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The Time Course of NMDA-and Kainate-induced cGMP Elevation and Glutamate Release in Cultured Neuron

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The levels of extracellualr glutamate, intracellular Ca^{2+} ($[Ca2+]_i$) and cGMP were determined for 1 h with the excitatory amino acids, N-methyl-D-aspartate (NMDA) or kainate in cultured cerebellar granule cells. Both NMDA and kainate produced a time-dependent release of glutamate, and kainate was more potent than NMDA in glutamate elevation. The elevation of extracellular glutamate was not purely governed by intracellular Ca^{2+} concentration. However, in opposite to the time-dependent elevation of glutamate, the elevation of cGMP by NMDA and kainate were at maximum level in short-time (1 min) incubation then remarkably decreased with longer incubation times. Post-applications (30 min after agonist) of EAA antagonst did not block EAAs-induced glutamate elevation. However, NMDA antagonist, phencyclidine (PCP), blocked NMDA-induced cGMP elevation at pre- or post-application, but kainate antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX), paradoxically augmented kainate-induced cGMP elevation for 1 h incubation. These results show that NMDA or kainate induces time-dependent elevations of extracellular glutamate, while the elevations of cGMP by these EAAs are remarkably decreased with longer incubation times. However, NMDA- and kainate-indcued glutamate release was blocked by pre-application of each receptor antagonist but not by post-application while EAA-induced $[Ca^{2+}]_i$ was blocked by post-application of antagonist. These observations suggest that EAA-induced elevation of $[Ca^{2+}]_i$ is not parallel with elevation of glutamate release or cGMP.

Key words: Excitatory amino acids, NMDA, Kainate, cGMP, Ca^{2+} influx

INTRODUCTION

It has been known that excitatory amino acids induced intracellular Ca^{2+} concentrations play a fundamental role in the regulation of many cellular processes, including vesicular exocytosis and synaptic transmission (Jassell and Kandel, 1993) and even neurotoxicity (Choi *et al.*, 1987). The role of Ca^{2+} in these macroscopic cellular functions is one of a second messenger, serving to regulate enzymes and glutamate receptor channels (Rosenmund and Westbrook, 1993), the expression of immediate early genes (Morgan and Curran, 1986), cell proliferation and growth (Brewer and Cotman, 1989) and long-term potentiation (Collingridge and Bliss, 1987). Under normal conditions, the Ca^{2+} concentration of the extracellular fluids is in the millimolar range (around 2.5 mM), whereas the intracellular free Ca^{2+} concentration is less than 100 nM (Kostyuk and Tepikin, 1991). Transient increases in the cytosolic Ca^{2+} concentration can result from the entry of Ca^{2+} from the external milieu through receptor-operated channels, voltage-activated channels, or ionic pumps, or from the release of Ca^{2+} from internal stores (Kostyuk and Tepikin, 1991).

Excitatory amino acids such as glutamate and aspartate are important and abundant neurotransmitters in CNS (Monaghan et al., 1989; Collingridg and Lester, 1989). Receptors for glutamate have been divided into two distinct groups: ionotropic receptors, which are permeable to cations and of which there are several subtypes (Jahr and Stevens, 1987; Cull-Candy and Usowicz, 1987), and metabotropic receptors, which are functionally linked to stimulation of inositol phospholipid metabolism via a G-protein coupling mechanism (Schoepp et al., 1990). Three classes of ionotropic glutamate receptors are permeable to cations and are further classified according to specific agonists: NMDA, kainate, a-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA).

The NMDA receptor plays a key role in synaptic plasticity and is thought to underlie memory, learning and development of the nervous system (Bliss and Collingridge, 1993; Bourne and Nicoll, 1993). The

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NMDA receptor is a ligand-gated/voltage-sensitive ion channel. The voltage dependence of NMDA receptors stems from a block of the ion channel by Mg^{2+}. Membrane voltage above about -40 mV releases the Mg^{2+} block allowing other ions to flow through the receptor channel. NMDA receptor has a high permeability to Ca^{2+} ions. The channel is 10 times more permeable to Ca^{2+} than to Na^{+} while kainate receptor is permeable to Na^{+} though Ca^{2+} is only 0.15 times as permeate as Na^{+} (Mayer and Westbrook, 1987). NMDA receptor is approximately 70 times more permeable to Ca^{2+} than kainate receptor. However, the influx of Na^{+} through kainate receptor elevates membrane potential and opens voltage sensitive Ca^{2+} channel, and removes Mg^{2+} from NMDA receptors results in rise of intracellular Ca^{2+}.

