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The potency of the fatty acid amide hydrolase inhibitor URB597 is dependent upon the assay pH

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Abstract

Inhibitors of the enzyme fatty acid amide hydrolase (FAAH), the principal enzyme involved in the metabolism of the endogenous cannabinoid anandamide, have potential utility in the treatment of disorders including inflammation and inflammatory pain. The carbamate compound URB597 (3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) potently and selectively inhibits FAAH by forming a covalent bond with a key serine residue of the enzyme. Little is known as to the pH dependency of this inhibition. Using a preincubation time of 10 min, URB597 inhibited rat brain anandamide hydrolysis with pI_{50} values of 7.19 ± 0.02 and 7.75 ± 0.06 at pH 6 and 8, respectively. The inhibition was time-dependent, and second order rate constants of $\sim 0.15 \times 10^6\ M^{-1}\ min^{-1}$ (pH 6) and $\sim 1.2 \times 10^6\ M^{-1}\ min^{-1}$ (pH 8) could be estimated. In intact C6 glioma cells and using a preincubation time of 10 min, URB597 inhibited the hydrolysis of 250 nM [^{3}H]AEA hydrolysis with pI_{50} values of 5.58 ± 0.07 and 6.45 ± 0.07 at extracellular pH values of 6 and 8, respectively. Since tissue pH is affected by inflammation, these data would suggest that the pH selectivity of the inhibition can contribute to the potency of the compound *in vivo*.

Keywords: Anandamide; Fatty acid amide hydrolase; URB597; pH dependency; Carbamylation

1. Introduction

Fatty acid amide hydrolase (FAAH) is the principal enzyme responsible for the metabolism of the endogenous cannabinoid anandamide (arachidonoylethanolamide, AEA) [1]. The enzyme has been cloned, and its physiological role in the body has been well characterized using both knockout mice and selective inhibitors [2–4]. Perhaps the most well-studied selective FAAH inhibitor is URB597 [5] (structure see Fig. 1), which produces potentially beneficial effects in models of inflammatory pain, inflammation, anxiety and depression [5–10].

Relatively little is known about the mechanism of action of the compound. In their original study, Kathuria et al. [5] reported that the inhibition was time-dependent, and could not be removed by dialysis, but the observed inhibition of brain FAAH measured ex vivo was lost 24 h after administration [11], which is shorter than seen with other irreversible compounds [12]. It was hypothesized that the compound interacts covalently with a key serine residue (Ser^{241}) of FAAH on the basis of interactions of carbamates with other enzymes [13], and recent data has supported this contention [14].

In a recent study using a limited number of data points, we found that both rat and mouse brain FAAH appeared to be more sensitive to inhibition by URB597 at pH 9 than at pH 7 following a 60 min preincubation [7]. This finding requires further investigation, since is not only of mechanistic interest, but also of pharmacological relevance in view of the fact that inflammation can affect both tissue and intracellular pH [15,16] and thereby potentially the potency of carbamate FAAH inhibitors like URB597. In consequence, in the present study, we have investigated the pH dependency of FAAH inhibition by URB597 using both homogenates and intact cells.

2. Materials and methods

2.1. Materials

[^{3}H-1,2-ethanolamine]AEA (specific activity 60 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc, St. Louis, USA. URB597 and non-radioactive anandamide were obtained from the Cayman Chemical Co., Ann Arbor, USA. Fatty acid free bovine serum albumin, active charcoal, and

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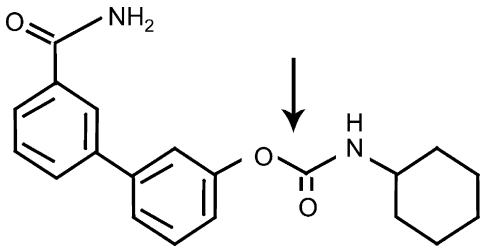

Fig. 1. Structure of URB597. The arrow indicates the part of the molecule involved in the carbamylation of the FAAH Ser^{241} residue [13,14].

indomethacin were obtained from Sigma Aldrich Inc, St. Louis, USA. C6 glioma cells were obtained from the European Collection of Cell Cultures, Porton Down, UK.

