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A second *N*-acylethanolamine hydrolase in mammalian tissues

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Abstract

It is widely accepted that fatty acid amide hydrolase (FAAH) plays a central role in the hydrolysis of anandamide. However, we found a second *N*-acylethanolamine hydrolase in animal tissues which hydrolyzed anandamide at acidic pH. This "acid amidase" was first detected with the particulate fraction of human megakaryoblastic CMK cells, and was solubilized by freezing and thawing without detergent. The enzyme was distinguishable from FAAH in terms of (1) the optimal activity at pH 5, (2) stimulation by dithiothreitol, (3) low sensitivity to two FAAH inhibitors (methyl arachidonyl fluorophosphonate and phenylmethylsulfonyl fluoride), and (4) high content in lung, spleen and macrophages of rat. The acid amidase purified from rat lung was the most active with *N*-palmitoylethanolamine among various long-chain *N*-acylethanolamines. To develop specific inhibitors for this enzyme, we screened various analogues of *N*-palmitoylethanolamine. Among the tested compounds, *N*-cyclohexanecarbonylpentadecylamine was the most potent inhibitor which does-dependently inhibited the enzyme with an IC_{50} value of 4.5 μM without inhibiting FAAH at concentrations up to 100 μM. The inhibitor was a useful tool to distinguish the acid amidase from FAAH with rat basophilic leukemia (RBL-1) cells that express both the enzymes.

Keywords: Anandamide; Endocannabinoid; *N*-acylethanolamine; Fatty acid amide hydrolase; Macrophage

1. Introduction

Anandamide (*N*-arachidonoylethanolamine) is an endocannabinoid (an endogenous ligand of cannabinoid receptor) isolated from the brain (Devane et al., 1992). Anandamide and other *N*-acylethanolamines (NAEs) are released from their corresponding *N*-acylphosphatidylethanolamines by a novel enzyme of the phospholipase D-type (Okamoto et al., 2004) (Fig. 1). It is generally accepted that in animal tissues anandamide is inactivated by cellular uptake by facilitated diffusion, followed by the hydrolysis to arachidonic acid and ethanolamine by fatty acid amide hydrolase (FAAH) (Fig. 1). FAAH was cloned from rat, human, mouse and pig (Cravatt et al., 1996; Giang and Cravatt, 1997; Goparaju et al., 1998) and has been extensively studied as described in detail in recent review articles (Fowler et al., 2001; Patricelli and Cravatt, 2001; Bisogno et al., 2002; Deutsch et al., 2002; Ueda, 2002; Cravatt and Lichtman, 2003).

Briefly, FAAH is a membrane-bound protein composed of 579 amino acids, and is widely distributed in various organs. The enzyme acts as an esterase as well as an amidase, thus hydrolyzing not only various long-chain NAEs including anandamide and *N*-palmitoylethanolamine and primary amides of fatty acids such as oleamide but also 2-arachidonoylglycerol, an ester. The enzyme has an atypical catalytic triad of Ser-241, Ser-217 and Lys-142, and is inhibited by non-specific serine hydrolase inhibitors such as phenylmethylsulfonyl fluoride (PMSF) (Deutsch and Chin, 1993) and methyl