A sustained increase in the intracellular free Ca^{2+} triggers a series of events; induces glutamate release and activates nitric oxide synthase. Glutamate is stored in small electroleucent synaptic vesicles which are distinct from the small and large dense-cored vesicles that contain amine transmitters or neuropeptides (De Camilli and Jahn, 1990). Plasma membrane depolarization and Ca^{2+} entry leads to release of glutamate to the extracellular space. The released glutamate from neurons by Ca^{2+}-dependent vesicular release is terminated ultimately by uptake carrier that cotransports two Na^{+} into the cell with each glutamate, while counter-transporting one K^{+} and one OH- out of the cell (Bouvier *et al.*, 1992; Attwell *et al.*, 1993). Therefore, depolarization of neurons with K^{+} evokes release of glutamate that is partly Ca^{2+}-dependent vesicular release, but also has a Ca^{2+}-independent component that is presumably mediated by reversal of uptake (Adam-Visi, 1992). Nitric oxide is produced by Ca^{2+}-dependent nitric oxide synthase (NOS) from L-arginine following activation of excitatory amino acid receptors, particularly those of the NMDA type (Garthwaite et al., 1988; Bredt and Snyder, 1989). Soluble guanylyl cyclase which contains heme as a prosthetic group can be activated by nitric oxide, therefore tissue levels of cGMP are elevated in a concentration dependent manner by activation of guanylyl cyclase with NO. The elevation of $[Ca^{2+}]_i$ has a major role to elevate cGMP levels but higher levels of Ca^{2+} may ultimately lower intracellular cGMP levels by activating phosphodiesterase, the cGMP degradative enzyme. Furthermore it has also been shown that higher intracellular Ca^{2+} (120 nM) inhibited guanylyl cyclase (Vincent and Hope, 1992). Therefore the elevations of cGMP level by EAAs may variable in accordance to exposing time with elevated $[Ca^{2+}]_i$. This experiment was aimed to determine the relationship of EAA-induced elevation of $[Ca^{2+}]_i$ with glutamate release and elevation of cGMP in long incubation.

MATERIALS AND METHODS

Cerebellar granule cell culture

Cerebellar granule cells were cultured as described by McCaslin and Morgan (1987) with slight modifications. Briefly, 8-day-old pups (Sprague-Dawley, Harlan, Indianapolis, IN) were decapitated, and the heads were partially sterilized by dipping them in 95% ethanol. The cerebellum was dissected from the tissue and placed in culture medium which lacks serum and bicarbonate. Growth medium (5 ml/60 mm dish, 300 mOsm) was Dulbecco's modified Eagle's medium (DMEM) supplemented with sodium pyruvate (0.9 mM), glutamine (3.64 mM), sodium bicarbonate (40 mM), glucose (22.73 mM), 6% bovine calf serum (Hyclone, Logan, UT) and 6% fetal bovine serum (JRH Bioscience, Lenexa, KS). After 2 days incubation (37°C, 10% CO_2), growth medium was aspirated from the cultures and new growth medium (5 ml/dish, 300 mOsm) containing 25 mM KCl was added with 5 μM cytosine arabinoside to prevent proliferation of non-neuronal cells. The cells were then incubated for 14 days at 37°C under 10% CO_2-90% filtered room air.

Glutamate measurement

After cells were grown for 10-14 days, growth medium was washed from the cells, and they were placed in a physiological saline HEPES (PSH) buffer solution containing the following mM concentrations: 135 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 40 bicarbonate, 10 glucose and 5 HEPES (pH 7.4, 300 mOsm). After a 30 min equilibriation period in PSH buffer (37°C, 10% CO_2), cells were washed and reincubated in the presence of various concentrations of compounds at 37°C for 1 h. The slides were removed after incubation and the cells were inactivated with 0.35 ml of hot 1 M perchloric acid (90°C) to determine cGMP levels. The amount of glutamate secreted into the buffer was separated and quantified by HPLC as described below (Ellison et al., 1987). A small amount of buffer (500 μl) was collected from the culture dish, and the glutamate concentration was quantified by HPLC (Bioanalytical Systems) with an electrochemical detector after precolumn derivatization of sample aliquots (50 μl) with 20 μl of o-phthalaldehyde/2-mercaptoethanol reagent. The C18, 5 mm, 4.6x150 mm, reverse-phase column (Rainin, CA) was eluted with mobile phase (pH 5.2) containing 0.1 M sodium phosphate buffer with 37% (v/v) HPLC-grade methanol at a rate of 1.0 ml/mein.

