively inhibited responses in striatal giant interneurons (blockade by 84 ± 5%, $n = 7$, Fig. 1, *a*) but had weak effects on responses in hippocampal pyramidal neurons (blockade by 10 ± 4%, $n = 6$, Fig. 1, *b*). Diminazene (100 μM) was also more effective in relation to calcium-permeable AMPA receptors in striatal giant interneurons (blockade by 64 ± 5%, $n = 7$) than calcium-impermeable AMPA receptors in hippocampal pyramidal neurons (blockade by 31 ± 7%, $n = 6$). We then studied the concentration dependence of the actions of diminazene on both main AMPA receptor subtypes. Representative examples of the inhibition of currents by diminazene (10–300 μM for calcium-permeable AMPA receptors and 30–300 μM for calcium-impermeable AMPA receptors) are shown in Fig. 1, *c* and Fig. 1, *d*, respectively. The inhibitory action of diminazene was rapid and reversible. Application of diminazene itself at concentrations of up to 300 μM did not induce currents in neurons, while higher concentrations led to cell death. IC_{50} values obtained by approximation of data to the Hill equation were 60 ± 11 ($n = 7$) and 160 ± 30 μM ($n = 6$) for calcium-permeable and calcium-impermeable AMPA receptors, respectively, and the Hill coefficients were 1.2 ± 0.2 and 1.5 ± 0.3. Thus, diminazene was more active in relation to calcium-permeable AMPA receptors than calcium-impermeable receptors, which is characteristic of antagonists acting via the ion channel blockade mechanism. For both receptor types, the curve shape suggested complete inhibition at high antagonist concentrations.

At the next stage, we elected to determine whether inhibition of AMPA receptors by diminazene is competitive or noncompetitive. We addressed this by comparing the efficacy of inhibition by a fixed concentration of substance at two different kainate concentrations: 50 and 500 μM (Fig. 2). The amplitude of the response to application of 500 μM kainate in the case of striatal giant interneurons averaged 3.9 ± 0.7 times greater than the amplitude of the response to application of 50 μM kainate. Figure 2, *a* shows a representative example of inhibition of calcium-permeable AMPA receptors in striatal giant interneurons by diminazene (60 μM). Responses to kainate (50 and 500 μM) were normalized on the plot for ease of interpretation. Diminazene was significantly more effective ($n = 6$, $p < 0.001$; Fig. 2, *c*) in blocking responses evoked by application of kainate (500 μM) (58 ± 5%) than by kainate (50 μM) (44 ± 4%). This provided unambiguous evidence for a noncompetitive mechanism of action. Analogous data were obtained for calcium-impermeable AMPA receptors in hippocampal pyramidal neurons (Fig. 2, *b*, Fig. 2, *d*; $n = 7$).

Two main types of noncompetitive AMPA receptor antagonists are currently known – negative allosteric modulators such as GYKI-52466 [21] and perampanel [15] and channel blockers such as the adamantane derivative IEM-1460 [11, 22], the phenylcyclohexyl derivative IEM-1925 [20, 23], argyrotoxins [24, 25], and philanthotoxins [26, 27]. The standard test for channel blockade for charged substances is analysis of the potential-dependence of their actions, which allows the depth of the binding site in the electric field of the membrane to be assessed. We compared the efficacy of blockade by a fixed diminazene concentration over the range of voltages +40 to –140 mV. Representative examples of the inhibition of kainate-induced currents by diminazene (60 μM) in striatal giant interneurons at