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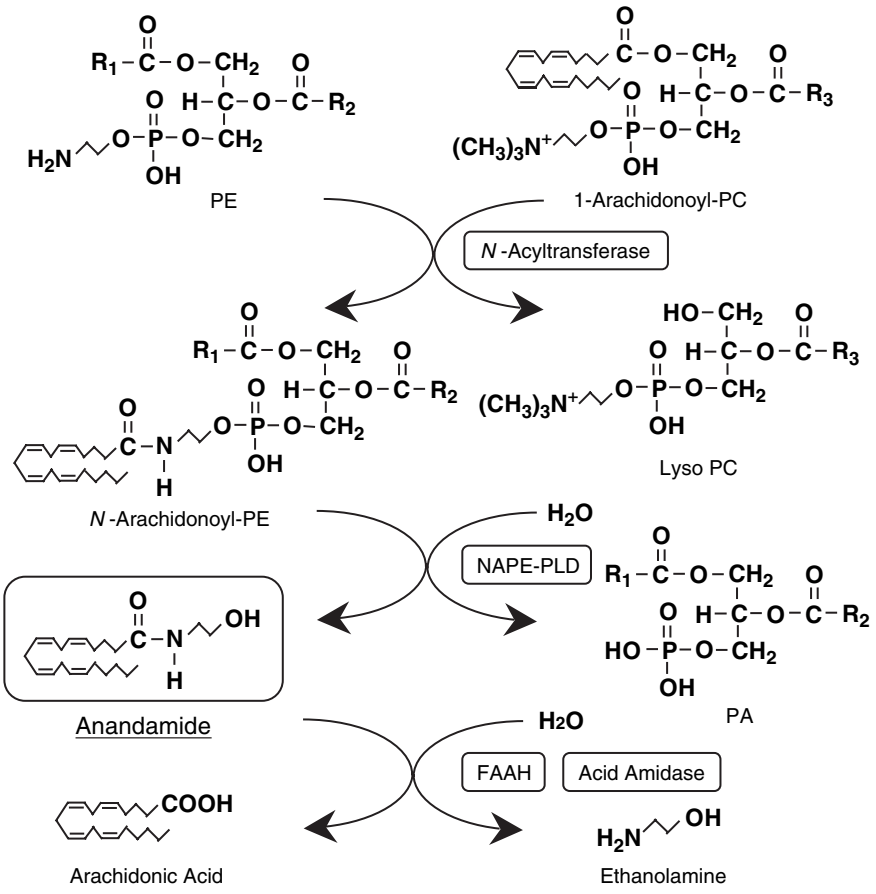

Fig. 1. A biosynthetic and degradative pathway of anandamide. NAPE-PLD, *N*-acylphosphatidylethanolamine-hydrolyzing phospholipase D; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

arachidonyl fluorophosphonate (MAFP) (De Petrocellis et al., 1997; Deutsch et al., 1997). Recent studies with FAAH gene-disrupted mice revealed that the mice showed the potentiated biological activity of exogenously added anandamide, high endogenous levels of NAEs including anandamide in the brain, and reduced pain sensation (Cravatt et al., 2001; Lichtman et al., 2002; Clement et al., 2003). Furthermore, the administration of a potent FAAH inhibitor URB597 to mice enhanced the brain level of anandamide, and exhibited anti-nociceptive and anxiolytic effects (Kathuria et al., 2003). These findings strongly supported the central role of this enzyme in the degradation of anandamide.

However, we found another enzyme catalyzing the same reaction, but catalytically distinguishable from FAAH. In this article we discuss our recent studies on the second NAE hydrolase and attempts to develop selective inhibitors of this enzyme.

2. Characterization of the acid amidase

The presence of a second NAE hydrolase was suggested for the first time based on the following observation (Ueda et al., 1999). When the whole cells of a human megakaryoblastic CMK cell line were incubated with ^{14}C-labelled anandamide, anandamide time-dependently decreased with a concomitant increase in radioactive neutral lipids and polar lipids. However, when we next incubated the homogenate of CMK cells with anandamide in a buffer at pH 9, the formation of arachidonic acid was not observed. The homogenate of rat basophilic leukemia cells RBL-1, expressing FAAH (Bisogno et al., 1997), generated arachidonic acid under the same assay conditions. On the other hand, with a buffer at pH 5, the CMK cell homogenate produced arachidonic acid. Thus, we presumed that the capability of hydrolyzing anandamide in CMK cells is derived from a novel enzyme distinct from FAAH in terms of pH dependency.

We compared the CMK cell enzyme with FAAH of RBL-1 cells under various assay conditions (Fig. 2). First, we examined pH dependency. The CMK cell enzyme showed the highest activity around pH 5, and was almost inactive at alkaline pH. This was in sharp contrast with the RBL-1 cell enzyme showing the optimal pH around 9. Second, dithiothreitol dose-dependently stimulated the CMK cell enzyme up to

