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Glutamate-and NMDA-induced calcium influx at synaptosomes and the difference of their actions

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ABSTRACT

Glutamate and aspartate may evoke an increase in membrane permeability to monovalent cations and Ca^{++}. However, it is uncertain whether Ca^{++} influx is mediated by voltage dependent Ca^{++} channels or by excitatory amino acid activated channels. In addition, the influences of excitatory amino acids on Ca^{++} uptake by neuronal tissues as well as the responses of their actions to extracellular Mg^{++} concentration are different.

K^+ induced Ca^{++} uptake by synaptosomes was dependent on extracellular Mg^{++} up to 5 mM and at concentration of 10 mM, Ca^{++} influx was rather reduced. In Na^+ rich media, glutamate-and aspartate-induced Ca^{++} uptake was increased by Mg^{++} in a dose independent manner. However, the response for NMDA was inhibited by Mg^{++} at concentrations above 2 mM. K^+-and glutamate-induced Ca^{++} influxs were inhibited by 2, 4-dinitrophenol, chlorpromazine and verapamil but not by tetraethylammonium chloride. Tetrodotoxin effectively inhibited the action of glutamate but did not affect that of K^+. The response for MNDA was inhibited by 2, 4-dinitrophenol and tetrodotoxin, slightly inhibited by verapamil, and not affected by tetraethylammonium chloride. In Na^+ rich medium, depolarizing action of glutamate, aspartate and MNDA on synaptosomes was not demonstrated, whereas these agents stimulated Ca^{++} uptake and caused Ca^{++} influx induced depolarization at mitochondria. On the other hand, the activities of synaptosomal ATPases were not affected by excitatory amino acids at 5 mM. The results suggest that glutamate or NMDA induced Ca^{++} influx at synaptosomes exhibits different responses for extracellular Mg^{++}. Excitatory amino acids induced Ca^{++} influx at synaptosomes may be associated with increased permeability of membrane for Na^+ and Ca^{++} except K^+ and membrane depolarization due to increased ionic permeability.

Key Words: Glutamate, NMDA, Aspartate, Ca^{++} influx at Synaptosomes

INTRODUCTION

Glutamate may be the principal neurotransmitter that mediate fast excitatory synaptic transmission in the vertebrate central nervous system (Watkins and Evans, 1981). It is suggested that glutamate not only has direct excitatory effects on neurones intrinsic to the striatum, but also stimulates the release of dopamine presumably at presynaptic receptor sites (Giorguieff *et al.*, 1977; Nieoullon *et al.*, 1978; Roberts and Anderson, 1979).

Glutamate seems to activate two or three distinct types of receptors, namely, N-methyl-D-aspartate (NMDA), quisqualate and kainate (Watkins and Evans, 1981; Ishida and Neyton, 1985). All these agonists increase membrane permeability to monovalent cations (Hablitz and Langmoen, 1982; Crunelli *et al.*, 1984), but NMDA also activates a conductance that per-

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mits significant calcium influx (MacDermott *et al.*, 1986) which is blocked in a voltage dependent manner by extracellular magnesium (Mayer *et al.*, 1984).

The excitatory action of glutamate upon neurones is shown to be accompanied by a considerable increase of aK^+e in the spinal cord of the frog (Sonnhof *et al.*, 1975) and in the cerebral cortex of the cat (Heinemann and Pumain, 1980). This increase of aK^+e is sufficient to depolarize cells in other neurone, causing them to release transmitters.

It is established that depolarization of the plasma membranes of synaptosomes which retain considerably functional integrity with either veratridine or high external K^+ leads to an enhanced uptake of Ca^{++} and consequent transmitter release (Blaustein, 1975). Since Ca^{++} uptake induced by K^+ depolarization is unaffected by tetrodotoxin, it appears that this Ca^{++} uptake dose not occur through the Na^+ channel (Nachshen and Blasustein, 1980).

Many of the neurotransmitters, hormones and drugs may modulate the efficiency of neurotransmitter release by modifying the permeability for Ca^{++} channels through their effects on cAMP-dependent protein kinases and subsequent phosphorylation in specific proteins at the plasma membrane (Reuter, 1983).

On the other hand, recently it is reported that exposure of mammalian brain cells to glutamate or its analogues causes enhanced hydrolysis of inositol phospholipids (Nicoletti *et al.*, 1986) and its metabolite, inositol triphosphate, triggers intracellular Ca^{++} mobilization (Michell, 1975).

Although it is well known that glutamate stimulates Ca^{++} uptake in brain slices (Ichida *et al.*, 1982) or in synaptosomes (Retz *et al.*, 1982), the mechanism of action involved in this effect is uncertain. The possibility that the stimulatory effect of glutamate on Ca^{++} uptake could be due to the effect of cell swelling is suggested (Terner *et al.*, 1950). Since electrophoretic administration of EDTA sometimes induces generalized excitation, chelating effect of glutamate is also proposed (Krnjević, 1970) as the mechanism. On the other hand, the synaptosomal accumulation of Ca^{++} induced by glutamate may result from decreased Ca^{++} efflux rather than from increased Ca^{++} influx. Furthermore, the different responses of glutamate of K^+ induced Ca^{++} uptake for tetrodotoxin is suggested (Retz and Coyle, 1984). Thus, the action of glutamate on Ca^{++} transport system at the plasma membrane is obscure.

In the present study, effects of glutamate, aspartate and NMDA on action sites which regulate Ca^{++} influx at synaptosomes were investigated. For this purpose, effects of inhibitors of membrane phosphorylation, calmodulin inhibitor and specific ionic channel blockers on the actions of exitatory amino acids were studied. influences of these agents on the membrane potential and activities of synaptosomal ATPases such as Na^+-K^+ ATPase and Ca^{++}, Mg^{++} ATPase were also examined.

