Involvement of AMPA receptors for *Mesobuthus tamulus* Pocock venom-induced depression of monosynaptic reflex in neonatal rat spinal cord *in vitro*

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Glutamate is a putative neurotransmitter at Ia-α motoneuron synapse in the spinal cord and mediate the action via N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors. Since NMDA receptors are not involved in *M. tamulus* Pocock (MBT) venom-induced depression of spinal monosynaptic reflex (MSR), the present study was undertaken to evaluate the role of AMPA receptors in mediating the depression of MSR by MBT venom. The experiments were performed on isolated hemisected spinal cord from 4-6 day old rats. Stimulation of a dorsal root with supramaximal voltage evoked MSR and polysynaptic reflex (PSR) potentials in the corresponding segmental ventral root. Superfusion of MBT venom (0.3 µg/ml) depressed the spinal reflexes in a time-dependent manner. The maximum depression of MSR (~ 66%) was seen at 10 min and it was 25 min for PSR (~ 75%). The time to produce 50% depression of MSR and PSR was 6.7 ± 1.5 and 10.8 ± 2.6 min, respectively. Pretreatment of the cords with 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 0.1 µM), an AMPA receptor antagonist, blocked the venom-induced depression of MSR but not PSR. The results indicate that venom-induced depression of MSR is mediated via AMPA receptors.

**Keywords**: CNQX, Indian red scorpion venom, Non-NMDA receptors, Spinal synaptic transmission.

Indian red scorpion (*Mesobuthus tumulus* Pocock, MBT) envenomation produces fatal abnormalities by involving various systems such as nervous, cardiovascular, respiratory, etc.1,5. The nervous system manifestations are presented as pain, paralysis, restlessness, autonomic overactivity and convulsions1,6-8. The convulsions represent the increased muscle activity while paralysis represents the decreased or loss of muscle activity. These alterations in muscle activity represent the alterations in spinal motoneurons. In the spinal cord, Ia-α motoneuron synapse is the final synaptic pathway and glutamate is a putative transmitter at this synapse. Recently it is reported that MBT venom produces depression of monosynaptic (MSR) and polysynaptic (PSR) reflexes in neonatal rat spinal cord in a time- and concentration-dependent manner9. Further, it is shown that venom-induced depression of spinal MSR is not mediated through N-methyl-D-aspartate (NMDA) receptors9, unlike aglycemia/ischemia or *Ptychodiscus brevis* toxin (PbTx)-induced depression of MSR10,11. It is known that spinal MSR is mediated by both NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors11-13. In physiological condition, glutamate activates AMPA receptors initially followed by NMDA receptors14. In absence of NMDA receptor involvement for venom-induced depression of MSR9, involvement of AMPA receptor remains a possibility. Therefore, this study has been undertaken to examine the role of AMPA receptors in mediating the venom-induced depression of spinal reflexes in isolated spinal cord from neonatal rats *in vitro*.

**Materials and Methods**

**Animals, anesthesia and isolation of spinal cord**—All the experiments were performed according to the guidelines of the Institute of Medical Sciences, Banaras Hindu University, Varanasi for conducting the animal experiments. Care was taken to minimize the number of animals and their suffering.

Method for the preparation of spinal cord has been described earlier11,15. Briefly, 4-6 day old Charles-Foster strain rats were anesthetized with diethyl ether. The vertebral column was quickly removed and placed in a Sylgard plated petridish containing physiological solution bubbled with 100% O₂. The vertebral column was dissected from thoracic to sacral.
region. The spinal cord was dissected out carefully keeping the integrity of corresponding dorsal and ventral roots. Cord was hemisected sagitally and transferred to a small Plexiglas bath (~1 ml) superfused with oxygenated physiological solution (3.5 ml/min) maintained at 25°C ± 0.5°C (pH = 7.3).

Preparation and attachment of suction electrodes—Suction electrodes were prepared by using borosilicate glass capillary tube as mentioned earlier.11,16 The cut ends of the corresponding dorsal/ventral roots between L3,5 segments were sucked gently in the capillary tubes filled with physiological solution. The preparation was allowed to stabilize in the experimental chamber for 1-2 h.

Stimulation and recording—The stimulation of a dorsal root with rectangular pulses with supramaximal strength (40-50 V; 0.5 ms duration) at 0.1 Hz evoked monosynaptic (MSR) and polysynaptic reflex (PSR) potentials in the segmental ventral root. The potentials were amplified by using Harvard AC-DC amplifier and digitized by using DMS 708A AD converter (Dynalog Microsystems, Mumbai, India) and stored in a personal computer for on-line or off-line analysis. The averaged potential of five consecutive reflexes was recorded and analyzed using UnkelScope software (MIT, Massachusetts, USA).

Drugs and solutions—MBT venom was obtained from Haffkine Institute, Mumbai, India and 6-cyano-3-nitroquinoxaline-2, 3-dione (CNQX) was obtained from Tocris Cookson Ltd., UK. The physiological solution had the following composition in mM (NaCl, 124.0; KCl, 5.0; KH2PO4, 1.2; CaCl2·2H2O, 2.5; NaHCO3, 4.5 and glucose, 11.0; pH, 7.3). Stock solution (10−5 M) of CNQX was prepared in dimethyl sulfoxide and venom was prepared in double distilled water. The solutions were refrigerated and thawed just before use.

