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2-Arachidonoylglycerol is a substrate for butyrylcholinesterase: A potential mechanism for extracellular endocannabinoid regulation

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ABSTRACT

2-Arachidonoylglycerol (2-AG) is a component of the endocannabinoid receptor pathway and is primarily hydrolyzed by monoacylglycerol lipase (MAGL) *in vivo*. We found that the non-specific serine esterase, butyrylcholinesterase (BChE), can hydrolyze 2-AG with reasonable affinity and may present a new compensatory mechanism for endocannabinoid regulation. *In vitro* hydrolysis reactions of 2-AG with equine BChE were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) positive/negative electrospray ionization (ESI±) to measure the formation of arachidonic acid (AA) and the loss of 2-AG over time (min). The resulting Michaelis–Menten approximations reveal that BChE has affinity towards 2-AG in phosphate buffer at neutral pH (7.4). The calculated V_{max}, K_m and k_{cat} were 12.1 nmol s^{-1}, 57.5 μM, and 0.074 s^{-1}, respectively, which produced a diffusion-controlled rate of association (k_{cat}/K_m) of 1.3 × 10^3 - M^{-1} s^{-1}. Human BChE 2-AG hydrolysis was measured by immunoprecipitating BChE from fresh plasma and monitoring 2-AG loss and AA formation over time. These findings show that BChE can hydrolyze 2-AG which may be evidence of a more specific role for BChE in endocannabinoid regulation.

Introduction

The two cholinesterase enzymes, acetylcholinesterase (AChE[2]; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8), are a versatile group of type-B carboxylesterases. They are characterized by their substrate activities and have a wide variety of biological influence. AChE and BChE share approximately 60% sequence homology and may have evolved separately through early gene duplication [1]; although they share similar properties, their influences on biological processes differ. AChE has clearly defined neurotransmitter activity at cholinergic synapses, but BChE acts more like a molecular scavenger, having a variety of activities towards ester containing molecules.

BChE is a globular, tetrameric enzyme with a molecular weight of approximately 340 kDa, it has differential distribution in tissue and is a circulating enzyme. It is known best for its detoxification activity on pharmaceutical compounds, bioactive agents and organophosphorus chemicals [2]. BChE is generally not related to specific biomaintenance roles like AChE but there is some evidence that BChE may play a bigger role in physiological processes than molecular scavenging, like those seen with the gut hormone ghrelin [3]. However, individuals with silent BChE do not have abnormal metabolism or brain function but they may lack the ability to rapidly clear bioactive esters like cocaine and aspirin, leading to slower clearance rates and potential toxicity [4].

2-arachidonoylglycerol (2-AG) is a calcium dependant endocannabinoid made by phospholipase C (PLC) and diacylglycerol lipase (DAGL). Its induction in the central nerve compartment increases intracellular calcium concentrations in neuroblastoma A glioma cells though the 7-transmembrane cannabinoid receptor type-1 (CB1) [5]. 2-AG induction alters cell polarization via ion channels and mediates analgesic effects. Monoacylglycerol lipase (MAGL; EC 3.1.1.23) is the primary enzyme that hydrolyzes 2-AG to arachidonic acid *in vivo* [6] and accounts for ~85% of total 2-AG hydrolysis [7]. MAGL is a prolyl aminopeptidase located in both the central nerve compartment and in circulation; similar to BChE.

2-AG is also a primary ligand for the 7-transmembrane CB2 receptors [8] expressed on immune cells, microglia cells, gastrointestinal cells and in the peripheral nervous system. It mediates biological effects in both the central nerve compartment and in circulation. From a clinical perspective, selective modulation of 2-AG may have multiple therapeutic benefits. The cannabinoids have been implicated in neurologic and autoimmune disorders, reward centers, and pain management [9]. Plasma 2-AG hydrolysis is primarily from MAGL; however BChE is also abundant in plasma

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[2] *Abbreviations used:* Δ9-THC, Δ9-tetrahydrocannabinoid; 1-AG, 1-arachidonoylglycerol; 2-AG, 2-arachidonoylglycerol; AEA, anandamide; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride); AA, arachidonic acid; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; CB1, cannabinoid receptor type-1; CB2, cannabinoid receptor type-2; DAGL, diacylglycerol lipase; MAGL, monoacylglycerol lipase; PLC, phospholipase C; UHPLC-MS/MS, ultra-high performance liquid chromatography tandem mass spectrometry.

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(~3 μg/ml) and linked to various exogenous bioactive esters, like benzoylmethylecgonine, which alters CB receptor cascades.

There are a few esterases that can hydrolyze 2-AG [7]; however, these have never been fully characterized with substrate velocities or expanded to include BChE. BChE concentrations are high endogenously and the enzyme has activity towards a wide variety of substrates, including agents that modulate CB receptor cascades. Romani and colleagues demonstrate that anandamide (AEA), a similar endocannabinoid to 2-AG, can inhibit BChE *in vivo* [10] and we show herein that both horse and human BChE can hydrolyze 2-AG to arachidonic acid and AEA influences rate of 2-AG to AA conversion at physiological pH. This work provides evidence for a potential maintenance or compensatory role for the cholinesterase family via the association between BChE, 2-AG and endocannabinoid signaling.