MATERIALS AND METHODS

Chemicals

Glutamic acid, N-methyl-D-aspartic acid (NMDA), arsenic acid, carbonyl cyanide m-chlorophenylhydrazone (CCCP), chlorpromazine, tetrodotoxin, tetraethylammonium chloride, lanthanum chloride, tris-ATP, choline chloride and arsenazo III were purchased from Sigma Chemical Co.. Verapamil was obtained from Knoll AG; 2, 4-dinitrophenol, aspartic acid, $MgCl_2$ and KCl from E. Merck; $CaCl_2$ and NaCl from Kanto Chemical Co.; safranine o from Junsei Chemical Co.. Other chemicals were of anlaytical reagent grade.

Preparation of rat cerebral synaptosomes

Synaptosomal particles were prepared from rat cerebrum by a modification of the method of Gray and Whittaker (1962). Male Sprague-Dawley rats weighing about 150 g were used. The animals were killed by decapitation. After removing blood clot, the brains were placed in 9 volume of cold 0.32 M sucrose and homogenized with a teflon glass homogenizer. A crude nuclear fraction was removed by centrifugation at 1,000 g for 15 min and the supernatant was transferred and centrifuged at 12,000 g for 60 min. The resulting residue was resuspended in cold 0.32 M sucrose and homogenized with a teflon glass homogenizer. Seven ml of suspension in 0.32 M sucrose (contained about 2 mg protein) was applied into a discontineous den-

sity gradient consisting of 13 ml layer of 0.8 M sucrose and 13 ml layer of 1.2 M sucrose. After centrifugation in BECKMAN L5-50B-ultracentrifuge using fixed 42.1 Ti rotor at 50, ultracentrifuge using fixed 42.1 Ti rotor at 50,000 g for 2 hr, a synaptosomal fraction was obtained at the interface of the 0.8-1.2 M sucrose layer. The synaptosomal band was crose and centrifuged at 15,000 g for 15 min. The synaptosomal pellet was resuspended in small volume of 0.32 M sucrose and protein concentration was determined by the method of Lowry et al. (1951).

In order to measure Ca^{++} uptake and release by synaptosomes, the synaptosomal band was diluted with 1.2 volume of distilled water and centrifuged at 15,000 g for 15 min. The synaptosomal pellet was resuspended in preincubation medium for Ca^{++} transport study (124 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$ and 20 mM Tris HCl, pH 7.4).

Measurement of Ca^{++} uptake by synpatosomes

The rate and extent of Ca^{++} uptake by and release from synaptosomes were measured through the absorbance changes of calcium sensitive dye, arsenazo III, at 675-685 nm with a dual wavelength-split beam spectrophotometer (Aminco Chance dual wavelength-split beam recording spectrophotometer, American Instrument Co., U.S.A.) (Ahmed and Connor, 1979; Akerman and Heinonen, 1983). Measurement of Ca^{++} uptake by synaptosomes was done in the reaction mixture consisting of 0.75 mg protein/ml synaptosomal particles, 130 mM KCl (or 124 mM NaCl), 1.3 mM $MgCl_2$, 20 mM Tris-maleate, pH 7.4, 100 μM arsenazo III and other compounds. After 10 min preincubation at 30℃, 20 μl of 4.5 mM $CaCl_2$ (final 30 μM) was added to above reaction medium and then absorbance change was measured for 1 min. Finally, Ca^{++} uptake by synaptosomes was initiated by 20 μl of 150 mM tris-ATP (final 1 mM) in a 3 ml of total reaction volume and the concentration of Ca^{++} was determined.

Measurement of membrane potential

The membrane potential was measured with the safranine method (Akerman and Wikaström 1976; Akerman, 1978). The spectrum of safranine changes in a way typical of stacking upon induction of potentials across the membranes of liposomes or mitochondria. The spectral shift was determined with Aminco Chance dual wavelength-split beam spectrophotmeter at 484-524 nm in the same rection medium as in Ca^{++} uptake measuring medium except that it contained 50 μM safranine o.

Measurement of ATPase activity

Synaptosomal particles (0.1 mg/ml) were incubated in the reaction medium containing 50 mM imidazole-HCl, pH 7.4; 150 mM NaCl, 5 mM $MgCl_2$ and 10 mM KCl for Na^+-K^+, Mg^{++} ATPase; 5 mM $MgCl_2$ and 1 mM ouabain for Mg^{++} ATPase; 2 mM $CaCl_2$, 5 mM $MgCl_2$ and 1 mM ouabain for Ca^{++}-Mg^{++} ATPase. Isoosmolarity of the reaction medium was adjusted with choline chloride. After 5 min preincubation at 37℃, the reaction was initiated by the addition of 0.1 ml of 20 mM tris-ATP (final 2 mM) and the total volume was 1.0 ml. After 10 min incubation at 37℃, the reaction was stopped with 1.0 ml of 15% cold trichloroacetic acid and the mixture was centrifuged at 1,000 g for 10 min. One ml of the supernatant was used for determination of Pi according to the method of Horwitt (1952). ATPase activity was expressed in μmoles Pi liberated per mg protein per hr.

RESULTS

Effects of Mg^{++} on the excitatory amino acid induced Ca^{++} uptake

Mg^{++} have been shown to antagonize the depolarizing action of NMDA on frog motoneurones (Evans *et al.*, 1977; Ault *et al.*, 1980). NMDA induced Ca^{++} influx is inhibited by extracellular Mg^{++} (MacDermott *et al.*, 1986). the response of glutamate for Mg^{++} is more complex because this agonist activates both NMDA receptors and other amino acid receptors linked to a voltage insensitive mechanism (Mayer and Westbrook, 1984). Furthermore, effects of excitatory amino acids on Ca^{++} uptake by striatal slices from brain are variable (Retz *et al.*, 1984). Thus, effects of Mg^{++} on K^+-and excitatory amino acid-induced Ca^{++} uptake by synaptosomes were investigated.

