Biosensors and Bioelectronics 74 (2015) 512–517

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Real-time monitoring of extracellular adenosine using enzyme-linked microelectrode arrays

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ARTICLE INFO

Article history:
Received 27 May 2015
Received in revised form
26 June 2015
Accepted 28 June 2015
Available online 7 July 2015

Keywords:
Amperometry
Voltammetry
Microdialysis

ABSTRACT

Throughout the central nervous system extracellular adenosine serves important neuroprotective and neuromodulatory functions. However, current understanding of the *in vivo* regulation and effects of adenosine is limited by the spatial and temporal resolution of available measurement techniques. Here, we describe an enzyme-linked microelectrode array (MEA) with high spatial (7500 μm^2) and temporal (4 Hz) resolution that can selectively measure extracellular adenosine through the use of self-referenced coating scheme that accounts for interfering substances and the enzymatic breakdown products of adenosine. *In vitro*, the MEAs selectively measured adenosine in a linear fashion ($r^2 = 0.98 \pm 0.01$, concentration range = 0–15 μM, limit of detection $= 0.96 \pm 0.5$ μM). *In vivo* the limit of detection was 0.04 ± 0.02 μM, which permitted real-time monitoring of the basal extracellular concentration in rat cerebral cortex (4.3 ± 1.5 μM). Local cortical injection of adenosine through a micropipette produced dose-dependent transient increases in the measured extracellular concentration (200 nL: 6.8 ± 1.8 μM; 400 nL: 19.4 ± 5.3 μM) [$P < 0.001$]. Lastly, local injection of dipyridamole, which inhibits transport of adenosine through equilibrative nucleoside transporter, raised the measured extracellular concentration of adenosine by 120% (5.6 → 12.3 μM) [$P < 0.001$]. These studies demonstrate that MEAs can selectively measure adenosine on temporal and spatial scales relevant to adenosine signaling and regulation in normal and pathologic states.

1. Introduction

Extracellular adenosine is an important neuromodulator of the central nervous system affecting heart rate (Drury and Szent-Gyorgyi, 1929; Tupone et al., 2013), sleep (Bjorness et al., 2009; Huang et al., 2014), and breathing (Spyer and Thomas, 2000). Accumulations of extracellular adenosine are also a vital part of a neuroprotective negative feedback loop to reduce synaptic activity and increase delivery of energy substrates (Dunwiddie and Masino, 2001). During periods of high energy demand, the extracellular concentration rises through dephosphorylation of adenine nucleotides by ecto-nucleotidases and transport from the intracellular environment (Illes and Zimmermann, 1999). In the extracellular space, activation of A_1 receptors suppresses synaptic transmission, while activation of A_{2A} receptors increases delivery of metabolic substrates (Dunwiddie, 1980; Pascual et al., 2005; Pedata et al., 2014). As a result, adenosine has exhibited neuroprotective effects in a variety of pathologic situations including: ischemia (Chen et al., 2014; Cui et al., 2013; Laghi Pasini et al., 2000; Weigand et al., 1999), trauma (Burnstock, 2015; Clark et al., 1997; Robertson et al., 2001), epilepsy (Dunwiddie and Worth, 1982; Masino et al., 2014; Zhang et al., 1990), cortical spreading depolarization (Lindquist and Shuttleworth, 2012, 2014), and seizure (During and Spencer, 1992; Miranda et al., 2014; Van Gompel et al., 2014).

Abbreviations: AA, Ascorbic Acid; ADA, Adenosine Deaminase; ATP, Adenosine-Triphosphate; BSA, Bovine Serum Albumin; DPR, Dipyridamole; ENT, Equilabrative Nucleoside Transporter; FSCV, Fast Scan Cyclic Voltammetry; IO, Inosine; LOD, Limit of Detection; MD, Microdialysis; MEA, Microelectrode Array; mPD, 1,3-phenylenediamine; NP, Nucleoside Phosphorylase; Pt, Platinum; XA, Xanthine; XO, Xanthine Oxidase

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http://dx.doi.org/10.1016/j.bios.2015.06.074
0956-5663/

Insights into the regulation of extracellular adenosine have been limited by a lack of selective *in vivo* techniques that are capable of quantifying the extracellular concentration with sufficient spatial/temporal resolution. Microdialysis (MD), a semi-permeable membrane that permits passive diffusion of neurochemicals from the extracellular space into the collection fluid for analysis, has been used over the past 30 years to measure extracellular adenosine (Hagberg et al., 1987). While MD is capable of selectively quantifying the extracellular concentration, the poor temporal (min–hrs) and spatial (mm) resolution of the technique limits the ability to discern changes on the temporal and spatial scales of physiologic and pathologic processes (Park and Gidday, 1990; Pedata et al., 1993). To overcome these methodological limitations, electrochemical detection methods have been developed that use a combination of three enzymes – nucleoside phosphorylase (NP), xanthine oxidase (XO), and adenosine deaminase (ADA) – to convert adenosine into the reporter molecule, H_2O_2 (Dale, 1998). The first generation probe, which consisted of two similar sensors one sensitive to adenosine and the other sensitive to the enzymatic breakdown products, provided a means to selectively quantify the extracellular concentration with improved spatial (surface area $\sim$200 mm^2) and temporal (1–2 min) resolution (Dale et al., 2000). However, the relatively large size of the probe limited the response time and produced considerable damage to the parenchyma, which may confound the physiological measurements (Llaudet et al., 2003). As such, second generation sensors were developed with improved spatial (surface area $\sim$25 mm^2) and temporal ($<$ 5 s) resolution (Llaudet et al., 2003), but suffered from reduced selectivity as they were also sensitive to the adenosine enzymatic breakdown products that inhibited quantification of the extracellular concentration (Llaudet et al., 2003). Recently, a new class of adenosine electrodes has been developed that utilize the specific oxidation properties of adenosine combined with fast scan cyclic voltammetry (FSCV) to produce a selective sensor with excellent spatial (surface area $\sim$1.5 mm^2) and temporal ($<$ 1 s) resolution (Nguyen et al., 2014; Swamy and Venton, 2007). While this technique enables rapid monitoring of extracellular adenosine in discrete functional circuits, methodological limitations of FSCV preclude quantification of the basal concentration (Nguyen and Venton, 2015).

