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Combined Effects of a Metabotropic Glutamate Receptor Agonist and an Ionotropic Glutamate Receptor Agonist on Cytosolic Ca^{2+} Concentrations in Cultured Hippocampal Cells

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Abstract Changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of cultured hippocampal cells from rat embryos were investigated using a fluorescent Ca^{2+} indicator, fura-2. An ionotropic and a metabotropic glutamate receptor agonist were applied singly or simultaneously to the same cell and $\Delta[Ca^{2+}]_i$ (the maximum increase in $[Ca^{2+}]_i$) after each application was calculated. In cells that responded well to 30 μM *N*-methyl-*D*-aspartate (NMDA), $\Delta[Ca^{2+}]_i$ induced by simultaneous application of trans-1-amino-cyclopentyl-1, 3-dicarboxylic acid (trans-ACPD) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) was 101.2 ± 40.3 nM, whereas the sum of $\Delta[Ca^{2+}]_i$ values induced by these agents alone was 87.6 ± 32.2 nM ($p=0.061$, $n=55$). In 18% of these cells, the mean $\Delta[Ca^{2+}]_i$ evoked by the simultaneous application of AMPA and trans-ACPD was at least twice the mean sum of $\Delta[Ca^{2+}]_i$ values evoked by these agents alone. In cells which only responded to 30 μM NMDA with small $[Ca^{2+}]_i$ changes, $\Delta[Ca^{2+}]_i$ induced by simultaneous application of AMPA and trans-ACPD was significantly higher than the sum of $\Delta[Ca^{2+}]_i$ values induced by these agents alone, 130.1 ± 35.2 and 41.2 ± 24.0 nM, respectively ($p<0.001$, $n=17$). These results suggest that activation of metabotropic glutamate receptors upregulates AMPA receptors in hippocampal cells.

Key Words : trans-ACPD, AMPA, NMDA, fura-2, cytosolic calcium concentration

Introduction

L-Glutamate is a major neurotransmitter in the central nervous system[1)2)] and is involved in a number of cell functions, including neuronal plasticity, synaptogenesis and neuronal death. Glutamate receptors can be divided into two classes: metabotropic, which are coupled to phosphatidylinositol metabolism and mobilization of cytosolic Ca^{2+}[3)], and ionotropic, which form ion channels. The latter are divided into two subclasses: *N*-methyl-*D*-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors. Recent studies revealed that co-stimulation of metabotropic and ionotropic glutarmate receptors is required for long term potentiation[4)5)6)], whereas activation of metabotropic glutamate receptors regulates the response of ionotropic glutamate receptors. Metabotropic glutamate receptor agonists potentiate the responses of NMDA receptors in hippocampal slices[4)7)] and *Xenopus* oocytes[8)] and of AMPA receptors in dissociated spinal neurons[9)], but not in

hippocampal slices[4]. In order to clarify the effects of cellular responses via biochemical cascades that are activated by stimulation of metabotropic glutamate receptors on responses mediated by excitatory ionotropic receptors, we investigated the influence of a selective agonist of metabotropic glutamate receptors, trans-1-amino-cyclopentyl-1,3-dicarboxylic acid (trans-ACPD)[10], on the changes in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in rat hippocampal cells evoked by AMPA. In this study, we used cultured cells, which enable drugs to be applied directly to the cells without penetrating the cell layers. We found that trans-ACPD potentiated the responses of 18% of the neurons studied to AMPA.

Materials and Methods

Hippocampal cells were isolated from Wistar rat embryos at gestational days 18 and 19 and cultured using the methods described by Shingai et al[11]. Briefly, the cells were dissociated with trypsin and plated in wells (15 mm in diameter) at a density of 5×10^5 cells/well. Each well comprised a collagen-coated glass cover slip with a silicone rubber wall (Heraeus, Flexiperm). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 6% (vol/vol) fetal calf serum (GIBCO) and 1 g/l glucose in a CO_2 incubator at 37℃ and the culture medium was changed every 3 days.

