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Enhancement of [^{3}H]D-aspartate release during ischemia like conditions in rat hippocampal slices: source of excitatory amino acids

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Abstract. Ischemic neuronal injury is supposed to be caused in part by the extracellular accumulation of excitatory amino acids (EAA). Neurotransmitter and metabolic EAA can be released from synaptic vesicles and cytoplasm of neurones and glial cells. In this study the release of the glutamate analogue [^{3}H]D-aspartate ([^{3}H]D-ASP), loaded into 500 μm slices of rat hippocampus, was investigated. The efflux of the label was measured during anoxic - aglycemic ("ischemic") and normoxic K^+ depolarization. To identify the pools from which [^{3}H]D-ASP is released we have estimated its calcium dependence and the effects of inhibitors of: (1) Na^+ - dependent transporter of amino acids (100 μM L-trans-pyrrolidine-2,4-dicarboxylic acid /L-trans-PDC/), (2) sodium channel (1 μM tetrodotoxin TTX), and (3) anion channel (1 mM furosemide). [^{3}H]D-ASP released upon normoxic depolarization was 40% inhibited by TTX, nearly 40% by L-trans-PDC and over 50% by furosemide. The "ischemic" release was in 40% calcium dependent, completely TTX independent and in approximately 50% blocked by furosemide treatment. Our data suggest that EAA accumulated in the synaptic cleft during ischemia are mainly released from the cytosolic compartment by mechanisms which are connected with the ischemic increase of extracellular potassium concentration.

Key words: excitatory amino acids, D-aspartate, hippocampal slices, ischemia *in vitro*, EAA compartments

INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the CNS, acting on various receptor-operated ion channels or signalling systems. It has been reported that glutamate may exist in different concentrations in at least five cellular compartments: synaptic vesicles, presynaptic cytoplasm, postsynaptic cytoplasm, astrocytes and extracellular space (Diemer et al. 1993, Mitani et al. 1994). The postsynaptic action of glutamate is rapidly terminated by reuptake systems located on both neurones and astrocytes surrounding the synaptic cleft.

The hippocampus, as well as the rest of cerebral cortex, undergoes drastic changes when subjected to ischemia, including decrease in ATP content, disruption of ion homeostasis and a massive release of neurotransmitters into the synaptic cleft (for review see Hara et al. 1993). It has been suggested that an increase in extracellular glutamate concentration may be responsible for ischemic degeneration of neurones (Simon et al. 1984; for review see Obrenovich and Richards 1995). Theoretically, glutamate released from any cellular compartment may contribute to this ischemic rise. By definition, neurotransmitter glutamate is released from synaptic endings, whereas "metabolic" glutamate responses may be located in both neuronal as well as in glial cells. The mechanism of EAA release from these compartments is undoubtedly different. Nicholls and Attwell (1990) proposed that the possible mechanisms for EAA release may be divided into two main groups: Ca^{2+} dependent and Ca^{2+} independent. It has been suggested that, in contrast to release of glutamate from the cytoplasmic pool, which may be a rather Ca^{2+} independent phenomenon (Szatkowski et al. 1990, Kimelberg et al. 1995), vesicular glutamate is exclusively released in a Ca^{2+} dependent manner. It seems that Ca^{2+} independent release of glutamate, although hardly activated under physiological conditions may play a dominant role in pathophysiology. Besides neuronal cytoplasm, glial cells can release glutamate mostly in a Ca^{2+} independent manner. However, there is some recent evidence showing that cultured astrocytes respond also to stimulus - coupled elevation of internal calcium by an extensive, non-vesicular, furosemide - sensitive glutamate release (Parpura et al. 1994). These data suggest, that these two pools of glutamate (vesicular and cytoplasmatic) can overlap in terms of their Ca^{2+} dependency. As an alternative, the determination of different amino acid pools is possible on the basis of their responsiveness to selective ion channel and transporter inhibition. Until now, this type of study has been conducted mainly on glial cells in culture and is still incomplete for whole brain slices.

Ischemia leads to disruption of the ion gradient and rapid cellular swelling (for review see Siesjö et al. 1993) which can activate, besides depolarization-induced vesicular exocytosis, an extensive release of EAA from the cytoplasmic pool through: (1) reversed direction of amino acid transporter system (Szatkowski et al. 1990, Phillis et al. 1994), which is sensitive to amino acids analogues, such as the competitive carrier inhibitors (L-trans-pyrrolidine-2,4-dicarboxylic acid /L-trans-PDC/ or dihydrokainate or (2) opening of anion channels, related to cell volume and activated by reduced osmolarity, which are sensitive to anion transporter inhibitors such as furosemide, bumetanide and L-644711 (Kimelberg and Frangakis 1985, Kimelberg et al. 1990, Attwell et al. 1993, Vitarella et al. 1994, Kimelberg et al. 1995).

