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N-Acetylaspartylglutamate acts as an agonist upon homomeric NMDA receptor (NMDAR1) expressed in *Xenopus* oocytes

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The electrophysiological effects of *N*-acetylaspartylglutamate (NAAG), an endogenous peptide restrictively distributed in the central nervous system, were studied using *Xenopus* oocytes injected with RNAs transcribed from cloned glutamate receptor cDNAs. NAAG induced an inward current, dose dependently, in oocytes injected with RNA for an *N*-methyl-D-aspartate receptor subunit (NMDAR1). In contrast, the oocytes injected with RNAs for AMPA-selective glutamate receptors (GluR1, GluR3, GluR1+GluR2 and GluR2+GluR3) scarcely responded to NAAG, and the oocytes injected with RNA for kainate receptor (GluR6) did not respond to NAAG. The half-maximal response (ED_{50}) value of NAAG on expressed NMDAR1 was 185 μM, which shows that NAAG is about 115-times less potent than L-glutamate (Glu), the ED_{50} of which value was 1.6 μM. The maximal current amplitude induced by NAAG was about 70% of that by Glu. NAAG-induced current in NMDAR1-injected oocytes was potentiated by glycine, dose-dependently antagonized by DL-2-amino-5-phosphonovaleric acid, and blocked by magnesium ions in a voltage-dependent fashion. These results suggest that NAAG is one of the endogenous agonists selective for NMDAR1.

N-Acetylaspartylglutamate; NMDA receptor; AMPA-selective glutamate receptor; Kainate receptor; *Xenopus* oocyte; Agonist

1. INTRODUCTION

N-Acetylaspartylglutamate (NAAG) is an endogenous peptide restrictively distributed in the central nervous system (CNS) [1–3]. In CNS, the content of NAAG is quite high, and almost similar to that of γ-aminobutyric acid (GABA) [4]. NAAG has been reported to evoke electrophysiological excitatory effects in the neurons of several brain regions [5–7], in cultured neuronal cells [8,9] and in *Xenopus* oocytes injected with cerebellar mRNA [10]. Since the excitatory effect of NAAG was suppressed by antagonists of glutamate receptors [6–9], it was suspected that NAAG was one of the endogenous agonists of central glutamate receptors. Three kinds of glutamate receptors have been suggested to mediate the excitatory effect of NAAG upon neurons; (i) *N*-methyl-D-aspartate (NMDA) receptors [7–9], (ii) quisqualate receptors (or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-selective glutamate receptors) [6] and (iii) 2-amino-4-phosphonobutyric acid-selective receptors [5].

Recently, cDNAs encoding three subtypes of glutamate receptors, AMPA-selective glutamate receptors (GluR1-4) [11–15], kainate receptors (GluR5-7) [16–18] and NMDA receptors (NMDAR1 and NMDAR2A-2C, or ζ1 and ε1–ε3) [19–23] were cloned. These clones were reported to form functional ligand-gated ion channels when expressed in *Xenopus* oocytes [11,12,14,15,17,19–21,23]. These findings have allowed us to study the effect of NAAG on each defined glutamate receptor subtype.

In this study, we expressed AMPA-selective glutamate receptors (GluR1-3), kainate receptor (GluR6) or NMDA receptor (NMDAR1) in *Xenopus* oocytes by injecting RNAs transcribed from their cDNAs, and studied the selectivity of NAAG for each receptor subtype.

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2. MATERIALS AND METHODS

2.1. *Molecular biological experiments*

The plasmid which carries cDNA for NMDAR1 was kindly provided by Dr. S. Nakanishi, Kyoto University, Kyoto, Japan. The plasmids which carry cDNAs for GluR1-3 and GluR6 were a gift from Dr. S. Heinemann, Salk Institute, San Diego, USA. Each plasmid was purified by CsCl–ethidium bromide ultracentrifugation, and the purified plasmid was linearized by an adequate restriction enzyme (New England Biochemicals), the recognition site of which was just downstream of the cDNA insert (*Not*I for NMDAR1, *Xho*I for GluR1-3 and *Xba*I for GluR6). The linearized plasmid was treated with proteinase K for 30 min at 37°C. In vitro transcription was carried out by T7 or T3 RNA polymerase (Stratagene) for NMDAR1 or for GluR1-3 and GluR6, respectively, in the presence of a cap analog, 5′,7-methyl Gppp 5′G (Stratagene), for 30 min at 37°C. The DNA template was digested by DNAse I for 5 min at 37°C and the reaction mixture was washed twice with a mixture of phenol and chloroform (1:1). RNA transcripts were precipitated by ethanol and the precipitate was dissolved in distilled water.