Cells that had been cultured for 7 to 10 days were used. The measurement of $[Ca^{2+}]_i$ was made by the methods described by Harada et al[12]. The culture medium was removed and Mg^{2+}-free solution comprising (in mM) 145 NaCl, 6 KCl, 2.5 $CaCl_2$, 0.5 NaH_2PO_4, 12.5 glucose, and 5 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES, pH 7.3) supplemented with 1.5 μM fura-2 acetoxymethylester (fura-2/AM) was introduced into each dish. The cells were incubated at 37℃ for 60 min, washed with Mg^{2+}-free solution and incubated for 10 min at 37℃, after which each dish was mounted on an inverted microscope. The cells were superfused continuously with the Mg^{2+}-free solution described above (22-24℃, pH 7.3) supplemented with 1.5 μM tetrodotoxin for all the experiments. Tetrodotoxin was used to prevent increases in $[Ca^{2+}]_i$ by spontaneous neuronal excitation[11]. Unless stated otherwise, the drugs were applied to each cell through a hole (about 50 μm in diameter) in a U-shaped polyethylene tube (inner diameter 0.3 mm)[12]. This pipe method of application enables drugs to be changed rapidly and sequentially and consequently, the concentrations of drugs around the cells can be controlled more accurately than when they are applied by superfusion. Only in the experiments in which the cells were pretreated with trans-ACPD, this agent was applied by superfusion before the application of AMPA plus trans-ACPD via the U-shaped tube. The cells were washed by superfusion with Mg^{2+}-free solution for 20 min before the application of each agonist.

Using fura-2, the $[Ca^{2+}]_i$ changes in response to drug application to the cells were investigated. The cells were exposed to light at excitation wavelengths of 340 and 360 nm (10-nm bandwidth) from a xenon lamp through the objective lens (Nikon Fluor ×20) of the microscope, which elicited fura-2 fluorescence. The fluorescence was interfaced with an Argus-100 computer (Hamamatsu Photonics) via a SIT camera. The emitted fluorescence (fluorescence intensity in response to excitation at 340 nm divided by that at 360 nm) gives the Ca^{2+} concentration after appropriate calibration[13]. The $[Ca^{2+}]_i$ values were calculated from the fura-2 ratio using the following formula

$$[Ca^{2+}]_i = K\,[(R - R_{min})/(R_{max} - R)]$$

where K is the dissociation constant for fura-2/Ca^{2+} (135 nM), R is the fluorescence intensity ratio at the two chosen wavelengths and R_{min} and R_{max} are the ratios at zero and saturation $[Ca^{2+}]_i$, respectively. A standard curve was constructed using calibration buffer solutions that contained Ca_2CO_3 and ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*,-tetraacetic acid (EGTA) in various calculated concentration ratios, 3.5 μM fura-2 pentapotassium salt, and (in mM) 130 KCl, 20 NaCl, and 10 3-(*N*-morpholino) propanesulfonic acid (MOPS, pH 7.05) at 20℃. The Ca^{2+} concentrations of the solutions ranged from 0 to 500,000 nM. However,

the parameters R_{min} and R_{max} determined inside cells have been reported to differ from those in conventional calibration buffers[14]. Therefore, corrected intracellular R_{min} and R_{max} values (0.85 times the values obtained in the nonviscous calibration buffers) were determined. This correction factor was derived from a consensus of several different correction methods for the cytoplasmic microviscocity of PtK1 cells[15]. The fluorescence microscopic images were stored in the Argus -100 compuer system at 3-s intervals for the analysis of $[Ca^{2+}]_i$ values, as described above.

Only culture dishes containing cells which showed a $[Ca^{2+}]_i$ increase in response to the application of 30μM NMDA for 15 s were included in the analysis. These cells were considered to be neurons[12)16], as NMDA has been demonstrated not to increase $[Ca^{2+}]_i$ in glial cells[17].

The $[Ca^{2+}]_i$ changes were expressed as means ± standard deviations and compared using Student's t-test for paired data. Differences at values of $p < 0.05$ were considered to be significant.