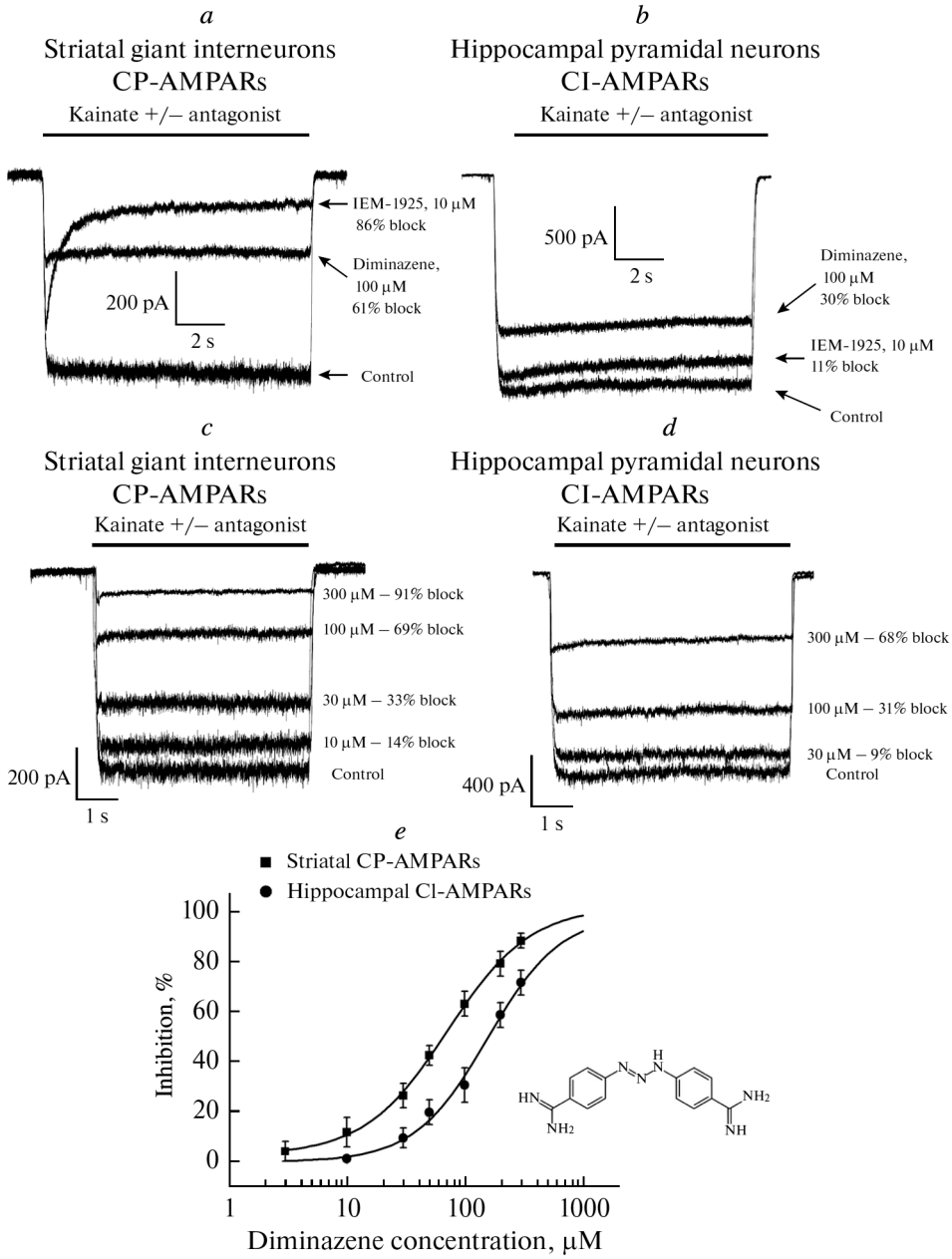

Fig. 1. Concentration dependence of the action of diminazene on AMPA receptors. (*a*, *b*) Representative examples of inhibition of kainate-induced currents by IEM-1925 (10 μM) and diminazene (100 μM) in striatal giant cholinergic neurons (*a*) and hippocampal field CA1 pyramidal neurons (*b*). *c*, *d*) Representative examples of the inhibition of kainate-induced currents by different diminazene concentrations in striatal giant cholinergic interneurons (*c*) and hippocampal field CA1 pyramidal neurons (*d*). *e*) Concentration dependence curves of the action of diminazene for calcium-permeable AMPA receptors in striatal giant interneurons and calcium-impermeable AMPA receptors in hippocampal pyramidal neurons.