One of the most important determinants for the above mechanisms is the extracellular ion balance. In brain ischemia a rapid increase in extracellular K^+ and decrease in Na^+ concentrations to about 60-70 mM is observed during the very first minutes (Siesjö et al. 1993). Therefore, in the present experiments, we have decided to perfuse hippocampal slices with the anoxic - aglycemic media containing K^+ and Na^+ concentrations adjusted in accordance to their extracellular concentrations observed under ischemia.

To further explore this problem and to evaluate the magnitude of vesicular vs. carrier - mediated, and/or volume dependent, cytoplasmic pools involved in ischemic EAA elevation, we have settled on a series of experiments using hippocampal slices *in vitro*.

The amino acid compartments have been studied under different experimental protocols. These consisted of:
- elimination of external Ca^{2+} in the absence or presence of the intracellular chelator BAPTA-AM;
- blocking of Na^+ dependent amino acid efflux by using tetrodotoxin (TTX); and
- elimination of different non-vesicular components of the release by the transporter and ion channel blockers L-trans-DPC and furosemide.

METHODS

Preparation of hippocampal slices

Transverse slices of 500 μm thickness were prepared from the rat hippocampus as has been described elsewhere (Zabłocka and Domańska-Janik 1994). Combined slices from four hippocampi were incubated in 1.5 ml of a standard, Krebs - Ringer - Henseleit (K-R-H) buffer, without calcium, containing 10 mM $MgSO_4$ for 20 min at 37°C under continuous gassing with 95% 0_2 - 5% CO_2. After that time, the buffer was supplemented with 1.0 μCi of [^{3}H]D-aspartate, ([^{3}H]D-ASP) and the incubation run for the next 25 min in the same conditions. Then, the slices were placed on Whatman GF/C glass fibre filters cut to fit a Brandell superfusion unit. Superfusion was carried out with a standard K-R-H buffer at the rate of 0.5 ml/min under continuous gassing. When anoxic and aglycemic conditions were required the glucose was omitted from the medium and oxygen was replaced by N_2. The radioactivity collected in each 0.75 ml fraction and that residing on the filters after superfusion was counted using a liquid scintillation counter Wallac 1409. The fractional release was calculated as a percentage of radioactivity lost during each 1.5 min superfusion for each time point.

The depolarization of slices was performed by 10 min application of a buffer containing 65 mM KCl, with concomitant lowering of Na^+ ions to obtain isosmotic, 295 mOsm medium. The hippocampal slices were depolarized in normoxic or hypoxic conditions in the presence of different modulators of ion channels or transporter systems:
- tetrodotoxin (TTX), the voltage gated Na^+ channel blocker (1 μM) was added to the standard medium 5 min prior to the KCl stimulation,
- L-trans-PDC was applied in the same schedule at a concentration of 100 μM ,
- furosemide (1 mM) was applied also 5 min before KCl stimulation.

In the same experiments, the superfusion buffer was calcium-deprived and in addition contained 100 μM BAPTA-AM.

Statistics

The data for the individual traces shown as bars were calculated as the mean fractional release from the 9 min period of high K^+ stimulation. Each experimental condition is represented by mean ±SD obtained from 8-12 traces based on three separate slice preparations. The differences between means were analysed by ANOVA followed by Student's [t] test, using the Pharmacological Calculation System Program.

RESULTS

[^{3}H]D-ASP efflux under normoxic vs. anoxic - aglycemic ("ischemic") depolarization

In our previous experiments (Zabłocka and Domańska-Janik 1994), when the hippocampal slices were superfused with glucose- and oxygen-free media we did not observe any elevation in the release of [^{3}H]D-ASP, in contrast to the known effect of anoxia i*n vivo* (Choi and Rothman 1990, Salińska et al. 1991). This strongly suggested that in our model of permanent superfusion we might have caused rinsing out of some endogenously produced substances which are crucial for triggering glutamate release. The great difference in the volume of the superfusion chamber (250 μl) and the slice's extracellular space could permit diffusion and dilution of these substances. However, as was previously mentioned, rapid elevation of extracel-

lular potassium in the first minutes of ischemia (Hansen 1985, Siesjö et al. 1993) has been suggested to participate in ischemic glutamate efflux (Szatkowski et al. 1990). Thus, in the present experiments we have decided to supplement the buffer with elevated, 65 mM potassium (with concomitant lowering NaCl concentration) during the onset of anoxic - aglycemic superfusion (later reffered to as the "ischemic" condition).

