Electrophysiological and Pharmacological Characteristics of Ionotropic Glutamate Receptors in Medial Vestibular Nucleus Neurons: A Whole Cell Patch Clamp Study in Acutely Dissociated Neurons

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ABSTRACT—A patch clamp study was performed to determine which subtype of ionotropic glutamate receptors is involved in the glutamate-induced excitation of the medial vestibular nucleus (MVN) neurons. Whole cell recording was performed on MVN neurons that were acutely dissociated by enzymatic and mechanical treatments. Application of glutamate at a concentration of 100 μM produced a current with a reversal potential of approximately 0 mV. The glutamate-induced current was completely blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), a non-N-methyl-D-aspartate (NMDA)-receptor antagonist. Application of α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) and kainic acid (KA), non-NMDA-receptor agonists, at concentrations of 30 and 100 μM produced a concentration-dependent depolarization concomitantly with an increase in firing rates during current clamp recording. During voltage clamp recording, glutamate, AMPA and KA elicited a concentration-dependent current with an equilibrium potential of approximately 0 mV. To clarify whether NMDA receptors are present in MVN neurons, the effects of glycine on the glutamate- and NMDA-induced current were examined. Two types of NMDA receptor-mediated current (types 1 and 2) were obtained in terms of the difference in sensitivity to both magnesium ion and MK-801, which act on the NMDA-receptor channel. In the type 1 neurons, the NMDA-induced current was not apparently blocked by magnesium ion or MK-801, although a larger current was obtained in the absence of magnesium ion. In the type 2 neurons, marked blockade of the NMDA-induced current was seen in the presence of magnesium ion and MK-801, as previously reported in other neurons of the central nervous system. These findings indicate the presence of both non-NMDA and NMDA receptors, which are involved in primary afferent transmission, in the MVN neuron, and two distinct types of NMDA receptors.

Keywords: Medial vestibular nucleus, Patch clamp, Acutely dissociated neuron, Non-NMDA receptor, NMDA receptor

The medial vestibular nucleus (MVN) is one of the main nuclei involved in the vestibulococular reflex. We have previously reported that L-glutamic acid diethyl ester hydrochloride (GDEE), a non-selective glutamate-receptor antagonist, blocks the afferent transmission in MVN neurons in vivo (1). In addition, MVN neurons have been found to show spontaneous firing, which is increased by glutamate in slice preparations (2, 3). These findings suggest that primary afferent transmission from nerves to MVN neurons is mediated by glutamate, since glutamate is reported to be present in the vestibular nerve (4).

The central nervous system contains two types of glutamate receptors, ionotropic and metabotropic receptors (5, 6). Ionotropic receptors, which contain integral cation-specific ion channels, are involved in excitatory synaptic transmission and formation of neural plasticity (7–9); they are subdivided into N-methyl-D-aspartate (NMDA) and non-NMDA receptors according to the permeability of cations and selectivity of agonists (5, 10). Recent molecular cloning studies of non-NMDA glutamate receptors has revealed the presence of three structurally subunit members (GluR1–4, GluR5–7 and KA1, 2)
These studies have revealed that $\alpha$-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainic acid (KA), which are non-NMDA-receptor agonists, can activate the same receptor channels (10, 13, 14, 18) and that various structurally and functionally distinct receptor channels can be assembled from the multiple KA/AMPA-receptor subunit gene.

Recent studies have also revealed molecular and functional diversity of the NMDA-receptor channel subunits, which were classified into the NMDAR1 (C1) and NMDAR2 (E) families according to the amino-acid sequence homology. At least five subunits of NMDA receptor (NMDAR1 and NMDAR2A-D for rats or C1 and E1-4 for mice) have been cloned (20-23). The NMDAR1 (C1) subunit is an essential component for the function of NMDA receptors, and multiple NMDAR2 (E) subunits are involved in the potentiation of the NMDA-receptor-mediated current and the differentiation of the characteristics of NMDA receptors by forming different heteromeric configurations with NMDAR1 (C1) (21-24).