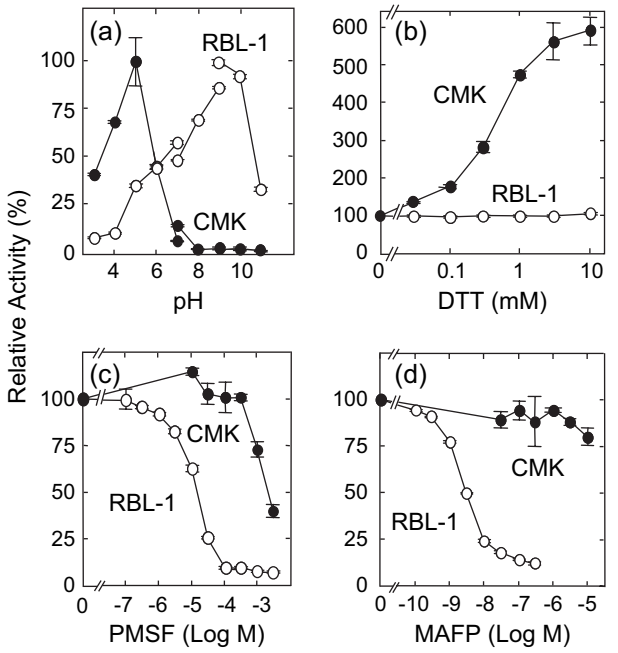

Fig. 2. Comparison between the CMK cell enzyme (acid amidase) and the RBL-1 cell enzyme (FAAH). The enzymes were assayed for the anandamide hydrolyzing activity at different pH (a) or in the presence of different concentrations of dithiothreitol (b), PMSF (c) and MAFP (d). Mean values ± SD are shown ($n = 3$).

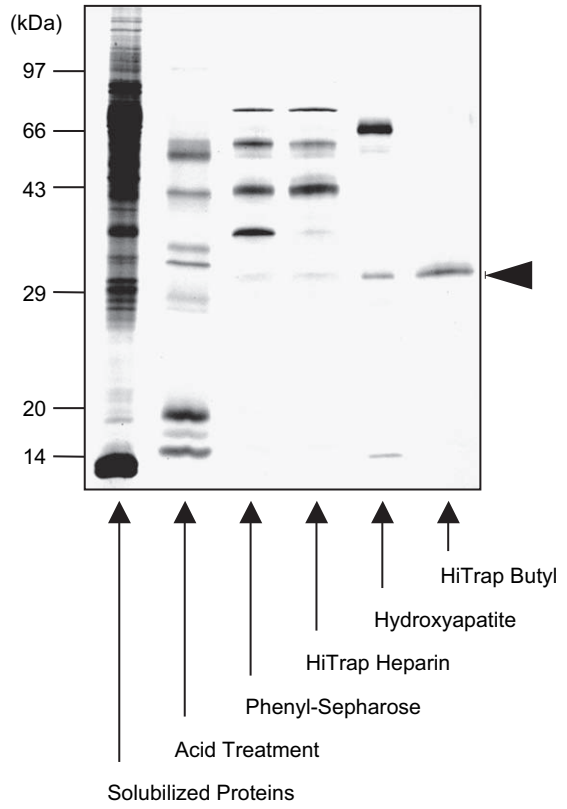

Fig. 3. Purification of the acid amidase from rat lung. The active fractions at each step were subjected to SDS-polyacrylamide gel electrophoresis. The major band in the final preparation is indicated by an arrowhead.

6-fold. Such a stimulatory effect was not observed with the RBL-1 cell enzyme. Third, inhibitors behaved differently. The RBL-1 cell enzyme was inhibited by PMSF and MAFP with IC_{50} values of 20 μM and 3 nM, respectively, as we expected. However, the CMK cell enzyme was much less sensitive to these compounds, and their IC_{50} values were as high as 3 mM and 10 μM, respectively. Fourth, intracellular location appeared to be different between the two enzymes. When cell homogenates were subjected to sequential centrifugation, the CMK cell enzyme showed the highest specific activity with the 12 000 × g pellet. Notably, the enzyme was effectively solubilized from the pellet by freezing and thawing without any detergent. Although FAAH of RBL-1 cells was also membrane-bound, detergent was indispensable to its solubilization from the membrane. Fifth, we examined the reactivity of the CMK cell enzyme with anandamide and *N*-palmitoylethanolamine. In contrast to FAAH, the CMK cell enzyme hydrolyzed *N*-palmitoylethanolamine faster than anandamide. These catalytic properties strongly suggested that the enzyme of CMK cells is an enzyme protein different from FAAH. The enzyme seemed to be one of lysosomal enzymes based on the optimal pH at 5 as well as its intracellular localization at the 12 000 × g pellet.

Since CMK cell was a leukemic cell line, it was interesting to examine whether mammalian tissues physiologically express an acid amidase corresponding to the CMK cell enzyme. We incubated proteins solubilized from the 12 000 × g pellet of various rat organs with *N*-palmitoylethanolamine at pH 5 in the presence of 1 μM MAFP (Ueda et al., 2001). The results exhibited wide distribution of the acid amidase. The highest activity was detected with lung, followed by spleen, small intestine, thymus, and cecum. In addition, we found a high activity in alveolar and peritoneal macrophages of rat. These results also revealed that the acid amidase was considerably different in organ distribution from FAAH, which was highly expressed in liver, small intestine, brain, and testis of rat (Katayama et al., 1997).