Comparing the extent of this "ischemic" stimulation of [^{3}H]D-ASP release with that evoked by the identical elevation of potassium but in normoxic slices, we have noticed its substantial reduction under "ischemia" from 3,45±0,68 to 2,64±0,54 respectively. Moreover, and in the agreement with our previous experience, anoxia - aglycemia alone did not change the basal amino acid release when 5 mM potassium was present in the superfusion buffer (Figs. 2 and 3).

Calcium dependency

For comparative purposes, at first we investigated the calcium dependency of normoxic and "ischemic" depolarization evoked-[^{3}H]D-ASP release in hippocampal slices. Figure 1 shows that, the Ca^{2+} free medium caused significant decrease in the efflux of [^{3}H]D-Asp., which was more accentuated in normoxic (panel A) than in "ischemic" (panel B) slices (by approximately 70% and 50%, respectively). Further deprivation of intracellular calcium by continuous perfusion with the permeable calcium chelator BAPTA-AM caused additional inhibition of amino acid release, but, in sharp contrast with the previous one, this reduction was enhanced in the "ischemic" slices. In consequence the Ca^{2+} independent [^{3}H]D-ASP release consisted of 28% of the total release in normoxic conditions and over 40% in "ischemia".

Effect of drugs

In order to clarify further the contribution of the different mechanisms and compartments underlying depolarization-evoked Ca^{2+} dependent as

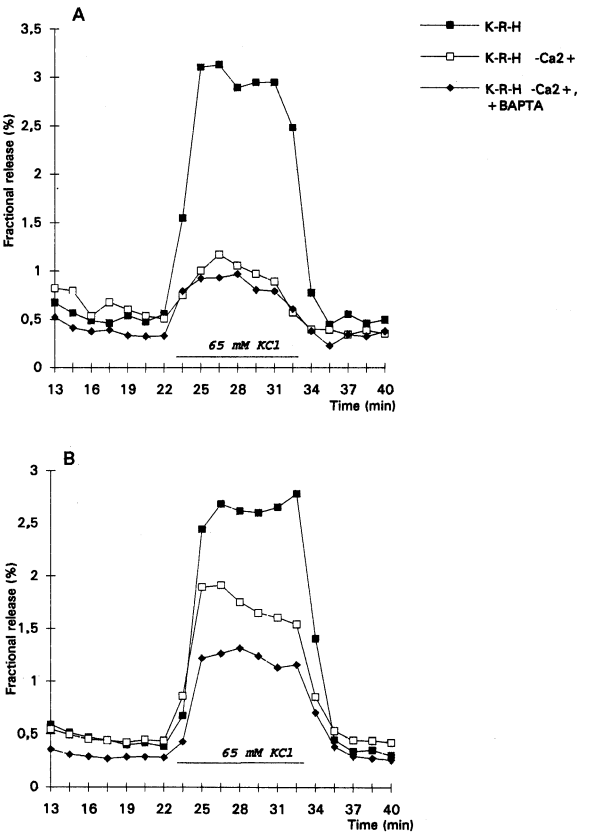

Fig. 1. Effect of Ca^{2+} free Krebs-Ringer-Henseleit (K-R-H) buffer on [^{3}H]D-ASP release from hippocampal slices in normoxic (A) and in anoxic-aglycemic (B) conditions. Bars indicate the time periods at which standard buffer was changed to that containing 65 mM KCl. Ca^{2+} free medium was applied 5 min before high K^+ stimulation. The trace represents the data obtained in one representative experiment and is the mean fractional release from 4-6 slices.

well as Ca^{2+} independent EAA releases in normoxic and "ischemic" conditions, we have probed the effects of various ion channel blockers and transporter inhibitors as described in the Introduction. All these chemicals were added to the superfusion buffer 5 min before the depolarization or "ischemia" had been started, then were kept constant to the end of stimulation.

Tetrodotoxin (TTX), the Na^+ channel blocker, applied at 1 μM concentration, significantly reduced the rate of [^{3}H]D-ASP release from nor-

moxic, K^+ stimulated slices by approximately 40% (Fig. 2). On the other hand, there was no significant alteration in the release activated by "ischemia" or in the basal levels of efflux in the presence of TTX (Fig. 3).