In our preliminary experiments, application of glutamate to dissociated MVN neurons produced concentration-dependent depolarizations and an inward current during voltage clamp recording (1). However, the receptor subtypes responsible for these responses remain to be clarified. Therefore, a further patch clamp study was performed to clarify the subtypes of receptors involved in the glutamate-induced excitation of the MVN neurons using acutely dissociated cells.

**MATERIALS AND METHODS**

**Preparation of acute dissociated MVN neurons**

The MVN neurons were dissociated according to the methods of Kay and Wong (25) and Akaike et al. (26) after minor modification. Adult male Wistar rats (80–200 g) were decapitated and the brain was rapidly excised. The slices of brainstem, cut coronally 400 μm in thickness, were made with a microslicer (DTK 1000; Dosaka E.M., Kyoto) at 0–4°C. These slices were incubated for 1 hr at 32°C in artificial cerebrospinal fluid (ACSF) of the following composition: 117 mM NaCl, 4.5 mM KCl, 1.3 mM MgSO₄, 0.92 mM NaH₂PO₄, 2.5 mM CaCl₂, 26.2 mM NaHCO₃, and 11 mM glucose that was bubbled with 95% O₂ and 5% CO₂ at room temperature. The ACSF was then flushed with 100% O₂ and 5% CO₂, pH 7.4, which was bubbled with 95% O₂ and 5% CO₂ at the rate of 1.5 to 2 ml/min at 25–30°C. The internal solution of the recording electrode contained 80 mM CsCl, 80 mM CsCH₃SO₃, 10 mM HEPES, and 46 mM KOH, pH 7.2, and tetrodotoxin in the perfusion solution was eliminated to observe action potentials.

**Recording**

For whole cell recording, the electrode was sealed against the cells and the underlying membranes were broken by suction pulses, allowing the recording and/or clamping of the intracellular membrane potential. The patch microelectrode had a resistance of 2–4 MΩ, and the series resistance was 5–15 MΩ under whole cell recording. No series resistance compensation was used in this study. Current clamp and voltage clamp recordings were done using an EPC-7 patch clamp system (List Electronics, Darmstadt, Germany). The signals filtered at 10 kHz were monitored on a digital oscilloscope (VC-10; Nihon Kohden, Tokyo) and a thermal array recorder (RTA-1100, Nihon Kohden). They were simultaneously stored on an FM data recorder (A-65; Sony, Tokyo) for later analysis.

To obtain an agonist-induced current-voltage (I-V) relation curve, ramp I-V curves were constructed by application of a voltage command that went from −110 mV to 20 mV in 0.5 sec. Data obtained in the absence of agonists were subtracted from those acquired during the steady-state component of the agonist response. Digitized data points were averaged for each 1.3 mV, and the average values were used for plotting the I-V curve. Ramp voltage application and data sampling were done by the Mac Lab system (AD Instruments, Castlehill, Australia).
Drug application

Whole cell currents were induced by fast application of glutamate and other related agonists, using a U-shaped tube (27) placed in the vicinity of the cell being recorded at a distance of approximately 50 to 100 µm, according to the method described previously (28). When the time required for exchanging solution was measured by conductance changes through a recording pipette during a switch of the solution from physiological saline to distilled water, the exchange was completed within 20–50 msec.

Drugs

The drugs used were: glutamic acid (Wako Pure Chemical Ind., Osaka), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; Research Biochemicals Co., Natick, MA, USA), kainic acid (KA; Sigma, St. Louis, MO, USA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris, Bristol, UK), N-methyl-D-aspartate (NMDA, Sigma), glycine (Nacalai Tesque, Kyoto), and (+)-MK-801 hydrogen maleate (MK-801, Research Biochemicals Co.). The stock solutions of the drugs were first dissolved in distilled water and then diluted in ACSF to obtain the desired final concentrations, except for CNQX that was first diluted with dimethyl sulfoxide (DMSO). Further details of the experimental conditions were similar to those described elsewhere (1, 26, 28).