Fig. 1 showed that K^+ induced Ca^{++} uptake

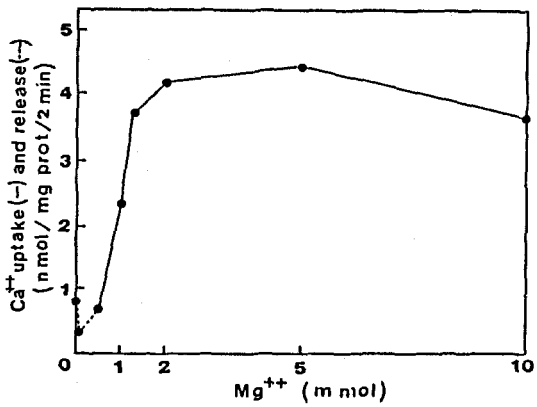

Fig. 1. K^+ induced Ca^{++} uptake in the presence of Mg^{++}, Ca^{++} transport at synaptosomes was spectrophotometrically measured through the absorbance changes of Ca^{++} sensitive dye, arsenazo III, at 675–685 nm. The reaction mixture contained 0.75 mg/ml of synaptosomes, varing concentration of $MgCl_2$, 130 mM KCl, 5 mM NaCl, 30 μM $CaCl_2$, 100 μM arsenazo III and 20 mM Tris-maleate, pH 7.4. Each point represents an average of 5 experiments.

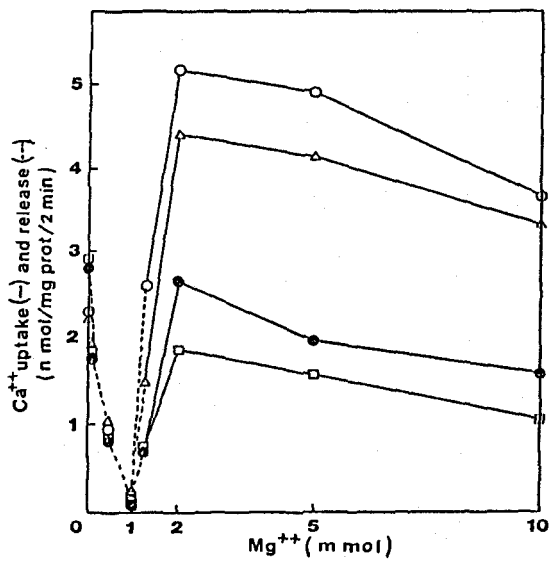

Fig. 2. Effect of Mg^{++} on excitatory amino acids induced Ca^{++} uptake in 96 mM NaCl and 44 mM KCl containing medium. Each point represents an average of 4–6 experiments. Concentration of excitatory amino acid is 5 mM.
●, no addition ; ○, glutamate ; △, aspartate ; □, NMDA.

by synaptosomes was stimulated by Mg^{++} at the concentration range of 0.5 mM-5.0 mM and at 10 mM of Mg^{++}, Ca^{++} influx was reduced rather than increased. On the other hand, at Mg^{++} concentrations below 0.5 mM the release of Ca^{++} from synaptosomes was observed.

To explore the difference between response of K^+ induced Ca^{++} uptake for Mg^{++} and that of excitatory amino acid induced Ca^{++} uptake for Mg^{++}, effects of Mg^{++} on glutamate, aspartate and NMDA induced Ca^{++} uptakes were observed in Na^+-rich medium. In reaction medium containing 44 mM KCl, 96 mM NaCl and $MgCl_2$ less than 1.0 mM, Ca^{++} release rather than uptake was observed. Accordingly, the presence of Mg^{++} may be required for glutamate or aspartate induced Ca^{++} uptake, however, these stimulatory effects were reduced by high concentration (10 mM) of Mg^{++}. Effect of NMDA was inhibited by Mg^{++} at concentrations above 2 mM (Fig. 2).

Similar findings were also investigated in Na^+-rich (124 mM) medium. Fig. 3 illustrated that NMDA induced response was significantly inhibited by Mg^{++}. Thus, it is suggested that Ca^{++} concentration.

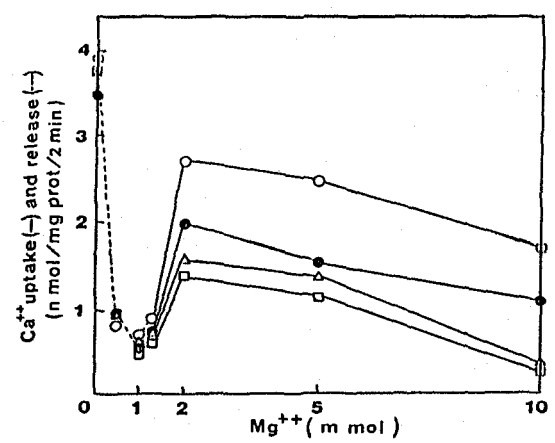

Fig. 3. Effect of Mg^{++} on excitatory amino acids induced Ca^{++} uptake in 132 mM NaCl and 5 mM KCl containing medium. Each point represents an average of 4–6 experiments. Concentration of excitatory amino acid is 5 mM.
●, no addition ; ○, glutamate ; △, aspartate ; □, NMDA.