The experiments to elucidate the participation of the reversed Na^+/K^+ dependent amino acid transporter in [^{3}H]D-ASP release triggered by normoxic or "ischemic" depolarization were performed using its specific inhibitor L-trans-PDC. While 100 μM concentration of this drug failed to influence the basal efflux, the stimulatory effect of depolarization was significantly attenuated. Figures 2 and 3 show that the release was reduced to 60% of control in normoxic and to 80% in "ischemic" slices.

The most significant decrease of [^{3}H]D-ASP release was achieved by the inhibition of the anion channels. The application of 1 mM furosemide, 5 min before the onset of depolarization or "ischemia", resulted in the significant lowering of amino

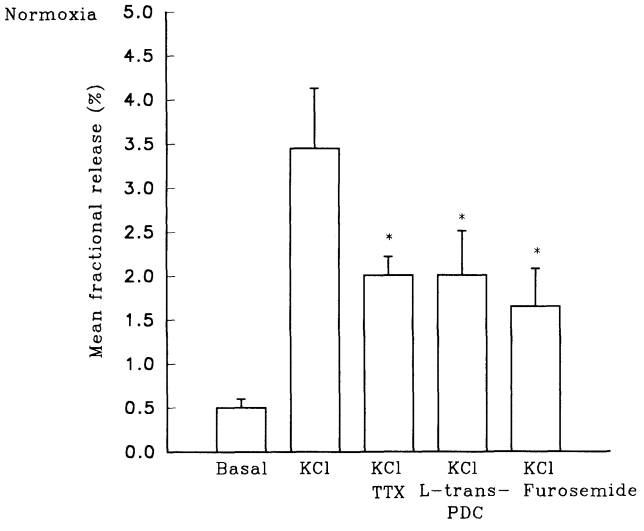

Fig. 2. Influence of channel blockers (1 μM TTX, 1 mM furosemide) and transporter inhibitor (100 μM L-trans-PDC) on 65 mM KCl stimulated release of [^{3}H]D-ASP from hippocampal slices in normoxic conditions. Slices were prepared and perfused as described in Methods. Bars represent the mean ± SD of fractional releases from at least 3 separate tissue preparations determined in triplicate traces. Each trace was calculated as a mean release obtained from 4-6 slices stimulated for 10 min with 65 mM KCl. Significant differences between treated and untreated stimulated slices (P<0.01) are marked by asterisks.

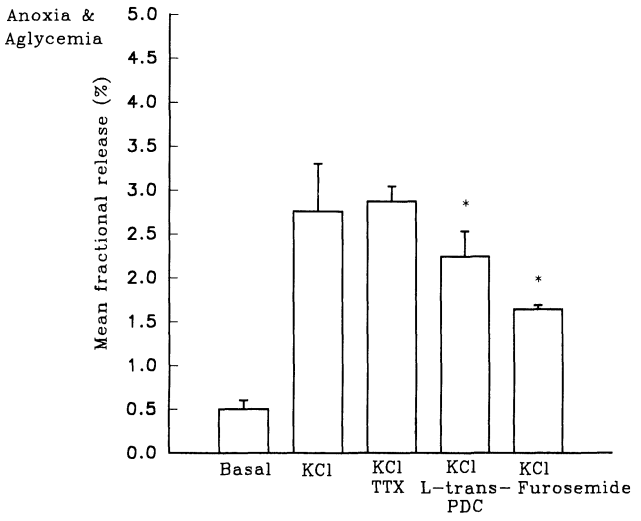

Fig.3. Influence of channel blockers (1 μM TTX, 1 mM furosemide) and transporter inhibitor (100 μM L-trans-PDC) on 65 mM KCl stimulated release of [^{3}H]D-ASP from hippocampal slices in anoxic - aglycemic conditions. Experimental conditions were as described in legend to Fig. 2.

acid efflux as compared to that observed in drug - untreated slices. As a result, a furosemide - insensitive portion of [^{3}H]D-ASP release evoked by either normoxic or "ischemic" depolarization amounted to 1,6±0,1 of the fractional release in both situations (Figs. 2 and 3).