RESULTS

Figure 1 shows a dissociated MVN neuron observed under a polarization microscope. The diameter of the cell body varied from 20 to 50 µm. They were usually devoid of distal dendrites but retained the proximal dendrite. To avoid space clamp errors, a patch clamp study was performed on the cells without large proximal dendrites. The present whole cell recording confirmed the previous findings (1) that the dissociated MVN neurons showed relatively regular, spontaneous firing with an interspike interval of 25 to 100 msec at the highest membrane potential of -55 to -70 mV under current clamp conditions and that the application of glutamate at 100 µM to the MVN neuron depolarized the membrane concomitantly with an increase in the firing rate. The glutamate-induced depolarization and increase in firing rate were completely blocked by the simultaneous application of GDEE, a non-specific glutamate-receptor antagonist, at 10 µM in all 4 neurons examined (data not shown) as described previously (1). The following examinations were performed only on the cells with suitable shape for patch clamp study and spontaneous firing.

Effects of glutamate and CNQX on MVN neurons

During the voltage clamp recording, 100 µM glutamate induced a voltage-dependent inward current at the membrane potential of -40, -60 and -80 mV (Fig. 2A). The glutamate (100 µM)-induced current had a near linear I-V relation with a putative equilibrium potential of approximately 0 mV (Fig. 2B). At the membrane potential of -70 mV, the inward current induced by glutamate (100 µM) was completely blocked by CNQX, a non-NMDA-receptor antagonist, at 10 µM in all 4 neurons tested (Fig. 2C).

Effects of non-NMDA-receptor agonists on MVN neurons

Both AMPA and KA at 30–100 µM applied to the same neuron under current clamp conditions induced depolarization of membrane potential and an increase in firing rate in a concentration-dependent manner (Fig. 3: A and B). The mean depolarization by AMPA and KA at a concentration of 100 µM under the current clamp conditions was 20.8±5.1 (mean±S.E.M., n=4) and 26.7±7.6 mV (mean±S.E.M., n=3), respectively.

Under voltage clamp conditions, application of AMPA at a concentration of 100 µM produced a voltage-dependent current in all 7 neurons tested. A nearly linear I-V curve was obtained with 100 µM AMPA while varying the membrane potential from -110 to 20 mV using ramp waves (Fig. 4A). KA at 100 µM also induced a voltage-
dependent current with a near-linear I-V relationship in all 8 neurons examined, although the current was larger than that obtained with AMPA at 100 μM (Fig. 4: A–C). Putative equilibrium potentials of both AMPA and KA were approximately 0 mV (Fig. 4A).

When either AMPA or KA was applied to the MVN neuron at a concentration of 10 to 100 μM, a concentration-dependent current was obtained (Fig. 4C). At the membrane potential of −70 mV, AMPA at a concentration of 10 and 30 μM induced larger inward currents than KA did in all 4 neurons tested, while the current produced by KA at 100 μM was larger than that produced by AMPA at 100 μM in all neurons tested (Fig. 4: B and C). In all 10 neurons tested, KA at 10 μM did not produce the current, whereas AMPA at 10 μM did. Comparing the peak current induced by 100 μM of AMPA with that by 100 μM of KA in the same neuron at the membrane potential of −70 mV, KA-induced currents were 1.2 to 5 times larger than the AMPA-induced currents in 10 neurons tested (Fig. 4: A–C).

The fast desensitizing component of currents was not seen by application of glutamate or AMPA in MVN neurons (Fig. 2A and Fig. 4: B and C), while a small fast desensitizing component of AMPA (100 μM)-induced current was observed in CA1 hippocampal pyramidal neurons, which was obtained by the same method for dissociation and drug application as in the MVN neurons (Fig. 4B).

