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The release of glutamate and aspartate from rat brain synaptosomes in response to domoic acid (amnesic shellfish toxin) and kainic acid

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Abstract

Kainic acid is known to stimulate the release of glutamate (GLU) and aspartate (ASP) from presynaptic neurons. It has been suggested that the enhanced release of these endogenous EAA's plays a significant role in the excitotoxic effects of KA. Domoic acid (DOM), a shellfish toxin, is structurally similar to KA, and has been shown to be 3–8 times more toxic than KA. In this study, effects of KA and DOM on the release of GLU and ASP from rat brain synaptosomes were investigated. Amino acid analysis was performed by the reversed phase HPLC, following derivatization with 9-fluorenylmethyl chloroformate (FMOC). Potassium chloride (40 mM) was used as a positive control, and stimulated GLU release from rat brain synaptosomes in presence or absence of Ca^{2+}. DOM enhanced the release of GLU, whereas KA stimulated the release of both GLU and ASP from synaptosomes in the presence of Ca^{2+}. However, their potency to stimulate GLU and ASP release was enhanced in absence of Ca^{2+}. These results indicate that different mechanisms may be involved in the release of GLU and ASP in response to DOM and KA, and that neurotransmitter release appeared to be highly specific for these agonists. It would appear that DOM and KA may interact with different receptors on the presynaptic nerve terminal, and/or activate different subtypes of voltage-dependent Ca^{2+} channels to promote influx of Ca^{2+} which is targeted for different pools of neurotransmitters. (Mol Cell Biochem **151:** 49–54, 1995)

Key words: excitotoxins, domoic acid, kainic acid, glutamate release, synaptosomes (rat brain)

Abbreviations: ANOVA – analysis of variance; ASP – aspartate; DOM – domoic acid; DHKA – dihydrokainic acid; EAA – excitatory amino acid; FMOC – 9-fluorenylmethyl chloroformate; GLU – glutamate; KA – kainic acid

Introduction

Kainic acid (KA) and domoic acid (DOM) are excitotoxic amino acids which produce selective neurodegeneration comparable to that seen with diseases such as epilepsy, Alzheimer's disease and Huntingtons chorea [1–3]. The molecular mechanism(s) by which DOM produces neurotoxicity is not defined. DOM resembles KA structurally, and both produce similar toxic responses in affected animals [4–7]. It has been proposed that DOM may produce neurotoxicity by mechanism(s) similar to KA, probably via interaction with KA receptors [4–7]. Kainic acid receptors are preferentially distributed in the CA3 region of the hippocampus where neurotoxic effects of KA and DOM are most evident [7].

Kainic acid has been demonstrated to increase extracellular concentrations of EAA neurotransmitters from different brain preparations [8–14]. It has been proposed that increases in extracellular glutamate (GLU) and aspartate (ASP) level may be fundamental to the neurotoxic action of KA [8–16]. Domoic acid has been shown to be 3–10 times more neurotoxic than

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KA [4, 6, 15], and this difference in potency may be due to different binding affinities of these neurotoxins for KA receptor subtypes [17].

An elevation in the extracellular levels of GLU or ASP could result from either the enhanced release of these neurotransmitters, and/or by inhibition of their reuptake into presynaptic nerve terminals. The enhanced release of EAA is caused by an interaction of KA with presynaptic EAA receptors [9, 10], whereas blocked uptake is likely due to interference with the acidic amino acid uptake carrier [13, 18].

Previous studies have shown that high potassium induces neurotransmitter release which is largely dependent on Ca^{2+} [11, 13, 19, 20], whereas results of Ca^{2+} dependence for KA-stimulated EAA release vary widely [8, 10–13, 18–20]. It is recognized that neurotransmitter released from secretory vesicles occurs by exocytosis, and is primarily dependent on extracellular Ca^{2+} [9, 16, 21, 22], whereas spontaneous release of neurotransmitters is either due to leakage of GLU from the cell cytoplasm, and/or inhibition of GLU reuptake by the presynaptic nerve endings [13, 23]. Kainic acid-induced GLU release from different brain preparations has been reported by some investigators to be dependent on Ca^{2+} [8–12, 19, 24], whereas others have reported it to be independent of Ca^{2+} [13, 18]. Such a variable response may result from differences in brain preparations containing variable pre- and post-synaptic elements, glial cells, and EAA receptor density [8–13, 18–20, 24].