potentials of +40 mV (*a*), –40 mV (*b*), –80 mV (*c*), and –120 mV (*d*) are shown in Fig. 3. The efficacy of inhibition by diminazene was minimal at a potential of +40 mV and reached a maximum at potentials of –40 to –60 mV and then decreased with hyperpolarization ($n = 5$, Fig. 3, *e*). This biphasic plot of inhibition vs. membrane potential is typical of blockers able to penetrate through channels into cells [28]. In the case of calcium-impermeable AMPA receptors, the relationship between inhibition effectiveness and membrane potential was also biphasic, though there was a more marked potential-dependent component ($n = 7$, Fig. 3, *e*). The data were approximated using an equation presented in [29] considering the potential for passage through channels into the cell and the existence of a potential-dependent component of inhibition:

$$B = (100 - A)/\{1 + (1/C)Kb\exp[FV/(RT)]z\delta m + \\ + (1/C)Kp\exp[-FV/(RT)]z\delta p\} + A.$$

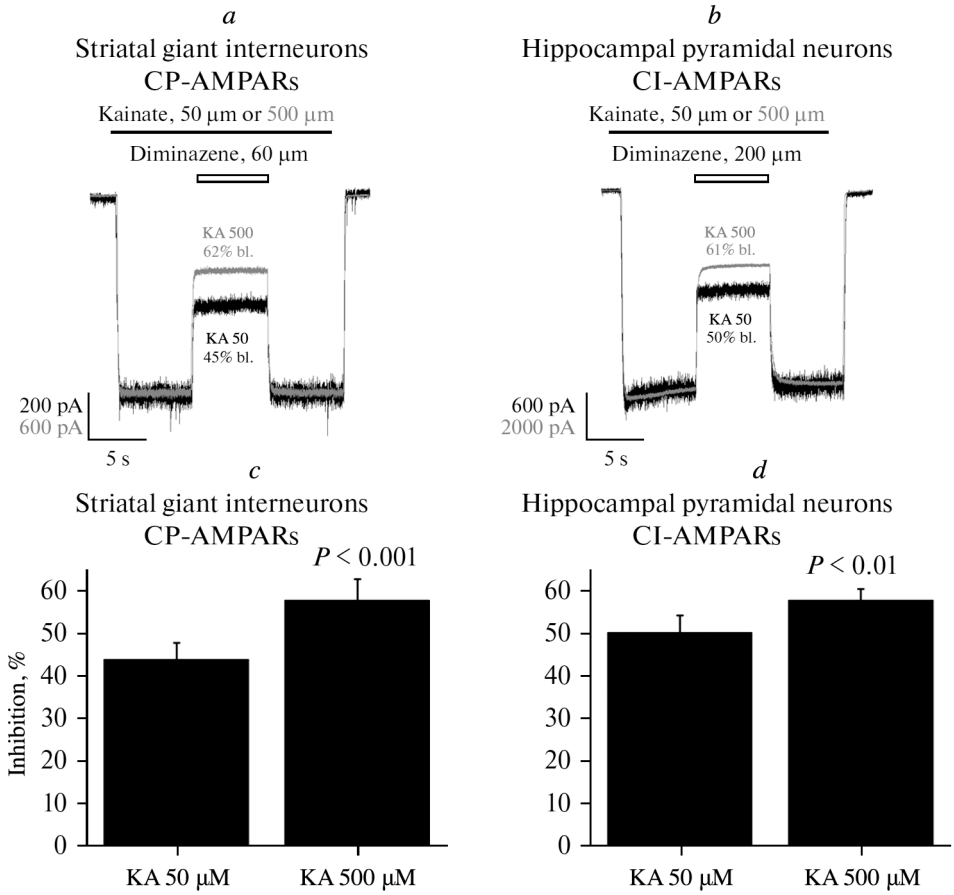

Fig. 2. Noncompetitive inhibition of AMPA receptors by diminazene. *a*, *b*) Representative examples of the inhibition of calcium-permeable (*a*) and calcium-impermeable (*b*) AMPA receptors at a fixed diminazene concentration at two different kainate concentrations (50 and 500 μM). Kainate response amplitudes at 50 and 500 μM were normalized for clarity. *c*, *d*) Mean percentage blockade by diminazene (60 μM) for calcium-permeable (*c*) and diminazene (200 μM) for calcium-impermeable AMPA receptors (*d*) at two different kainate concentrations (50 and 500 μM). Diminazene was more effective at the high agonist concentration for both types of receptor ($p < 0.001$ for calcium-permeable AMPA receptors and $p < 0.01$ for calcium-impermeable AMPA receptors).