Cyclic GMP measurement

Cyclic GMP levels were determined via radioimmunoassay as described by Harper and Brooker (1975). Briefly, after stimulating cells with an EAA and inac-

tivating enzymes with hot (90°C) perchloric acid (1 M), cells were sonicated and then centrifuged at 5000 g for 5 min. cGMP levels were determined in the supernatant after neutralization (pH 7.4) with 2M $KHCO_3$ using radioimmunoassay kits (Amersham, IL). Each cGMP levels are presented as p moles/mg protein. The protein content was determined according to Bradford (Bradford, 1976) using bovine serum albumin as the standard.

Measurement of intracellular Ca^{2+} levels

Calcium influx was determined by ratio fluorometry as described by others (Grynkiewicz et al., 1985; Cai and McCaslin, 1992). The cells were grown on glass cover slides, loaded with 10 mM fura-2 AM for 1 hr in PSH buffer at 37°C, and washed with PSH buffer. Cell culture slides were cut and mounted into spectrophotometer cuvettes containing 2.5 ml PSH buffer (without bicarbonate). Fluorescence was measured with a FLUOROLOG-2 spectrophotometer (SPEX Ind. Inc., Edison, NJ) by exciting cells at 340 and 380 nm and measuring light emission at 505 nm. Baseline $[Ca^{2+}]_i$ was measured for 60 sec before the addition of various experimental compounds. R_{min} and R_{max} were determined by addition of EGTA (20 μM) and ionomycin (10 μM), respectively. Calcium concentrations were calculated according to the method of Grynkiewicz *et al.*, (1985) using a K_D of 224 nM by using TM 3000 software (SPEX).

RESULTS

The representative curves showing changes in $[Ca^{2+}]_i$ in response to application of NMDA and kainate (each 100 μM) are shown in Fig. 1. NMDA and kainate induced large increase over baseline $[Ca^{2+}]_i$ which were sustained for 1 h. In short time (6-7 min after application), kainate-induced elevation of $[Ca^{2+}]_i$ was peak and gradually become plateau level while NMDA did not show sharp peak curve. Each antagonist, PCP (10 μM) and DNQX (10 μM), remarkably decreased $[Ca^{2+}]_i$ levels which was induced by NMDA and kainate, respectively (Fig 1B). These suppressed $[Ca^{2+}]_i$ by antagonist was sustained up to 1 h (data not shown).

The incubation of cells for 1 h with NMDA or kainate (each 100 μM) resulted in elevation of glutamate release (Fig. 2). NMDA and kainate gradually elevated glutamate levels in accordance with the exposure time up to 1 h. After 1 h incubation, kainate showed 4 times higher elevation of glutamate than NMDA. In contrast to the glutamate release, the levels of cGMP were remarkably elevated by NMDA and kainate in early exposure time and gradually decreased to the baseline with time lapse (Fig. 3). After 30 min exposure, kainate-induced cGMP level was not elevated significantly, but NMDA-induced cGMP level was elevated significantly even after 1 h incubation.

To determine the effect of exposure time with EAA agonist on glutamate release and cGMP elevation, each antagonists were applied in different time; antagonist was applied either 5 min before (pre-application) or 30 min after (post-application) agonist adding. Pre-application of NMDA antagonist (PCP, 10 μM) blocked NMDA induced glutamate release and cGMP elevation (Fig. 4A, 5A). However, 30 min delayed appli-

