Korean J Physiol Pharmacol
Vol 2: 41−48, February, 1998

Differential Effect of Homocysteic Acid and Cysteic Acid on Changes of Inositol Phosphates and $[Ca^{2+}]i$ in Rat Cerebellar Granule Cells

Won-Ki Kim and Young-Sook Pae

Department of Pharmacology, College of Medicine; Division of Neuroscience, Medical Research Center, Ewha Womans University, Seoul 158−056, Korea

The present study was undertaken to characterize homocysteic acid (HCA)-and cysteic acid (CA)-mediated formation of inositol phosphates (InsP) in primary culture of rat cerebellar granule cells. HCA and CA stimulated InsP formation in a dose-dependent manner, which was prevented by the *N*-methyl-D-aspartate (NMDA) receptor antagonist D,L-2-amino-5-phosphopentanoic acid (APV). CA-, but not HCA-, mediated InsP formation was in part prevented by the metabotropic glutamate receptor antagonist α-methyl-4-carboxyphenylglycine ((±)-MCPG). Both HCA- and CA-mediated increases in intracellular calcium concentration were completely blocked by APV, but were not altered by (±)-MCPG. CA-mediated InsP formation was in part prevented by removal of endogenous glutamate. In contrast, the glutamate transport blocker L-aspartic acid-β-hydroxamate synergistically increased CA responses. These data indicate that in cerebellar granule cells HCA mediates InsP formation wholly by activating NMDA receptor. In contrast, CA stimulates InsP formation by activating both NMDA receptor and metabotropic glutamate receptor, and in part by releasing endogenous glutamate into extracellular milieu.

Key Words: Homocysteic acid, Cysteic acid, Cerebellar granule cell, Primary culture, Inositol phosphates, $[Ca^{2+}]i$, *N*-methyl-D-aspartate (NMDA) receptor, Metabotropic glutamate (mGlu) receptor

INTRODUCTION

Sulfur-containing amino acids (SAAS) have been demonstrated to be heterogenously distributed in the central nervous system (Do et al, 1986; Kilpatrick & Mozley, 1986). SAAs may play important roles in neurotransmission (Zhang & Lipton, 1992; Do et al, 1986; Kilpatrick & Mozley, 1986). SAAs are released from neurons by depolarization and they also stimulate release of other neurotransmitters (Lehman et al, 1988; Dunlop et al, 1989, 1991 and 1992). Once released, SAAs are rapidly transported into neurons and non-neuronal cells (Grieve et al, 1991).

In the central nervous system the excitatory amino acid glutamate is involved in many of the physiological and pathological events through ionotropic and metabotropic excitatory amino acid receptors (Monaghan et al, 1989). SAAs have been demonstrated as endogenous ligands for the *N*-methyl-D-aspartate (NMDA), kainate and D,L-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors of ionotropic glutamate receptors (Santhosh-Kumar et al, 1994; Cuenod et al, 1990). SAAs increase intracellular levels of Ca^{2+} by activating NMDA and/or non-NMDA receptors of dissociated hippocampal neurons, cerebral cortical neurons and retinal ganglion cells (Patneau & Mayer, 1990; Zhang & Lipton, 1992; Frandsen et al, 1993). Moreover, excess amount of SAAs can cause neuronal cell death, possibly by the enormous influx of calcium (Kim et al, 1987; Pullan et al, 1987; Frandsen et al, 1993). In addition to the increase in $[Ca^{2+}]i$, SAAs have also been shown to stimulate phosphoinositide hydrolysis in cerebral cortex and cerebellar granule cells (Porter & Roberts, 1993; Gorman & Griffiths,

Corresponding to: Won-Ki Kim, Department of Pharmacology, College of Medicine, Ewha Womans University, 911-1 Mok-6-dong, Yangchun-ku, Seoul 158-056, Korea (Tel) 82-2- 650-5745, (Fax) 82-2-653-8891 e-mail: Won-K@mm.ewha.ac.kr

1994; Toms et al, 1995). However, different subtypes of glutamate receptors may be involved in some SAAs- mediated phosphoinositide hydrolysis in the neurons.

In the present study we compared differential action of HCA and CA in ionotropic and metabotropic glutamate receptor-mediated inositol phosphate formation and $[Ca^{2+}]i$ rise. We also investigated if release of endogenous glutamate plays a role in HCA- and CA-mediated phosphoinositide hydrolysis.