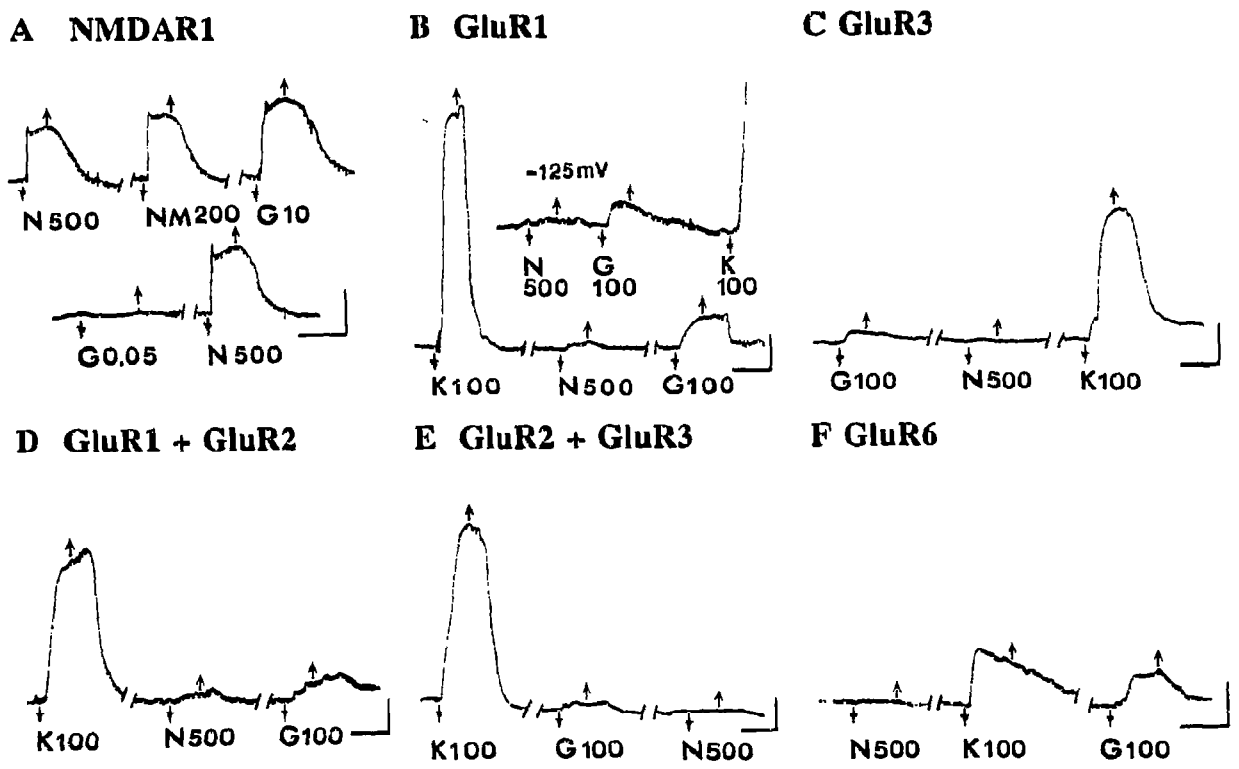

Fig. 1. Electrophysiological effect of NAAG (N), Glu (G), NMDA (NM) and Kainate (K) upon oocytes injected with RNA transcribed from cDNA encoding NMDAR1 (A), AMPA-selective glutamate receptors (B–E) or kainate receptor (F). In this and subsequent figures, the downward and upward arrows indicate the initiation and termination of the application of reagents, respectively, the denoted numbers are concentration (in μM) of reagents, and the upward deflection indicates inward current. Glycine (10 μM) was added to a solution of NAAG, Glu and NMDA only in the case of A. All records except the insert in B, were obtained at a holding potential of −70 mV; the record in the insert in B was obtained at −125 mV from a different oocyte in B. Vertical bar 25 nA for A and F, 10 nA for B and 20 nA for C–E; horizontal bar 30 s for all.

