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URB754 Has No Effect on the Hydrolysis or Signaling Capacity of 2-AG in the Rat Brain

Brief Communication

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Summary

Previous studies indicate that in brain tissue the endocannabinoid 2-AG is inactivated by monoglyceride lipase (MGL)-catalyzed hydrolysis, and a recent report has indicated that MGL activity could be specifically inhibited by URB754 [1]. In the present study, URB754 failed to inhibit 2-AG hydrolysis in rat brain preparations. In addition, brain cryosections were employed to assess whether URB754 could facilitate the detection of 2-AG-stimulated G protein activity. Nevertheless, whereas pretreatment with PMSF readily allowed detection of 2-AG-stimulated G protein activity, URB754 was ineffective. In contrast to previous claims, brain FAAH activity was also resistant to URB754. Thus, in our hands URB754 was not able to block the endocannabinoid-hydrolyzing enzymes and cannot serve as a lead structure for future development of MGL-specific inhibitors.

Introduction

2-Arachidonoyl-glycerol (2-AG), a monoglyceride of arachidonic acid esterified at the *sn*-2 position, is currently recognized as the most abundant and potent endocannabinoid. The synthesis, release, and degradation of neuromodulatory 2-AG is thought to occur in close proximity to the cannabinoid CB1 receptor-enriched neuronal structures. This guarantees a restricted spatial coordination for the action of this unique lipid messenger. In vivo, the effects of 2-AG are transient, partly due to its rapid and efficient enzymatic hydrolysis. The main enzyme responsible for 2-AG hydrolysis in brain tissue is thought to be monoglyceride lipase (MGL) [2, 3]. Since the role of MGL as a 2-AG hydrolyzing enzyme in the cannabinoid system has only recently been elucidated, few MGL inhibitors have been developed. However, it is known that purified MGL, as well as 2-AG-degrading enzymatic activity in rat cerebellar membranes, can be inhibited by various nonspecific serine hydrolase inhibitors, most notably MAFP, HDSF, and PMSF [2, 4]. Additionally, sulfhydryl-specific compounds, including *p*CMB, mercury chloride, and NEM have previously been shown to inhibit MGL [5, 6]. We have also reported that 2-AG hydrolysis in rat cerebellar membranes was relatively potently (IC_{50} 140 nM) inhibited by a maleimide analog of 2-AG, *N*-arachidonylmaleimide (NAM) [7]. A recent report claimed that MGL activity can be specifically and noncompetitively inhibited by two compounds, URB754 and URB602 (IC_{50} values 200 nM and 75 μM, respectively) [1]. In the present study, URB754 and URB602 were tested for their potential to inhibit 2-AG hydrolysis in rat brain homogenates and membrane preparations. In addition, brain sections were employed to assess whether URB754 could promote endogenous 2-AG accumulation or whether treatment with this compound could facilitate the detection of 2-AG-stimulated G protein activity in brain regions enriched with the CB1 receptor and MGL. Our results provide no evidence to support the claim that URB754 and URB602 are inhibitors of 2-AG hydrolysis in the rat brain.