We then tried to purify the acid amidase. Starting from the proteins solubilized from the 12 000 × g pellet of rat lung, the enzyme was purified by acid treatment and four steps of chromatography (Phenyl-Sepharose, HiTrap Heparin, hydroxyapatite, and HiTrap Butyl). The final preparation showed a specific activity of about 1.8 μmol/min/mg protein. SDS-polyacrylamide gel electrophoresis revealed a major band with a molecular mass of about 31 kDa in the final preparation (Fig. 3). When the purified enzyme was allowed to react with various NAEs with different acyl groups, *N*-palmitoylethanolamine was the most reactive substrate, followed by *N*-myristoylethanolamine (Fig. 4). Anandamide was hydrolyzed only at 8% the rate of the

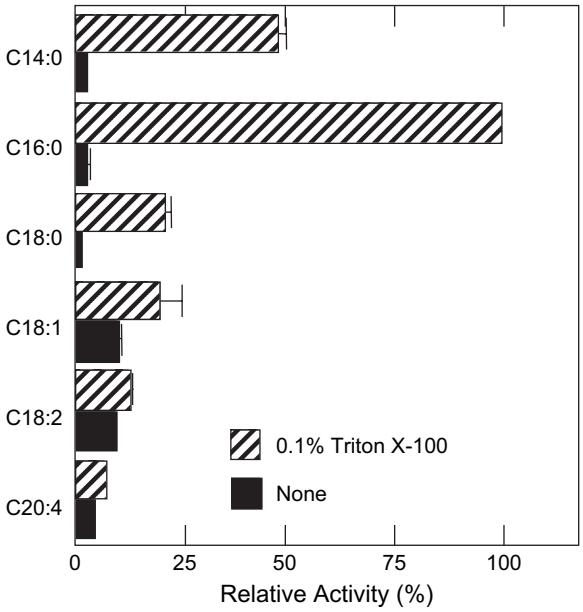

Fig. 4. Reactivities of the purified acid amidase with various *N*-acylethanolamines at 100 μM. The enzyme reactions were performed in the presence of 0.1% Triton X-100 or in its absence. Mean values ± SD are shown ($n = 3$). C14:0, *N*-myristoylethanolamine; C16:0, *N*-palmitoylethanolamine; C18:0, *N*-stearoylethanolamine; C18:1, *N*-oleoylethanolamine; C18:2, *N*-linoleoylethanolamine; C20:4, anandamide.

N-palmitoylethanolamine hydrolysis. However, when 0.1% Triton X-100 was removed from the reaction mixture, the reactivity with saturated NAEs including *N*-palmitoylethanolamine was markedly reduced.

3. Development of selective inhibitors of the acid amidase

To elucidate a physiological role of the acid amidase, selective inhibitors without inhibitory effects on FAAH should be useful. For this purpose, we synthesized various analogues of the most reactive substrate *N*-palmitoylethanolamine and examined their inhibitory effects on rat lung acid amidase (Vandevoorde et al., 2003). We first screened many analogues in which the ethanolamide moiety was replaced by other amides. However, these amide compounds at 100 μM inhibited the enzyme only by less than 30%. We next tested various ester analogues, and observed higher inhibitory activities with most of them. Cyclohexyl hexadecanoate (compound **1**) was the most potent, and resulted in 84% inhibition at 100 μM (Fig. 5). Moreover, *N*-(3-hydroxypropionyl)pentadecanamide (**2**), a 'retroamide' in which the amide bond was inverted, caused 77% inhibition. Two 'retroester' compounds, hexadecyl propionate (**3**) and hexadecyl acetate (**4**) also inhibited the enzyme by about 70%. By testing different concentrations of the inhibitors, IC_{50} values of **1**, **2** and **3** were calculated to be 19, 32 and 54 μM, respectively. Interestingly, these compounds showed no or lower inhibitory activity towards FAAH.