In the present study, effects of KA and DOM on the extracellular levels of GLU and ASP in presynaptic nerve endings (synaptosomes) were investigated. DOM stimulated GLU release, whereas KA enhanced the release of both GLU and ASP from rat brain synaptosomes. These results indicate that different mechanisms are involved in GLU and ASP release in response to KA and DOM, and that neurotransmitter release from rat brain synaptosomes is highly specific for these agonists.

Methods

Male Sprague Dawley rats (Charles River, St-Constant, Quebec), weighing between 250 and 300 g were housed in AVC animal quarters with a 12 h cycle of light and dark, and provided food and water *ad libitum*. The animals were decapitated, brain excised, and synaptosomes prepared by the method of Booth and Clarke [25]. Aliquots (150 µl) of synaptosomal suspension were placed in test tubes, Krebs Henseleit HEPES medium (140 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 5 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 1 mM Tris, 10 mM D-glucose, 10 mM HEPES, 1 mM NaH_2PO_4, pH 7.2) added to yield a final protein concentration of approximately 6 mg/ml. Tubes were placed in a water bath at 30°C, and agitated gently. After incubation for 10 min, 350 µl aliquots of reaction mixture were removed and placed in microcentrifuge tubes, and 30 µl of test media containing agonists in HEPES medium, were added to the reaction mixture. The incubation was continued for another 10 min, when 350 µl samples were obtained for analysis. All samples were centrifuged for 1 min at 1470 × g in a Beckman ultra-centrifuge (model 12, Beckman Canada, Dartmouth, NS). The resulting supernatants were removed to storage vials, acidified with 20 µl 16% TCA, and frozen at –80°C for subsequent HPLC analysis.

Estimation of proteins

Proteins were measured colorimetrically by the method of Lowry *et al.* [26], using bovine serum albumin (Fraction V) as standards.

High performance liquid chromatography

Glutamate and aspartate were measured according to the procedure of Pocklington *et al.* [27]. Previously acidified and frozen supernatant samples were allowed to thaw at room temperature, and their pH adjusted to 7.0 with 1 N NaOH. Aliquots (300 µl) of sample were placed in a test tube and diluted with distilled water to a total volume of 400 µl. To each sample, 100 µl of 1 M boric acid (pH 6.2), and 40 µl of 10 µM dihydrokainic acid (DHKA, an internal standard in 10% acetonitrile), were added. The mixture was vortex-mixed for 10 sec. FMOC (500 ul of 15 mM 9-fluorenylmethyl chloroformate) was added, and mixed for exactly 45 sec. Ethyl acetate (1 ml) was added, and mixing continued for additional 10 sec. Ethyl acetate is not soluble in aqueous solution and stops the reaction by removing excess free FMOC. Test tubes containing reaction mixture were centrifuged for 2 min at 10,500 × g in a clinical centrifuge (International Equipment Company, model CL, MA). The top (organic) layer was discarded, and the aqueous layer was washed with 1.0 ml ethyl acetate, and recentrifuged. The bottom aqueous layer was transferred to 2 ml autosampler vials, and subjected to HPLC [27]. Amino acids were eluted with a concentration gradient, and detected by fluorescence detector (Model RF-535, Shimadzu Corp, Japan).

Statistics

Four trials were carried out, with all measurements in duplicate. Results for each sampling time were examined separately by analysis of variance (ANOVA) using the general linear model (GLM). Treatment groups were compared by Student Newman-Keuls (SNK) method [28].