Results and Discussion

Makara et al. [1] reported that URB754 and URB602 could inhibit recombinant rat brain MGL in HeLa cells with IC_{50} values of 200 nM and 75 μM, respectively. Additionally, that report claimed that URB754 was able to elevate 2-AG levels in rat forebrain slice cultures and to prolong depolarization-induced suppression of inhibition (DSI) in hippocampal slices in a CB1 receptor-dependent manner. We tested these two compounds in various experimental settings assessing enzymatic degradation and signaling capacity of 2-AG in brain preparations. As is evident from Figure 1, the hydrolysis of exogenously added 2-AG (5×10^{-5} M) in rat cerebellar membranes (CbM) was not inhibited by URB754 (100 μM). The outcome was essentially the same regardless of whether BSA (0.5% w/v) was included (as in Figure 1) or not (data not shown). Moreover, less than 10% of 2-AG hydrolyzing activity in homogenates of rat forebrain (BrH) and cerebellum (CbH), two brain regions with prominent expression of mRNA encoding MGL [2], was sensitive to URB754 (100 μM) (Figure 1). The structure of URB754 used in the present study was confirmed by ^{1}H NMR spectroscopy to be identical to that reported by Garin et al. [8] and by Makara et al. [1] (data not shown). Similarly, URB602 (1 mM) did not inhibit 2-AG hydrolysis in rat cerebellar membranes, and only ~12% inhibition was evident in brain homogenates. Consistent with our previous findings [7], NAM (1 mM) inhibited ~90% of 2-AG hydrolyzing activity in cerebellar membranes, and this was also the case with brain homogenates (Figure 1). The negative outcome with these novel putative inhibitors was rather surprising, especially in the light of the previous reports that the brain 2-AG hydrolyzing activity (assumed to be MGL) [9] as well as that of recombinant MGL [2] is found in both cytosolic and particulate fractions. Further, immunodepletion experiments have indicated that MGL accounts for at least 50% of the 2-AG-hydrolyzing activity in rat brain soluble fractions [3]. One explaining factor could be that MGL, which in the hippocampus and the cerebellum is localized to the presynaptic terminals in close proximity with the CB1 receptors [10], might only represent a minor fraction of the total

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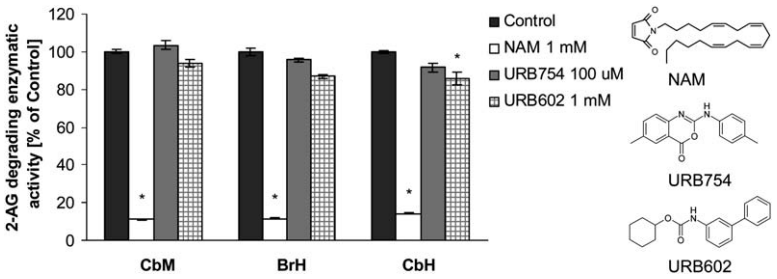

Figure 1. Hydrolysis of 2-AG in NAM, URB754, and URB602 Pretreated Rat Cerebellar Membranes, Brain Homogenate, and Cerebellar Homogenate

The enzyme activity of control samples was expressed as 100%. The data represent the mean ± SD from three independent experiments performed in duplicate. An asterisk denotes a statistically significant (*p < 0.05) difference from the respective control. CbM, pretreated rat cerebellar membranes; BrH, brain homogenate; CbH, cerebellar homogenate.

2-AG-hydrolyzing enzymatic capacity in the presently used brain preparations. To test this hypothesis, the effectiveness of URB754 in potentiating 2-AG-stimulated and CB1 receptor-dependent G protein activity was assessed using the functional approach of [^{35}S]GTPγS autoradiography with rat brain cryostat sections. Our previous studies have indicated that brain sections effectively degrade 2-AG. In fact, this phenomenon has complicated attempts to visualize 2-AG-stimulated responses, in contrast to the robust responses evoked by the synthetic agonist CP55,940 [11]. As shown in Figure 2, pretreatment with PMSF (1 mM, at concentrations that almost totally eliminated 2-AG hydrolyzing activity in the cerebellar membranes) [4] allowed clear visualization of 2-AG-stimulated G protein activity throughout the CB1-responsive brain regions, whereas a similar treatment with URB754 (10 μM) was ineffective. Additional studies revealed that incubation of brain sections in Tris-HCl-based buffer with submicromolar concentrations of MAFP approximately quadrupled the tissue 2-AG content, as determined by GC-MS analysis of chloroform-methanol-extracted brain sections, but 2-AG levels were not elevated following treatment with URB754 (10 μM) (V.P. et al., unpublished data).