**Fig. 2.** Glutamate-induced current in dissociated medial vestibular nucleus neurons. A: Glutamate (100 μM)-induced current at the holding potential of −40, −60 and −80 mV. B: I-V relationship of glutamate (100 μM)-induced current constructed by application of a ramp voltage from −110 to 20 mV in 0.5 sec. The putative equilibrium potential was approximately 0 mV. C: Inward current induced by application of glutamate (100 μM) and antagonism by CNQX (10 μM) against glutamate (100 μM)-induced current.
Effects of glycine on glutamate-induced current and CNQX on glutamate plus glycine-induced current

To characterize NMDA receptors in MVN neurons, glycine at 3 μM was simultaneously applied with glutamate at 100 μM to open NMDA-receptor channels. As a result, two types (types A and B) of responses were observed in terms of the difference in the sensitivity to glycine.

In type A, 3 μM glycine augmented the glutamate-induced current even in the presence of magnesium ion at 1.3 mM. In the absence of magnesium ion, the glutamate-induced current was further potentiated by glycine (Fig. 5). These effects of glycine were observed in 7 of the 12 neurons tested. In the presence of 1.3 mM magnesium ion, the current induced by glycine (3 μM) plus glutamate (100 μM) was reduced to approximately 60–70% of the control value by CNQX (10 μM), which completely blocked the glutamate-induced current in the absence of glycine (Fig. 2C and Fig. 6: type A), suggesting that the remaining current was mediated by NMDA receptors with low sensitivity to magnesium ion.

The remaining 5 neurons showed the type B response: that is, 3 μM glycine did not significantly potentiate the glutamate (100 μM)-induced currents in the presence of magnesium ion at 1.3 mM. Significant enhancement of glutamate-induced current by glycine was not seen even in the absence of magnesium ion (Fig. 5). In the presence of 10 μM CNQX and absence of magnesium ion, the glycine plus glutamate-induced current almost disappeared, suggesting that this current was mediated mainly through non-NMDA receptors (Fig. 6: type B).

To exclude the possibility that glycine itself produced the current in MVN neurons, the effects of glycine alone on the MVN neurons were examined. Glycine at the concentration of more than 10 μM produced an inward current at a membrane potential of −60 mV. However, glycine at 3 μM did not induce a current in any of the 8 cells tested (data not shown).

The NMDA-induced current in MVN neurons

To further characterize the NMDA receptor in MVN neurons, whether or not NMDA applied to MVN neurons induces a current was examined. NMDA at 100–500 μM in the presence of glycine at 3 μM produced a current in 45 of 73 neurons. However, in the absence of glycine, NMDA at 100 μM could not produce a current in any of the 10 tested cells (data not shown).

MVN neurons were classified into type 1 and type 2

![Fig. 3. AMPA (A: 30 and 100 μM) and KA (B: 30 and 100 μM)-induced, concentration-dependent depolarization concomitantly with an increase in firing in the MVN neuron under current clamp conditions.](image-url)
neurons, according to the sensitivity difference to magnesium ions of the NMDA-mediated current. In type 1 neurons, NMDA (100 µM) induced a current regardless of the presence or absence of magnesium ions (1.3 mM) even under the membrane potential of -10 mV, although a larger current was noted in the absence than in the presence of magnesium ions (Fig. 7: A and B). Such responses to NMDA were found in 18 out of 34 neurons. The remaining 16 neurons were classified as type 2, in which NMDA (100 µM) did not induce a current in the presence of magnesium ions at a concentration lower than 0.5 mM (Fig. 7). There was no morphological difference

Fig. 4. AMPA- and KA-induced current in the MVN neuron under voltage clamp conditions. A: Ramp I-V relation curve of AMPA (100 µM)- and KA (100 µM)-induced currents. Putative equilibrium potentials of AMPA- and KA-induced current were both approximately 0 mV. B: AMPA (100 µM)- and KA (100 µM)-induced current (a and b) in MVN and hippocampal CA1 (c) neurons at a voltage clamp of -70 mV. C: Concentration-dependent increase in current induced by AMPA (A: 10, 30 and 100 µM) and KA (B: 10, 30 and 100 µM) at the membrane potential of -70 mV.
between type 1 and type 2 neurons. In both types, the putative equilibrium potential of the NMDA-induced current was approximately $-10\, \text{mV}$.