Results

The HPLC profile of amino acids in the incubation medium (extracellular) illustrates satisfactory resolution of different amino acids from each other, and the usefulness of this method to measure the release of GLU and ASP from synaptosomal preparation (Fig. 1). Glutamate and aspartate eluted from the column with retention times of 12.5 and 13 min, respectively, whereas DHKA (an internal standard) eluted at 21 min (Fig. 1). Amino acid concentration was determined using peak area for each amino acid, and a linear relationship between peak area and GLU and ASP standards. The correlation coefficient between the amino acid concentration and peak area for GLU and KA was above 98%.

There was no significant difference between GLU and ASP released in samples collected at 10 min (control, Table 1). At 20 min of incubation, GLU and ASP released into the medium was somewhat diminished in the control samples, although this decrease was not statistically significant ($p > 0.05$). High potassium (40 mM) stimulated the release of GLU and ASP ($p < 0.01$). Replacement of 40 mM KCl with choline chloride to maintain comparable osmolarity in the incubation medium, had no effect on GLU and ASP release (data not reported).

Domoic acid stimulated GLU release from the synaptosomes in the presence of Ca^{2+}, and had no effect on ASP release. In contrast, KA (1 mM) stimulated the release of both GLU and ASP (Table 1).

The incubation medium lacking Ca^{2+} contained no exogenous Ca^{2+}, but 1 mM EGTA. The release of GLU and ASP from synaptosomes in the control medium, was not affected by the absence or presence of Ca^{2+}, indicating that the addition of EGTA to the medium had no adverse effect on the neurotransmitter release (Table 2). Similarly, the lack or presence of Ca^{2+} did not influence the release of GLU and ASP in response to 40 mM K^+ (Table 2). However, the absence of Ca^{2+} from the incubation medium enhanced the release of GLU and ASP in response to KA and DOM (Table 2).

Discussion

The results of the present study show that 40 mM K^+ stimulated the release of GLU and ASP from the rat brain synaptosomes. These findings are similar to the observations of many other investigators, indicating that the present preparation of synaptosomes was viable, and appropriate for studies examining the release of GLU and ASP in response to DOM.