Effects of inhibitors of phosphorylation and ion channel blockers on K^+ induced Ca^{++} uptake

Changes of ion permeability of synaptosomes retaining functional integrity during K^+ induced depolarization are relatively not clarified. Thus, character of Ca^{++} transport in K^+ induced depolarization was examined.

As can be seen in Table 1, K^+ induced Ca^{++} uptake was remarkably inhibited by inhibitors of protein phosphorylation, such as 2,4-dinitrophenol, arsenate and CCCP. Therefore, these results suggest that phosphorylation of protein at the plasma membrane opens K^+ activated Ca^{++} channels. K^+ induced Ca^{++} uptake was inhibited by a calmodulin inhibitor, chlorpromazine and Ca^{++} channel blocker, La^{+++} and verapamil but not by Na^+ channel blocker, La^{+++} and verapamil but not by Na^+ channel blocker tetrodotoxin and K^+ channel blocker, tetraethylammonium chloride.

Thus, it is suggested that K^+ may activate action sites which are directly linked to Ca^{++} channels.

Table 1. Effects of inhibitors of membrane phosphorylation and ion channel blockers on K^+ induced Ca^{++} uptake

Additions		Ca^{++} uptake during 2 min (nmol/mg protein)
None		4.14 ± 0.67
Dinitrophenol	10 μM	1.70 ± 0.23
Arsenate	10 μM	1.78 ± 0.17
CCCP	1 μM	1.58 ± 0.28
CPZ	10 μM	3.47 ± 0.16
TTX	10 μM	4.23 ± 0.27
TEA	100 μM	4.11 ± 0.11
La^{+++}	10 μM	3.50 ± 0.15
Verapamil	0.5 mM	2.96 ± 0.24

The reaction mixtures were the same as described in Fig. 1. Each value represents mean ± S.E. of 6 experiments. The agents were present during preincubation period of 10 min.

Effects of inhibitors of phosphorylation and ion channel blockers on excitatory amino acid induced Ca^{++} uptake

Although opening of Ca^{++} channels during membrane depolarization and the depolarizing action of excitatory amino acids are well known, the conductance mechanism underlying their depolarizing action has not been clearly defined. In the present study, Ca^{++} transport process stimulated by excitatory amino acids was compared with K^+ induced Ca^{++} uptake.

The results represented in Table 2 showed that glutamate-induced Ca^{++} uptake was inhibited by 2, 4-dinitrophenol, tetrodotox in and verapamil but not by chlorpromazine and tetraethylammonium chloride. NMDA induced Ca^{++} influx was inhibited by 2, 4-dinitrophenol and

Table 2. Effects of inhibitors of membrane phosphorylation and ion channel blockers on excitatory amino acids induced Ca^{++} uptake

Additions		Ca^{++} uptake during 2 min (nmol/mg protein)		
		Without	+ Glutamate	+ NMDA
None		2.63 ± 0.16	6.08 ± 0.12	2.77 ± 0.18
Dinitrophenol	10 μM	2.09 ± 0.23	3.58 ± 0.48	1.79 ± 0.12
CPZ	100 μM	2.67 ± 0.19	5.03 ± 0.10	2.54 ± 0.19
TTX	10 μM	2.37 ± 0.24	3.54 ± 0.07	1.51 ± 0.10
TEA	1 mM	2.34 ± 0.19	4.91 ± 0.29	3.08 ± 0.20
Verapamil	0.5 mM	1.82 ± 0.13	3.40 ± 0.21	2.08 ± 0.16

The reaction mixture contained 0.75 mg/ml of synaptosomes, 96 mM NaCl, 44 mM KCl, $MgCl_2$ (2 mM for glutamate , 1.3 mM for NMDA), 30 μM $CaCl_2$, 100 μM arsenazo III and 20 mM Tris-maleate, pH 7.4 with 5 mM of amino acid or not. Each value represents mean ± S.E. of 6 experiments. The agents were present during preincubation period of 10 min.

tetrodotoxin, and slightly reduced by verapamil.

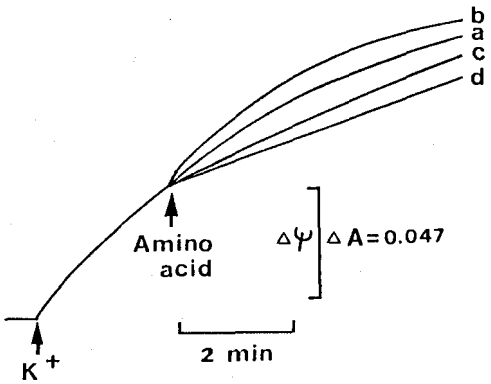

Fig. 4. Effects of excitatory amino acids on synaptosomal membrane potential. The spectral shift of positive charged safranine o was measured with dual wavelength-split beam spectrophotometer at 484–524 nm. The reaction mixtures were same as described in Table 2. a, no addition ; b, glutamate ; c, NMDA ; d, aspartate.

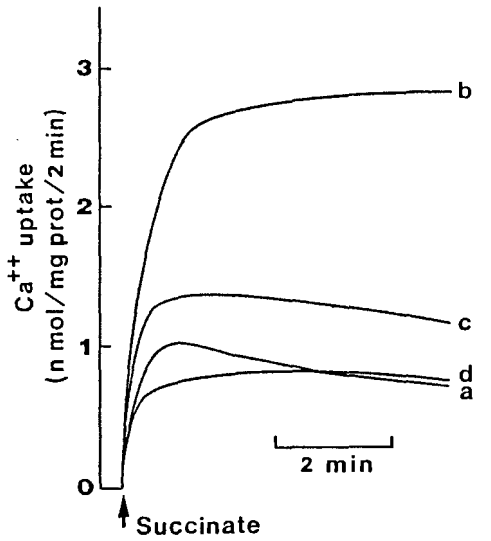

Fig. 5. Effects of excitatory amino acids on energized mitochondrial Ca^{++} uptake. Ca^{++} uptake was spectrophotometrically measured at 675–685 nm. The reaction mixture contained 0.5 mg/ml of mitochondria, 5 mM succinate, 250 mM sucrose, 100 μM arsenazo III and 20 mM HEPES-tris, pH 7.4. a, no addition ; b, 1 mM glutamate ; c, 5 mM NMDA ; d, 5 mM aspartate.