Effects of MK-801 on NMDA-induced current in MVN neurons

To clarify the difference between NMDA-induced current in type 1 and type 2 neurons, the effects of MK-801, a channel blocker of NMDA receptors, on the NMDA-induced current were examined. In all 4 type 1 neurons in which an NMDA-induced current was observed, MK-801 at 1 $\mu\text{M}$ did not block the NMDA current (Fig. 8). In addition, MK-801 even at 10 $\mu\text{M}$ did not block NMDA current (data not shown). In all type 2 neurons in which NMDA-induced current was obtained, MK-801 at 1 $\mu\text{M}$ inhibited the NMDA-induced current, and 10 $\mu\text{M}$ MK-801 completely blocked the current (Fig. 8).

Characteristics of non-NMDA receptor in MVN neurons

Glutamate alone, AMPA and KA, which activate non-NMDA receptors, induced a voltage-dependent current in our dissociated MVN neurons with an equilibrium potential of approximately 0 $\text{mV}$ with a linear I-V relationship. These currents induced by the non-NMDA-receptor agonists were similar to those obtained in other neurons of the central nervous system such as hippocampal, cerebral cortical, cerebellar cortical Purkinje and granule cells (29–37). In addition, the current induced by glutamate alone was completely blocked by CNQX, a non-NMDA-receptor antagonist. These findings indicate the presence of non-NMDA receptors in the MVN neurons.

Both AMPA and KA produced a current in MVN neurons, indicating that non-NMDA receptors expressed in MVN neurons have the subunits and/or its assembly which can be activated by both AMPA and KA. In addition, 10 $\mu\text{M}$ AMPA, but not 10 $\mu\text{M}$ KA, induced the current, suggesting that non-NMDA receptors expressed in MVN neuron have higher sensitivity to AMPA than KA. On the contrary, 100 $\mu\text{M}$ KA induced a larger current and depolarization than 100 $\mu\text{M}$ AMPA.

In many kinds of non-NMDA-receptor subunits, both AMPA-sensitive subunits including GluR1, 2, 3, 4 and KA-sensitive subunits including GluR5, 6 and 7 were reported to be present in MVN neurons by immunocytochemistry (38, 39). Our results almost corresponded to these previous immunocytochemistry findings.

In our study, glutamate, AMPA or KA-induced current had no rapidly desensitizing components. In contrast to the present results, fast application of glutamate, AMPA and quisqualate to neurons reportedly elicits currents that exhibit a fast rise-time and then quick decay to a plateau value in the continuous presence of the agonists (9, 40, 41). To clarify the reason why a rapidly desensitizing component of AMPA-induced current was not observed, we tried to observe AMPA-induced current in acutely dissociated hippocampal CA1 pyramidal neurons. Our previous reports indicated that non-NMDA-receptor agonists including glutamate, AMPA and quisqualate induced an apparent rapidly desensitizing component in cultured hippocampal CA1 pyramidal neurons using a U-tube method (28). However, we could only obtain a tiny desensitizing component of AMPA-induced current in these cells (Fig. 4B). This result suggested that a slow switching of the solutions may mask the rapidly desensitizing phase or the enzymatic treatment of MVN neurons may alter the property of non-NMDA-receptor channels, although the possibility that the non-NMDA-receptor-mediated current without a rapid desensitizing component was a specific characteristic of non-NMDA-

![Fig. 5. Glycine (3 $\mu\text{M}$) plus glutamate (100 $\mu\text{M}$)-induced current in MVN neurons at $-70\, \text{mV}$. In type A neurons, glycine potentiated the glutamate-induced current even in the presence of magnesium ions at a physiological concentration of 1.3 mM. Further potentiation was observed in the absence of magnesium ions. In type B neurons, glycine did not augment the glutamate-induced current in both presence and absence of magnesium ions.](image-url)
receptors in MVN neurons can not be completely excluded.