Makara et al. [1] reported that URB754 could also inhibit native rat brain FAAH with an IC_{50} value of 31.8 μM. However, in our test system, URB754 did not inhibit rat brain FAAH by more than 10% at the highest concentration (100 μM) tested (n = 2, data not shown). Nevertheless, the previously reported potent FAAH inhibitor URB597 [12] was found to inhibit FAAH with an IC_{50} value of 3.8 nM [13], which is comparable to the previously reported IC_{50} value of 4.6 nM [12].

There are two possibilities to explain the discrepant findings between our study and that of Makara et al. [1]. The first explanation is that the previously characterized 2-AG hydrolyzing activity in rat brain must be different from the URB754-sensitive MGL activity described by Makara et al. [1]. However, this line of argument is not supported by several pieces of evidence. Previous immunodepletion experiments indicate that MGL accounts for at least 50% of the 2-AG-hydrolyzing activity in rat brain soluble fractions [3], and this activity is expected to be fully preserved in the brain homogenates and tissue sections used in this study. As clearly shown here, URB754 only marginally affected 2-AG hydrolysis in brain homogenates and was devoid of activity in brain sections assessing the CB1 receptor-dependent signaling capacity of 2-AG. This means that it is unlikely that the majority of 2-AG hydrolase activity in rat brain would correspond to some MGL-like activity mediated by a pharmacologically similar but nonetheless different enzyme from the purified MGL [2, 14], with the only difference being sensitivity of MGL to URB754. The second, and to our opinion more likely, explanation is that for some unknown reason, the effects attributed to URB754 [1] in fact represent activity related to another chemical entity. This is supported by the fact that the human recombinant MGL, which at the amino acid level is highly homologous to the rat and mouse MGL, does not appear to be sensitive to URB754 [15].

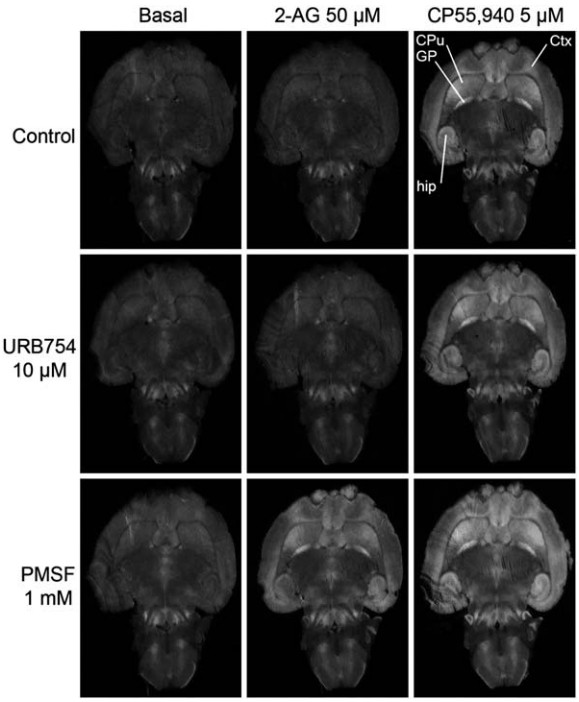

Figure 2. Treatment of Rat Brain Sections with PMSF Allows Detection of 2-AG-Stimulated G Protein Activity throughout the CB1 Receptor-Enriched Brain Regions, Whereas Similar Treatment with URB754 Is Ineffective

Horizontal brain sections were processed for [^{35}S]GTPγS autoradiography using a three-step assay protocol as detailed in the Experimental Procedures. PMSF (1 mM) was present in step 2, and URB754 (10 μM) throughout steps 2 and 3. [^{35}S]GTPγS labeling was conducted for 90 min under basal conditions or in the presence of the cannabinoid agonists. Note that in PMSF-treated sections, 2-AG and the synthetic cannabinoid agonist CP55,940 stimulate [^{35}S]GTPγS binding to the same CB1 receptor-enriched brain regions, most notably in the caudate putamen (CPu), the globus pallidus (GP), the hippocampus (hip), and the cerebral cortex (Ctx). Note also that URB754 totally lacks such a 2-AG signal-enhancing effect.