2.2. *Electrophysiological methods*

Oocytes were removed from *Xenopus laevis* (Nasco) and treated with collagenase (3 mg/ml, Sigma Type 1a) with gentle agitation for 2 h at room temperature in a Ca^{2+}-free frog Ringers' solution composed of 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES (pH 7.4), and 0.1 mM EGTA. After 2 h, oocytes were transferred to modified Barth's medium (MBM) to wash out collagenase. The composition of MBM was 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM HEPES, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 μg/ml sodium penicillin and 10 μg/ml streptomycin sulfate. Immediately after this treatment, an oocyte was impaled with a glass micropipette filled with the RNA transcript solution and 50 nl each of the solution (400 ng of RNA per μl) was injected. When two different RNA transcripts were injected, the transcripts were mixed prior to injection so that the final concentration of each RNA was 400 ng/μl. The oocytes were incubated for 3 days at 15–20°C in MBM.

Three days after injection, an oocyte was transferred to the center of a round chamber, of diameter 7 mm and depth 3 mm (115 μl in volume), and was continuously perfused at a flow rate of 0.5 ml/min with frog Ringers' solution (115 mM NaCl, 10 mM HEPES, 2 mM KCl and 1.8 mM $CaCl_2$, pH 7.2). Voltage-clamp recordings were carried out using AXOCLAMP-2A (Axon Instruments) by impaling two microelectrodes filled with 3 M KCl, one for voltage monitoring (input resistance was about 10 MΩ) and the other for voltage clamping (about 1 MΩ).

The Hill coefficient was obtained as the slope of the regression line determined from the double-logarithm plots (log[concentration] vs. log{response/(maximal response−response)}).

All drugs were dissolved in frog Ringers' solution and applied by perfusion. When the effect of agonists on NMDAR1-RNA-injected oocytes was investigated, glycine (10 μM) was added to the solutions of NAAG, Glu and NMDA unless otherwise specified. NAAG was dissolved into 50 ml frog Ringers' solution at a concentration of 10 mM and the pH of the solution was adjusted to 7.2 by several drops of 10 M NaOH. This concentrated solution was stored at 4°C and diluted with frog Ringers' solution for use. Furthermore this solution was subjected to amino acid analysis (Peptide Technologies) to determine the concentration of free amino acid contaminants.

NAAG, kainate and Glu were purchased from Sigma. DL-2-Amino-5-phosphonovaleric acid (APV) and NMDA were purchased from Cambridge Research Biochemicals.

3. RESULTS

3.1. *Analysis of the NAAG sample*

No peak of free Glu and aspartate was detected in the 10 mM NAAG sample used in the electrophysiological study. The detection limit of the amino acid analyzer used was 5–10 pmol, and 20 μl of 10 mM NAAG solution was assayed. Therefore, the maximum concentration of Glu contaminating the 10 mM NAAG sample is 500 nM and the maximum Glu concentration in the

500 μM NAAG solution used for electrophysiology is 25 nM.

3.2. *Electrophysiology*

At a holding potential of −70 mV, an inward current was elicited by NAAG in all the oocytes injected with the RNA transcript for NMDAR1 (Fig. 1A). As shown in Fig. 1A, the current response by NAAG resembled that of Glu or NMDA. Little or no current was observed after the application of NAAG (up to 1 mM) in the uninjected control oocytes (data not shown). 500 μM NAAG induced inward currents in the oocytes, while 50 nM Glu, which was 2-times higher than the maximum concentration expected to contaminate the 500 μM NAAG solution, induced no inward current in the same oocytes (Fig. 1A).

Because the lowest concentration of NAAG, Glu and NMDA inducing maximal current amplitude in NMDAR1-RNA-injected oocytes was determined to be about 500 μM, 10 μM (Fig. 2) and 200 μM (unpublished observation), respectively, the potency of the three agonists to open the channel formed by NMDAR1 receptors was compared by using each agonist at this concentration. As shown in Fig. 1A and summarized in Table I, the potency of NAAG was weaker than that of Glu and NMDA. The mean current amplitude induced by 500 μM NAAG, 10 μM Glu and 200 μM NMDA in the presence of 10 μM glycine at the holding potential of −70 mV was 30 ± 5, 40 ± 8 and 35 ± 8 nA, respectively, in NMDAR1-RNA-injected oocytes, as shown in Table I.