**Characteristics of NMDA receptors in MVN neurons**

In our study, two types of NMDA-induced current were observed according to the sensitivity difference to magnesium ion and to MK-801. In type 1 neurons, 1.3 mM magnesium ion did not sufficiently block the NMDA-induced current, although a larger current was obtained in the absence of magnesium ions. On the contrary, the NMDA-induced current in type 2 neurons were markedly blocked by magnesium ion even at concentrations lower than 1 mM. In addition, MK-801 did not block NMDA-induced current in type 1 neurons, whereas MK-801 blocked NMDA-induced current in type 2 neurons. These findings suggested the presence of two distinct NMDA receptors that have different properties with respect to magnesium ion and MK-801 in MVN neurons. In this regard, type A responses as shown in Figs. 5 and 6 may be mediated via type 1 NMDA receptor, but not type 2 NMDA receptor.

The blockade by magnesium ions and MK-801 of the NMDA-induced current is the common response in other central nervous systems as described previously (42, 43). Therefore, the responses of type 1 neurons to magnesium ions and MK-801 are unique. Recent molecular studies have shown that the NMDAR2 (α) subunits assembled with NMDAR1 (ζ) subunit differentiated the sensitivity of the NMDA channels to magnesium ions and MK-801 (21, 22, 24). These studies have revealed that the heteromeric NMDAR1/NMDAR2A receptors have high sensitivity to magnesium ions and MK-801, whereas the heteromeric NMDAR1/NMDAR2C receptors have low sensitivity to them (21, 22, 24). Therefore, the NMDA receptors observed in the present study in type 1 MVN neurons that have low sensitivity to magnesium ions and MK-801 are suggested to consist of heteromeric NMDAR1/NMDAR2C receptors, since both NMDAR1 and NMDAR2C subunits have been reported to be expressed in MVN neurons as determined by immunocytochemistry and in situ hybridization (24, 44–46). In contrast, type 2 MVN neurons may have NMDA receptors that mainly consist of NMDAR1/NMDAR2A receptors, since NMDAR2A are also expressed in the MVN neurons (46).

However, the possibility that NMDA receptors with low sensitivity to magnesium ions and MK-801 may be assembled with a new type of NMDA receptor subunits in
Fig. 7. NMDA-induced current in types 1 and 2 MVN neurons in the presence and absence of magnesium ions. All experiments were performed in the presence of 3 μM glycine. A: I-V relationship of two types of NMDA-induced current constructed by the ramp method in MVN neurons in the presence and absence of magnesium ions. B: Examples of NMDA-induced current in the presence and absence of magnesium ions in type 1 and type 2 neurons. In type 1 neurons, the physiological concentration of magnesium ions at 1.3 mM did not sufficiently block the NMDA-induced current under the membrane potential of -10 mV. In contrast, in the type 2 neurons, the NMDA-induced current was concentration-dependently blocked by magnesium ions under the membrane potential of -10 mV. The putative equilibrium potentials were approximately -10 mV in both type 1 and type 2 neurons.
MVN neurons can not be completely excluded at present. Site-directed mutagenesis studies of NMDA receptor subunits have indicated that the conserved asparagine residue in segment M2, which was considered to be the portion of channel pores, constitutes both the blocking sites for magnesium ions and MK-801 (47). Thus, type 1 neurons may have a new type of NMDA-receptor subunit whose M2-segment amino-acid sequence is different from those found previously, although the possibility that the properties of NMDA receptors were altered by enzymatic treatment could not be completely excluded.

In conclusion, both non-NMDA and NMDA receptors are present in MVN neurons involved in the primary afferent transmission from the vestibular nerve to MVN neurons. In addition, there are two distinct NMDA receptors in the MVN neurons, one having low sensitivity to magnesium ions and MK-801 and the other having high sensitivity to them.

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