Significance

In this present study, the newly introduced MGL inhibitors URB754 and URB602 showed no significant inhibitory effect on the hydrolysis of 2-AG in rat brain preparations. In addition, URB754 failed to potentiate 2-AG signaling in rat brain sections. In the light of the present findings, we do not believe that URB754 will serve as a lead structure for further development of MGL-specific inhibitors. The development of specific inhibitors for the enzyme responsible for 2-AG hydrolysis may provide valuable molecular tools to clarify the physiological role of 2-AG. Furthermore, such inhibitors could offer a rational approach for the treatment of certain pathological states, such as pain and anxiety.

Experimental Procedures

Materials

2-AG, URB754, and URB602 were purchased from Cayman Chemical (Ann Arbor, MI). BSA (essentially fatty acid free) was purchased from Sigma (St. Louis, MO).

Animals and Preparation of Membranes and Homogenates

The animals and membrane preparations have been described previously [7]. Briefly, rat brain cerebella from 4-week-old male Wistar rats were homogenized and centrifuged first at 1,000 × g for 10 min and subsequently at 100,000 × g for 10 min (at 4°C). The resulting pellet was referred to as the rat cerebellar membranes (CbM). Homogenates from forebrains and cerebella from 8-week-old male Wistar rats were centrifuged at 10,000 × g for 20 min (at 4°C), and the resulting supernatants were referred to as the rat brain homogenate (BrH) and the rat cerebellum homogenate (CbH), respectively [13].

Enzyme Assay for MGL Activity

The assay for MGL has been described previously [7]. Briefly, CbM (10 μg protein), BrH (35 μg protein), and CbH (35 μg protein) were incubated with the test compounds at 25°C (pH 7.4) for 30 min. After the preincubations, the substrate 2-AG was added, and incubations were continued for 90 min prior to addition of acetonitrile (the pH of the samples was simultaneously decreased to 3.0 with phosphoric acid) and HPLC analysis (described previously [4]). The final incubation volume contained 5 μg CbM, 17.5 μg of BrH or CbH protein, 0.5% (w/v) BSA (fatty acid free), and 50 μM of 2-AG.

Enzyme Assay for FAAH Activity

The assay for FAAH has been described by Saario et al. (2006) [13]. Briefly, BrH (18 μg protein) was incubated with test compound for 10 min at 37°C (pH 7.4). At the 10 min time point, the substrate, AEA, was added so that its final concentration was 2 μM (containing 50×10^{-3} μCi of 60 Ci/mmol [^{3}H]AEA). The incubations were performed in the presence of 0.5% (w/v) BSA (essentially fatty acid free). Ethyl acetate was added at the 20 min time point to stop the enzymatic reaction. Additionally, unlabeled ethanolamine was added as a "carrier" for radioactive ethanolamine, which was measured by liquid scintillation counting.

[^{35}S]GTPγS Autoradiography

20 μm thick horizontal cryosections from 4-week-old male Wistar rats were incubated in three sequential steps (20, 60, and 90 min) in Tris-HCl buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$) at 20°C, essentially as previously described [16, 17]. Throughout steps 2 and 3, the buffer was supplemented with GDP (2 mM), BSA (0.5%), 8-cyclopentyl-1,3-dipropylxanthine (1 μM) along with the vehicle (DMSO), URB754 (10 μM), or PMSF (1 mM, only step 2). [^{35}S]GTPγS binding was conducted for 90 min in the presence of DTT (1 mM) and either vehicle (ethanol), 2-AG (50 μM), or CP55,940 (5 μM). Sections were washed twice (5 min each time) in buffer (50 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 0°C) and briefly dipped in Millipore water (0°C, 30 s). The sections were exposed on BioMax MR, Kodak Scientific Imagin Film for 4 days, and film was developed using Kodak D-19 developer and scanned with HP digitalized 7400c.

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