Inhibitory Action of Nebracetam on Various Stimuli-Evoked Increases in Intracellular Ca$^{2+}$ Concentrations in Cultured Rat Cerebellar Granule Cells

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ABSTRACT—Nebracetam (10 - 100 $\mu$M) dose-dependently inhibited increases in intracellular Ca$^{2+}$ concentrations evoked by various stimuli in cultured rat cerebellar granule cells. The magnitude of the nebracetam (100 $\mu$M)-induced inhibition of L-glutamate- and N-methyl-D-aspartate-evoked Ca$^{2+}$ responses was 1.5-fold and 1.7-fold greater, respectively, than the inhibition of the high K$^+$-evoked response. These findings suggest that in cultured cerebellar granule cells, nebracetam attenuates the external Ca$^{2+}$ influx derived from the activation of N-methyl-D-aspartate receptor-gated rather than voltage-gated Ca$^{2+}$ channels.

Keywords: Nebracetam, Intracellular Ca$^{2+}$ concentration, Cerebellar granule cell (cultured)

Nebracetam has been proposed to have neuroprotective action and cognitive enhancing effects (1-3). The compound protected against hypoglycemia/hypoxia-induced striatal damage (1) and improved the disruption of spatial cognition and memory impairment induced by various treatments (2, 3). Excessive Ca$^{2+}$ entry, an etiological event in neuronal injury, occurs largely through Ca$^{2+}$ channels gated by the N-methyl-D-aspartate (NMDA) receptor, a subtype of glutamate receptor, but also through voltage-gated Ca$^{2+}$ channels (VGCC) (4). We have provided an in vitro model of ischemic brain damage to facilitate the distinction between L-glutamate/NMDA receptor-mediated and VGCC-mediated neuronal dysfunction (5, 6), and we have found that nebracetam significantly protected against striatal dopaminergic impairment induced by L-glutamate and NMDA, but that it did not protect against such impairment induced by BAY K 8644, an L-type VGCC agonist (6). This evidence suggested that the neuroprotective action of nebracetam was due to its inhibition of inappropriate Ca$^{2+}$ influx through NMDA receptor-gated Ca$^{2+}$ channels. Cultured rat cerebellar granule cells have been widely accepted to be a useful brain neuronal model for neuroscience research. The cerebellum contains a relatively small number of cell types organized in a highly stereotyped, geometric pattern. The majority of these cell types develop postnatally, making them easily accessible. They are morphologically distinct in size and shape (7). Thus, we examined the effect of nebracetam on high K$^+$-, L-glutamate- and NMDA-evoked increases in intracellular Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_i$) in cultured rat cerebellar granule cells.

Cells were prepared from the cerebella of 8-day-old Wistar rats according to the method of Gallo et al. (8), with a minor modification, as described elsewhere (9). Unless otherwise specified, the reagents used in culture and in the measurement of [Ca$^{2+}$]$_i$ were obtained from Gibco BRL (Grand Island, NY, USA) and Wako Pure Chemical Industries (Osaka), respectively. In brief, the cerebella were minced and incubated with 0.0250$\%$ trypsin in phosphate-buffered saline, followed by trituration in a DNAase solution (0.0008$\%$) with a trypsin inhibitor (0.004$\%$). The final cell suspension was plated, at a density of 2.5 x 10$^6$ cells/2 ml/dish, in a culture dish ($\phi$ 25 mm) equipped with a poly-L-lysine-coated coverslip glass ($\phi$ 25 mm). Cultures were maintained in Modified Basal Eagle Medium (Kyokuto Pharmaceutical Industries, Tokyo) supplemented with 10% fetal calf serum (Bocknek, Toronto, Canada), 25 mM KCl, 4 mM glutamine and 0.2% gentamycin under a humidified atmosphere of 95% air/5% CO$_2$ at 37°C for 3-7 days before use. To prevent the proliferation of non-neuronal cells, the culture was treated with cytosine arabinoside (10 $\mu$M) for 16 hr on the
1st day.

The granule cells cultured on coverslips were incubated for 45 min at 37°C with 5 μM fura-2 acetoxymethylester (Dojin, Kumamoto) in Krebs-Ringer solution of the following composition: 136 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl₂, 10 mM glucose and 20 mM HEPES, at pH 7.4 with NaOH. Fura-2-loaded cells were then placed in a chamber mounted on the fluorometer (CAF-100; Jasco, Tokyo) and perfused with Krebs-Ringer solution, at a flow rate of 1 ml/min, at 37°C. [Ca²⁺]ᵢ was quantitatively measured by a dual-beam (340 and 380 nm) excitation method (10), with a computerized monitoring system (CAF-200 software, Jasco), as described previously (11). The absolute Ca²⁺ concentration was estimated from the ratio of emitted fluorescence (F340/F380) according to a calibration curve obtained by using a calcium calibration buffer kit with 1 mM Mg²⁺ (C-3721; Molecular Probes, Eugene, OR, USA).