In this equation, B is the percentage blockade at potential V by an antagonist concentration C, A is the percentage potential-dependent inhibition, Kb is the dissociation constant for the potential-dependent component at a potential of 0 mV, δm is the depth of the binding site in the electric field of the membrane, z is the charge on the blocker, and F, R, and T are the Faraday constant, the gas constant, and the absolute temperature, respectively. Parameters Kp and δp describe passage through the channel into the cell [28].

Data on blockade of calcium-permeable AMPA receptors by 60 μM diminazene was quite well approximated without consideration of the potential-dependent component of the action; δm was 09 ± 0.2 and $\delta p = 0.06$, which corresponds to the depth of the binding site of classical cationic channel blockers [28, 29]. Data on blockade of calcium-impermeable AMPA receptors by 200 μM diminazene were well approximated by consensus values of $\delta m = 0.6$ and $\delta p = 0.05$ for this type of receptor and cationic channel blockers [29]. The relatively high values of $\delta p = 0.05$–0.06 for both types of AMPA receptor and the weak flexion in the voltage-dependence curves at voltages of –80 to –140 mV are evidence that diminazene, although able to penetrate into cells through channels, its entry is hindered. It should be noted that in our experiments we did not add endogenous polyamines to the intrapipette solution, with the aim of ensuring that the pure effect of diminazene was addressed rather than a mixture of the effects of diminazene and polyamines. In the absence of polyamines in the intracellular solution and making measurements more than 5 min after establishing the whole cell configuration, the IV curve in striatal giant interneurons is linear in nature [30].

Discussion. This study obtained the first description of the inhibition of AMPA receptors by diminazene. This compound produces more active blockade of calcium-permeable AMPA receptors then calcium-impermeable (Fig. 1, *e*), which is characteristic of cationic antagonists acting via the channel pore blockade mechanism. The lower activity of similar compounds in relation to calcium-impermeable AMPA receptors is associated with the fact that the selective filter of the GluA2 subunit includes an arginine resi-