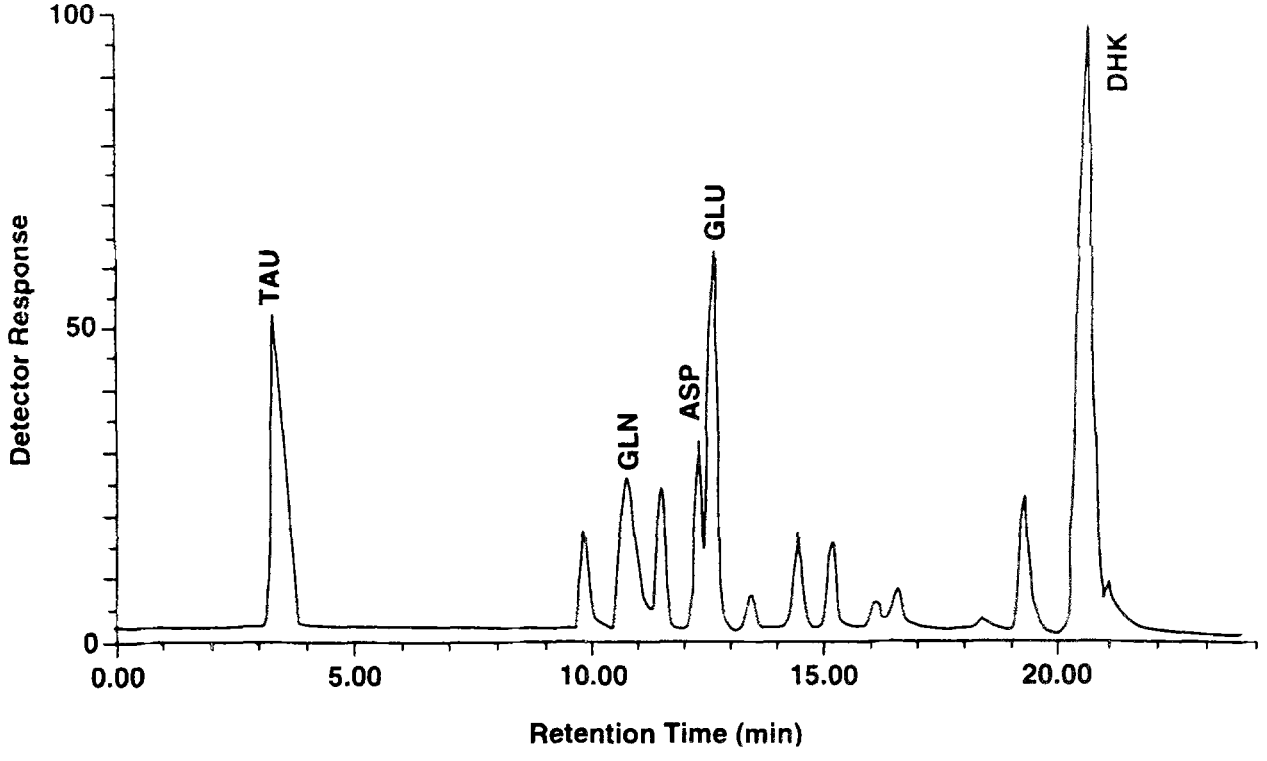

Fig. 1. An HPLC profile of amino acids following derivatization with FMOC as described in Methods. The supernatant resulting from centrifugation of incubation medium was treated with FMOC to derivatize amino acids, and subjected to HPLC on a C_{18} column (25 cm × 4.6 mm, ID, Phenomenex, Torrance, CA), and resolved into individual amino acid peaks, which were detected with a fluorescence detector (Shimadzu Corp., Kyoto, Japan). TAU-taurine; GMINE-glutamine; ASP-aspartate; GLU-glutamate; DHK-dihydrokainic acid (internal standard)

Table 1. Effects of high potassium, kainic, and domoic acid on glutamate and aspartate release from rat brain synaptosomes

Time (min)	Amino acid	Control	Potassium (40 mM)	Kainic acid (0.5 mM)	Kainic acid (1.0 mM)	Domoic acid (0.5 mM)	Domoic acid (1.0 mM)
10	ASP	0.48 ± 0.4	0.36 ± 0.2	0.32 ± 0.1	0.40 ± 0.1	0.43 ± 0.3	0.41 ± 0.3
	GLU	0.41 ± 0.2	0.40 ± 0.2	0.59 ± 0.5	0.58 ± 0.2	0.62 ± 0.4	0.69 ± 0.4
20	ASP	0.24 ± 0.1	6.35 ± 5.4[1]	0.35 ± 0.3	0.75 ± 0.62	0.43 ± 0.3	0.53 ± 0.3
	GLU	0.28 ± 0.2	7.28 ± 3.4[1]	0.76 ± 0.3[1]	1.00 ± 0.6[1]	0.83 ± 0.3[1]	1.4 ± 0.3[1]

Data are expressed as nmol/mg protein; ± SD, n = 4; 1- $P < 0.01$; 2- $P < 0.05$

Table 2. Glutamate and Aspartate release from rat brain synaptosomes in response to different agonists in the presence or absence of calcium

Treatment	Amino acid	+ Ca^{2+}	− Ca^{2+}
Control	GLU	0.21 ± 0.0	0.23 ± 0.0
	ASP	0.22 ± 0.2	0.18 ± 0.1
Potassium (40 mM)	GLU	3.78 ± 0.4	3.77 ± 1.6
	ASP	2.57 ± 0.3	2.36 ± 1.1
Kainate (1 mM)	GLU	0.71 ± 0.8	1.14 ± 0.7*
	ASP	0.52 ± 0.4	0.85 ± 0.3*
Domoate (1 mM)	GLU	0.69 ± 0.5	1.01 ± 0.4*
	ASP	0.50 ± 0.3	0.80 ± 0.3*

Data are expressed as nmol/mg protein, ± SD, n = 3 *significantly different from the corresponding values in presence of 1.3 mM Ca^{2+} ($P < 0.05$).