Materials and methods

Reagents

Arachidonic acid (AA), 2-arachidonoylglycerol (2-AG) and anandamide (AEA) were purchased from Sigma–Aldrich (St. Louis, MO). Protein A/G agarose beads (sc-2003) and goat anti-human polyclonal BChE antibody (sc-46801) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pefabloc (AEBSF, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) was from Roche Diagnostics (Indianapolis, IN). Solvents were of the highest analytical quality purchased from J.T. Baker (Phillipsburg, NJ). Sodium citrate solution (20 mM), *equine* butyrylcholinesterase (CAS number 9001-08-5; catalog number C1057; purity 7.6 mg solid and 987 units/mg enzyme), phosphate buffered saline (50 mM phosphate, 0.154 M NaCl), RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless stated otherwise.

Human blood plasma collection

Human blood was collected at Pfizer Inc. following international good laboratory practices and occupational health guidelines for blood donor sample collection. Blood was collected from three male donors in a 10 ml K_2 EDTA vacutainer tube (366643) placed on ice and centrifuged for 20 min (4 °C, 350 RCF). This plasma was pooled and split into two groups: one received a 1/100 dilution of saturated AEBSF (200 mg/ml) for a final 2 mg/ml concentration and the other received nothing. The human plasma matrices described above were used for all experiments described herein.

Assay of 2-AG hydrolysis

For *in vitro* studies of 2-AG hydrolysis to arachidonic acid, 2-AG (150 μg/ml; 40 μmol) was incubated in 3 independent experiments with BChE in 50 mM phosphate buffer containing 5 mM EDTA, in the presence of equimolar AEA (40 μmol) at pH 7.4 and 37 °C and in crash solution (600 nM BChE in acetonitrile containing 1% formic acid) at 37 °C. For Michaelis–Menten enzyme kinetics, 2-AG (0.1 up to 150 μg/ml) was incubated in 3 independent experiments with BChE (50 mM phosphate buffer, 5 mM EDTA, 37 °C) up to 20 min. Enzyme rate studies were initiated by preparing a 1 mg/ml enzyme (based on purity) stock solution and mixing it with an equal volume of substrate solution prepared at 2× concentration. For these studies, a 150 μl enzyme aliquot (1 mg/ml) was mixed with a 150 μl aliquot of substrate solution (2×) to achieve a 300 μl (1.75 nmol BChE active site per vial) incubation.

For BChE immunoprecipitation activity, a 500 μl 2-AG (100 μg/ml in 50 mM phosphate buffer with 5 mM EDTA) aliquot was added to and incubated with six-independent human BChE immunoprecipitants and up to 3 h at 37 °C. All reactions were quenched by aliquoting 25 μl of the reaction mixture into an LC-autosampler vial containing 150 μl of acetonitrile with 1% formic acid (final pH ~4.5). Samples were vortexed, centrifuged at 3000 rpm and placed in the autosampler for analysis of AA formation and 2-AG degradation by LC-MS/MS.

Immunoprecipitation of BChE from human plasma

A 200-μl plasma aliquot was added to eight 1.5 ml siliconized centrifuge tubes containing 800 μl RIPA buffer. The samples were precleared by adding 20 μl of protein G agarose beads with agitation at 4 °C for 1 h. After centrifugation at 10,000 rpm for 4 min, the supernatant was transferred to fresh 1.5 ml centrifuge tubes, followed by further incubation with 6 μg of goat anti human BChE polyclonal-antibody for 1.5 h at 4 °C. Protein G agarose beads (20 μl) were added followed by incubation at 4 °C for 30 min and room temperature for an additional 30 min. The beads were washed with 1 ml of RIPA buffer followed by two washes with 1 ml of PBS (pH 7.4) using centrifugation at 2000 rpm in between. Beads were either diluted in SDS sample loading buffer containing DTT (Invitrogen) for 1-D PAGE gel electrophoreses or in PBS containing 100 μg/ml of 2-AG for enzyme kinetics as described above. Approximately 500 ng of control horse BChE was loaded on the gel as a positive control.

SDS PAGE of BChE immunoprecipitations

The agarose beads were suspended in SDS denaturing buffer with DTT and were boiled for 20 min at 110 °C followed by centrifugation at 10,000 rpm for 10 min. The resulting supernatant was loaded onto an 8% Bis-Tris gel (Invitrogen) and electrophoresed with MOPS buffer at 150 V for ~2 h. The gel was washed 3 times with nano pure water and stained with 50 ml of Simply Blue safe stain (Invitrogen, LC6060) for 2 h. The gel was washed three times 100 ml nano pure water over an 8 h period before a 5 min equilibration in Gel-Dry™ drying solution (Invitrogen, LC4025) before drying between two nitro cellophane membranes ~24 h.

UHPLC-MS/MS analysis of 2-AG and AA

A 50 μl sample aliquot was injected onto a Kinetex C18 (3 × 30 mm) column using a Shimadzu Prominence XLT autosampler coupled to an AB-4000 (AB Sciex, Foster City, CA). Mobile phase A was 1% formic acid and mobile phase B was acetonitrile with 1% formic acid. Analytes were eluted using a linear gradient from 10–90% mobile phase B over 3.2 min at a flow rate of 1 ml/min and a column temperature of 50 °C. 2-AG and AEA were monitored using a positive ion (ESI+) method before switching to negative ion mode (ESI−) for AA detection. All analytes were detected in multiple reaction monitoring mode (MRM) using the following transitions: the ESI+ MRM transition for 2-AG was 379.2 > 287.2, AEA was 348.2 > 62.1; for ESI− MRM, the AA transition was 303.2 > 259.4. The electrospray voltage was 3.5 kV in ESI+ and −3.5 kV in ESI− and the source temperature was 600 °C for both methods. 2-AG and AA levels were determined by isotope dilution tandem mass spectrometry using analyte peak area response.