Effects of excitatory amino acids on mebrane potential

Since isolated synaptosomes have a potassium diffusion potentials similar to those found in intact nerve cells and it is suggested that they contain specific mechanisms for the transport of Ca^{++}, Na^{+} and K^{+} across the plasma membrane (Blaustein *et al.*, 1972; Swanson *et al.*, 1974; Blaustein, 1975), effects of excitatory 1974; Blaustein, 1975), effects of excitatory amino acids on ionic permeability of synaptosomes were investigated in relation to their depolarizing actions. Depolarizing action of glutamate, aspartate and NMDA was obser- Na^{+} and K^{+}, but their action was not detected in the medium containing 44 mM KCl and 90

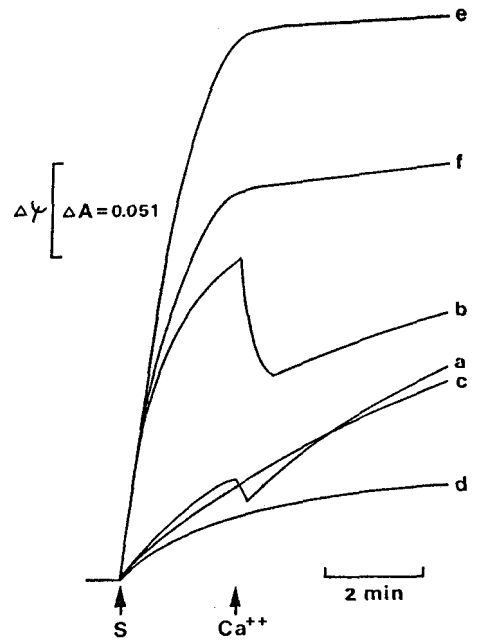

Fig. 6. Effects of excitatory amino acids on mitochondrial membrane potential. The spectral shift of positive charged safranine o was measured at 484–524 nm. The reaction mixtures contained safranine o instead of arsenazo III were the same as described in Fig. 4. S indicates succinate. a, no addition ; b, 5 mM glutamate ; c, 5 mM glutamate plus 10 μM 2, 4-dinitrophenol; d,10 μM 2,4-dinitrophenol; e, 5 mM glutamate plus 5 μM ruthenium red ; f, 5 μM ruthenium red.

Table 3. Effects of excitatory amino acids on synaptosomal Na^+–K^+, Mg^{++} ATPase, Mg^{++} ATPase and Ca^{++}–Mg^{++} ATPase activities

Additions	Na^+–K^+, Mg^{++} ATPase	Mg^{++} ATPase	Ca^{++}, Mg^{++} ATPase
None	20.08 ± 0.26	10.46 ± 0.16	10.90 ± 0.21
Glutamate	20.72 ± 0.29	9.90 ± 0.32	11.03 ± 0.22
Aspartate	22.24 ± 0.28	10.59 ± 0.38	11.98 ± 0.45
NMDA	20.69 ± 0.13	10.78 ± 0.11	11.11 ± 0.32

Concentration of excitatory amino acid is 5 mM. Experimental conditions were the same as described in Materals and Methods. All values are expressed in μmol Pi/mg protein/hr and represent mean ± S.E. of 6 experiments.

mM NaCl (Fig. 4). Thus, excitatory amino acids induced depolarization was studied with mitochondria, in which the spectral shift to safranine, the cationic membrane potential probe was well established.

The driving force of mitochondrial Ca^{++} transport seems to be a membrane potential with negative intramitochondrial polarity (Akerman, 1978). As can be seen in Fig. 5 and 6, excitatory amino acids stimulated Ca^{++} uptake in energized conditions and facilitated a Ca^{++} uptake in enrgized conditions and facilitated a Ca^{++} influx mediated depolarization which was effectively prevented by ruthenium red and 2, 4-dinitrophenol. Thus, the result indicated that glutamate, aspartate and NMDA increase Ca^{++} permeability of the mitochondrial membrane.

Effects of excitatory amino acids on Na^+-K^+, Mg^{++} ATPase activities

Action of excitatory amino acids on regulatory apparatus of ion transport across the plasma membrane was examined. Glutamate, aspartate and NMDA at concentration of 5 mM did not affect the activities of Na^+-K^+, Mg^{++} ATPase, Mg^{++} ATPase and Ca^{++}, Mg^{++} ATPase (Table 3).

DISCUSSION

Excitatory amino acids such as L-glutamate and L-aspartate are thought to act as fast synaptic transmitters, the ionic mechanism of which is not well understood. It has been suggested that neurones of the mammalian central nervous system possess several different receptors including NMDA receptor for acidic amino acids (Watkins and Evans, 1981), which in turn are coupled to seperate conductance mechanisms (MacDonald and Porietis, 1982). Excitatory amino acids may cause neuronal depolarization by increasing the membrane permeability to Na^+ (Bührle and Sonnhof, 1983). Glutamate evokes influx of Na^+ and Ca^{++} into motoneurones (Bührle and Sonnhof, 1983). NMDA receptors gate channels that are permeable to Na^+, K^+ and Ca^{++} (Mayer and Westbrook, 1985). The voltage dependence of the NMDA receptor-linked conductance is reported (Dingledine, 1983). Various experiments suggest that Ca^{++} influx is also associated with the activation of excitatory amino acid receptors on vertebrate neurones (Pumain and Heinemann, 1985). Thus, whether Ca^{++} enters through voltage dependent Ca^{++} channels or through excitatory amino acid activated channels of one or more subtype is unclear.

