Comparative Studies on the Inhibitory Effects of Calcium Antagonists on Cytosolic Ca\textsuperscript{2+} Levels Increased by High-Potassium or Glutamate in Cultured Rat Cerebellar Granule Cells

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ABSTRACT—The inhibitory effects of calcium antagonists on high-potassium or glutamate (Glu) enhanced intracellular calcium ion ([Ca\textsuperscript{2+}]) levels were studied in cultured cerebellar granule cells. Dosages between 0.5 and 10 \textmu M of flunarizine, nicardipine, SM6586 and SM12565 reduced the rise in [Ca\textsuperscript{2+}] induced by 50 mM KCl in a dose-dependent manner, although diltiazem, verapamil and nifedipine showed less effects on such [Ca\textsuperscript{2+}] increase. SM6586, SM12565 and flunarizine at dosages below 10 \textmu M each reduced the magnitude of the [Ca\textsuperscript{2+}] increase induced by 25 \textmu M Glu, but the other examined calcium antagonists were less effective. These results suggest the dissimilar efficacy of calcium antagonists on the inhibition of [Ca\textsuperscript{2+}] levels increased by high-potassium and Glu.

Keywords: Calcium antagonist, Receptor operated Ca\textsuperscript{2+} channel, Cerebellar granule cell (cultured)

Some calcium antagonists have been reported to prevent the neuronal damage of ischemia in vivo (1, 2). The prevention of neuronal dysfunction by these antagonists were explained as follows: A) they could increase the cerebral blood flow through a direct action on the cerebral vessels and B) they could reduce the entry of Ca\textsuperscript{2+} into the cell by blocking the voltage-sensitive calcium channels (VOC). Furthermore, studies on the mechanism of neuronal death suggest that ischemia/hypoxia-induced neuronal death is due to the release of glutamate (Glu) from brain tissue during hypoxia, and the excessive extracellular accumulation of Glu results in a Ca\textsuperscript{2+} overload which leads to neuronal degeneration (3–5). For these reasons, the direct effects of calcium antagonists which block the VOC and/or receptor operated calcium channel (ROC) may be beneficial and expected to improve the neuronal dysfunction either by blocking [Ca\textsuperscript{2+}] increase or modulating Glu release (6).

In this experiment, we compared the direct inhibitory effects of flunarizine, nicardipine, nifedipine, SM12565, SM6586, diltiazem and verapamil on both the high-potassium and Glu induced increase in [Ca\textsuperscript{2+}] levels in cultured rat cerebellar granule cells to determine their effects and potential on VOC and ROC in central neurons.

Cultured cells were prepared by our previously described method (7), which was slightly modified from the method of Gallo et al. (8). Briefly, 10 cerebella from 8-day-old Wistar rats were dissected and chopped into 400-\textmu m blocks. Cells were isolated by mild trypsinization (0.025%, trypsin) followed by trituration in a DNAase solution (0.0008%) with a trypsin inhibitor (0.004%). Finally, the cells were suspended in Modified Basal Eagle Medium (Kyokuto Pharm. Ind. Co., Ltd., Tokyo), containing 10% fetal bovine serum (Bocknek, Toronto, Canada), 25 mM KCl, 4 mM glutamine and 0.2% gentamicin, at a density of 1.25 \times 10^6 cells/ml. Then a 2-ml aliquot of the cell suspension was put into each 35-mm culture dish containing a coverslip glass (\phi 25 mm) coated with 20 \textmu g/ml poly-l-lysine. Fifty microliters of cytosine arabinoside (5\textmu M) was added to each culture in 16 hr to prevent non-neuronal cell proliferation. Cultures were incubated at 37\textdegree C in a humidified atmosphere containing 5% CO\textsubscript{2} for 3 to 5 days in vitro before use. The alteration of [Ca\textsuperscript{2+}] was measured with a fluorescent Ca\textsuperscript{2+} indicator, Fura-2, as reported previously (9). The coverslip glass containing granule cells was washed twice with Krebs' HEPES (KH) buffered solution (136 mM NaCl, 10 mM glucose, 20 mM HEPES, 5 mM KCl, 2 mM

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CaCl₂, 1.3 mM MgCl₂, and sufficient NaOH bring the pH to 7.4). Then cells were incubated in 5 μM Fura-2AM (Dojin Chem., Ltd., Kumamoto) in KH for 45 min at 37°C. Loaded cells were then transferred into the measuring chamber of the fluorimeter (CAF-100, Jasco, Tokyo) and were superfused with KH (1 ml/min at 37°C). A group of cells was alternately illuminated with 340/380 nm excitation, emitted light was collected at 500 nm, and the fluorescence was monitored and recorded by means of a PC-9801 personal computer (NEC, Tokyo) with CAF-200 software (Jasco). The value of [Ca²⁺] was determined by the following formula as described by Grynkiewicz et al. (10):

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[Ca^{2+}] = K_d (R - R_{min})/(R_{max} - R)(F_0/F_s)
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The values for R_{max}, R_{min} and F_0/F_s were obtained by measuring the fluorescence of 20 nM Fura-2 in a solution of 150 mM KCl, 10 mM HEPES, 2 mM MgSO₄, 10 mM EGTA, and sufficient NaOH to bring the pH to 7.1. R_{min} was the 340/380 ratio without added calcium; R_{max} was the ratio when saturating calcium, 15 mM, was added; F_0/F_s was the 380 nm intensity in saturating calcium, and the K_d for Fura-2 was 224 nM (10).