Results

1) Simultaneous application of AMPA and trans-ACPD to the cells responding to NMDA

Cells that responded to both AMPA and trans-ACPD with increases in $[Ca^{2+}]_i$ of over 15 nM were selected for analysis. Application of 1-10 μM trans-ACPD or 1-10 μM AMPA for 15 s to the cells induced $[Ca^{2+}]_i$ increases and $\Delta[Ca^{2+}]_i$ indicates the maximum increase in $[Ca^{2+}]_i$ evoked by application of a drug to a cell. The mean $\Delta[Ca^{2+}]_i$ values evoked by 1 and 10 μM AMPA were 59.1± 31.7 (n=35 cells) and 106.7±18.2 nM (n=7), respectively, and those evoked by 1 and 10 μM trans-ACPD were 22.1±12.6 (n=22) and 58.1±22.4 nM (n=22), respectively.

In 55 cells, application of 30 μM NMDA increased $[Ca^{2+}]_i$ by 30 nM or more. These cells were considered to be neurons. The $\Delta[Ca^{2+}]_i$ induced by mixtures of 1-10 μM AMPA and 1-10 μM trans-ACPD was 101.2±40.3 nM, whereas the sum of the $\Delta[Ca^{2+}]_i$ values induced by these agents alone was 87.6±32.2 nM ($p=0.061$, n=55). Application of mixtures of 1-10 μM AMPA and 1-10 μM trans-ACPD to the cells for 15 s resulted in approximately the same $\Delta[Ca^{2+}]_i$ value as the sum of the responses after exposure to the agents alone for 15 s in 23 neurons (Fig. 1), although in 5 neurons, the $\Delta[Ca^{2+}]_i$ values induced by mixtures of the two agents were at least twice the sum of the $\Delta[Ca^{2+}]_i$ values evoked by these agents alone. In 5 neurons, the $\Delta[Ca^{2+}]_i$ values induced by the simultaneous application of AMPA and trans-ACPD after application of 1-2 μM trans-ACPD by superfusion for 30 s were at least twice the sum of those in

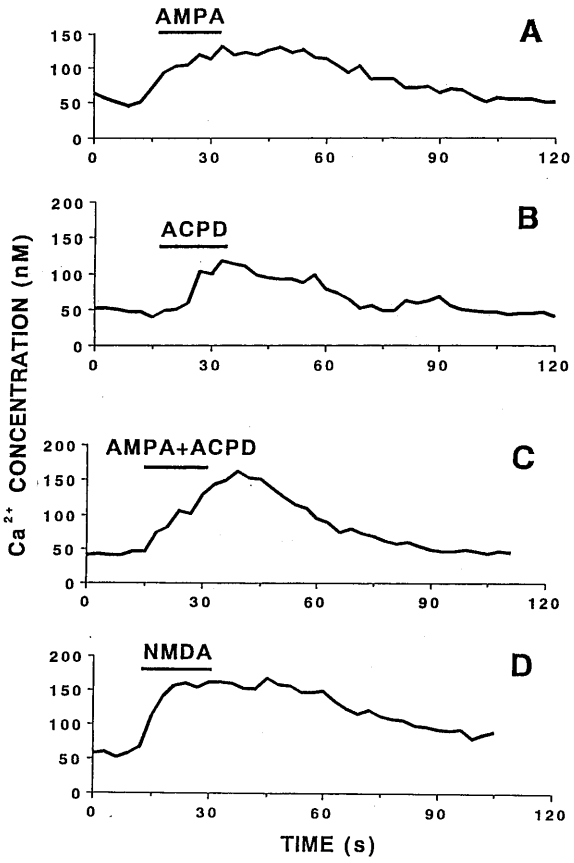

Fig. 1 Changes in $[Ca^{2+}]_i$ induced by glutamate receptor agonists in a neuron that responded to 30 μM NMDA. (A) 3 μM AMPA, (B) 10 μM trans -ACPD, (C) 3 μM AMPA and 10 μM trans-ACPD simultaneously and (D) 30 μM NMDA were applied to the same neuron by the pipe method. The ratio R* (see text) was 0.84.

response to exposure to these agents alone for 15 s (Fig. 2). The ratios (R*) of $\Delta[Ca^{2+}]_i$ values induced by simultaneous application of trans-ACPD and AMPA to the sum of those induced by these agents alone in the same cells are listed in Table 1. There was no significant difference between the R* values with and without 1-2 μM trans-ACPD applied for 30 s before application of the mixture of the two agents. The R* value was greater than 2.0 for 18% (10/55) of these neurons and the average R* for the remainder was close to 1.0.

