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Evidence for the existence of pyrimidinergic transmission in rat brain

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ABSTRACT

The uridine nucleotides uridine-5′-triphosphate (UTP) and uridine-5′-diphosphate (UDP) have previously been identified in media from cultured cells. However, no study to date has demonstrated their presence in brain extracellular fluid (ECF) obtained in vivo. Using a novel method, we now show that UTP and UDP, as well as uridine, are detectable in dialysates of striatal ECF obtained from freely-moving rats. Intraperitoneal (i.p.) administration of uridine or exposure of striatum to depolarizing concentrations of potassium chloride increases extracellular uridine, UTP and UDP, while tetrodotoxin (TTX) decreases their ECF levels. Uridine administration also enhances cholinergic neurotransmission which is accompanied by enhanced brain levels of diacylglycerol (DAG) and inositol trisphosphate (IP_3) and blocked by suramin, but not by PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid) or MRS2578 suggesting a possible mediation of P2Y2 receptors activated by UTP. These observations suggest that uridine, UTP and UDP may function as pyrimidinergic neurotransmitters, and that enhancement of such neurotransmission underlies pharmacologic effects of exogenous uridine on the brain.

1. Introduction

Uridine, the principal circulating pyrimidine nucleoside in humans (Wurtman et al., 2000) and its nucleotide products uridine-5′-diphosphate (UDP), uridine-5′-triphosphate (UTP), UDP-glucose, UDP-galactose (Lecca and Ceruti, 2008) and cytidine-5′-triphosphate (CTP) (Cansev et al., 2005) affect numerous physiological functions, including the syntheses of RNA (Lecca and Ceruti, 2008) and membrane phosphatides (Ulus et al., 2006; Wurtman et al., 2006; Cansev and Wurtman, 2007; Sakamoto et al., 2007), and the differentiation of neuron-related cells (e.g. neurite outgrowth) (Pooler et al., 2005). Uridine's effects on phosphatide synthesis are mediated by CTP, an intermediate in the Kennedy Cycle (Kennedy and Weiss, 1956), while its stimulation of neuronal differentiation (Pooler et al., 2005) involves the activation by UTP of brain P2Y2 receptors (Lustig et al., 1993; Pooler et al., 2005).

The uridine nucleotides UTP and UDP are ligands to several P2Y receptors (Abbracchio et al., 2006). UTP activates P2Y2 (Lustig et al., 1993) and P2Y4 (Communi et al., 1995) receptors while UDP activates P2Y6 (Communi et al., 1996) and P2Y14 receptors (Carter et al., 2009). Activation of these cell surface receptors in the brain requires that UTP and UDP are released from cells into the extracellular fluid (ECF) (Cansev, 2007; Lecca and Ceruti, 2008). Therefore, intensive work has been done to identify uridine nucleotides in brain ECF. Although previous in vitro studies reported the detection of UTP in the culture medias of a variety of cells in low nanomolar ranges (1 ± 10 nM in 0.5 ml medium bathing 2.5 cm^2 dish) (Lazarowski et al., 1997; Lazarowski and Harden, 1999), to date, only uridine (Dobolyi et al., 1998), but not UTP or UDP, has been identified in brain ECF in vivo.

Therefore, the aim of the present study was to detect and quantify uridine and its nucleotides UTP and UDP in brain ECF by in vivo microdialysis. We initially aimed to solve the possible

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problem of rapid hydrolysis of extracellular nucleotides by membrane-bound ecto-nucleotidase enzymes (Zimmermann, 1996) which might have been the reason for prior failure to detect uridine nucleotides in brain ECF in vivo. We have thus developed a method – based on blocking the ecto-nucleotidase enzymes that would destroy the UTP and UDP in ECF dialysates – for quantifying these nucleotides in brain ECF.

Using that method, we confirmed their presence in rat brain ECF in vivo and observed increases in their levels following intraperitoneal (i.p.) administration of uridine in doses known to raise (Cansev et al., 2005) plasma and brain uridine levels and brain levels of UTP and UDP. We further observed that uridine, UTP and UDP are released from excitable cells in experimental designs including potassium chloride-induced neuronal depolarization and tetrodotoxin (TTX) blockade of action potentials. The enhanced release of these pyrimidines subsequently enhanced cholinergic neurotransmission, manifested by increased acetylcholine release; this effect was blocked by suramin, a non-selective antagonist of P2Y2 and P2Y6 receptors, but not by PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid), a non-selective antagonist of P2Y4 and P2Y6 receptors or MRS2578, a selective antagonist of P2Y6 receptors.

