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THE ANION CHANNEL BLOCKER, 4,4′-DINITROSTILBENE-2, 2′-DISULFONIC ACID PREVENTS NEURONAL DEATH AND EXCITATORY AMINO ACID RELEASE DURING GLYCOLYSIS INHIBITION IN THE HIPPOCAMPUS *IN VIVO*

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Abstract—Neuronal death associated with cerebral ischemia and hypoglycemia is related to increased release of excitatory amino acids (EAA) and energy failure. The intrahippocampal administration of the glycolysis inhibitor, iodoacetate (IOA), induces the accumulation of EAA and neuronal death. We have investigated by microdialysis the role of exocytosis, glutamate transporters and volume-sensitive organic anion channel (VSOAC) on IOA-induced EAA release. Results show that the early component of EAA release is inhibited by riluzole, a voltage-dependent sodium channel blocker, and by the VSOAC blocker, tamoxifen, while the early and late components are blocked by the glutamate transport inhibitors, L-trans-pyrrolidine 2,4-dicarboxylate (PDC) and DL-threo-beta-benzyloxyaspartate (DL-TBOA); and by the VSOAC blocker 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS). Riluzole, DL-TBOA and tamoxifen did not prevent IOA-induced neuronal death, while PDC and DNDS did. The VSOAC blockers 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB) and phloretin had no effect either on EAA efflux or neuronal damage. Results suggest that acute inhibition of glycolytic metabolism promotes the accumulation of EAA by exocytosis, impairment or reverse action of glutamate transporters and activation of a DNDS-sensitive mechanism. The latest is substantially involved in the triggering of neuronal death. To our knowledge, this is the first study to show protection of neuronal death by DNDS in an *in vivo* model of neuronal damage, associated with deficient energy metabolism and EAA release, two conditions involved in some pathological states such as ischemia and hypoglycemia.

Key words: anion channels, exocytosis, glutamate transporters, iodoacetate, hypoglycemia, neuronal death.

The basal concentration of glutamate in the extracellular space is regulated by several release pathways including the spontaneous calcium-dependent release from synaptic terminals (Takahashi and Momiyama, 1993), the activity of the cystine-glutamate exchanger (Baker et al., 2002) and the activation of astrocytic hemichannels (Ye et al., 2003). An increase in glutamate extracellular levels and the consequent over-activation of its receptors induces excitotoxic neuronal death (Choi, 1988), a cell-death mechanism triggered during ischemia and hypoglycemia (Benveniste et al., 1984; Sandberg et al., 1985; Wieloch, 1985; Sandberg et al., 1986; Smith and Meldrum, 1992; Takagi et al., 1993; Auer, 2004). The extracellular accumulation of the excitatory amino acids (EAA), aspartate and glutamate, during ischemia involves an early calcium-dependent release, possibly from neurons (Drejer et al., 1985; Nelson et al., 2003). After exocytotic release glutamate removal from the synaptic cleft is carried out by glutamate transporters coupled to the sodium electrochemical gradient generated by the Na^+,K^+-ATPases. One glutamate or aspartate molecule is co-transported with three sodium ions while one potassium ion is countertransported to the extracellular medium (Huang and Bergles, 2004). Collapse of the sodium/potassium gradient during ischemia or hypoglycemia induces an intracellular Na^+ overload, promoting the reverse function of glutamate transporters and the increase in amino acid extracellular basal levels (Rossi et al., 2000; Mitani and Tanaka, 2003). This mechanism has been suggested to be involved in EAA release during energy failure associated with cerebral ischemia (Seki et al., 1999; Phillis et al., 2000). An additional release pathway for EAA during ischemia involves the action of volume-sensitive organic anion channels (VSOAC) activated in response to cell swelling (Seki et al., 1999; Kimelberg et al., 2004). Although several studies suggest the participation of these routes in glutamate and aspartate release during ischemia (Drejer et al., 1985; Phillis et al., 1994, 1998, 2000; Seki et al., 1999; Dawson et al., 2000), their relation to neuronal death is still unclear. On the other hand, the role of these pathways on amino acid efflux and cell damage during hypoglycemia has received less attention.