All image data stored in the computer system were investigated in order to determine the effects of neighboring cells on the $[Ca^{2+}]_i$ changes of the observed neurons. The neurons were divided into two groups. One consisted of neurons touching neighboring cells, and the neighboring cells responded to trans-ACPD, but not to NMDA. The other one consisted of neurons that were separated from these neighboring cells. In 40 neurons touching neighboring cells which responded to trans-ACPD, but not to 30 μM NMDA, the $\Delta[Ca^{2+}]_i$ induced by simultaneous application of AMPA and trans-ACPD was greater than the sum of the $\Delta[Ca^{2+}]_i$ values induced by these agents alone, 105.0±43.6 and 85.2±34.0 nM, respectively ($p<0.05$). In 15 neurons separated from cells which responded to trans-ACPD, but not to NMDA, the $\Delta[Ca^{2+}]_i$ induced by simultaneous application of AMPA and trans-ACPD and the sum of the $\Delta[Ca^{2+}]_i$ values induced by these agents alone were 91.8±32.0 and 94.3±26.5 nM, respectively ($p=0.84$).

2) Simultaneous application of AMPA and trans-ACPD to the cells that did not respond to NMDA

Seventeen cells essentially did not respond to 30 μM NMDA ($[Ca^{2+}]_i$ increase <30 nM).

Table 1 Ratio(R*) of the maximum $[Ca^{2+}]_i$ change induced by simultaneous application of trans-ACPD and AMPA to the sum of the maximum $[Ca^{2+}]_i$ changes induced by each agent alone.

	R*	No. of cells with R*>2.0 (and R*>1.5)
1) Cells responding to 30 μM NMDA[a]		
Without pre-application[b]		
Total	1.41±0.81 S.D. (n=28)	5(6)
Cells with R*≦2.0	1.07±0.36 S.D. (n=23)	
With pre-application[c]		
Total	1.34±0.62 S.D. (n=27)	5(7)
Cells with R*≦2.0	1.08±0.37 S.D. (n=22)	
2) Cells which did not respond to 30 μM NMDA[d]	4.46±2.86 S.D. (n=17)	12(14)

a) The maximum $[Ca^{2+}]_i$ increase in each cell was 30 nM or more.
b) The concentrations of AMPA and trans-ACPD were 1-10 μM.
c) 1-2 μM trans-ACPD was applied for 30 s before the simultaneous application of 1-10 μM trans-ACPD and 1-10 μM AMPA for 15 s.
d) Includes cells in which the $[Ca^{2+}]_i$ increase was less than 30 nM.