Mg^{++} depresses depolarizing responses of motoneurones of the immature rat evoked by carbachol, norepinephrine and substrance p (Ault *et al.*, 1980). It has also been reported that Mg^{++} depresses acetylcholine-as well as amino acid-induced excitation in the cat central nervous system (Davis and Watkins, 1977). The excitatory action of acidic amino acids acting at NMDA receptors is remarkably sensitive to the membrane potential and it has been suggested that the NMDA receptor is coupled to a voltage sensitive conductance (Mayer *et al.*, 1984). The blockade of inward current flow through ion channels linked to NMDA receptors by Mg^{++} is observed (Ault *et al.*, 1980). Glutamate and aspartate also induce a voltage dependent conductance increase (Nowak *et al.*, 1984; Pumain and heinemann, 1985). However, the responses of glutamate, aspartate and

NMDA induced Ca^{++} uptake by neuronal tissues for extracellular Mg^{++} concentration are different. Thus, in synaptosomes of rat brain the changes of K^+-and excitatory amino acids-induced Ca^{++} uptake by Mg^{++} were examined.

The result represented in Fig. 1 indicates that in K^+ rich medium Mg^{++} have not inhibitory effect on Ca^{++} flux into synaptosomes because K^+ induced Ca^{++} uptake by synaptosomes was stimulated by extracellular Mg^{++} at concentrations of 0.5 mM through inhibitory action of Mg^{++} was observed at 10 mM. This finding does not correspond with the report of Nachshen (1984). Moreover, in Mg^{++} free medium release of Ca^{++} from synaptosomes occured. Thus, it is suggested that K^+ induced Ca^{++} uptake is dependent upon Mg^{++} which activates ATPases such as Na^+-K^+ ATPase and Ca^{++}, Mg^{++} ATPase. Fig. 3 shows that although stimulatory effect of glutamate is rather reduced by high concentration of Mg^{++} compared to low concentration, glutamate induced Ca^{++} uptake may be dependent upon extracellular Mg^{++}. However, actions of NMDA on Ca^{++} influx in Na^+ rich media were antagonized by extracellular Mg^{++}. The results suggest that Ca^{++} influx associated with NMDA actions may be inhibited by Mg^{++} induced voltage dependent channel block, whereas glutamate affects other ion transport systems than the voltage dependent channel.

Since action of glutamate on Ca^{++} transport system at synaptosomes was uncertain, effects of uncouplers of membrane phosphorylation and specific ion channel blockers on glutamate induced Ca^{++} uptake were investigated, along with their effects on K^+ induced Ca^{++} influx. Glutamate-and K^+-induced Ca^{++} influx was inhibited by 2, 4-dinitrophenol, inhibitor of membrane phosphorylation and verapamil, a Ca^{++} channel blocker but not by tetraethylammonium chloride, a K^+ channel blocker. On the other hand, tetrodotoxin, a Na^+ channel blocker effectively inhibited glutamate's action, but tetrodotoxin did not affect K^+ induced Ca^{++} influx. The insensitivity of glutamate to tetrodotoxin in the present experiment is in agreement with earlier studies in striatal synaptosomes from the rat (Retz *et al.*, 1982). Therefore, Table 1 and 2 suggest that the increased permeability for Ca^{++} by glutamate and K^+ may be mediated through their actions on membrane phosphorylation, and these agents activate divalent cation selective pathways for Ca^{++} entry. Stimulated Ca^{++} uptake by glutamate may be partially attributable to the increased Na^+ permeability and to the membrane depolarization because tetrodotoxin inhibits Ca^{++} influx by glutamate. NMDA induced Ca^{++} influx change was also reduced by 2, 4-dinitrophenol and tetrodotoxin but slightly inhibited by verapamil. Some experiments reported that Ca^{++} influx is assocated with the activation of excitatory amino acid receptors and excitatory amino acids increase the intracellular Ca^{++} activity by activation of NMDA receptor channels rather than actions on voltage dependent Ca^{++} channels (MacDermott *et al.*, 1986). Accordingly, although opening of voltage dependent Ca^{++} channel chiefly regulates NMDA induced Ca^{++} influx, activation of NMDA receptor channels in part also may have a minor role.

The preceding article shows that synaptosomes may have membrane potentials which behave like K^+ diffusion potentials. It is established that veratridine and high K^+, which increase Ca^{++} transport across membranes, through a mechanism involving membrane depolarization (Blaustein, 1975). The experiment of Cho et al. (1988) showed that in the reaction mixture containing Ca^{++} without K^+ the addition of ATP induced the synaptosomal Ca^{++} uptake only slightly. The further addition of K^+ is followed by a marked increase in calcium uptake with concomittent development of membrane depolarization. The depolarizing action of glutamate and NMDA can be easily demonstrated in electrophysiological studies using either cultured neurones or brain slices. However, in the present reaction medium containing Na^+ and K^+ depolarizing actions of glutamate, aspartate and NMDA would not be detected by the safranine method. It is reported that glutamate causes depolarization of synaptosomes in the presence of Ba^{++} or 4-aminopyridine, whereas in Na^+ rich media it causes no or insignificant changes of membrane potential (Akerman *et al.*, 1987). thus, this finding suggests that ouflow of K^+ limits the membrane potential changes induced by glutamate and the changes of safranine spectrum shift in synaptosomes by excitatory amino acids may be relatively smaller than K^+ and measurement of excitatory amino acids induced depolarization using safranine is limited. In addition, examination for sensitivity differences between