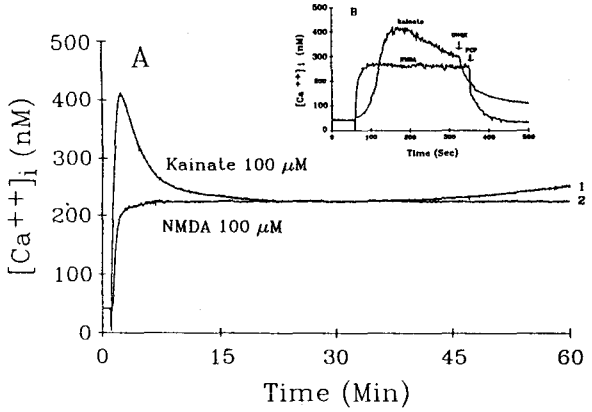

Fig. 1. A comparison of the NMDA and kainate induced $[Ca^{2+}]_i$. Cells were loaded with fura-2 AM for 60 min and washed PSH buffer. (A). Intracellular Ca^{2+} levels were measured for 60 min by stimulation with NMDA (100 μM, curve 2) or kainate (100 μM, curve 1) at the break in the curves (60 sec). (B). Intracellular Ca^{2+} levels were measured as (A) for 500 sec. EAA antagonist, PCP (10 μM) or DNQX (10 μM), was applied as indicated by arrows (around 350 sec). Each curve represents the average of at least three experiments.

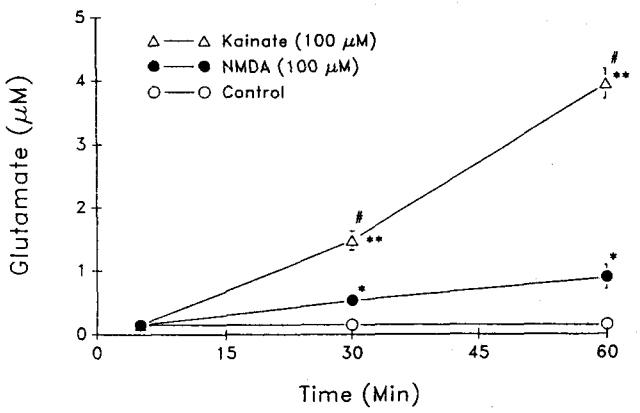

Fig. 2. Time course for the elevation of extracellular glutamate concentrations in NMDA or kainate (each 100 μM) treated cells. Cerebellar granule cells were grown for 14 days in vitro. Cells were washed with PSH buffer and equilibriated for 30 min before adding compounds. Cells were incubated with NMDA or kainate for 30 to 60 min. Values present the mean±SEM (bars) of μM in 3 ml of extracellular buffer (n=5). Data were analyzed by Student's t-test. *<0.05, **<0.01 from the control group. #<0.05 from the NMDA treatment group.

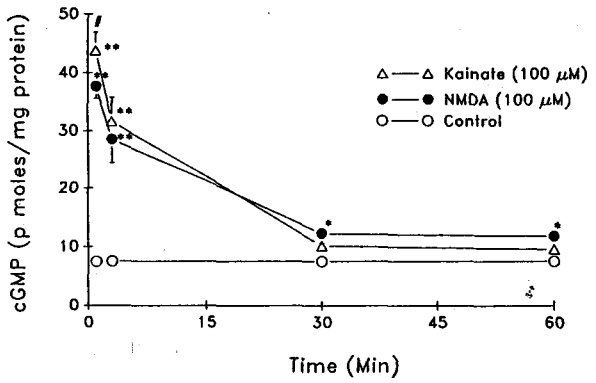

Fig. 3. Time response for the elevation of cGMP levels in NMDA or Kainate (each 100 μM) treated cells. Cerebellar granule cells were grown for 14 days in vitro and then washed with PSH buffer. After a 5 min (for 1, 3 min measurement groups) or 30 min (for 30, 60 min measurement groups) equilibriation period in PSH buffer and incubated with NMDA or kainate. Values present the mean±SEM (bars) of p moles/mg protein (n=5). Data were analyzed by Student's t-test. *<0.05, **<0.01 from the control group. #<0.05 from the NMDA treatment group.