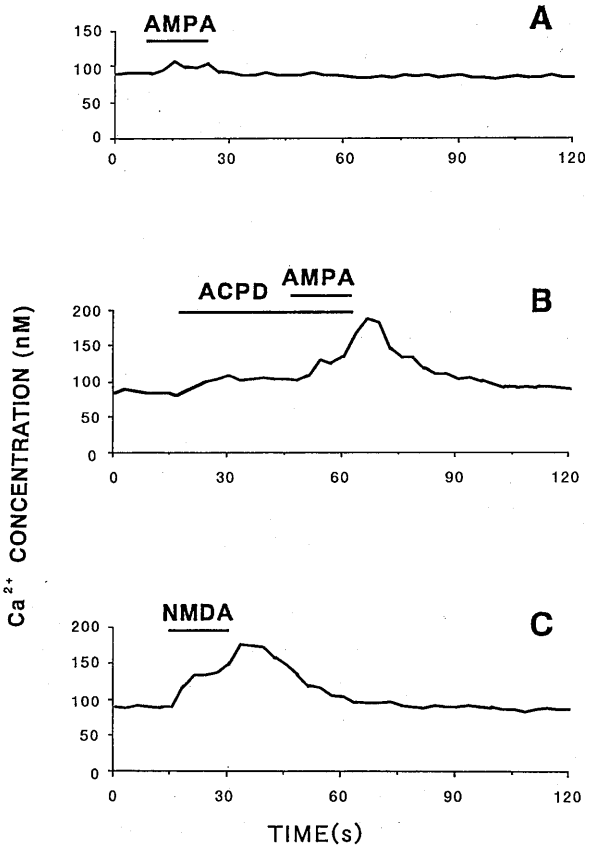

Fig. 2 The $[Ca^{2+}]_i$ responses after prior application of trans-ACPD of a neuron that responded to 30 μM NMDA. (A) 2 μM AMPA applied by the pipe method, (B) 2μM trans-ACPD applied by superfusion, followed by a mixture of 2 μM trans-ACPD plus 2 μM AMPA by the pipe method and (C) 30 μM NMDA applied by the pipe method to the same neuron. R* was 2.2.

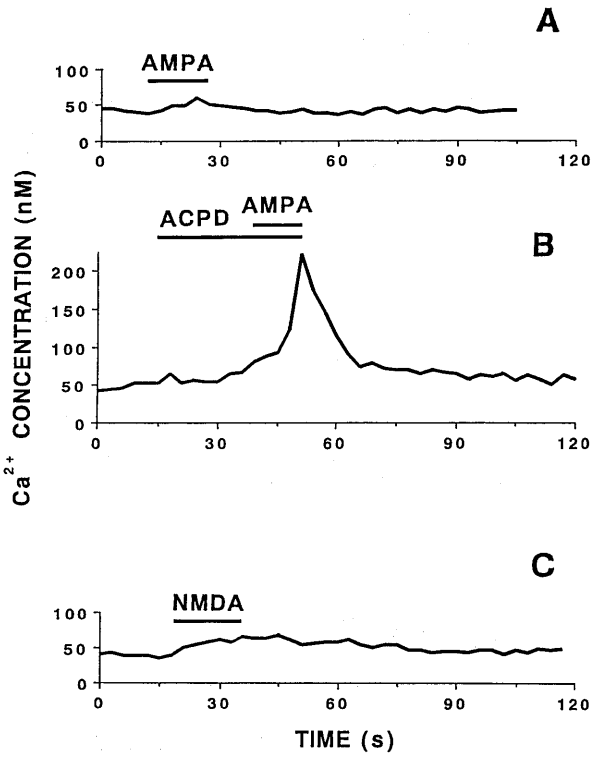

Fig. 3 The $[Ca^{2+}]_i$ responses of a cell in which the maximum $[Ca^{2+}]_i$ change induced by 30 μM NMDA was small (18 nM). (A) 2 μM AMPA applied by the pipe method, (B) 2 μM trans-ACPD applied by superfusion, followed by a mixture of 2 μM trans-ACPD plus 2 μM AMPA by the pipe method and (C) 30 μM NMDA applied by the pipe method to the same cell. R* was 4.1.

Simultaneous application of 1-10 μM AMPA and 1-2 μM trans-ACPD to these cells induced the potentiated responses (Fig. 3). The $\Delta[Ca^{2+}]_i$ evoked by the mixtures and the sum of the $\Delta[Ca^{2+}]_i$ values evoked by these agents alone were 138.2±31.3 and 44.5±24.5 nM, respectively ($p<0.001$), and the R* value was greater than 2.0 for 71% (12/17) of these cells (Table 1).

3) Simultaneous application of NMDA and trans-ACPD to neurons

The $\Delta[Ca^{2+}]_i$ induced by simultaneous application of 2-5 μM NMDA and 2-5 μM trans-ACPD and the sum of the $\Delta[Ca^{2+}]_i$ values induced by these agents alone were 99.4±35.6 and 69.3±20.9 nM, respectively ($p<0.05$, $n=16$), and the R* value was greater than 2.0 for 31% (5/16) of these neurons (Fig. 4).