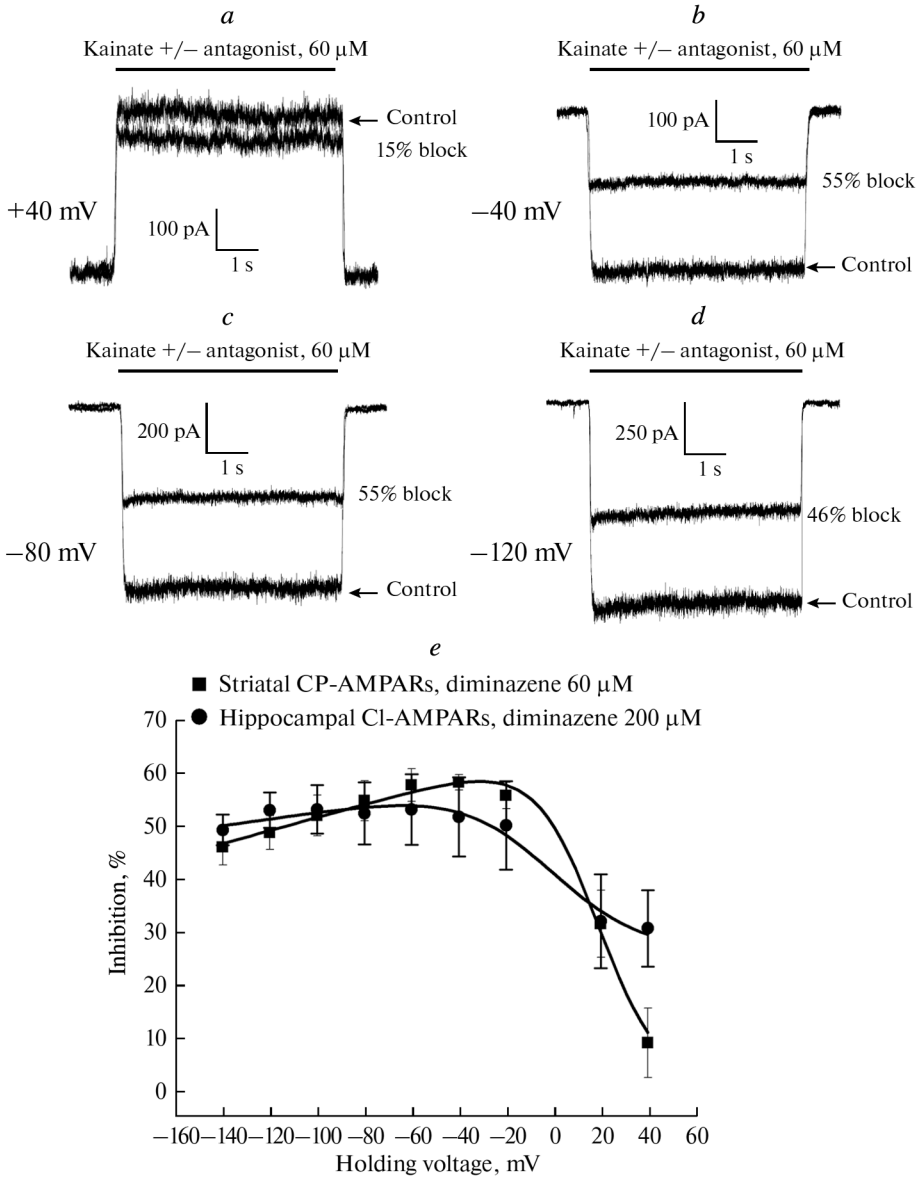

Fig. 3. Potential dependence of AMPA receptor inhibition by diminazene. *a–d*) Representative examples of the inhibition of kainate-induced currents in striatal giant interneurons by diminazene (60 μM) at membrane potentials of +40 (*a*), –40 (*b*), –80 (*c*), and –120 (*d*) mV. *e*) Potential dependence plots for inhibition by diminazene (60 μM) for calcium-permeable AMPA receptors in striatal giant interneurons and diminazene (200 μM) for calcium-impermeable AMPA receptors in hippocampal pyramidal neurons.

due, which repels positively charged blocker molecules. Diminazene acts noncompetitively (Fig. 2) and demonstrated biphasic potential-dependent blockade effectiveness at a fixed concentration (Fig. 3). The δm value of 0.9 ± 0.2 obtained by approximating the data on the voltage dependence of the effectiveness of blockade of calcium-permeable AMPA receptors is evidence that the diminazene binding site is located deep in the electric field of the membrane, while decreases in blockade effectiveness on hyperpolarization demonstrate the ability to penetrate through channels into cells. The rapid kinetics of the action of diminazene (Fig. 2, *a*, *b*) do not allow us to determine whether it is able to remain in the cavity of the closed AMPA receptor channel (the trap effect). It should be noted that in the case of NMDA receptors, no diminazene trap is seen – it acts by the "foot-in-the-door" mechanism [8]. No AMPA receptor blockers acting by the foot-in-the-door-mechanism are known.

It is interesting that diminazene in our experiments was more effective on activation of AMPA receptors by high agonist concentrations. This could potentially have the result that such compounds would provide weaker inhibition of normal synaptic transmission and stronger inhibition in conditions of pathological glutamate excess seen in a number of neurodegenerative diseases [31]. What might explain this dependence of effectiveness on agonist concentration? This may be linked with the ability of diminazene to penetrate

through channels into cells [32]. In fact, if a blocker is able to bind only with open channels and remain in the channel pore after closing and then diffuse into the cell cytoplasm, the blocking activity will be determined by the balance between binding with open channels and release from closed channels. Thus, blocking activity will be higher in conditions promoting channels being in the open state, including in the presence of high agonist concentrations. Further studies will answer the question of whether this dependence of effectiveness on agonist concentration is characteristic of other compounds of the diarylamidine group and for other blockers able to penetrate channels into cells.

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The authors declare that they have no conflicts of interests related to publication of this report.

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