We initially examined the effects of either high-potassium or Glu on [Ca²⁺] levels in cultured neurons. The rest of the text continues with the description of the inhibitory effects of the calcium antagonists on [Ca²⁺] levels in cultured cerebellar granule cells…

The inhibitory effects of calcium antagonists on [Ca²⁺] levels in cultured cerebellar granule cells are represented by the solid columns and oblique columns, respectively. Each data shows the mean ± S.E.M. of 3 to 6 separate experiments (each experiment consisted of one culture dish). Ten micromolar nifedipine, SM6586 and SM12565 each inhibited more than 80% of the increase in [Ca²⁺]. levels by 50 mM KCl, although 100 μM nifedipine only caused about a 43% inhibition of this rise (Fig. 1). In the case of the 25 μM Glu-induced [Ca²⁺] increase, dosages from 1 to 10 μM of SM6586 and SM12565 showed a similar inhibitory potency as with 50 mM KCl; however, 500 μM nicardipine caused a 30% inhibition of such an increase. Unlike the case of the 50 mM KCl-induced [Ca²⁺] increase, 10 μM nifedipine was found to inhibit the 25 μM Glu-enhanced [Ca²⁺] increase, and 50 μM nifedipine showed about 50% inhibition of the [Ca²⁺] increase (Fig. 1). In Fig. 2, 0.5 to 5 μM of flunarizine inhibited the 50 mM KCl-induced [Ca²⁺] elevation in a dose-dependent manner. Ten micromolar flunarizine showed 48% inhibition of the 25 μM Glu-enhanced [Ca²⁺] elevation. In addition, at 10 to 100 μM flunarizine, there was no dose-dependent relationship, all of the test doses in this range giving about 50% inhibition of the 25 μM Glu enhanced [Ca²⁺] elevation (data are not shown). Diltiazem and verapamil have no effect on the 50 mM KCl- or 25 μM Glu-induced [Ca²⁺] increase except at doses above 10 μM. The fifty percent inhibition value (IC₅₀) of each examined calcium antagonists was calculated by the probit test. In the case of the 50 mM KCl-induced [Ca²⁺] increase, the IC₅₀ values were: 1.05 ± 0.10 μM for flunarizine, 1.74 ± 0.30 μM for nicardipine, 2.78 ± 0.10 μM for SM6586, and 3.39 ± 0.40 μM for SM12565. The IC₅₀ values of the examined potent calcium antagonists to the 25 μM Glu enhanced [Ca²⁺] increase were 2.32 ± 0.32 μM for SM12565 and 2.70 ± 0.70 μM for SM6586. These data suggest that the inhibitory potentiation of the examined calcium antagonists to the 50 mM KCl depolarization-induced [Ca²⁺] increase was in the
Fig. 1. Inhibitory effects of 1,4-dihydropyridines on 50 mM KCl (solid column) or 25 μM Glu (oblique column) increased [Ca^{2+}]_i levels in cultured cerebellar granule cells. Each dose of the examined drugs was tested on 3 to 6 culture dishes, and data represent the mean ± S.E.M.

Fig. 2. Inhibitory effects of non-dihydropyridines on 50 mM KCl (solid column) or 25 μM Glu (oblique column) increased [Ca^{2+}]_i levels in cultured cerebellar granule cells. Each dose of the examined drugs was tested on 3 to 6 culture dishes, and data represent the mean ± S.E.M.
order of: flunarizine > nicardipine > SM6586 > SM12565  
>> verapamil > diltiazem > nifedipine; and for the case of  
25 μM Glu, it was SM12565 = SM6586 > flunarizine >  
nifedipine >>> verapamil = diltiazem = nicardipine.

The present data suggest that the pharmacological  
potency of the examined calcium antagonists are different  
from the order that is seen in the peripheral nervous sys-  
tem (11, 12) and clearly different from the cardiac circula-
tory system (9, 11–13). This might be explained by differ-
cences between the calcium channels in the neurons com-
pared to those of other cells (14). However, the present  
results also indicate that calcium antagonists, for ex-
ample, even the similar 1,4-DHP derivatives, SM6585,  
SM12565, nicardipine and nifedipine, produce different  
pharmacological potentiation on VCO and ROC in the  
central nervous system. Whether or not these differences  
are due to the different binding affinities of these an-
tagonists is yet to be identified.

Additionally, the novel 1,4-DHPs, SM6586 and  
SM12565, were reported to have less effect on the cardiac  
blood system (13), but in this experiment, they effectively  
inhibited not only VCO but also ROC in cultured cerebel-
lar granule cells in a dose-dependent manner. These data  
indicate that SM6586, SM12565 and flunarizine have the  
same efficacy for the protection against increased [Ca2+],  
levels produced by high-potassium or excessive amounts  
of Glu.

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