To the best of our knowledge, our data provide the first comprehensive evidence for a pyrimidinergic neurotransmission, the enhancement of which enhances cholinergic neurotransmission via, probably, P2Y2 receptors.

2. Material and methods

2.1. Animals

Adult male Sprague–Dawley rats (300–350 g; Experimental Animals Breeding and Research Center, Uludag University Medical School, Bursa, Turkey) were housed in groups of four in a temperature controlled room with free access to standard rat chow and water under a 12 h light/dark cycle. The experimental protocol was approved by the Animal Care and Use Committee of Uludag University, Bursa, Turkey (Approval ID: 2008-12/7), and all experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

2.2. Surgical procedures

Experiments were carried out in two settings; in vivo microdialysis, for obtaining brain ECF, and, in vivo freezing for obtaining samples of blood and of brain tissue.

For in vivo microdialysis experiments, skulls of rats which had been anesthetized with Ketamine and Xylazine (80 mg/kg and 10 mg/kg, respectively) and then placed in a stereotaxic frame were exposed, and a small hole was drilled over the left striatum, using the bregma as the reference point (coordinates were: AP 1.0 mm; ML 2.8 mm; V 6.0 mm). A hand-made probe (molecular weight cutoff of dialysis membrane was 13,000 Da and length was 1 mm) was implanted and then fixed to the skull, using acrylic cement. After surgery the rats were placed in individual cages and allowed to recover from anesthesia for 24 h. During this period, they remained calm and showed no evidence of pain.

For in vivo freezing experiments, rats were anesthetized with Ketamine and Xylazine (80 mg/kg and 10 mg/kg, respectively) and their skulls were exposed. Blood sampling was performed by heart puncture immediately before the rats' heads were dipped into liquid nitrogen. Blood sampling lasted for less than 10 s within which period the heart puncture probably resulted in a cessation of circulation such that the rats were technically dead.

2.3. In vivo microdialysis study

In vivo microdialysis experiments were carried out 24 h after surgery to allow rats to recover from the effects of anesthesia and surgery. The dialysis probe was perfused at a rate of 2 μl/min with artificial cerebrospinal fluid (CSF; pH = 7.4) of the following composition: 148 mM NaCl, 3.0 mM KCl, 1.4 mM $CaCl_2$, 0.8 mM $MgCl_2$, 1.3 mM NaH_2PO_4, 0.2 mM Na_2HPO_4. The artificial CSF also contained PSB069 (Tocris Bioscience, Bristol, UK), a non-selective nucleoside triphosphate diphosphohydrolase (NTPDase) inhibitor, which reportedly inhibits rat NTPDases 1, 2 and 3 with similar potencies (Baqi et al., 2009). Addition of PSB069 to the perfusion fluid protected extracellular uridine nucleotides from hydrolysis, and enabled us to measure these nucleotides in extracellular fluid by in vivo microdialysis technique.

In experiments that we tested the effect of uridine (1 mmol/kg; i.p.) on acetylcholine and choline release, 10 μM of physostigmine was included in the standard artificial CSF in order to block acetylcholinesterase and thus detect acetylcholine, as reported previously (Westerink, 2000). In follow-up experiments that we investigated the mediation of P2Y receptor stimulation on enhanced acetylcholine release, an intracerebroventricular (i.c.v.) cannula directed to the lateral ventricle was implanted along with the microdialysis probe one day prior to the experiments. The next day 10 μl of artificial CSF containing suramin (1 μmol; Sigma, St. Louis, MO, USA), a non-selective antagonist of P2Y2 and P2Y6 receptors, PPADS (0.1 μmol; Sigma, St. Louis, MO, USA), a non-selective antagonist of P2Y4 and P2Y6 receptors, or MRS2578 (1 μmol; Sigma, St. Louis, MO, USA), a selective antagonist of P2Y6 receptors was slowly injected 15 min prior to uridine (1 mmol/kg; i.p.) administration (Suramin at 5 and 10 μmol doses and PPADS at 0.5 and 1 μmol doses caused convulsions in the rat).

In separate experiments we tested the effect of high potassium stimulation on the release of uridine and its nucleotides into brain ECF. The concentration of KCl (Sigma, St. Louis, MO, USA) in the perfusion fluid was 52 mM and equimolar amount of NaCl (Sigma, St. Louis, MO, USA) was removed from the perfusion fluid during high potassium stimulation. Perfusion with 52 mM KCl was continued for 2 h and the medium was then replaced with the regular artificial CSF.