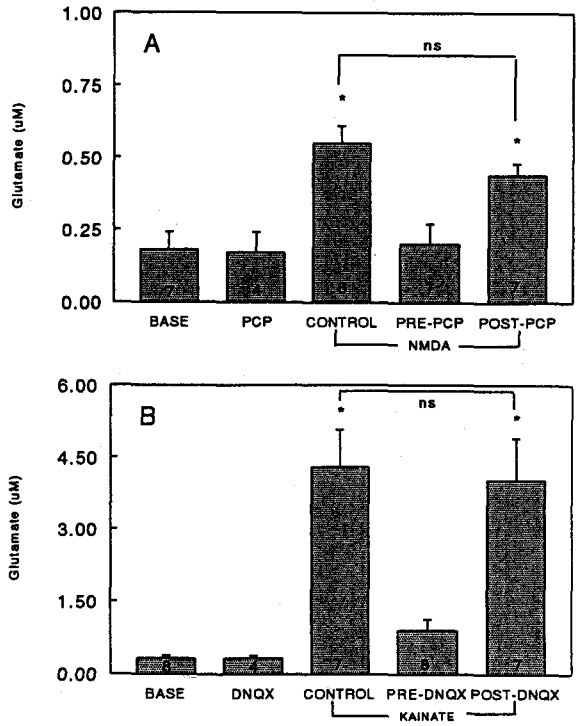

Fig. 4. Effects of antagonists on the NMDA- and kainate-induced release of glutamate for 60 min incubation (NMDA, kainate 100 μM; PCP, DNQX 10 μM). Cells were washed with PSH buffer and equilibriated for 30 min before adding compounds. Each antagonist was applied 5 min prior to agonist adding in pre-application group but antagonist was applied 30 min after agonist adding in post-application group. The numbers for each group are shown in the columns. Values present the mean±SEM of μM in 3 ml of extracellular buffer. Data were analyzed by an ANOVA followed with Newman-Keuls test. * <0.05 from the control group.

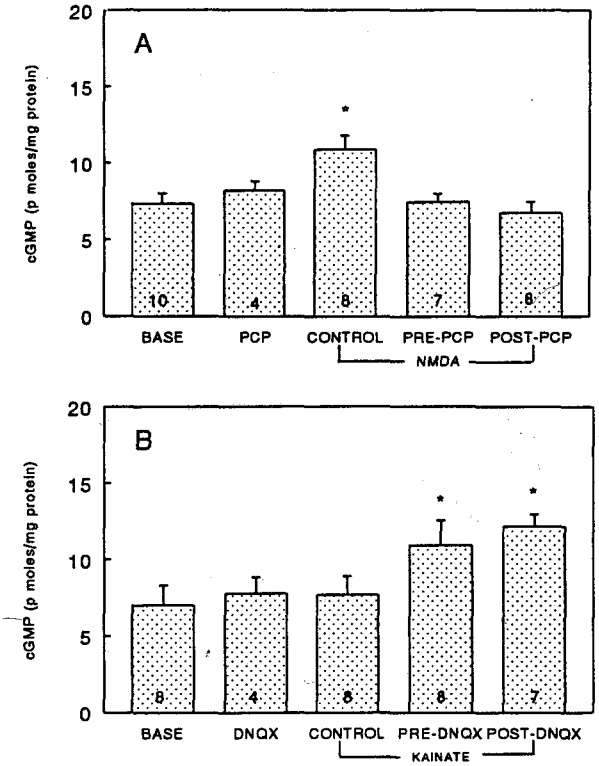

Fig. 5. Effects of antagonists on the NMDA- and kainate-induced cGMP elevation for 60 min incubation (NMDA, kainate 100 μM; PCP, DNQX 10 μM). Experiments were performed as described in the legend of figure 4. The numbers for each group are shown in the columns. Values present the mean±SEM of p moles/mg protein. Data were analyzed by an ANOVA followed with Newman-Keuls test. * <0.05 from the control group.

cation of PCP failed to decrease NMDA-induced glutamate release although elevation of cGMP level was blocked. Analogous to NMDA receptor function, kainate antagonsit (DNQX, 5 μM) only blocked kainate-induced glutamate release in case of pre-application but failed to block in case of post-application (Fig. 4B). Unexpectedly, DNQX failed to block kainate induced cGMP elevation rather augmented cGMP levels both pre- and post-application (Fig. 5B). However, the other kainate receptor antagonst, GYKI 52466 (10 μM) blocked both kainate-induced glutamate release and cGMP elevation (unpublished data).

