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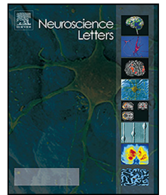

Research paper

Extracellular levels of ATP and acetylcholine during lithium-pilocarpine induced status epilepticus in rats

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HIGHLIGHTS

- Extracellular levels of ATP and acetylcholine were measured in rat hippocampus.
- ATP release is reduced by calcium-free conditions, but not by neostigmine or tetrodotoxin.
- Status epilepticus leads to an increase of extracellular ATP which is only measurable when 5′-endonucleotidase is inhibited.
- There was no evidence of crosstalk between acetylcholine and ATP.

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ABSTRACT

Acetylcholine (ACh) and ATP are rapidly acting neurotransmitters with a putative role in epileptic seizures. In the present study we investigated extracellular concentrations of both neurotransmitters in parallel by microdialysis in rat hippocampus. We found that infusion of neostigmine increases, while calcium-free perfusion and infusion of tetrodotoxin (TTX) decreases, ACh levels. Calcium-free perfusion also decreased ATP levels which were, however, not affected by neostigmine or TTX. During status epilepticus, ACh levels were increased threefold but returned to baseline after the termination of seizures by diazepam. ATP levels were unchanged during status epilepticus but a several-fold increase was seen when AOPCP, an inhibitor of 5′-endonucleotidase, was infused. The results demonstrate an increase of ATP levels during epileptic seizures which, however, was not of neuronal origin.

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1. Introduction

Acetylcholine (ACh) and ATP are two rapidly acting neurotransmitters which activate ionotropic as well as metabotropic receptors. The actions of ACh have been investigated in great detail in peripheral as well as in central nervous systems [1,2]. ATP has been recognized as a neurotransmitter more recently and has attracted much interest in the last three decades [3,4]. In fact, both transmitters share many characteristics; they can be co-stored and co-released, and, in contrast to other small molecule transmitters, their actions are terminated by extracellular hydrolysis [4]. While vesicular ACh release and its hydrolysis by acetylcholinesterase (AChE) has been investigated in great detail [4], the knowledge of ATP metabolism remains sketchy, partly because specific inhibitors of ATP metabolism are scarce. It is known that extracellular ATP, once released, is hydrolyzed by ecto-nucleosidtriphosphate-diphosphohydrolases (NTPDase) to ADP and AMP. The enzyme 5′-ectonucleotidase degrades AMP to the final product, adenosine [5]. Adenosine can be taken up by the nucleoside transporter into the nerve terminal followed by resynthesis of ATP and its vesicular storage. The nucleotides AMP, ADP and ATP as well as adenosine are agonists on purinergic receptors classified into P1 receptors (adenosine receptors A1–A3) and P2 receptors, i.e., nucleotide receptors which are subdivided into ligand-gated ion channels (P2X receptors) and G-protein coupled P2Y receptors [6]. In addition to their action as neurotransmitters, both ACh and ATP have been ascribed non-neuronal roles. Thus, ACh can be released from non-

Abbreviation: ACh, acetylcholine; AChE, acetylcholinesterase; aCSF, artificial cerebrospinal fluid; AOPCP, adenosine 5′(α,β-methylene)diphosphate; AMP, adenosine mono-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; MWCO, molecular weight cut-off; TTX, tetrodotoxin.

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neuronal cells such as epithelial cells through cation transporters [7,8], and ATP can be released from non-neuronal cells through connexin and pannexin hemichannels as well as via anion channels [6,9].

The relationship of cholinergic and purinergic neurotransmission is poorly characterized. Part of this lack of knowledge is due to analytical limitations; while the intracellular concentrations of ATP are in the millimolar range and the intravesicular ATP concentration can reach 150 mM [6], the extracellular ATP concentration is in the low nanomolar range. Nevertheless, highly sensitive bioluminescence methods can now measure extracellular ATP, for instance in microdialysates [10,11]. In the present study, we used microdialysis in rat hippocampus to investigate the origin of extracellular ATP in the brain and to test a potential crosstalk between ACh and ATP release. In addition, we measured both transmitters during neuronal hyperexcitation, using the lithium-pilocarpine model to evoke status epilepticus in rats [12,13].

2. Material and methods

2.1. Materials

AOPCP (Adenosine 5'(α,β-methylene) diphosphate), lithium chloride, neostigmine bromide, pilocarpine and the chemicals for artificial cerebrospinal fluid (aCSF) were purchased from Sigma–Aldrich (Munich, Germany). Tetrodotoxin citrate (TTX) was obtained from Abcam (Cambridge, UK). The chemicals for the mobile phase of the HPLC analysis were obtained from Merck, Darmstadt, Germany ($KHCO_3$), from Carl Roth, Karlsruhe, Germany (EDTA-2Na), from Alfa Aesar, Karlsruhe, Germany (sodium decane-1-sulfonate) and from Sigma–Aldrich, Munich, Germany (RotisolV HPLC gradient grade water).