Measurement of [Ca²⁺]ᵢ was begun after the cells had been washed for 5 min. Cultured cells were stimulated

![Fig. 1. Traces showing the typical response to various stimuli with a dual-beam excitation method (top, 380 nm; middle, 340 nm in each panel) in fura-2-loaded cultured rat cerebellar granule cells. The absolute Ca²⁺ concentration was estimated from the ratio of emitted fluorescence (F340/F380) (bottom in each panel) according to a calibration curve obtained by using a calcium calibration buffer kit. Cells were stimulated with 100 μM NMDA (A), 25 μM L-glutamate (B) and 25 mM KCl (C) for 1 min in the absence and presence of nebracetam.](image-url)
with 25 mM KCl, 25 μM L-glutamate HCl (Sigma, St. Louis, MO, USA) and 100 μM NMDA (Sigma) for 1 min at 10-min intervals. The application of nebracetam (Nippon Boehringer Ingelheim, Hyogo) was initiated 5 min after the end of the first stimulation (S1)-evoked response and terminated at the end of the second stimulation (S2)-evoked response, that is, S2 was given after 5-min pre-incubation with nebracetam. L-Glutamate and NMDA were dissolved in Mg2+-depleted Krebs-Ringer solution to avoid the blockade of NMDA receptor-gated Ca2+ channels by Mg2+ (12). Granule cells cultured for 3–5 days were used for the experiment with high K+, while, for the experiment with L-glutamate and NMDA, the cells were cultured for 7 days, because of the delayed expression of NMDA receptors (13). When extracellular Ca2+ was depleted, 2 mM CaCl2 in Krebs-Ringer solution was replaced with 1 mM ethyleneglycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid. The effects of nebracetam on the elevated [Ca2+]i, evoked by various stimulators were expressed as a percentage of the S1-evoked response, an effect compared with the control experiment carried out in the absence of nebracetam. Values are represented as means±S.D. Statistical analyses were carried out by one-way analysis of variance followed by Dunnett’s test for multiple comparisons. Differences were regarded as statistically significant at P < 0.05.

Figure 1 shows typical records of the various stimuli-elevated [Ca2+]i in the absence and presence of nebracetam in cultured rat cerebellar granule cells. Stimulation with high K+ (25 mM), L-glutamate (25 μM) and NMDA (100 μM) produced almost the same degree of increase (about 300 nM) in [Ca2+]i over the resting level (100±8.6 nM, n = 5) (Fig. 1). In the control experiment, without the application of nebracetam, an S2-elevated [Ca2+]i equivalent to the S1-evoked response was obtained with each stimulator (Fig. 2). The resting level of [Ca2+]i, and the fluorescence of fura-2 were not altered by nebracetam at any of the examined concentrations. Nebracetam, 10 to 100 μM, produced a concentration-dependent inhibition of the increased [Ca2+]i evoked by the various stimuli in the cultured rat cerebellar granule cells (Fig. 2). Nebracetam, at 10, 50 and 100 μM, inhibited the NMDA-evoked increase in [Ca2+]i by 15.8±5.9%, 33.0±6.4% and 49.0±8.9%, respectively. This inhibitory action of nebracetam was equipotent to that seen with the L-glutamate-evoked Ca2+ response (10 μM: 23.3±7.6%, 100 μM: 55.7±1.2%) (Fig. 2). The magnitude of inhibition induced by nebracetam (100 μM) in the L-glutamate- and NMDA-elevated [Ca2+]i, was 1.5-fold and 1.7-fold greater, respectively, than that of the inhibition of the high K+ -evoked response (33.5±1.3%) (P < 0.01, Fig. 2). This differential effect of nebracetam on the increased [Ca2+]i, evoked by various stimuli was also observed in a separate batch of cells. The effective dose of nebracetam in the present study was in good accord with that producing a neuroprotective action (1, 6). When Ca2+-depletion instead of the application of nebracetam was used in the above-mentioned testing procedure, the increase in [Ca2+]i evoked by the various stimuli was below the limits of detection of our present system. These findings suggest that high K+ and L-glutamate/NMDA stimulate VGCC (14) and NMDA-gated Ca2+ channels (12), respectively, in granule cells to deliver external Ca2+ into cells, resulting in an elevation in [Ca2+]i.

The present observations together with the preferential inhibitory action of nebracetam on the L-glutamate- and NMDA-evoked Ca2+ response, indicate that the interaction of nebracetam with NMDA receptor-gated Ca2+ channels rather than with VGCC is likely to be the predominant factor in attenuating external Ca2+ entry. The preferential neuroprotective action of nebracetam on glutamate- and NMDA-evoked striatal dopaminergic impairment has already been described (6), and this finding was supported by the evidence that nebracetam protected against the impairment of 2-deoxyglucose uptake induced by L-glutamate in cortical slices (15). Thus, the present results underline the importance of NMDA receptor-gated Ca2+ channels in mediating the neuroprotective action of nebracetam. The contribution of VGCC to this event, however, must be taken into account. L-Glutamate and NMDA seem to easily depolarize the membrane, since Mg2+ was depleted from the medium during the exposure of these reagents (11). In addition, nebracetam
significantly inhibited the high K⁺-evoked increase in 
[Ca²⁺], although this inhibition was significantly smaller
than that seen with the NMDA-evoked elevated Ca²⁺
response.

In light of these findings, the possibility that nebracet-
am inhibits inappropriate Ca²⁺ influx into neurons due to
pathological events such as hypoxia/ischemia by interact-
ing predominantly with NMDA receptor-gated Ca²⁺ chan-
nels, and to some extent with VGCC, would have to be
considered. These findings, we believe, will aid in the un-
derstanding of the mechanisms whereby nebracetam ex-
erts its neuroprotective action.

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