GLYCINE ENHANCES GLUTAMATE-INDUCED EXCITATION IN VENTROMEDIAL HYPOTHALAMIC NEURONS IN AWAKE RATS

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Abstract: The finding that glycine potentiates N-methyl-D-aspartate (NMDA) receptor-mediated responses, has tremendously changed our understanding of glutamatergic synaptic transmission in the brain. Although the phenomenon has been confirmed in number of preparations, it is yet to be demonstrated in awake animals. Further, the controversy that glycine binding sites of NMDA receptor are saturated in vivo or not, can be best verified in awake animals. Here, we have demonstrated that glycine enhanced glutamate-induced neuronal discharges in the ventromedial nucleus of the hypothalamus of awake behaving rats using microiontophoresis technique, suggesting that the glycine binding sites of NMDA receptor are not saturated under physiological conditions.

Key words: glycine site NMDA receptor co-agonist synapti transmission hypothalamus microiontophoresis

INTRODUCTION

Glycine, the simplest of the amino acids, serves a dual role in the central nervous system. It is not only the primary inhibitory neurotransmitter in the spinal cord, retina and brain stem, but also an obligatory co-agonist for N-methyl-D-aspartate (NMDA)
receptor/channel complex of excitatory neurotransmitter glutamate (1, 2). This glycine binding site of the NMDA receptor complex is strychnine-insensitive and is widely distributed in the brain (3). Enhancement of NMDA receptor-mediated responses by exogenously applied glycine was first shown in cultured mouse brain neurons by Johnson and Ascher in 1987 (1). The phenomenon has been observed thereafter in number of preparations including brain slices and in vivo brains of anesthetized animals (4–7).

Extracellular glycine is a prerequisite for channel activation by glutamate or NMDA (2, 8–10). In the brain, extracellular glycine concentration is in the micromolar range in vivo, while measured affinity for the glycine site for NMDA receptor is in the sub-micromolar range ($K_i = 0.1–0.3 \mu M$) (11, 12), suggesting that the glycine site is already saturated in vivo and hence glycine plays no role under physiological conditions. However, recent findings suggest that extracellular glycine levels near the NMDA receptor is efficiently lowered to sub-saturating concentrations by an astroglial high-capacity glycine transporter adjacent to the NMDA receptor (13). Despite the fact that co-agonist concept of glycine has tremendously changed our understanding of glutamatergic neurotransmission in the brain, the role of extracellular glycine modulating of NMDA responses is yet to be confirmed in the brain of awake animals.

The present study aimed at investigating modulatory effects of microiontophoretically (juxtaneuronally) applied glycine on glutamate-induced single neuronal activities in the ventromedial nucleus of the hypothalamus (VMH) of awake behaving rats. Effects of glycine on spontaneous neuronal activities were also investigated for comparison. The reason why we choose the VMH as a recording site depends largely on its low spontaneous activity (14) and moreover the existence of silent neurons in awake conditions (15), which enabled us to investigate glycine effects on glutamate-induced neuronal discharges using microiontophoretic technique.

MATERIALS AND METHODS

Six male adult Wistar rats (SLC, Hamamatsu, Japan), weighing 200-220 g at the beginning of the study, were used. All experiments were approved by the local committee on Ethics for Animal Experimentation and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and with guiding principles for the Care and Use of Animals in the Field of Physiological Sciences recommended by the Physiological Society of Japan. All necessary actions were undertaken to minimize animal suffering and reduce the number of animals used.

Before neuronal recording, clanioplastic surgery and skull opening surgery were performed on separate days as described earlier (14, 15). Briefly, under pentobarbital sodium anesthesia (40 mg/kg, i.p.), four impression indentations for fake ear bars were formed on the skull with dental cement anchored to five stainless steel screws. These indentations were used later to fix the rat’s head painlessly in the stereotaxic apparatus during recording of extracellular neuronal activities. A five mm long stainless steel
tubing (27-gauge) was embedded in the cranioplastic acrylic near bregma to serve later as a reference coordinate pin. After recovery (7–10 days) from the surgery, the rats were placed on a 24 h water-deprivation regimen for training (2 weeks) of water ingestion during neuronal recording. Each rat was placed in a restraining cage with the head fixed rigidly and painlessly in the stereotaxic apparatus by the fake ear bars. While restrained, rats had access to a spout from which they learned to take water reward. Rats were trained for increasingly longer periods, initially 1 h to eventually 4 h per day. The day before beginning of neuronal recording, under pentobarbital anesthesia (40 mg/kg, i.p.) a Burr hole (2- to 3-mm diameter) was made through the skull over the intended recording site of the VMH (A, -2.3 to -3.3 from bregma; L, 0.3-1.0; V, 8.0-10.0) (15). Between recording sessions on different days, the exposed dura mater was covered with hydrocortisone ointment (Rinderon-VG® ointment, Shionogi, Osaka, Japan). The hole was covered with sterile cotton and sealed with epoxy glue.