METHODS

Materials

Tetrodotoxin (TTX), glycine, D,L-homocysteic acid (HCA), L-cysteic acid (CA), *N*-methyl-D-aspartate (NMDA), poly-D-lysine, glutamate, glutamine, glutamate pyruvate transaminase and L-aspartic acid-β -hydroxamate (AβH) were purchased from Sigma Chemical Company (St. Louis, MO). D,L-2-amino-5-phosphonopentanoic acid (APV), 6-cyano-7nitroquinoxaline-2,3-dione (CNQX) and α-methyl-4-carboxyphenylglycine ((±)-MCPG) were obtained from Research Biochemicals International (RBI; Natick, MA). Dulbecco's minimum essential media (DMEM), trypsin, DNAase, Heat-inactivated horse serum, penicillin and streptomycin were purchased from Gibco BRL (Life Technologies, Inc. NY). The acetoxymethyl ester of fura-2 (fura-2/AM) was obtained from Molecular Probes (Eugene, OR). *Myo*-[2-^{3}H] inositol was purchased from DuPont/New England Nuclear (Boston, MA). All other chemicals were obtained from standard commercial sources.

Cell culture

Primary culture of cerebellar granule cells was prepared as described in our previous report (Kim & Pae, 1996). In brief, cerebella from 2- to 4-day old rats (Spragae-Dawley) were freed of meninges and minsed into small pieces in Dulbecco's minimum essential medium (DMEM). Cells were dissociated by 0.1% trypsin/0.05% DNAase-containing DMEM and incubated for 10 min at 37°C. After removal of supernatant, cerebella pieces were washed thrice with DMEM, resuspended, and triturated thoroughly in the growth media (DMEM supplemented with 24.5 mM KCl, 50 mM glucose, 1 μg/ml para-aminobenzoic acid, 10% heat-inactivated fetal calf serum). The dissociated cells were passed through 135 μm nylon-mesh, and preplated onto the culture flask coated with poly-D-lysine (10 μg/ml) for 10 min at 37°C to remove astrocytes. The medium containing the unattached neurons was then passed through two sizes of sterile nylon mesh (80 and then 25 μm). Cells (approximately 1.5 million cells) were plated on poly-D-lysine (100 μg/ml)-coated 12 mm coverslips sitting in 35 mm culture dishes or onto poly-D-lysine (50 μg/ml)-coated 12 well culture plates. After 1 day in culture cells were treated for 24 h with 10 μM cytosine arabinoside and then again after 5 days in order to remove proliferating cells. On average, less than 5% of the cells were immunoreactive for glial fibrillary acidic protein (GFAP), an astrocyte-specific marker. Experiments were carried out using the cells maintained for three to five days following the second cytosine arabinoside treatment.

For the primary culture of non-neuronal cells, prefrontal cortices from 2- to 4-day old rats (Sprague-Dawley) were dissociated by mild trypsinization (0.1% trypsin/0.05% DNAase-containing DMEM) and passed through sterile nylon sieves (80 μm pore size) into DMEM containing 10% heat-inactivated fetal calf serum. Cells were then counted and plated (about 50,000 cells) on poly-D-lysine (2 μg/ml)-coated 12 mm coverslips sitting in 35 mm culture dishes. For non-neuronal cell culture, cells were not treated with cytosine arabinoside. Experiments were carried out using the cells in culture for 10 days.

[^{3}H]inositol phosphates formation

Phosphoinositide metabolites were measured using the methods previously described by Kim et al. (1993). In brief, cells in 12-well culture plates were incubated for 18-24 hr with *myo*-[2-^{3}H] inositol (2 μCi/ml). Cells were washed thrice with Krebs-Henseleit buffer (KRH buffer; 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 6 mM glucose, 1 mM $CaCl_2$, 25 mM $NaHCO_3$, pH 7.4) and then incubated for 15 min at 37°C in KRH buffer containing 10 mM LiCl and 1 mM inositol. Phosphoinositide hydrolysis was measured at 37°C in a final volume of 0.8 ml containing KRH, 10 mM LiCl, 1 mM inositol, and appropriate agonists. All agonists were always coapplied with glycine (1 μM) and TTX (1 μM). The reaction was terminated after 15 min with 500 μl of ice-cold 9% perchloric acid, and cells were incubated on

ice for 15 min. Cells were harvested, sonicated for 2 min, and then centrifuged at 1,000 × g for 10 min. The resulting pellets were solubilized with 0.1 N NaOH and used for the determination of protein content by the method of Lowry et al. (1951). The supernatants were neutralized with a solution containing 0.5 M KOH, 9 mM $Na_2B_4O_7$ and 1.9 mM EDTA, and then the [^{3}H]InsP were separated by anion exchange chromatography on Dowex columns. Briefly, the neutralized supernatants were added to 0.75-ml columns of Dowex 1-X8 resin (100~200 mesh, formate form; Bio-Rad). Inositol monophosphate (InsP1)was eluted with 0.2 M ammonium formate plus 0.1 M formic acid. The more polar inositol 4,5-bisphosphate (InsP2) and inositol 1,4,5-trisphosphate (InsP3) were eluted with 0.4 M ammonium formate plus 0.1 M formic acid and 1 M ammonium formate plus 0.1 M formic acid, respectively. Radioactivity in each fraction was determined by liquid scintillation spectrophotometry. Data were expressed as dpm/mg protein.