Here, we describe a novel technique using enzyme-linked microelectrode arrays (MEA) and a self-referencing methodology to selectively quantify the extracellular adenosine concentration with excellent temporal (4 Hz) and spatial (surface area 7.5 mm^2) resolution that is capable of monitoring discrete functional brain circuits, such as sub-regions of the hippocampus (Hinzman et al., 2010a; Stephens et al., 2011), prefrontal cortex (Mattinson et al., 2011; Miller et al., 2015), and whisker-barrel cortex (Thomas et al., 2012). Our initial results suggest MEAs are capable of selectively monitoring the extracellular adenosine concentration with sufficient temporal resolution to study the intrinsic regulatory mechanisms.

2. Methods

2.1. Animals

Six male Sprague-Dawley rats weighing 325–400 g (Harlan Laboratories, Inc.) were used in the experiments. Animals were exposed to a 12 h light/dark cycle, with food and water available *ad libitum* in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures were performed during the light cycle, were approved by the University of Cincinnati Institutional Animal Care and Use Committee, and conformed to the Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council).

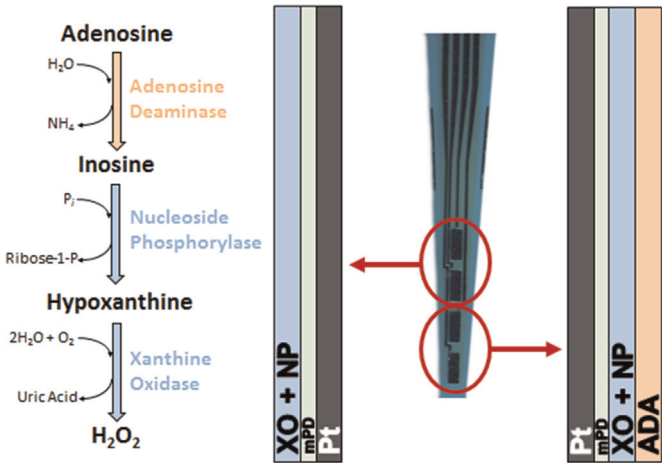

Fig. 1. Microelectrode array coating scheme for adenosine measures. Diagram depicts chemical reactions and enzymatic coating scheme applied to the platinum (Pt) recording sites of each microelectrode array (MEA). Generation of the reporter molecule, H_2O_2, from extracellular adenosine required three enzymes (ADA, NP, XO). The distal recording sites were coated with ADA, XO, and NP, while the proximal sites were coated with only XO and NP. An exclusion layer (mPD) prevented large-molecule interferents (ascorbic acid) from reaching the Pt surface, while still allowing passage of H_2O_2 for oxidation. Pt recording sites 50 $\times$ 150 μm^2.

2.2. Microelectrode Array Preparation

MEAs consisted of four platinum (Pt) recording sites (50 $\times$ 150 μm^2) arranged vertically on the tip of the electrode with 50 μm spacing between each site (Quanteon, LLC, Lexington, KY). The MEAs were configured in a self-referenced manner, where the two distal electrodes were sensitive to adenosine and the two proximal electrodes served as sentinel sites that were sensitive to both interfering molecules and the enzymatic breakdown products of extracellular adenosine, but not adenosine (Fig. 1). This provided a more selective measure and permitted determination of basal extracellular adenosine concentrations (Burmeister and Gerhardt, 2001; Burmeister et al., 2002). The distal recording sites were coated with an ADA enzyme solution that consisted of 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO), 0.125% glutaraldehyde (Sigma-Aldrich), 0.5 units of xanthine oxidase (XO) (Sigma-Aldrich) and 0.5 units of nucleoside phosphorylase (NP) (Sigma-Aldrich), and 2 units of adenosine deaminase (ADA) (Worthington Biochem, Lakewood, NJ). A small drop, approximately 0.1 μL, of the ADA containing solution was applied to the distal pair of Pt recording sites manually using a dissecting microscope and a microsyringe. The solution was allowed to dry for one minute, and repeated two more times for a total of three coats. For the sentinel sites a similar solution was prepared including all of the above components except for ADA. This solution was used to coat the two proximal sites of the MEA in the same fashion. The MEAs were then cured for 24 h at 25 °C. To restrict interference from large molecules *in vivo* (*e.g.*, ascorbic acid), a size-exclusion layer of 1,3-phenylenediamine (mPD) was electroplated onto the Pt recording sites prior to *in vitro* calibration. The electroplating procedure was as follows: MEAs were connected to the FAST-16 mkI system (Fast Analytical Sensor Technology Mark I; Quanteon, L.L.C., Nicholasville, KY) and the Pt recording site area (tip) of the MEA was placed in a 5 mM mPD solution (Acros Organics, Morris Plains, NJ). A triangular potential wave with $\pm$0.25 V peak amplitude, offset of $-$0.5 V, and frequency of 0.05 Hz was then applied for 20 min (Hinzman et al., 2010b, 2012).