DISCUSSION

These experiments show that NMDA or kainate elevates glutamate release in a time-dependent manner but produces inverse elevation of cGMP levels. On the other hand, both EAA-induced rises of $[Ca^{2+}]_i$ were maintained plateau level although kainate induced transient elevation $[Ca^{2+}]_i$ after 1-2 min and maintained plateau with longer incubation times. It is in-

teresting that kainate elevated extracellular glutamate concentration 4 times higher than NMDA with a same concentration of agonist although both EAA-induced elevation of $[Ca^{2+}]_i$ was not so big different. These suggest that glutamate release is not purely proportional to the elevation of $[Ca^{2+}]_i$. The elevated glutamate is removed by reuptake into neurons by a sodium-dependent but Ca^{2+}-independent transport (Flot and Seifert, 1991). Normally, the transporter carries one glutamate and two Na^+ into the cell while one K^+ and one OH- or HCO_3- are transported into the cell, so that one net positive charge is transported into the cell. Therefore, uptake is reduced by membrane depolarization, presumably due to a more positive internal voltage (Bouvier *et al.,* 1992; Brew and Attwell, 1987). Furthermore, depolarization of cell has been shown to produce the release of glutamate by the reversal of the transporter (Attwell *et al.,* 1993). This could explain why kainate is more potent than NMDA in elevating the release of glutamate since kainate induces Na^+ influx. The possibility of cell damage by kainate was tested. However, cultured cell was not damaged by activation with kainate for 1 h evidenced by trypan blue exclusion test and lactate dehydrogenase (LDH) release test (data not shown). In other hand, EAA-induced cGMP elevation was not maintained for 1 h rather decreased with longer incubation times while elevated $[Ca^{2+}]_i$ by EAA was maintained. These cGMP results are similar to those reported by Garthwaite (1982) showing increases in cGMP induced by glutamate, aspartate or NMDA were transient in cerebellar slices, reaching a maximum after 2-5 min and decreasing gradually with longer incubation times. The kainate-induced cGMP elevation for 30 and 60 min incubations is less than NMDA in contrast to the higher elevation of cGMP in short time incubation (Fig. 3). It is presumably that EAA-induced $[Ca^{2+}]_i$ have a dual effect on cGMP levels; influxed Ca^{2+} activate the nitric oxide synthase in short-term, subsequently produce NO results in elevation of cGMP, on the other hand, elevated higher levels of $[Ca^{2+}]_i$ may ultimately decrease intracellular cGMP levels by activating the cGMP degradative enzyme, cyclic nucleotide phosphodiesterase. Furthermore it has also been shown that guanylyl cyclase is inhibited by higher intracellular Ca^{2+} (120 nM) (Vincent and Hope, 1992). Therefore, pre-exposure of NMDA receptor antagonist (PCP) could block NMDA receptor result in blocking Ca^{2+} influx so elevation of cGMP subsequently could be blocked (Fig. 5A) while 30 min delayed exposure (post-PCP) of antagonist failed to NMDA-induced elevation of cGMP and release of glutamate. These results suggest that certain level of Ca^{2+} is needed for elevation and maintaining of cGMP level, so $[Ca^{2+}]_i$ balance may be an important factor for maintaining of cGMP level. Unexpectedly, pre- and post-exposure of DNQX failed to block kainate-induced cGMP elevation rather significantly augmented cGMP elevation (Fig. 5B) although pre-application of DNQX blocked kainate-induced glutamate release. This may be the specific activity of DNQX since the other kainate receptor antagonist (GYKI 52466) blocked kainate-induced elevation of glutamate and cGMP levels by pre- and post-application. Probably this paradoxical effect of DNQX is due to its structure character which contains nitrite (-NO_2) group like a thiol dependent nitric oxide generating compound (e.g. glycery trinitrite) since thiol containing neurocompound such as cystein could be released by activation of kainate receptor in long-term incubation. Indeed, DNQX blocked kainate-induced elevation of cGMP in short-term (3 min) incubation (data not shown).

Collectively, both NMDA and kainate produced a time-dependent release of glutamate, and kainate was more potent than NMDA in glutamate release. The EAA-induced glutamate release was blocked by pre-application of EAA antagonist but not by post-application. The EAA-induced glutamate release was not correlated with elevation of $[Ca^{2+}]_i$ since the levels of $[Ca^{2+}]_i$ induced by these EAAs were almost same and could be blocked by post-application of antagonist. Furthermore, the pattern of EAA-induced cGMP elevation was not parallel with glutamate release. NMDA and kainate elevated cGMP at maximum level in short time (1 min) incubation but EAA-induced cGMP levels were remarkably decreased with longer incubation times (30-60 min).

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