Domoic acid is a shellfish toxin which was found in steamed mussels (Mytilus edulis) cultivated in the east coast of Prince Edward Island. The ingestion of contaminated mussels with DOM toxin produced gastrointestinal distress, followed by severe deficits in short term memory in seriously affected patients [29]. The mechanism by which DOM produces dysfunction of the central nervous system (CNS), is not defined at present.

Domoic acid and KA stimulated the release of GLU from the synaptosomes, both being equipotent in stimulating the release of EEA's. Interestingly, DOM at low and high concentrations stimulated the release of GLU, and had no effect on ASP release. In contrast, KA stimulated the release of both GLU and ASP (Table 1). It would appear that the mechanisms of GLU and ASP release from synaptosomes are different, and highly specific for DOM and KA. The neurotransmitter release in response to DOM and KA may be mediated by different receptor subtypes located on the presynaptic nerve terminal, and/or may originate from different subcellular pools i.e. cytoplasm and/or vesicles. Since DOM and KA are structurally similar, it has been assumed that these agonists share the same mechanism of action on their target tissue. In fact, recent evidence suggests that DOM may produce its toxic effects via KA receptors [3–8]. Kainic acid neurotoxicity appears to be mediated by excess release of GLU which is known to be toxic in brain tissue [1, 8–10, 15, 16]. However, the present findings show different specificities for these agonists to stimulate the release of GLU, and aspartate from rat brain synaptosomes, and warrant further investigations into characterization of receptors subtypes that may mediate their effects on neurotransmitter release in brain tissue.

While KA has been shown to stimulate GLU release from brain preparations by several other investigators, effects of DOM on GLU release has not been studied in brain tissue, except a recent study [30]. Terrain *et al.* have reported a lack of stimulation of GLU release from hippocampal mossy fibres synaptosomes in response to 300 uM DOM. However, DOM enhanced K^+-induced GLU release in guinea pig hippocampal mossy fiber synaptosomes. The discrepancy between the results of present study and that of Terrain *et al* may be due to the fact that synaptosomes from different regions of brain were used, and that synaptosomes prepared from different regions of brain may have totally different receptor profiles and density. Secondly, the experimental conditions of the two studies are different. In our study, we used Krebs-Hanseleit medium for incubation of synaptosomes, whereas Terrain et al preincubated their synaptosomes in a medium containing 50 uM D-aspartate to "reduce the level of cytoplasmic glutamate and thereby enhance the calcium dependence of measured glutamate release". This treatment of synaptosomes stimulates the exchange between the cytoplasmic GLU and extracellular D-ASP via acidic amino acid carrier, reducing the cytoplasmic concentration of GLU [30]. If DOM stimulates GLU release from the cytoplasmic pool, effects of DOM on GLU release would be masked by treatment of synaptosomes with D-aspartate. In addition, Terrain et al employed a superfusion incubation system in which increases in extracellular amino acid concentrations due to blockage of the reuptake of GLU into presynaptic elements, are not evident. Also, Terrain et al used 300 uM DOM, whereas we used 500 uM and 1000 uM DOM, and DOM at both concentrations stimulated GLU release from synaptosomes.

Dakshinamurti et al have reported that domoic acid enhanced KCl-induced glutamate release from the rat hippocampal slices [34]. Administration of domoic acid into the

caudal vein of pregnant mice produced profound impairment in hippocampal functions in the offsprings [35]. This was accompanied by an elevation in glutamate with a corresponding decrease in GABA levels in cerebral cortex and hippocampus. Domoic acid treatment resulted in a significant reduction in glutamatic acid decarboxylase (GAD) activity. Domoic acid inhibition of GAD results in reduced synthesis of GABA and increased seizure activity, followed by neuronal excitotoxicity [35]. It was concluded that domoic acid-induced hippocampal pathology is mediated by significantly reduced GABA, and elevated glutamate levels in brain regions. Domoic acid-induced neurotoxicity may be further aggravated by (a) the enhanced release of glutamate, (b) an increased receptor binding to synaptosomal membranes, and (c) by the enhanced calcium influx into neuronal cells [35].