Glucose oxidation provides the energy necessary for the regulation of ambient glutamate concentration *in vivo* (Voutsinos-Porche et al., 2003; Shulman et al., 2004). Impairment of the glycolytic pathway induces excitotoxic cell death, possibly resulting from increased EAA release and failure of its removal. *In vitro* inhibition of the glycolytic enzyme, glyceraldehyde 3-phosphate dehydro-

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Abbreviations: DIDS, diisothiocyanostilbene-2,2′-disulfonic acid; DL-TBOA, DL-threo-beta-benzyloxyaspartate; DNDS, 4,4′-dinitrostilbene-2,2′-disulfonic acid; EAA, excitatory amino acids; IOA, iodoacetate; MK801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NBQX, 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo[f]quinoxaline-7-sulfonamide disodium; NMDA, *N*-methyl-D-aspartate; NPPB, 5-nitro-2-(3-phenylpropyl-amino) benzoic acid; PDC, L-trans-pyrrolidine 2,4-dicarboxylate; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; VSOAC, volume-sensitive organic anion channel.

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genase (GAPDH) by iodoacetate (IOA), promotes the elevation of extracellular glutamate through the activation of exocytotic release, the inverse function of glutamate transporters and hemichannel permeation (Ogata et al., 1995; Zeevalk et al., 1998; Kulik et al., 2000; Contreras et al., 2002; Sundstrom and Mo, 2002). In previous *in vivo* studies we have demonstrated that IOA perfusion through a microdialysis probe into the rat hippocampus induces the elevation of extracellular EAA levels and neuronal death (Massieu et al., 2000), suggesting an important role of glycolysis in the control of glutamatergic neurotransmission and neuronal survival. In the present study we have investigated whether EAA release during IOA perfusion into the hippocampus is related to exocytotic release, the inverse action of glutamate transporters, or VSOAC activation. To test these alternatives we have used the voltage-dependent sodium channel blocker, riluzole, to inhibit exocytotic EAA release, the substrate and no-substrate glutamate uptake blockers, L-trans-pyrrolidine-2,4-dicarboxylate (PDC) and DL-threo-β-benzyloxyaspartate (DL-TBOA), respectively, and the VSOAC blockers, 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), tamoxifen and phloretin. We have also studied the involvement of each one of these release pathways on the induction of neuronal death.

EXPERIMENTAL PROCEDURES

Chemicals

IOA, riluzole, DIDS, phloretin and tamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO, USA); PDC, DL-TBOA, NPPB, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK801), and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo[f]quinoxaline-7-sulfonamide disodium (NBQX) were obtained from Tocris (Ellisville, MO, USA), and DNDS from Molecular Probes, Inc. (Eugene, OR, USA).

Microdialysis experiments

Male Wistar rats (250–300 g) were used throughout the study. They were handled according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and the local Animal Care Committee approved all animal treatments. All efforts were made to minimize the number of animals used and their suffering. Rats were anesthetized with halothane (1.5%) and fixed on a stereotaxic frame. Microdialysis probes, 2 mm long and 0.5 mm in diameter (CMA12; Bioanalytical Systems, West Lafayette, IN, USA, were implanted in the CA1 region of the hippocampus using the following coordinates: AP −3.6 mm from Bregma, L +2.0 mm from midline, and V −4.0 mm from dura (Paxinos and Watson, 1986) and animals were maintained under low anesthesia (0.5% halothane) throughout the experiment. Body temperature was kept at 36.5–37.5 °C by means of a heating pad throughout the implantation of dialysis probes and the duration of the microdialysis experiment.