safranine and cyanine dye in synaptosomal depolarization may be necessarily. The changes in membrane potential during Ca^{++} influx across the mitochondrial membrane are well demonstrated using safranine. The extent of safranine spectral change correlates linearily to the magnitude of membrane potential at least up to 200 mV and thus accurate estimates of the mitochondrial membrane potential in different conditions can be obtained (Akerman and Wikström, 1976). As can be seen in Fig. 5 and 6, glutamate, aspartate and NMDA stimulated mitochondrial Ca^{++} uptake and induced Ca^{++} diffusion potential. Accordingly, depolarizing action of excitatory amino acids is investigated as the changes of mitochondrial membrane potential.

Finally, increases of intracellular Ca^{++} ion concentration evoked by excitatory amino acids are not explained by their action on synaptosomal Na^{+}-K^{+} ATPase and Ca^{++}, Mg^{++} ATPase.

The results suggest that excitatory amino acids induced Ca^{++} influx may be associated with increased permeability of membrane to ions other than K^{+}.

REFERENCES

Ahmed Z and Connor JA: *Measurement of calcium influx under voltage clamp in molluscan neurones using the metallochromic dye arsenazo III. J Physiol 286:61-82, 1979*

Åkerman KEO: *changes in membrane potential during calcium ion influx and efflux across the mitochondrial membrane. Biochim Biophys Acta 502: 359-366, 1978*

ÅKerman KEO and Heinonen E: *Qualitative measurements of cytosolic calcium ion concentration within isolated guinea pig nerve endings using entrapped arsenazo III. Biochim Biophys Acta 732:117-121, 1983*

Åkerman KEO, Scott IG, Heikkilä JE and Heinonen E: *Ionic dependence of membrane potential and glutamate receptor-linked responses in synaptoneurosomes as measured with a cyanine dye, Dis-C_2-(5). J Neurochem 48: 522-559, 1987*

Åkerman KEO Wikström MK: *Safranine as a probe of the mitochondrial membrane potential. FEBS Lett 68(2):191-197, 1976*

Ault B, Evans RH, Francis AA, Oakes DJ and Watkins JC: *Selective depression of excitatory amino acid induced depolarizations by magnesium ions in isolated spinal cord preparations. J Physiol 307:413-428, 1980*

Blaustein MP: *Effects of potassium, veratridine and scorpion venom on calcium accumulation and transmitter release by nerve terminals in vitro. J Physiol 247:617-655, 1975*

Blaustein MP, Johnson EMJr and Needleman P: *Calcium-dependent norepinephrine release from presynaptic nerve endings in vitro. Proc Natl Acad Sci USA 69:2237-2240, 1972*

Bührle ChPh and Sonnhof U: *The ionic mechanism of the excitatory action of glutamate upon the membranes of motoneurones of the frog. Pflügers Arch 396:154-162, 1983*

Cho SJ, Lee CS, Shin YK and Lee KS: *Modulation of synaptosomal calcium transport by catecholamines through adrenergic receptors. Chung-Ang J Med in press*

Crunelli V, Forda S and Kelly JS: *The reversal potential of excitatory amino acid action on granule cells of the rat dentate gyrus. J Physiol 351:327-342, 1984*

Davis J and Watkins JC: *Effects of magnesium ions on the responses of spinal neurones to excitatory amino acids and acetylcholine. J Physiol 124: 370-384, 1977*

Dingledine R: *N-methyl aspartate activates voltage dependent calcium conductance in rat hippocampal pyramidal cells. J Physiol 343:385-405, 1983*

Evans RH, Francis AA and Watkins JC: *Selective antagonism by Mg^{2+} of amino acid-induced depolarization of spinal neurones. Experientia 33:489-491, 1977*

Giorguieff MR, Kenel ML and Glowinski J: *Presynaptic effects of L-glutamic acid on the release of dopamine in rat striatal slices. Neurosci Lett 6:73-77, 1977*

Gray EG and Whittaker VP: *The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. J Anat 96:79-87, 1962*

Hablitz JJ and Langmoen IA: *Excitation of hippocampal pyramidal cells by glutamate in the guinea pig and rat. J Physiol 325:317-331, 1982*

Heinemann U and Pumain R: *Extracellular calcium activity changes in cat sensorimotor cortex induced by iontophoretic application of amino acids. Exp Brain Res 40:247-250, 1980*

Horwitt BN: *Determination of inorganic serum phosphate by means of stannous chloride. J Biol Chem 199:537-541, 1952*

Ichida S, Tokunaga H, Moriyama M, Oda Y, Tanaka S and Kita T: *Effects of neurotransmitter candiates on ^{45}Ca uptake by cortical slices of rat brain: stimulatory effect of L-glutamic acid. Brain Res 248:305-311, 1982*

Ishida AT and Neyton J: *Quisqualate and L-glutamate inhibit retinal horizontal-cell responses to kainate. Proc Natl Acad Sci USA 82:1837-1841, 1985*

Krnjevic K: *Glutamate and γ-aminobutyric acid in brain. Nature 228:119-124, 1970*

Lowry OH, Rosebrough NJ, Farr AL and Randal RJ: *Protein measurement with the folin phenol reagent. J Biol Chem 193:266-275, 1951*

MacDermott AB, Mayer ML, Westbrook GL, Smith SJ and Barker JL: *NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. Nature 321:519-522, 1986*

MacDonald JF and Porietis AV: *DL-Quisqualic and L-aspartic acids activate separate excitatory conductances in cultured spinal cord neurones. Brain Res 245:175-178, 1982*