[Ca^{2+}]i measurement

Neuronal [Ca^{2+}]i was analyzed with fura-2 acetoxymethyl ester (fura-2/AM; Molecular Probes) using a protocol for digital Ca^{2+} imaging that has been detailed previously (Kim et al, 1994). In brief, cells on the 12-mm glass coverslips were rinsed twice with KRH buffer and were loaded with 10 μM fura- 2/AM for 30 min at 37°C. Cells were washed thrice with KRH buffer and were left in KRH for 20 min at room temperature before measuring [Ca^{2+}]i. Reagents were administered by superfusion for 8 s using pneumatic puffer pipettes. All sulfur-containing amino acids and NMDA were always coapplied with glycine (1 μM) and TTX (1 μM). Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 and 380 nm were determined at room temperature using an image processing system (Quantex QX7-210, Sunnyvale, CA) interfaced to an IBM personal computer. [Ca^{2+}]i was determined as described by Grynkiewicz et al (1985).

Statistical analysis

Data are presented as mean±standard error of the mean (s.e.m.) and were analyzed for statistical significance using a paired-*t* test or analysis of variance [ANOVA] and Dunnett's multiple comparison test. Concentration-response relationships were analyzed

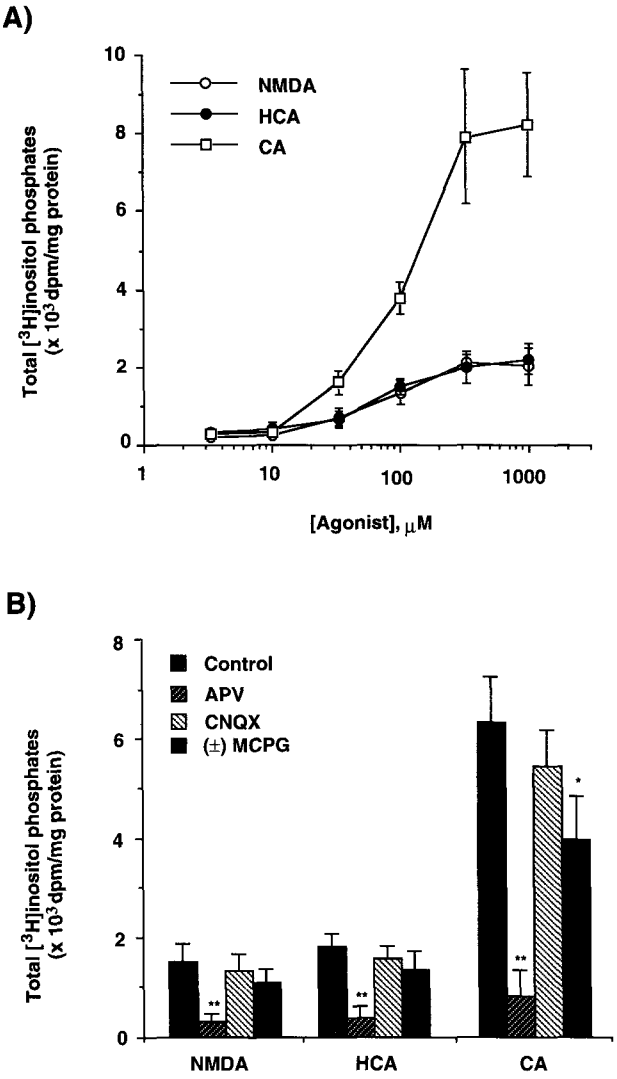

Fig. 1. HCA- and CA-mediated InsP formation.
A) Concentration-response relationships of SAAs-mediated inositol phosphate formation. [^{3}H]InsP formation was quantified in the presence of various concentrations of HCA, CA and NMDA. Data are representative of 4 separate experiments and are expressed as mean±s.e.m. **B)** Effect of ionotropic and metabotropic glutamate receptor antagonists on SAAs-mediated inositol phosphate formation. [^{3}H]InsP formation evoked by HCA, CA, and NMDA (each 333 μM) was quantified in the absence and presence of the NMDA receptor antagonist APV (200 μM), the AMPA/kainate receptor antagonist CNQX (10 μM) and the metabotropic excitatory amino acid antagonist (±)-MCPG (1 mM). Data are expressed as the sum of InsP1, InsP2, and InsP3 and are plotted as mean±s.e.m. from 8 separate experiments. *P<0.05; **P<0.01, compared with the responses evoked by agonist alone.

using a standard set of pharmacologic programs (Tallarida & Murray, 1981).