Microdialysis probes were continuously perfused with a Ringer–Krebs medium containing (in mM): NaCl 118, KCl 4.5, $MgSO_4$ 2.5, NaH_2PO_4 4.0, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 10, at a flux rate of 2 μl/min via a microinjection pump (BAS MD 1001, West Lafayette, IN, USA), during a 90-min period in order to obtain the stabilization of the probe. Animals were assigned to four experimental groups: the first group was perfused with 1 mM IOA; the second with 1 mM IOA+1 mM Riluzole; the third group received 1 mM IOA+either one of the glutamate uptake blockers studied (100 μM or 500 μM DL-TBOA; or 1 mM or 5 mM PDC); and animals in the fourth group were perfused with 1 mM IOA+either one of the VSOAC inhibitors tested (10 mM DNDS, 250 μM tamoxifen, 1 mM phloretin or 1 mM NPPB). The doses used of the different anion channel blockers were chosen according to previous studies showing their effectiveness in blocking ischemia-induced amino acid release (Singh et al., 1991; Rutledge et al., 1998; Seki et al., 1999; Fan et al., 2001; Franco et al., 2001; Al-Nakkash et al., 2004; Haskew-Layton et al., 2005; Abdullaev et al., 2006). Since the real concentration of the different compounds in the tissue is not known, we assumed a 10% delivery of the drugs according to the recovery of amino acids by the microdialysis probe previously calculated (Massieu et al., 1995).

After the stabilization of the probes 10 fractions of 25 μl were continuously collected. When the effect of IOA alone was studied, the three first fractions were perfused with Ringer–Krebs alone, then IOA was administered in Ringer–Krebs medium during the four following fractions, and during the last three fractions Ringer–Krebs medium alone was perfused again. When the effect of the different inhibitors on IOA-induced EAA release was studied, basal levels were monitored during the first collected fraction, afterward the corresponding inhibitor was perfused alone in Ringer–Krebs medium for two fractions, and during the next four fractions perfusion media contained inhibitor+IOA. The last three fractions corresponded to Ringer–Krebs medium alone. In a series of experiments 100 mM KCl was administered after IOA perfusion in order to discard amino acid depletion from releasable pools.

DNDS is a stilbene disulfonic acid related to SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid) and DIDS and was used instead of SITS or DIDS because they were found to interfere with the fluorescence emission of amino acid o-phthaldialdehyde-derivatives studied by high-performance liquid chromatography (Seki et al., 1999).

The effects of PDC and DL-TBOA on basal amino acid levels, have been previously studied (Montiel et al., 2005). In the present work the effect of DNDS (10 mM) and NPPB (1 mM) on EAA basal concentrations was studied. In these experiments Ringer–Krebs was perfused during the first fraction, during the six following fractions DNDS or NPPB was added to Ringer–Krebs medium, and Ringer alone was perfused again during the three last fractions.

Twenty-four hours after the end of the microdialysis experiment rats were anesthetized with sodium pentobarbital anesthesia and transcardially perfused with 250 ml of 0.9% saline solution followed by 250 ml of 5% formaldehyde in 0.1 M phosphate buffer (pH 7.3). Brains were removed and placed in fixative solution for additional 24 h. They were then transferred successively to a 20% and 30% sucrose solution and coronal sections (40 μm) were made in a cryostat and stained with Cresyl Violet. Lesion size was calculated by examination of all brain sections where neuronal damage was evident in each experimental animal. Tissue damage was determined by the presence of pyknotic nuclei and the absence of cells with a visible cytoplasm. The damaged area was delineated manually and measured with the aid of an image analyzer (NIH Macintosh Image 1.6). The lesion volume was calculated by the sum of measured areas in all sections multiplied by the slice thickness. Results are expressed as means±S.E.M. of lesion volume per each animal group.

Quantification of amino acid levels

Amino acid content in dialysates was determined by HPLC as previously reported by (Antoine et al., 1999), with slight modifications. Briefly, the 25-μl fractions were derivatized with the same volume of *o*-phthaldialdehyde and 3 min later a 10-μl volume of this mixture was injected into an HPLC system (Waters 600; Waters, Millford, MA, USA) equipped with an ODS column