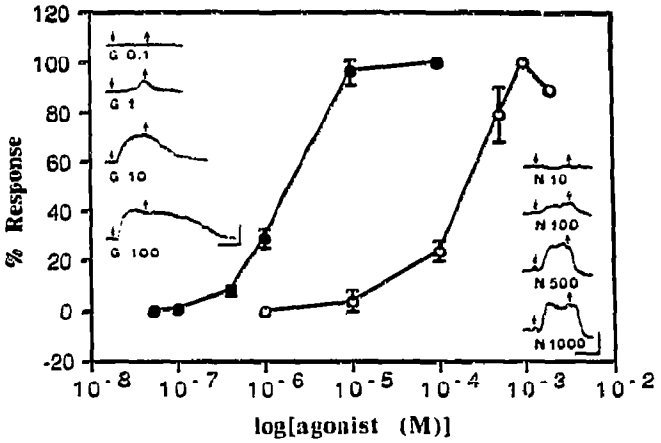

Fig. 2. Dose-response curves for NAAG (open circles) and Glu (closed circles) in oocytes injected with RNA for NMDAR1. NAAG and Glu (10 μM glycine was added) were applied at the various concentrations as illustrated in the inserts (N, NAAG; G, Glu; vertical bar 25 nA; horizontal bar 20 s), and the amplitude of each current was normalized as the fraction of maximal current (the current obtained at 1 mM for NAAG (30 ± 4 nA) and at 100 μM for Glu (35 ± 5 nA)). Each point represents the mean ± S.E.M of 4–6 experiments. At the points where the error bar is not indicated, S.E.M. is smaller than the symbol. For other symbols see Fig. 1.

In contrast, as shown in Fig. 1B–F, NAAG (500 μM) scarcely induced an electrophysiological response in the oocytes injected with RNA for AMPA-selective glutamate receptors (Fig. 1B–E) or for kainate receptors (Fig. 1F), at the holding potential of −70 mV, while Glu

Table I

Amplitude of the current induced by NAAG, Glu, NMDA and kainate in oocytes injected with RNA for various glutamate receptors

RNA injected	Agonist-evoked current (nA)			
	Kainate	Glu	NMDA	NAAG
NMDAR1	–	40 ± 8 (100)	35 ± 8 (84)	30 ± 5 (78)
	–	(6/6)	(6/6)	(6/6)
GluR1	41 ± 7 (421)*	10 ± 4 (100)	–	1 ± 1 (16)
	(6/6)**	(6/6)	–	(2/6)
GluR3	66 ± 9 (1,434)	5 ± 1 (100)	–	0 ± 0 (0)
	(5/5)	(5/5)	–	(0/5)
GluR1 + GluR2	103 ± 11 (1,306)	10 ± 3 (100)	–	2 ± 1 (22)
	(4/4)	(4/4)	–	(3/4)
GluR2 + GluR3	140 ± 21 (1,455)	10 ± 2 (100)	–	2 ± 2 (18)
	(4/4)	(4/4)	–	(2/4)
GluR6	34 ± 7 (198)	17 ± 3 (100)	–	0 ± 0 (0)
	(3/3)	(3/3)	–	(0/3)

Inward currents induced by kainate (100 μM), glutamate (10 μM for NMDAR1 and 100 μM for others), NMDA (200 μM) and NAAG (500 μM) were recorded at −70 mV in the oocytes injected with the RNAs for various glutamate receptors. Glycine (10 μM) was added to the agonists solution only in the case of NMDAR1. Agonist-evoked current is represented as the mean ± S.E.M. in nA.

*Relative responses, representing the relative value of each response when the Glu response is standardized as 100.