RESULTS

Effects on phosphoinositide hydrolysis

D,L-homocysteic acid (HCA) and L-cysteic acid (CA) stimulated the formation of [^{3}H]inositol phosphates (InsP) in a concentration-dependent manner in rat cerebellar granule cells (Fig. 1A). The concentrations of drugs resulting in half-maximal stimulation (EC50 values) for the formation of InsP by HCA and CA were 87±14.2 μM and 108±21.3 μM, respectively. However, HCA and CA did not significantly stimulate the InsP formation in nonneuronal cells (data not shown). CA was more efficacious in stimulating phosphoinositide hydrolysis than HCA and NMDA; CA (300 μM) increased the formation of InsP (as sum of InsP1, InsP2 and InsP3) 8.2±0.72-fold higher than basal level. HCA (300 μM) and NMDA (300 μM) elicited less than a 2.3-fold increase in InsP formation.

The possible contribution of the NMDA receptor-ion channel complex to the agonist-stimulated InsP formation was studied using the selective NMDA receptor antagonist APV. HCA-, CA- and NMDA-mediated formation of InsP were blocked 80±7.7%, 86±5.1% and 79±8.9%, respectively, by APV (200 μM) (Fig. 1B). The AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) did not alter the HCA- and CA-mediated InsP formation (data not shown). The involvement of metabotropic glutamate receptors in the agonist-stimulated InsP formation was further quantified using the selective metabotropic glutamate receptor antagonist (±)-MCPG. CA-mediated formation of InsP was blocked 378.2% by (±)-MCPG (1 mM) (Fig. 1B). However, the HCA-, and NMDA-mediated InsP formation was not significantly blocked by (±)-MCPG.

Effects on [Ca^{2+}]i

The effects of agonists on [Ca^{2+}]i were measured fluorometrically using fura-2/AM. For these experiments the Na$^+$ channel blocker TTX was included to prevent depolarization-induced influx of calcium. Because glycine (1 μM) increases potency and efficacy of NMDA in rising [Ca^{2+}]i, this compound was also included in the measurement. HCA, CA and NMDA caused concentration-dependent increases in [Ca^{2+}]i (Fig. 2A). Although it stimulated PI hydrolysis more efficaciously than HCA and NMDA as shown in Fig.

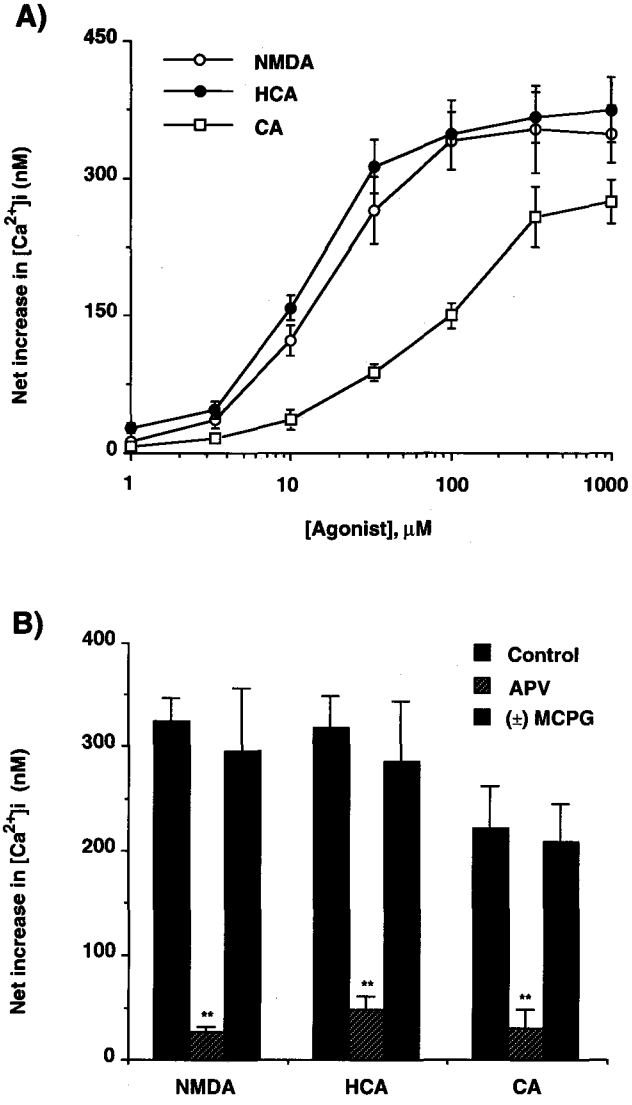

Fig. 2. HCA- and CA-mediated rises in [Ca^{2+}]i.
A) Concentration-response relationships of SAAs-mediated rises in [Ca^{2+}]i. [Ca^{2+}]i rises were measured in the presence of various concentrations of HCA, CA and NMDA. Data are representative of 4 separate experiments and are expressed as mean standard error. **B)** Effect of ionotropic and metabotropic glutamate receptor antagonists on HCA- and CA-mediated calcium responses. [Ca^{2+}]i rises evoked by HCA, CA and NMDA (each 333 μM) were quantified in the absence and presence of APV (200 μM) and (±)-MCPG (1 mM). Data are expressed as mean s.e.m. from 7 separate experiments. **P<0.01, compared with the responses evoked by agonist alone.