As the second screening, we synthesized several analogues of compound **1** with cyclohexyl group or phenyl group (Fig. 6) (Tsuboi et al., 2004). As compared at 10 μM, two 'retroamide' compounds *N*-cyclohexanecarbonylpentadecylamine (**5**) and *N*-benzoylpentadecylamine (**6**) were found to be more potent inhibitors than the parent compound **1**, resulting in 66 and 61% inhibition, respectively. We then focused on compound **5** to be the most potent inhibitor.

Compound **5** dose-dependently inhibited the acid amidase, and its IC_{50} value was calculated to be 4.5 μM (Fig. 7). In contrast, the compound did not inhibit FAAH at least at concentrations up to 100 μM. To examine inhibition manner, the acid amidase was allowed to react with different concentrations of *N*-palmitoylethanolamine in the presence of compound **5** or in its absence. Based on double reciprocal plot, the inhibition manner was judged as non-competitive type. We examined the inhibitory effect of compound **5** with rat alveolar macrophages which show a potent activity of the acid amidase as described above. When the homogenate of macrophages was allowed to react with *N*-palmitoylethanolamine at pH 5, the enzyme was

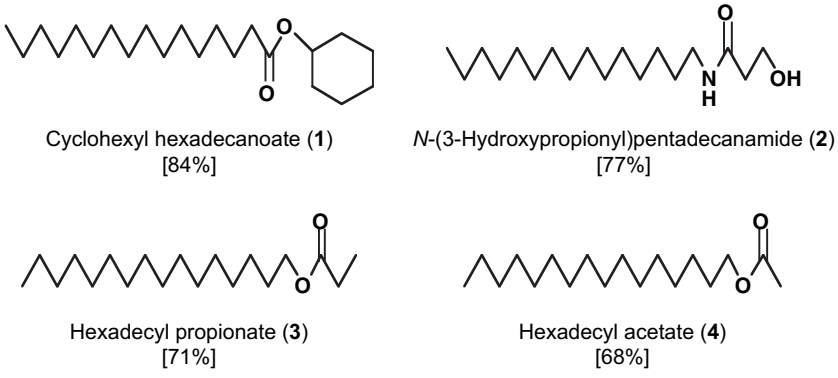

Fig. 5. The first screening of inhibitors for rat lung acid amidase. Inhibitory activities (% inhibition) of each compound at 100 μM are shown in brackets.

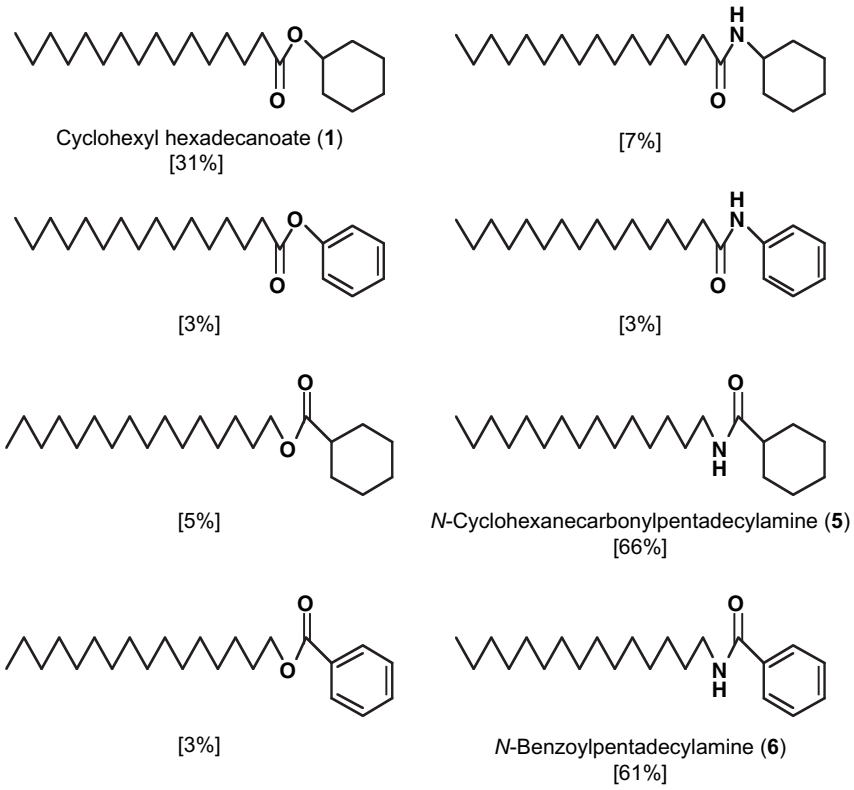

Fig. 6. The second screening of inhibitors for rat lung acid amidase. Inhibitory activities (% inhibition) of each compound at 10 μM are shown in brackets.