Mayer ML and Westbrook GL: *Mixed-agonist action of excitatory amino acids on mouse spinal cord neurones under voltage clamp. J Physiol 354:29-53, 1984*

Mayer ML and Westbrook GL: *The action of N-methyl-D-aspartic acid on mouse spinal neurones in culture. J Physiol 361:65-90, 1985*

Mayer ML, Westbrook GL and Guthrie PB: *Voltage-dependent block by Mg^{2+} of NMDA responses in spinal cord neurones. Nature 309: 261-263, 1984*

Michell RH: *Inositol phospholipids and cell surface receptor function. Biochim Biophys Acta 415:81-147, 1975*

Nachshen DA: *Selectivity of the Ca binding site in synaptosome Ca channels: Inhibition of Ca influx by multivalent metal cations. J Gen Physiol 83:941-967, 1984*

Nachshen DA and Blaustein MP: *Some properties of potassium-stimulated calcium influx in presynaptic nerve endings. J Gen Physiol 76:709-728, 1980*

Nicoletti F, Meek JL, Iadarola MJ, Chuang DM, Roth BL and Costa E: *Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. J Neurochem 46:40-46, 1986*

Nieoullon A, Cherany A and Glowinski J: *Release of dopamine evoked by electrical stimulation of the motor and visual areas of the cerebral cortex on both caudate nuclei and in the substantia nigra in the cat. Brain Res 145:69-83, 1978*

Nowak L, Bregestovski P, Ascher P, Herbel A and Prochiantz A: *Magnessium gates glutamate-activated channels in mouse central neurones. Nature 307:462-465, 1984*

Pumain R and Heinemann U: *Stimulus and amino acid induced calcium and potassium changes in rat neocortex. J Neurophysiol 53:1-16, 1985*

Retz KCD and Coyle JT: *The differential effects of excitatory amino acids on uptake of $^{45}CaCl_2$ by slices from mouse striatum. Neuropharmacol 23:89-94, 1984*

Retz KC, Young AC and Coyle JT: *Glutamate stimulation of ^{45}Ca uptake by rat striatal synaptosomes. Eur J Pharmacol 79:319-322, 1982*

Reuter H: *Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature 301:569-574, 1983*

Roberts PJ and Anderson SD: *Stimulatory effect of L-glutamate and related amino acids on [^3H] dopamine release from rat striatum. An in vitro model for glutamate actions. J Neurochem 32: 1539-1545, 1979*

Sonnhof U, Grafe P, Krumnikl J, Linder M and Schindler L: *Inhibitory postsynaptic actions of taurine, GABA and other amino acids of motoneurons of the isolated frog spinal cord. Brain Res 100:327-341, 1975*

Swanson PD, Anderson L and Stahl WL: *Uptake of calcium ions by synaptosomes from rat brain. Biochim Biophys Acta 356:174-183, 1974*

Terner C, Eggleston LV and Krebs HA: *The role of glutamic acid in the transport of potassium in brain and retina. Biochem J 47:139-140, 1950*

Watkins JC and Evans RH: Excitatory amino acid transmitters. Ann Rev Pharmacol Toxicol 21: 165-204, 1981

＝국문초록＝

Glutamate와 NMDA에 의한 Synaptosome에서의 칼슘 유입과 이들의 작용의 차이

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Glutamate와 aspartate는 단가 양이온과 칼슘에 대한 세포막의 투과성을 증가시키는 것으로 시사되고 있다. 그러나 칼슘 유입이 voltage에 의존적인 칼슘 통로에 의하여 또는 흥분성 아미노산에 활성적인 통로에 의하여 이루어지는가는 분명하지 않다. 더우기, 신경세포의 칼슘 유입에 미치는 흥분성 아미노산의 영향과 세포의 마그네슘에 대한 이들의 반응이 다른 것으로 추정하고 있다.

Synaptosome에서 포타슘에 의한 칼슘 흡수는 세포외 마그네슘에 의존적이었으나 10 mM 농도에서는 그 이하의 농도에서보다 오히려 감소하였다. 소듐이 주된 반응액에서 glutamate와 aspartate에 의한 칼슘 흡수는 마그네슘에 의하여 용량에 비의존적인 양상으로 증가하였다. 그러나 NMDA의 작용은 2 mM 이상의 마그네슘에 의하여 억제되었다. 포타슘과 glutamate에 의한 칼슘 흡수는 2,4-dinitrophenol, chlorpromazine과 verapamil에 의하여 억제되었으나 tetraethylammonium chloride의 영향은 받지 아니하였다. Tetrodotoxin은 효과적으로 glutamate의 작용을 억제하였으나 K^+의 작용에는 영향을 주지 않았다. NMDA의 작용은 2, 4-dinitrophenol과 tetrodotoxin에 의하여 억제되었고 verapamil에 의하여 약간 억제되었으며 tetraethylammonium chloride의 영향은 받지 아니하였다. 소듐이 주된 반응액에서 glutamate, aspartate와 NMDA에 의한 synaptosome의 탈분극은 관찰되지 않았으나 이들은 mitochondria에서 칼슘 유입에 따른 탈분극을 초래하였다. 한편, 흥분성 아미노산은 synaptosome의 ATPase 활성도에 영향을 나타내지 않았다.

이상의 결과로부터 glutamate 또는 NMDA에 의한 synaptosome의 칼슘 흡수는 세포외 마그네슘에 각기 다른 양상을 나타내며 이들에 의한 칼슘 흡수는 포타슘을 제외한 소듐과 칼슘에 대한 세포막 투과성의 증가 그리고 이에 따른 탈분극에 연관이 있을 것으로 시사되었다.