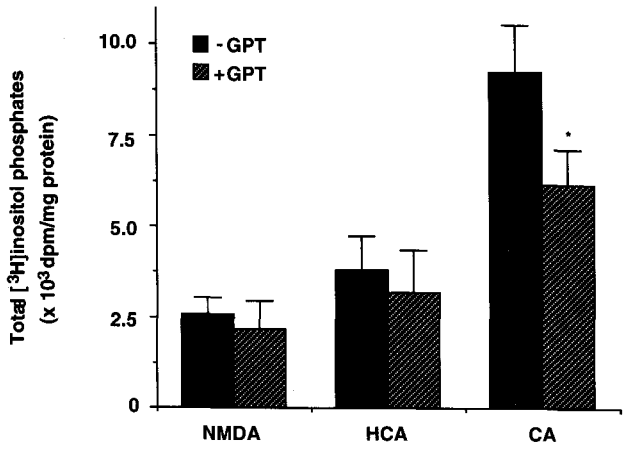

Fig. 3. Removal of endogenous glutamate by glutamate pyruvate transaminase (GPT) prevented CA-, but not HCA- and NMDA-, mediated InsP formation.
As shown in our previous report (Kim and Pae, 1996), cells were thoroughly washed at least 5 times (5 min each) with the glutamine-free growth media and incubated overnight with the media supplemented with 5 mM pyruvate and then 10 U/ml of GPT. Cells were stimulated with HCA, CA and NMDA in the presence of 5 mM pyruvate and 10 U/ml of GPT. Data are expressed as mean s.e.m. from 7 separate experiments. *P<0.05, compared with the response evoked by CA in the absence of GPT.

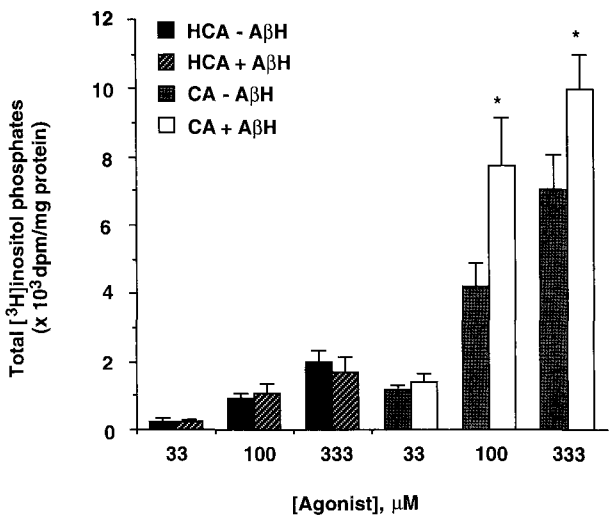

Fig. 4. Effect of the glutamate transport blocker L-aspartic acid-β-hydroxamate on HCA- and CA-mediated InsP formation.
Cells were incubated with various concentrations of HCA and CA in the absence and presence of 1 mM AβH. Data are expressed as mean s.e.m. from 5 separate experiments. *P<0.05, compared with the response evoked by CA in the absence of AβH.

1, however, CA was less potent than HCA in increasing $[Ca^{2+}]i$: the EC50 values for the increase of $[Ca^{2+}]i$ by HCA and CA were 14±5.2 μM and 97±31.2 μM, respectively. Both HCA (333 μM)- and CA (333 μM)-stimulated calcium responses were completely blocked 86±8.1% and 85±7.7%, respectively, by the selective NMDA receptor antagonist APV (Fig. 2B). However, HCA- and CA- mediated increases in $[Ca^{2+}]i$ were not altered by the metabotropic glutamate receptor antagonist (±)-MCPG (1 mM).

Role of endogenous glutamate

Agonist-induced InsP formation could be mediated by the release of endogenous glutamate. To test this hypothesis, endogenous glutamate was removed by treatment with glutamate pyruvate transaminase (GPT), which catalyzes transamination of glutamate into α-ketoglutarate in the presence of pyruvate. The enzyme treatment has been shown to suppress completely the level of extracellular glutamate released by cerebellar cultures after a K^+ depolarization (Didier et al, 1993). Under this condition, CA-, but not HCA- and NMDA-, mediated InsP formation was decreased by 33±8.2% (Fig. 3). The basal InsP formation was not altered by GPT (data not shown).