A multibarrel glass micropipette assembly was used for recording of extracellular single neuronal activities and for microiontophoresis of chemicals. The method of microiontophoresis is well established for investigating effects of various bioactive substances on recorded neurons in the brain (16) and the application for awake animals is described by Kondoh et al (15). With this technique, charged molecules or ions can be ejected without negligible injury. This approach has a merit of bypassing the major diffusion barriers and of limiting the potential site of chemical action to the recorded neuron. Briefly, an indwelling carbon fiber (7-μm diameter) protruding 15–20 μm from the central barrel of a multibarrel glass micropipette assembly was used for neuronal recording. The outer barrels were filled with monosodium L-glutamate (0.05 M, pH 8.5), glycine (0.05 M, pH 8.5) and NaCl (0.15 M) for microiontophoresis. Separate two barrels were filled with 4 M NaCl for current balancing. During recording sessions, the electrode assembly was lowered stereotaxically to the VMH by a microdrive (SM-21, Narishige, Tokyo, Japan) for recording either spontaneous or glutamate-induced single neuronal activity and for microiontophoresis. The chemicals were ejected randomly using current strength of 10–70 nA of appropriate polarity and for the duration of 30–60 s by a microiontophoretic apparatus (DIA Medical System, Tokyo, Japan). Inter-trial intervals of at least 2 min were allowed for neurons to recover from the effects of chemicals. To exclude contribution of current effects, the null baseline neuronal responses to Na⁺ and Cl⁻ by application of either positive or negative current from the NaCl barrel, respectively, were verified during the initial trials. A neuronal response of more than 30% from the basal firing rate was considered as significant responses to the chemicals applied (15, 17).

After the last recording sessions, several iron deposits were made stereotaxically around the recording sites by passing positive current (20 μA, 30 s) through a stainless steel electrode (0.2 mm diameter). The neuronal recording sites were verified microscopically on 50-μm thick coronal brain sections stained with cresyl violet.
RESULTS

The effects of L-glutamate and glycine were tested in a total of 36 VMH neurons. Of these, 11 (31%) were spontaneously firing and another 25 (69%) were silent neurons.

In the spontaneously firing neurons, glutamate application for brief duration produced further excitation. The response was reproducible and appeared to be dose dependent (Fig. 1A). The effects of glutamate were not due to current ejection as verified by application of Na⁺ and Cl⁻ (data not shown). In any of these neurons, application of glycine produced no change in the discharge rate.

While tracking through glass microelectrodes, silent neurons were encountered occasionally by ejection of glutamate. Once firing was initiated in these neurons, a steady level of firing was maintained by continuous background application of low doses of glutamate (typically 10–20 nA). The firing continued as long as the glutamate was made available and the discharges of these neurons ceased if glutamate application was discontinued. In contrast to spontaneously firing neurons, addition of glycine on these glutamate-induced excitable neurons markedly enhanced neuronal discharge (Fig. 1B). The enhancing action of glycine was reversible and appeared to be dose-dependent. Of 25 silent neurons, 16 (64%) neurons showed enhancement of glutamate-induced activity and another 9 (36%) showed no response to glycine applications.

DISCUSSION

In the present study, we showed enhancing action of glycine on glutamate-induced neuronal activities in awake behaving rats. The enhancing actions of glycine on NMDA receptor activities have been demonstrated so far either in cultured cells, brain slices or anesthetized animals.
The results suggested that glycine binding site of NMDA receptors is not saturated in vivo and hence glycine levels in the brain have important physiological roles via modulation of NMDA-mediated glutamatergic activities. In contrast to glycine, lysine and arginine (basic amino acids) suppress glutamate-induced neuronal activities in the VMH of awake rats (15). In the rat cerebrospinal fluid, glycine levels are low while lysine and arginine levels are high (19). The levels of some of these specific amino acids (glycine, lysine, and arginine) in the synaptic cleft may cooperatively modify glutamatergic activities in the brain under physiological conditions.

The absence of glycine response in spontaneously firing VMH neurons is consistent with the results reported in cultured mouse brain neurons (1). It has been recently shown that binding of NMDA agonist or glycine alone is insufficient to cause current flow through the ion channel. For activation of NMDA receptors, binding of both glycine and glutamate sites are required (9). In the present study, it could be possible that the synaptic transmission in the spontaneously firing neurons was not glutamatergic or majority of microiontophoretically applied glycine could not reach efficiently to NMDA receptors at the synapses.

In contrast to the action of glycine on spontaneously firing neurons, the action on silent neurons was strikingly different. When these silent neurons were excited by glutamate, glycine application markedly enhanced neuronal activities. Although direct activation of non-NMDA receptors by glycine (20) could not be excluded in the present study, glutamate binds with more affinity to NMDA receptors than it binds to non-NMDA ones (5). There is conflicting evidence that glycine sites are normally saturated in vivo. Available evidences have provided both presence and absence of potentiation (18, 20–23). In the present study when the silent neurons were driven by glutamate, glycine was not added from exogenous sources. It has been shown in cultured neurons and brain slice preparation that glycine is obligatory for potentiation for NMDA receptors (1, 9). It could be possible that endogenous glycine in the synaptic cleft initiated glutamate action. Alternatively, there could be spill over of glycine from nearby inhibitory glycinergic synapse (24). It is unlikely that glycine was available from the presynaptic terminals, as in this case the postsynaptic neurons were excited by exogenous glutamate. Glycine transporters at the synaptic cleft can remove glycine below submicromolar concentrations (25). In addition, glycine site in vivo might be blocked by endogenous antagonist such as kynurenic acid, a tryptophan metabolite (20, 22). The fact that glutamate-induced firing and its further potentiation by exogenous glycine suggested that the glycine binding site of NMDA receptor is not saturated in vivo.

Glutamate is the most widely accepted excitatory amino acid neurotransmitter in the central nervous system. Its role has been implicated in number of physiological and pathological processes, which include neural development, learning and memory, synaptic plasticity, schizophrenia and ischaemic brain damage (26–29). Excessive application of glutamate not only increase neuronal firing but also leads to neuronal death. The finding of the present study offers a potentially safe and feasible approach for augmentation of NMDA receptor mediated neurotransmission by glycine. Glycine can act as an alternative for potentiating glutamate action in situations where excessive glutamate is not desired.
REFERENCES