It is widely recognized that both Ca^{2+}-dependent and -independent mechanisms contribute to GLU release from different brain preparations [9, 11, 13, 16–20, 23]. Some investigators have reported 53–75% dependence on Ca^{2+} for K^+-stimulated GLU release [20]. In the present study, K^+-stimulated GLU release was not affected by the lack or presence of Ca^{2+} (Table 2). This discrepancy in results appears to be related to experimental conditions. We incubated synaptosomes with the test medium agonists for 10 min, when the effects of Ca^{2+} on K^+-stimulated GLU release had already diminished [20, 23]. Apparently, the optimal effect of Ca^{2+} was noted 4 min after the incubation of synaptosomes with the agonist [20, 23].

On the basis of findings that Ca^{2+} was required for KA stimulation of GLU release from brain preparations [8–13], it has been proposed that KA interaction with receptors at presynaptic membranes results in the activation of calcium channels, thereby, allowing the entry of Ca^{2+} into the presynaptic nerve terminal. A rise in intracellular Ca^{2+} promotes aggregation of microtubules, facilitating the transfer of vesicles containing neurotransmitter to the periphery of cell where vesicle membrane fuses with plasma membranes. The fused membranes lyse at the site of adhesion, allowing the extrusion of neurotransmitters into a synapse by the process of exocytosis [21, 23].

Glutamate release enhanced by high K^+ was not affected in a medium lacking Ca^{2+} and containing 1 mM EGTA (Table 2). In contrast, DOM- and KA-induced GLU release from synaptosomes was potentiated by the absence of Ca^{2+} (Table 2). Domoate and KA are negatively charged molecules at the physiological pH (7.4), and thus, are capable of binding Ca^{2+} in the medium. The simultaneous presence of either DOM or KA, with EGTA (a negatively charged molecule which specifically binds to Ca^{2+}) in the incubation medium, may have variably reduced the chelation of Ca^{2+} by EGTA. Effectively, more Ca^{2+} would be freed from Ca-EGTA complex to form Ca-DOM or Ca-KA complexes. This would reduce negative surface charge on DOM and KA molecules, and may facilitate their interaction with specific receptors on the pre-synaptic nerve terminals resulting in the activation of Ca^{2+} channels, and enhancement of Ca^{2+} influx [18]. The transfer of Ca^{2+} intracellularly may be also enhanced by the exchange mechanism between the amino acid carrier and Ca^{2+} [18]. Consequently, Ca^{2+}-mediated GLU release would be enhanced. Alternatively, K^+ in the incubation medium depolarizes the synaptosomal membranes, thereby, reducing the electrochemical potential gradient across synaptosomal membranes, and affecting the influx of Ca^{2+} [30, 32].

In conclusion, while DOM stimulated only GLU release, KA stimulated the release of both GLU and ASP from rat brain synaptosomes. These results indicate that the mechanisms by which DOM and KA stimulated the neurotransmitter release from brain synaptosomes are different, and highly specific for the agonists involved. A differential inhibition of GLU and ASP release from hippocampal tissue has been noted by other investigators [33]. These results imply that either receptors mediating the stimulatory effects of DOM and KA on the presynaptic nerve terminal are different, or DOM and KA activate different subtypes of voltage-sensitive calcium channels (N and P) which facilitate rapid and slow rise in intrasynaptosomal Ca^{2+} [18, 21, 32, 33]. The rapid initial release of GLU in response to high K^+ does not appear to be linked with the rapid rise in intracellular Ca^{2+} [18]. However, the latter slow and prolonged GLU release is linked with the plateau phase of Ca^{2+} entry [18]. Ca^{2+} entry during these two phases may occur in different populations of synaptosomes [18, 33]. Alternatively, channels responsible for Ca^{2+} entry may lie in different active zones, directing calcium to different pools of GLU and ASP in the presynaptic nerve terminal [18, 32, 33].

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