To further investigate the role of release of glutamate in HCA- and CA-mediated InsP formation, cells were incubated with HCA and CA in the presence of the glutamate transport inhibitor L-aspartic acid-β-hydroxamate (AβH). AβH (1 mM) enhanced CA-mediated InsP formation by 84±13.6% (for 100 μM CA) and 42.5±11.3% (for 333 μM CA) (Fig. 4). The basal InsP formation was not altered by AβH (data not shown).

DISCUSSION

Homocysteic acid (HCA) appeared to activate a NMDA receptor subtype in cerebellar granule cells to increase InsP formation. Thus, HCA-mediated increases in InsP formation and $[Ca^{2+}]i$ were completely blocked by the NMDA receptor antagonist APV and not by the metabotropic antagonist (±)-MCPG. The kainate/AMPA receptor antagonist CNQX did not alter the HCA-mediated InsP formation. A previous report also demonstrated that HCA activated the

NMDA receptor subtype of rat retinal ganglion cells and did not activate kainate-like currents (Zhang & Lipton, 1992). However, other patch-clamp studies of dissociated hippocampal neurons and cerebellar Purkinje cells have revealed that high concentrations of HCA activated both NMDA and non-NMDA receptors (Lee et al, 1988; Patneau & Mayer, 1990). Therefore, non-NMDA receptors in different regions of the CNS are thought to have different characteristics in their preference of HCA.

Ca^{2+} influx has been reported to activate directly a variety of phospholipase C (PLC) isozymes. In cultured cortical neurons, the increase in PLC-δ immunoreactivity was prevented by removal of extracellular Ca^{2+} or the application of an NMDA receptor antagonist MK-801 (Shimohama et al, 1995). Calcium stimulated InsP formation in guinea-pig synaptosomes (Griffin & Hawthorne, 1978) and rat cerebral membranes (Narang et al, 1996). Calcium also activated PLC in non-neuronal cells such as rat vascular smooth muscle cells (Griendling et al, 1991) and human erythrocytes (Downes & Michell, 1982). Thus, HCA may regulate the activity of PLC by this mechanism.

CA was also found to activate the NMDA receptor subtype. Dissimilar to HCA, however, CA seems to activate both ionotropic (NMDA) and metabotropic glutamate receptor subtypes. Thus, CA-mediated formation of inositol phosphates was prevented by APV as well as by (±)-MCPG. The CA-mediated InsP formation was not further inhibited by (±)-MCPG (2 and 4 mM), probably due to the remaining calcium-dependent InsP formation through the NMDA receptor. Maximal effect of CA on InsP formation was found to be much greater than that of HCA. This difference might be due to simultaneous stimulation of ionotropic and metabotropic glutamate receptor subtypes by CA. In agreement with our result, Gorman and Griffiths (1994) reported that the metabotropic glutamate receptor agonist trans-1-aminocyclopantane-1,3-dicarboxylic acid (*trans*-ACPD) alone weakly stimulated the formation of InsP and the combination of HCA and *trans*-ACPD resulted in a stimulation of InsP formation comparable to that observed with CA alone. It implies that simultaneous stimulation of ionotropic and metabotropic glutamate receptor subtypes leads to a synergistic activation of phospholipase C. Coactivation of ionotropic and metabotropic glutamate receptors has been shown to be involved in various neuronal functions. For example, the activation of metabotropic glutamate receptor by *trans*-ACPD produces a potent NMDA receptor-mediated transmission at the synapse of mossy fiber and granule cell (Kinney & Slater, 1993). The induction of long-term potentiation in the CA1 region of the hippocampus (Musgrave et al, 1993) and in the granule cells of the dentate gyrus (O'Connor et al, 1995) also require coactivation of metabotropic and ionotropic glutamate receptors. Thus, certain SAAs-mediated coactivation of ionotropic and metabotropic glutamate receptors may play important roles in the synaptic transmission in the CNS. Moreover, CA has a unknown additional, not through the activation of metabotropic glutamate receptor, mechanism for the synergistic enhancement of the Ca^{2+} influx through the NMA receptor-ion channel complex. This conclusion was deducted by the finding that CA-mediated rise in intracellular calcium level was not so much inhibited by (±)-MCPG as by APV.