Discussion

The significant increase in $[Ca^{2+}]_i$ evoked by the simultaneous application of NMDA and trans-ACPD compared with the sum of the increases evoked by these agents alone may be similar to the phenomena reported

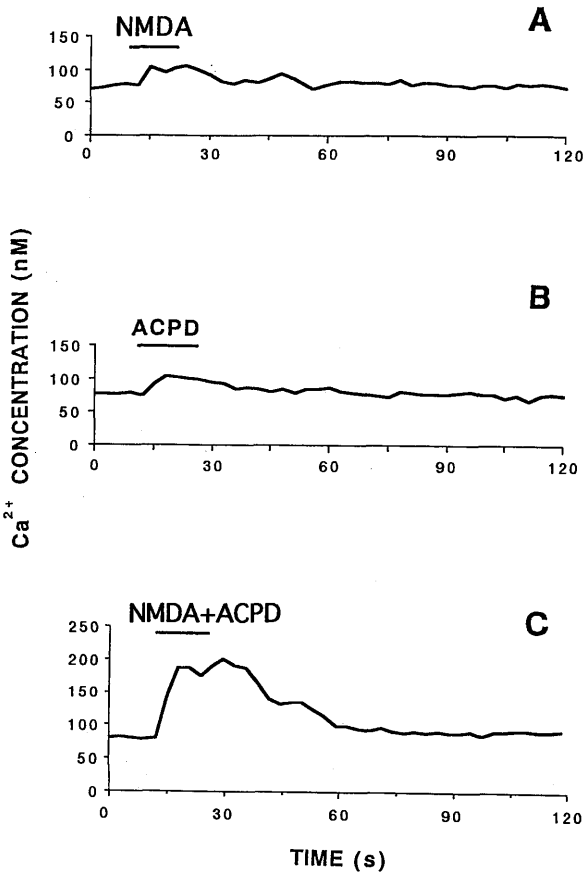

Fig. 4 Changes in $[Ca^{2+}]_i$ induced by NMDA and trans-ACPD. (A) 2 μM NMDA, (B) 2 μM trans-ACPD and (C) 2 μM NMDA and 2 μM trans-ACPD simultaneously were applied to the same neuron by the pipe method. R* was 2.3.

with hippocampal slices[4], *Xenopus* oocytes[8] and enzymatically dissociated spinal dorsal horn neurons[9], in which the ionic currents induced by NMDA were potentiated by trans-ACPD application. These findings are thought to be attributable to the upregulation of NMDA receptors by the activation of metabotropic glutamate receptors. Contrary to the results of a previous study[4], our results indicate that there are hippocampal neurons, in which activation of the metabotropic glutamate receptors by trans-ACPD potentiates AMPA-induced elevation of $[Ca^{2+}]_i$.

The activation of metabotropic glutamate receptors by trans-ACPD is linked to an increase in cyclic AMP[5] and cyclic AMP-dependent protein kinase A upregulates AMPA receptors in hippocampal neurons[18][19]. This potentiaion of AMPA receptor responses by trans-ACPD is thought to facilitate long term potentiation in hippocampal neurons[5][6].

In the cells that showed only small increases in $[Ca^{2+}]_i$ in response to application of 30 μM NMDA, which may, therefore, have included glial cells, marked potentiation of AMPA receptor response was observed. The neurons adjacent to cells which responded to trans-ACPD, but not to NMDA, showed significant increases in $[Ca^{2+}]_i$ in response to simultaneous application of both agents, compared with the summed responses to the agents alone. A recent study[20] indicated that $[Ca^{2+}]_i$ increases of a glial cell raise the $[Ca^{2+}]_i$ of its neighboring neurons directly. Increases in glial cellular $[Ca^{2+}]_i$ induced by trans-ACPD may, therefore, stimulate neuronal activity.

Modulation of AMPA receptors by the activation of metabotropic glutamate receptors would appear to play an important role in signal transmission in hippocampal cells.

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