DISCUSSION

The present results show that [^{3}H]D-ASP marks the EAA pools which may be released partially in a Ca^{2+} dependent and partially in a Ca^{2+} independent manner, in both normoxic and "ischemic" conditions. Based on experiments where not only external but also internal calcium was eliminated in the presence of BAPTA, we have found approximately 70% of the evoked outflow in normoxia and 50% in "ischemia" to be Ca^{2+} dependent. The normoxic percentages are similar to those reported previously (Zabłocka and Domańska-Janik 1994), even assuming that depolarizing pulses were much more extended in the present experiments. The relatively small effect of BAPTA chelation in normoxia, together with the high responsiveness to TTX

inhibition, would indicate that this [^{3}H]D-ASP which is releasable from the vesicular, TTX - sensitive compartment depends on the influx of external Ca^{2+} through voltage - activated channels. On the other hand, Ca^{2+} dependent release under "ischemia" differs substantialy from that in normoxia not only by its extend but also by the greater reaction on BAPTA and the complete lack of TTX inhibition. Therefore, Ca^{2+} dependent [^{3}H]D-ASP release during "ischemia" seems to involve some other than vesicular compartments. It was reported that, under hypoxia-hypoglycemia *in vitro*, the amount of EAA released from astroglia can exceed many times that released from co-cultured neurones (Ogata et al. 1992). Moreover, ischemia *in vivo* preferentially depletes immunochemically localised glutamate from the cytosol of cell bodies and not from presynaptic vesicles of hippocampus (Torp et al. 1992). Also, Ca^{2+} free conditions fail to alter the ischemia - induced efflux of either glutamate or aspartate, implying further that vesicular release plays only a minor role under these conditions (Ikeda et al. 1989, Phillis et al. 1994). Recently Parpura et al. (1994) showed that efflux of EAA from cultured astrocytes can be mediated by receptor - coupled stimulation of these cells by the neuroligand bradykinin. Moreover, this [^{3}H]D-ASP outflow was highly dependent on internal calcium mobilization and completely blocked by furosemide. Supposedly, taking into account the inhibitory effect of furosemide observed also in our slices, such a ligand - activated compartment would be responsible for calcium - dependent but TTX - insensitive release during "ischemia".

Present results are in disagreement with other reports showing that ischemia can stimulate amino acid release mainly in a Ca^{2+} independent manner (Ikeda et al. 1989). However these experiments were based on a different method where glutamate release was directly estimated by means of HPLC analysis. Whether [^{3}H]D-ASP, this most widely used radiolabelled glutamate analog, is in fact a marker of exocytotic release has been called into question. There are reports showing that it does not appear to enter isolated synaptic vesicles as glutamate does (Naito and Ueda 1985). As a compromise we can assume that there are certain pools of glutamate which are not as easily labelled by D-ASP as others are and this would be responsible for these differences in results.

Among non-vesicular release, the participation of the reversed amino acid transporter does not seem to be dominant under ischemia. Its inhibition by L-trans-PDC accounts only for approximately 20% of total [^{3}H]D-ASP release, whereas about 40% was inhibited under normoxia. The reason for this difference is still unclear and will be addressed in our further investigations. It must be stressed that, as discussed in the Introduction, persistent depolarization produces highly unusual non-physiological conditions for normoxic slices as well. This may be compared with that occurring during non-compensated, extensive nerve cell excitation or spreading depression *in vivo* (Leao 1944). Both of these circumstances were suggested to contribute to brain ischemic damage (Swanson 1969, Hossman 1994). This is consistent with the facilitated cellular swelling of depolarized slices (Joanny and Hillman 1963) as well as with the relatively large furosemide-inhibited, [^{3}H]D-ASP -releasing compartment observed in the present work. Therefore it seems that enhancement of amino acid release evoked by "ischemia" in our model *in vitro* could be compared rather with the basal [^{3}H]D-ASP efflux from unstimulated slices as more representative for the physiological status of brain tissue.

On the other hand, persistent depolarization, apart from the induction of osmotic imbalance and cellular swelling seems to be metabolically compensated under normoxia. In contrast with anoxia - aglycemia, the high energy stores are well preserved in these slices (Verhage et al. 1994), probably because of a compensatory increase of oxidative metabolism (Domańska-Janik and Zalewska 1979). Nevertheless, on the basis of present results it can be suggested that deteliorous effect of high extracellular potassium, accompanying anoxia - aglycemia *in vitro* and *in vivo* (Domańska-Janik and Zalewska 1979, Hossman 1994) as well as being a causative factor in spreading depression *in vivo*

(Leao 1944) may involve a hazardous excitotoxic input. This may be due to reversal of the amino acid transporter and opening of furosemide-sensitive, volume regulated large anion channels, which in concert with exocytosis of synaptic vesicles would release glutamate in this condition.

Finally, the positive trials proving neuroprotective effects of these inhibitors of non-vesicular glutamate release on postischemic outcome will be of great clinical value, supporting the contribution of these mechanisms/compartments in excitotoxic ischemic damage. The first reports showing their protective role in animal models of ischemia have been published (Kohut et al. 1992).

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