In addition to receptor-mediated actions, CA appears to increase the formation of InsP at least in part by releasing endogenous glutamate into media. Thus, CA-mediated increases in InsP formation were decreased by removing endogenous glutamate and increased by blocking the glutamate transport. Certain SAAs have been shown to stimulate neurotransmitter release in a Ca^{2+}-dependent manner in the absence of other depolarizing agents (Dunlop et al, 1989). Although the physiological relevance remains uncertain, SAAs have also been shown to stimulate non-vesicular release from cytosol in a Ca^{2+}- independent manner (Dunlop et al, 1992). The cellular mechanisms involved in the glutamate release are unknown. However, it is possible that CA mediates the release of endogenous glutamate by receptor-mediated, depolarization-induced reversal of the cellular glutamate transport (Dunlop et al, 1991) and by presynaptic action inducing a vesicle release (Herrero et al, 1994; Brandstatter et al, 1996). Our finding that the glutamate transport blocker L-aspartic acid-β-hydroxamate enhances CA-mediated InsP formation appears to be in agreement with the previous report that the blocker increased some SAAs (e.g., CA and cysteine sulphinic acid (CSA))-mediated neurotoxicity in cultured cerebral cortical neurons (Frandsen et al, 1993).

In summary, the present data indicate that HCA stimulates InsP formation and $[Ca^{2+}]i$ increase wholly by activating NMDA receptor. In contrast, CA synergistically increases InsP formation by activating both NMDA receptor and metabotrophic glutamate

receptor. In addition to its direct action on receptors, CA evokes InsP formation indirectly by releasing endogenous glutamate. These findings provide evidence to support a role for acidic SAAs in excitatory neurotransmission.

REFERENCES

Brandstatter JH, Koulen P, Kuhn R, van der Putten H, Wassle H. Compartmental localization of a metabotropic glutamate receptor (mGluR7): two different active sites at a retinal synapse. *J Neurosci* 16: 4749−4756, 1996

Cuenod M, Do KQ, Streit P. Homocysteic acid as an endogenous excitatory amino acid. *Trends Pharmacol Sci* 11: 477−478, 1990

Didier M, Heaulme M, Gonalons N, Soubrie P, Bockaert J, Pin JP. 35 mM K^+-stimulated $^{45}Ca^{2+}$ uptake in cerebellar granule cell cultures mainly results from NMDA receptor activation. *Eur J Pharmacol* 244: 57−65, 1993

Do KQ, Mattenberger M, Streit P, Cuenod M. In vitro release of endogenous excitatory sulfur-containing amino acids from various rat brain region. *J Neurochem* 46: 779−786, 1986

Downes CP, Michell RH. The control by Ca^{2+} of the polyphosphoinositide phosphodiesterase and the Ca^{2+} pump ATPase in human erythrocytes. *Biochem J* 202: 53−58, 1982

Dunlop J, Grieve A, Damgaard I, Schousboe A, Griffiths R. Sulphur-containing excitatory amino acid-evoked Ca^{2+}-independent release of D-[^{3}H]aspartate from cultured cerebellar granule cells: The role of glutamate receptor activation coupled to reversal of the acidic amino acid plasma membrane carrier. *Neurosci* 50: 107−115, 1992

Dunlop J, Grieve A, Schousboe A, Griffiths R. Neuroactive sulphur amino acids evoke a calcium dependent transmitter release from cultured neurons that is sensitive to excitatory amino acid receptor antagonists. *J Neurochem* 52: 1648−1651, 1989

Dunlop J, Grieve A, Schousboe A, Griffiths R. Stimulation of [^{3}H]GABA release from cultured mouse cerebral cortex neurons by sulphur-containing excitatory amino acid transmitter candidates : Receptor activation mediates two distinct mechanisms of release. *J Neurochem* 57: 1388−1397, 1991

Frandsen A, Schousboe A, Griffiths R. Cytotoxic actions and effects on intracellular Ca^{2+} and cGMP concentrations of sulfur-containing excitatory amino acids in cultured cerebral cortical neurons. *J Neurosci Res* 34: 331−339, 1993

Herrero I, Miras-Portugal MT, and Sanchez-Prieto J. Rapid desensitization of the metabotropic glutamate receptor that facilitates glutamate release in rat cerebrocortical nerve terminals. *Eur J Neurosci* 6: 115−120, 1994

Gorman A, Griffiths R. Sulphur-containing excitatory amino acid-stimulated inositol phosphate formation in primary cultures of cerebellar granule cells is mediated predominantly by *N*-methyl-D-aspartate receptors. *Neurosci* 59: 299−308, 1994

Griendling KK, Taubman MB, Akers M, Mendlowitz M, Alexander RW. Characterization of phosphatidylinositol-specific phospholipase C from cultured vascular smooth muscle cells. *J Biol Chem* 266: 15498−15504, 1991

Grieve A, Dunlop J, Schousboe A, Griffiths R. Kinetic characterisation of excitatory sulphur amino acid transport in synaptosomes and in primary cultures of different brain cells. *Biochem Soc Trans* 19: 5S, 1991