2.2. Preparation of rat brain homogenates

Adult male Wistar and Sprague Dawley rats were used in the study. Whole brains (minus cerebellum) were homogenized (20 strokes, hand held glass homogeniser) in 20 mL of buffer (20 mM HEPES, 1 mM $MgCl_2$, pH 7.0). Samples were then centrifuged at ~35 000 × *g* for 20 min (4 °C), resuspended in 20 mL of buffer and centrifuged again. After the second centrifugation, the pellets were resuspended in 10 mL of buffer and incubated at 37 °C for 15 min in order to hydrolyse all endogenous FAAH substrates. They were then centrifuged a final time at ~35 000 × *g* for 20 min (4 °C). Supernatants were discarded and samples were resuspended in 1 mL. 200 mg^{-1} (original tissue weight) of Tris–HCl buffer (50 mM Tris–HCl, 1 mM EDTA, 3 mM $MgCl_2$, pH 7.4) and frozen at −80 °C in 250 μL aliquots. A modified Lowry procedure [17] was used to determine protein content in each sample with bovine serum albumin as standard. Ethical permission for the animal experiments was obtained from the local animal ethics committee.

2.3. Assay of FAAH activity in brain homogenates

FAAH was assayed as described by Boldrup et al. [18]. Briefly, membrane homogenates were diluted with buffer (10 mM Tris–HCl, 1 mM EDTA, pH either 6 or 8) to give protein contents of 2.5 μg (pH 6) or 0.8 μg (pH 8) per assay. The lower protein content at the higher pH reflects the pH profile of FAAH [19]. Test compounds were diluted with ethanol and compared against controls which used the same amount of ethanol (10 μL $assay^{-1}$). Tubes were preincubated at 37 °C for 10 min (unless otherwise stated). Aliquots (25 μL) of [^{3}H]AEA (in 10 mM Tris–HCl, 1 mM EDTA, 1% (w/v) fatty acid free bovine serum albumin, pH 6 or 8 as appropriate) were added to give an assay substrate concentration of 2 μM. The tubes (assay volumes 200 μL) were then incubated for 10 min at 37 °C before being put on ice and having 400 μL of active charcoal mixture (80 μL charcoal + 300 μL 0.5 M HCl) added. Each tube was corked, vortexed three times and left at room temperature for 30 min. Samples were then centrifuged to sediment the charcoal, and aliquots (200 μL) of the supernatants were taken to determine tritium content by liquid scintillation spectroscopy with quench correction. Blanks were in all cases tubes with buffer in place of membranes.

2.4. FAAH activity in C6 glioma cells

The assay used was based on the method of Jonsson et al. [20]. Briefly, C6 glioma cells (passage range 16–25) were seeded into 24 well culture plates (2×10^5 cells $well^{-1}$, culture medium F-10 Ham with 10% foetal bovine serum, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) and incubated for 18 h at 37 °C, 5% CO_2 in humidified atmospheric pressure. On the day of assay, the wells were washed twice with 500 μL assay buffer (116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM HEPES, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$ in milliQ deionized water, pH 6 or 8) containing 1% (w/v) bovine serum albumin. After removal of the buffer by aspiration, 345 μL buffer containing 0.1% (w/v) fatty acid-free bovine serum albumin was added followed by 5 μL of URB597 or ethanol vehicle. The wells were incubated for 10 min at 37 °C after which 50 μL [^{3}H]AEA was added, to give a final assay substrate concentration of 0.25 μM. After incubation for 5 min at 37 °C, the culture plates were placed on ice, and methanol (400 μL) was added. The cells were collected by scraping the wells. Aliquots (400 μL) were transferred to glass tubes, chloroform (200 μL) was added, and the samples were vortex mixed twice. After centrifugation (1400 × *g*, 5 min) to separate the phases, aliquots (200 μL) of the aqueous phase were taken to determine tritium content by liquid scintillation spectroscopy with quench correction. Blanks were in all cases wells not containing cells.

2.5. Analysis of data

pIC_{50} values [$-\log_{10}$(IC_{50} values)] were analyzed for data expressed as % of control using the built-in equation 'sigmoid dose-response' (variable slope) of the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, USA) with 'top' (uninhibited) values fixed at 100 and 'bottom' (minimum activity) fixed at 0. Confidence intervals of the slopes for the time-dependency data were also determined using the GraphPad Prism computer programme.

3. Results

3.1. pH-dependent inhibition of rat brain FAAH by URB597

Concentration–response curves for the inhibition of rat brain [^{3}H]AEA hydrolysis by URB597 following a preincubation time of 10 min are shown in Fig. 2A. The compound was less potent at pH 6 than at pH 8, with pI_{50} values of 7.19 ± 0.02 and 7.75 ± 0.06, respectively, being found. These values correspond to IC_{50} values of 64 and 18 nM, respectively. Under the same conditions, the acidic non-steroidal anti-inflammatory drug indomethacin was found to exhibit the reverse pattern, with pI_{50} values of 4.43 ± 0.03 and 4.05 ± 0.03 (corresponding to IC_{50} values of 37 and 89 μM, respectively) (data not shown), a result in line with previous data [21].