2.2. Animals

Male Sprague-Dawley rats (Janvier labs, Saint Berthevin, France) were housed in a facility with 20–22 °C controlled temperature, 60–65% humidity and a day/night cycle of 12/12 h. They had free access to food (Altromin 1320, Lage, Germany) and water. 7–8 week old rats (220–300 g) were used for experiments after 1 week of acclimatization in the animal facility room. All experiments were performed in accordance with the guidelines of the local animal committee and approved by the responsible government agency (Regierungspräsidium Darmstadt, Germany).

2.3. Microdialysis experiments and drug treatment

Rats were anesthetized with isoflurane (induction dose 5%, maintenance dose 1.5–2% v/v) in synthetic air (Air Liquide, Düsseldorf, Germany) and placed in a stereotaxic frame. Infiltration anesthesia with 1% lidocaine solution was performed in the area surrounding the operation field. Self constructed, I-shaped, concentric dialysis probes with a molecular weight cut-off (MWCO) of 30 kDa and an exchange length of 3.5 mm [14,15] were implanted in the right ventral hippocampus with the following coordinates (from bregma): AP −5.2 mm; L −5.2 mm; DV −7.0 mm [16]. Bupivacaine gel was applied to the surgical wound as an analgesic, and the animal recovered over night.

On the following day, the probes were perfused with aCSF (147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$), or with calcium-free aCSF (omission of $CaCl_2$), aCSF supplemented with neostigmine (1 μM) or aCSF supplemented with TTX (1 μM). After 60 min equilibration time, basal values were collected for 45 min, then the perfusion fluid was switched and samples were collected for another 45 min. The perfusion rate was 2 μl/min and collection intervals were 15 min.

For induction of status epilepticus [17], rats received an injection of lithium chloride (127 mg/kg i.p) immediately after probe implantation. On the next day, probes were perfused with aCSF; in some experiments aCSF was supplemented with AOPCP (1 mM). The perfusion rate was 2 μl/min and the dialysate samples were collected in intervals of 15 min. After 60 min equilibration time, basal values were collected for 45 min. Subsequently, rats received an injection of pilocarpine (30 mg/kg s.c.) in order to induce status epilepticus [17] which set in approx. 30 min after pilocarpine injection. From 45 to 90 min after pilocarpine injection, i.e., when status epilepticus was fully developed, three samples were collected for data analysis. 90 min after pilocarpine, diazepam (10 mg/kg i.p.) was given to terminate seizures and three dialysates were sampled for another 45 min.

2.4. Acetylcholine and ATP determinations

Acetylcholine was analyzed by HPLC using an Eicom HTEC-500 system (Kyoto, Japan) consisting of degasser, low-speed pump, pre- and separation column, enzyme reactor and electrochemical detector with a platinum electrode operating at 0.5 V [18]. The mobile phase contained 50 mM $KHCO_3$, 134.3 mM EDTA-2Na and 1.64 mM sodium decane-1-sulfonate in RotisolV HPLC gradient grade water (pH 8.5) and was set to a flow rate of 150 μl/min. The enzyme reactor carried immobilized acetylcholine esterase and choline oxidase. The detection limit of this system was 1–2 fmol/10 μl.

ATP was measured by bioluminescence (ATPlite^{TM} Luminescence Assay System, PerkinElmer, Rodgau, Germany). 50 μl substrate buffer solution was diluted with 300 μl MilliQ water, then 20 μl dialysate or standard solution was added and mixed at 700 rpm, 25 °C for 5 min. The reaction solution remained in the dark reaction chamber for 10 min and was then analyzed for bioluminescence for 30 s (LB 9508 Lumat^3, Berthold Technologies, Bad Wildbad, Germany). Detection limit was 0.1 nM. As the dialysate was protein-free, no cell lysis reagent was required. A calibration curve was made daily for each experiment.

2.5. Statistics

Time courses of ACh and ATP concentrations were compared by one-way ANOVA with Tukey post-test using the Prism® 5 software (GraphPad, San Diego, CA). All data are given as means ± S.D. or S.E.M. The number of experiments is indicated in the figure legends. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Experiments in untreated rats

Fig. 1 summarizes the time courses of acetylcholine (ACh) and ATP concentrations in rat hippocampal microdialysates before and during the infusion of drugs. In the absence of neostigmine, basal ACh levels were in the low nanomolar range. The infusion of 1 μM neostigmine (Fig. 1A) caused a significant increase in ACh levels from 0.95 ± 0.54 nM to 40.95 ± 7.36 nM ($p < 0.001$). At 0.50 ± 0.09 nM, ATP levels were similar to ACh levels but were unchanged during neostigmine infusion (Fig. 1B). In contrast, calcium-free conditions decreased both ACh and ATP levels: ACh was reduced from 0.82 ± 0.09 nM to 0.18 ± 0.07 nM (Fig. 1C; $p < 0.001$) and ATP from 0.54 ± 0.12 nM to 0.25 ± 0.07 nM (Fig. 1D; $p < 0.01$). Tetrodotoxin (TTX), a blocker of voltage-dependent sodium channels, reduced ACh levels to almost undetectable levels (0.16 ± 0.03 nM) (Fig. 1E; $p < 0.01$) whereas ATP levels were not affected at 0.47 ± 0.09 nM (Fig. 1F).

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