**Number of oocytes responding/number of oocytes tested

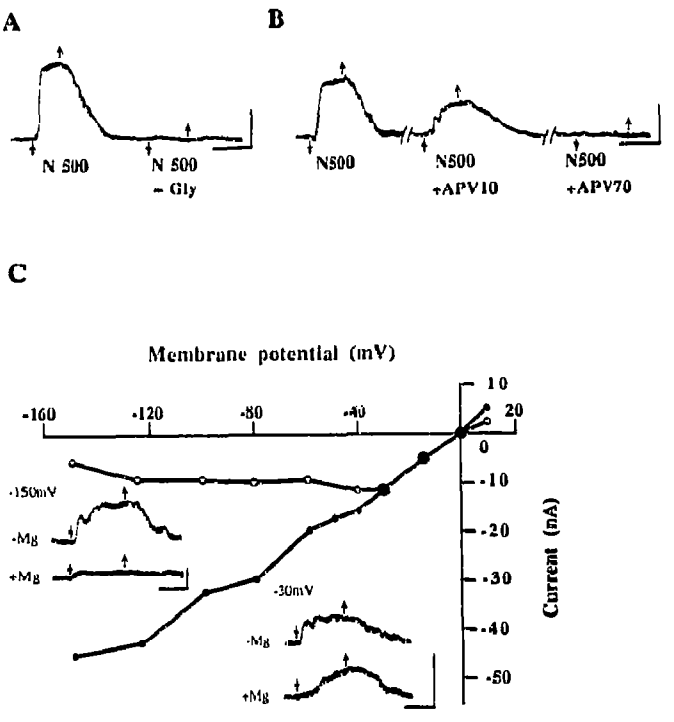

Fig. 3. Properties of NAAG-induced current in the oocytes injected with RNA for NMDAR1. (A) Effect of removing of glycine on NAAG (N, 500 μM)-induced current. (B) Effect of APV (10 and 70 μM) on NAAG (500 μM)-induced current. Vertical bar 15 nA; horizontal bar 20 s. (C) Current–voltage relationship of NAAG-induced current in the absence (closed circles) and presence (open circles) of 100 μM $MgCl_2$. The inserts were sample records at −150 and −30 mV. Vertical bar 20 nA; horizontal bar 30 s.

(100 μM) or kainate (100 μM) induced an inward current in these oocytes. In homomeric AMPA-selective glutamate receptors, only 2 out of 6 oocytes injected with RNA for GluR1 responded to NAAG with a current smaller than 4 nA (Fig. 1B and Table I), and no detectable current was observed in the remaining 4 oocytes even at a membrane potential of −125 mV, as shown in the Fig. 1B insert. Furthermore, in the case of the oocytes injected with RNA for GluR3, no detectable current was induced by NAAG in all 5 oocytes tested (Fig. 1C and Table I). In heteromeric AMPA-selective glutamate receptors, 3 out of 4 oocytes or 2 out of 4 oocytes injected with a RNA mixture of GluR1 and GluR2 (Fig. 1D and Table I) or Glu R2 and GluR3 (Fig. 1E and Table I), respectively, responded to NAAG with a current smaller than 5 nA, and the other oocytes did not respond to NAAG. On the other hand, for the kainate receptor (GluR6, Fig. 1F and Table I), no detectable current was observed after the application of NAAG. The mean amplitudes, including those of non-responsive oocytes, and relative responses at the holding potential of −70 mV are summarized in Table I.

Fig. 2 shows the dose–response curve of NAAG (open circles) and Glu (closed circles) for NMDAR1-injected oocytes and the inserts are the sample records (G, Glu; N, NAAG; μM) As illustrated in Fig. 2, the inward current induced by NAAG and Glu in NMDAR1-injected oocytes was dose dependent, and the apparent half-maximal response (ED_{50}) value was 185 and 1.6 μM for NAAG and Glu, respectively. The ED_{50} value for Glu is slightly higher than the reported value [19]. The Hill coefficient calculated from these data is 1.7 for Glu and 1.0 for NAAG.

Fig. 3 shows the properties of an NAAG-induced current in the oocytes injected with RNA for NMDAR1. The NAAG-induced current was reduced by removing glycine (Fig. 3A, −Gly) and was antagonized, dose dependently, by APV (Fig. 3B). Fig. 3C is the current– voltage relationship of the NAAG-induced current in the absence (closed circles) and the presence (open circles) of 100 μM Mg^{2+}, and the inserts are the sample records. As shown in Fig. 3C, the NAAG-induced current was blocked by Mg^{2+} in a voltage-dependent manner.

4. DISCUSSION

We demonstrate in the present study that *Xenopus* oocytes injected with RNA for NMDAR1 respond to NAAG electrophysiologically, while the response to NAAG of oocytes injected with RNAs for AMPA-selective glutamate receptors or kainate receptors was very low or absent, respectively.

Because a 2-times higher concentration of Glu than that of the maximal contamination of Glu in the NAAG sample used induced no response in NMDAR1–RNA-injected oocytes, it is unlikely that the NAAG-induced current is due to Glu present in the NAAG sample. It is possible that the NAAG which is applied to oocytes might be quickly hydrolyzed by peptidases on the surface of oocytes and that the Glu produced through this procedure might activate NMDAR1, but this possibility appears unlikely because NAAG induced an inward current at a latency as short as that induced by Glu (Fig. 1A). The fact that NAAG and Glu have different Hill coefficients also provides evidence that the effect of NAAG was due to NAAG itself.

NAAG has been reported to activate NMDA receptors in neurons [7–9]. Results of the present study provide further confirmation of the previous reports. The present study also indicates that NAAG induces the typical response elicited by NMDA receptors: potentiation by glycine [24,25], suppression by APV [26] and voltage-dependent blockade by Mg^{2+} [27,28]. Furthermore, it was found that NAAG scarcely activated AMPA-selective glutamate receptors and did not activate kainate receptors. These basic properties of NAAG upon glutamate receptors will be helpful to address the physiological role of NAAG in the CNS.

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