Griffin HD, Hawthorne JN. Calcium-activated hydrolysis of phosphatidyl-myo-inositol 4phosphate and phosphatidyl-myo-inostol 4,5-bisphosphate in guinea-pig synaptosomes. *Biochem J* 176: 541−552, 1978

Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440−3450, 1985

Kilpatrick IC, Mozley LS. An initial analysis of the regional distribution of excitatory sulphur containing amino acids in the rat brain. *Neurosci Lett* 72: 189−193, 1986

Kim JP, Koh JY, Choi DW. L-homocysteate is a potent neurotoxin on cultured cortical neurons. *Brain Res* 437: 103−110, 1987

Kim W-K, Hawthom M, Rabin RA. Differential effects of chronic ethanol exposure on ATPand Bradykinin-induced increases in intracellular calcium levels in PC 12 cells. *Mol Pharmacol* 44: 405−411, 1993

Kim W-K, Johnson RG, Izu LT, Rabin RA. Chronic ethanol exposure inhibits ATP-stimulated but not KCl-stimulated dopamine release in PC 12 cells. *J Pharmacol Exp Ther* 270: 336−341, 1994

Kim W-K, Pae Y-S. Involvement of N-methyl-D-aspartate receptor and free radical in homocysteine-mediated toxicity on rat cerebellar granule cells in culture. *Neurosci Lett* 216: 117−12, 1996a

Kinney GA, Slater NT. Potentiation of NMDA receptor-mediated transmission in turtle cerebellar granule cells by activation of metabotropic glutamate receptors. *J Neurophysiol* 69: 585−594, 1993

Lee M, Strahlendorf HK, Strahlendorf JC. Differential effects of N-methyl-D-aspartic acid and L-homocysteic acid on cerebellar Purkinje neurons. *Brain Res* 456: 104−112, 1988

Lehmann J, Tsai C, Wood PL. Homocysteic acid as a

putative excitatory amino acid neurotransmitter: I. Postsynaptic characteristics at N-methyl-D-aspartate receptors on striatal cholinergic interneurons. *J Neurochem* 51: 1765−1770, 1988

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. *J Biol Chem* 193: 265−275, 1951

Monaghan DT, Bridges RJ, and Cotman CW. The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. *Ann Rev Pharmacol Toxicol* 29: 365−402, 1989

Musgrave MA, Ballyk BA, Goh JW. Coactivation of metabotropic and NMDA receptors is required for LTP induction. *Neuroreport* 4: 171−174, 1993

Narang N, Joseph JA, Ayyagari PV, Gerber M, Crews FT. Age-related loss of cholinergic-muscarinic coupling to PLC: Comparison with changes in brain regional PLC subtypes mRNA distribution. *Brain Res* 708: 143−152, 1996

O'Connor JJ, Rowan MJ, Anwyl R. Tetanically induced LTP involves a similar increase in the AMPA and NMDA receptor components of the excitatory postsynaptic current: investigations of the involvement of metabotropic glutamate receptors. *J Neurosci* 15: 2013−2020, 1995

Patneau DK, Mayer ML. Structure-activity relationships for amino acid transmitter candidates acting at N-methyl-D-aspartate and quisqualate receptors. *J Neurosci* 10: 2385−2399, 1990

Porter RHP, Roberts PJ. Glutamate metabotropic receptor activation in neonatal rat cerebral cortex by sulphur-containing excitatory amino acids. *Neurosci Lett* 154: 78−80, 1993

Pullan LM, Olney JW, Price MT, Compton RP, Hood WT, Michle J, Monahan JB. Excitatory amino acid receptor potency and subclass specificity of sulphur containing amino acids. *J Neurochem* 49: 1301−1307, 1987

Santhosh-Kumar CR, Hassell KL, Deutsche JC, Kolhouse JF. Are neuropscychiatric manifestations of folate, cobalamin and pyridoxine deficiency mediated through imbalances in excitatory sulfur amino acids? *Medical Hypotheses* 43: 239−244, 1994

Shimohama S, Akaike A, TAmura Y, Matsushima H, Kume T, Fujimoto S, Takenawa T, Kimura J. Glutamate-induced antigenic changes of phospholipase C-delta in cultured cortical neurons. *J Neurosci Res* 41: 418−426, 1995

Tallarida RJ, Murray RB. *Manual of Phannacologic Calculations,* Springer-Verlag, New york, 1981

Toms NJ, Jane DE, Tse H-W, Roberts PJ. Characterization of metabotropic glutamate receptor-stimulated phosphoinositide hydrlysis in rat cultured cerebellar granule cells. *Br J Pharmacol* 16: 2824−2827, 1995

Zhang D, Lipton SA. L-homocysteic acid selectively activates N-methyl-D-aspartate receptors of rat retinal ganglion cells. *Neurosci Lett* 136: 173−177, 1992