Experimental Protocol—The experiments were divided into two groups. In group-I, the control reflex recordings were obtained after stabilization. The cord was then exposed to a concentration of venom (0.3 µg/ml) for 30 min and the recordings were performed at every 5 min. Subsequently, the preparation was exposed to venom (0.3 µg/ml) in presence of CNQX for 30 min and recordings were made at every 5 min. At the end, the cord was washed with normal physiological solution for 30 min and the recordings were made.

Analysis of data—Reflex activity was quantified by measuring the peak amplitude of the reflex potentials. The responses after stabilization were taken as the initial response for normalization. Normalized amplitude of reflexes (after 30 min pretreatment with saline/CNQX) at various time intervals after venom were presented as mean±SE. Differences between the groups were compared by using two-way ANOVA followed by Student-Newman-Keul test for multiple comparisons. Student’s t-test was also used as required. P < 0.05 was considered significant.

Results

MBT venom depressed the spinal reflexes—Superfusion of MBT-venom (0.3 µg/ml) depressed MSR and PSR in a time-dependent manner (Fig. 1). The depression of reflexes began within 2 min and at 5 min, the depression of MSR was 31% and PSR was 45%. The maximum depression of MSR was seen at 10 min (63±4.8%) and remained at that level up to 30 min. In case of PSR, time-dependent depression was observed. The time to produce 50% depression (T-50) of MSR and PSR was 6.7±1.5 and 10.8±2.6 min, respectively. The effect of venom was partially reversed in case of MSR by washing the cord with normal physiological solution for 30 min (Fig. 1).

CNQX antagonized the venom-induced depression of MSR—Pretreatment of the spinal cord with CNQX (0.1 µM), AMPA receptor antagonist, for 30 min decreased the amplitude of MSR (by 25.8±4.7%; P < 0.05, Student’s t-test for paired observations) and PSR (by 19.1±11.1%). In the presence of CNQX, MBT venom (0.3 µg/ml) -induced depression of the MSR was blocked at various time intervals significantly (P < 0.05, two-way ANOVA followed by Student-Newman-Keul test; Fig. 1). The depression of MSR was 5% at 5 min, 28% at 15 min and remained at that level up to 30 min. However, the time course of the depression of PSR was not different from the venom only group (Fig. 1). The maximum depression of PSR was 78% at 25 min. The T-50 value for MSR could not be determined while for PSR it was 9.8±2.6 min and was not different from the venom only group. The effect of venom was not reversed by washing the cord with normal physiological solution for 30 min (Fig. 1).
Discussion

The findings of the present experiments while confirming the depression of spinal reflexes by venom in a time-dependent manner further provide evidence for the involvement of AMPA receptor for the venom-induced depression of MSR.

The depression of spinal reflexes can be produced by the inhibition of excitatory system or excitation of inhibitory system or by depolarizing the post synaptic site as reported for 5-hydroxytryptamine (5-HT)\textsuperscript{17,18}. Since venom increases the excitation of nerve fibers\textsuperscript{6}, the inhibition of excitatory system appears unlikely. Thus, the venom-induced depression can be mediated by enhancing the excitatory transmission that modulate the inhibitory interneurons as observed in Ptychodiscus brevis toxin (PbTx) or aglycemia/ischemia-induced depression\textsuperscript{10,19}. In PbTx-induced depression, both NMDA and non-NMDA mechanisms are involved\textsuperscript{19}. Earlier, it was shown that NMDA receptors are not involved for the venom-induced depression of spinal MSR\textsuperscript{9}. Thus, the involvement of non-NMDA receptors appears a possibility. The antagonistic action of CNQX on the venom-induced depression of MSR in the present experiments confirms the involvement of AMPA (non-NMDA) receptors.

The spinal MSR is mediated by glutamate utilizing both NMDA and non-NMDA receptors\textsuperscript{12,13}. Non-NMDA receptors include AMPA and kainate receptors. AMPA receptors are clustered at post synaptic site on motoneurons\textsuperscript{20}. Glutamate binding to AMPA receptor opens the cation channel to produce fast excitatory synaptic transmission\textsuperscript{13}. Multiple protein kinases and phosphatases are shown to modulate AMPA receptor functions\textsuperscript{21,22}. AMPA receptors are implicated in synaptic plasticity and synaptic desensitization\textsuperscript{20}. However, the present results exclude the long lasting effects such as synaptic plasticity/desensitization produced by AMPA receptors.

Glutamate is known to involve 5-HTergic system in the spinal cord\textsuperscript{23}. MBT venom-induced depression involves 5-HTergic system\textsuperscript{16}. In a report elsewhere, descending tract stimulation produced both ipsilateral and contralateral alterations in the segmental reflexes in neonatal rat spinal cord involving serotonergic pathway\textsuperscript{23}. Further, all these responses were blocked by CNQX\textsuperscript{23}. These findings indicate that AMPA receptors primarily modulate the descending 5-HT transmission in the spinal cord. Thus with the present results, it is likely that the depression of MSR induced by venom involve AMPA receptors via 5-HTergic system to produce depolarization and depression as suggested. The partial recovery of MSR in venom only group after washing supports the possibility of depolarizing effect. However, the recovery after wash (Fig. 1) was not complete indicating the involvement of other mechanisms.

In summary, it is proposed that venom activates glutamate receptors which in turn produce
depolarization via AMPA receptors to produce depression. Further, involvement of additional transmitters, like 5-HT, has been implicated.

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