In additional experiments the effect of TTX perfusion on the release of uridine and its nucleotides into brain ECF was investigated. The concentration of TTX in the perfusion fluid was 1 μM. Perfusion with TTX was continued for 2 h and the medium was then replaced with the regular artificial CSF.

Dialysate samples of approximately 60 μl were collected at 30-min intervals for up to 5.5 h after the i.p. injection of uridine (0.1–1.0 mmol/kg) or perfusions of high KCL or TTX. The dialysis probes were perfused with artificial cerebrospinal fluid for an initial 60-min stabilization period, and then three consecutive basal samples were collected. The uridine, UTP and UDP levels in the three basal samples did not differ by more than 10%

2.4. Blood collection and brain tissue sampling by in vivo freezing method

A separate set of experiments was performed to determine serum uridine concentrations, and brain uridine and uridine nucleotide levels, after i.p. uridine administration. For analyses of serum uridine concentrations 200 μl blood samples were obtained by heart puncture using a 22G injector needle before (at time zero) and at 1, 2, 4, and 8 h after i.p. injection of uridine (0.1–1 mmol/kg). In vivo brain freezing was performed at the same time points immediately after collection of the blood samples. Bloods were centrifuged immediately at 10,000 *g* and 4 °C to obtain serum samples which were kept frozen until analysis for uridine.

In order to prevent rapid degradation of nucleotides after decapitation, we utilized an in vivo brain freezing method (Wiegand et al., 1999; Cansev et al., 2005) to measure brain concentrations of uridine and its nucleotides. Immediately after blood collection by heart puncture, as described above, heads of anesthetized rats were dipped into liquid nitrogen for 45 s at the same time points as those used for blood collection (i.e., before or 1, 2, 4 and 8 h after i.p. uridine administration). Samples of brain tissue were then obtained using a tissue homogenizer (Polytron PT 10–35, Kinematica AG, Lucerne, Switzerland). The homogenizer has two rotating knives, 1.1 cm apart, which extend 4 mm beyond its shaft vertically; when operated at high speed, it cuts the frozen skull enabling removal of a circular 1 cm-diameter piece of frozen cortex. This freezing method minimized the breakdown of brain nucleotides, as confirmed by the fact that several-fold greater nucleotide levels were found after in vivo freezing than after conventional brain dissection (data not shown) in which the brain is removed from the skull after decapitation and samples for assay are excised within minutes.

Brain tissues were homogenized using the same tissue homogenizer (Polytron PT 10–35, Kinematica AG, Lucerne, Switzerland), in 50 volumes of ice-cold deionized water also containing the PSB069 (10 μM); aliquots were kept frozen for analysis of brain uridine and the uridine nucleotides. Serum and brain samples were extracted using 80% methanol; dried under vacuum; and reconstituted with deionized water before analysis of uridine and uridine nucleotides. Tissue levels of uridine and its nucleotides were expressed as picomoles per mg tissue.

2.5. Analyses of uridine and uridine nucleotides in serum and brain tissue samples

Serum and brain tissue samples were analyzed for uridine and uridine nucleotides using High Performance Liquid Chromatography (HPLC), as described previously (Cansev et al., 2005). The HPLC (Hitachi, Tokyo, Japan) was attached to a diode-array detector on a reversed-phase column (Thermo Scientific Hypersil ODS, 5 μm, 150 mm × 4.6 mm I.D.) which was connected to a guard cartridge (Supelco Discovery HS C18 Supelguard, Sigma–Aldrich, St. Louis, MO, USA). Uridine in serum and brain samples was eluted using 4 mM potassium phosphate buffer containing 0.1% methanol at pH 5.5. Individual peaks were detected by UV absorption at 260 nm and their identities confirmed by comparison with the positions of authentic standards.

Uridine nucleotides (UTP and UDP) in brain were analyzed by HPLC (Hitachi, Tokyo, Japan) using a strongly basic anion-exchange column (Macherey-Nagel Nucleosil SB, 5 μm, 150 mm × 4.6 mm I.D.) attached to a guard cartridge (Partisil SAX 7.5 × 4.6 mm, Alltech, Deerfield, IL, USA) with an isocratic buffer containing 200 mM NaH_2PO_4 at pH 2.8 flowing at 1 ml/min. Individual peaks were detected by UV