inhibited by 100 μM of compound **5**, but not by 1 μM MAFP as an FAAH inhibitor. In contrast, at pH 9, which is optimal for FAAH, the *N*-palmitoylethanolamine-hydrolyzing activity was hardly detectable. These results indicated that the acid amidase rather than FAAH is mostly responsible for the *N*-palmitoylethanolamine hydrolysis in rat alveolar macrophages. In addition, we examined the inhibitory effect of compound **5** with intact macrophage cells. Preincubation of the suspended cells with the compound caused 85% inhibition for the hydrolysis of exogenously added *N*-palmitoylethanolamine.

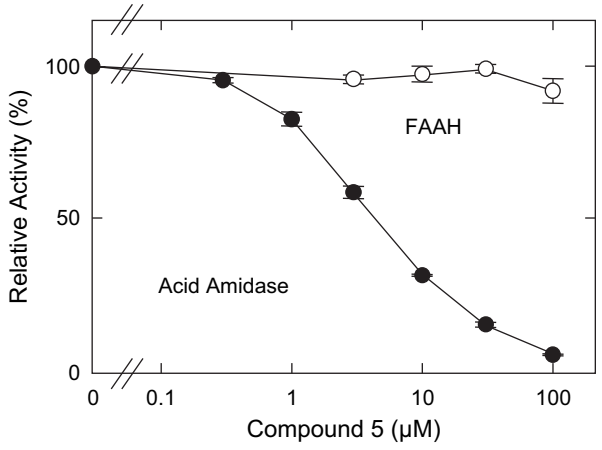

Fig. 7. Selective inhibition of the acid amidase by compound **5**. Rat lung acid amidase or rat liver FAAH was allowed to react with 100 μM *N*-palmitoylethanolamine in the presence of the increasing concentrations of compound **5**. Mean values ± SD are shown ($n = 3$).

Although RBL-1 cells express FAAH as described above, we examined whether or not this cell line also possesses the acid amidase. The cells were subjected to freezing and thawing, and centrifuged to separate the cytosol from the particulate fraction. The *N*-palmitoylethanolamine-hydrolyzing activity in the cytosol was higher at pH 5 than at pH 9, and was inhibited by compound **5**, but not by MAFP (Fig. 8). In contrast, the enzyme activity in the particulate fraction was higher at pH 9, and was inhibited by MAFP rather than by compound **5**. These results indicated that RBL-1 cells express not only FAAH but also the acid amidase, and compound **5** was found to be a useful tool to distinguish the acid amidase from FAAH even with a preparation containing both the enzymes.

4. Conclusions and perspectives

A series of our enzymological studies including the development of selective inhibitors confirmed that animal tissues have a second NAE hydrolase distinguishable from FAAH. Even if FAAH plays a central role in the degradation of anandamide, the unique tissue distribution, characterized by high content in immune

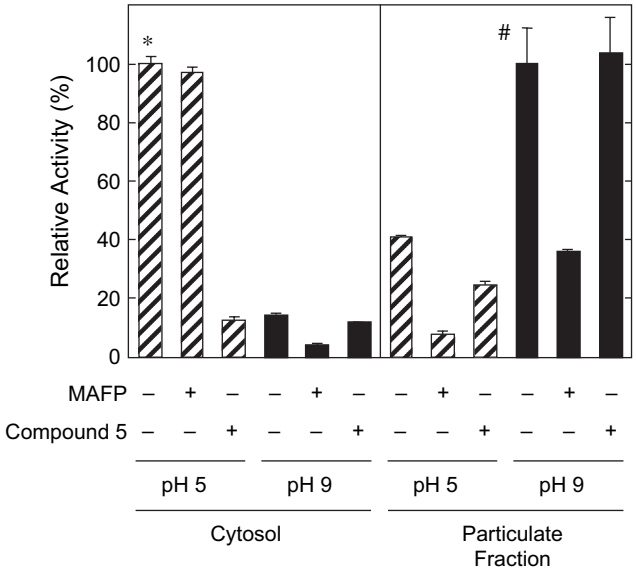

Fig. 8. Detection of the acid amidase and FAAH in RBL-1 cells. The homogenates of RBL-1 cells were subjected to freezing and thawing, followed by centrifugation at 105 000 x g. The supernatant (cytosol) and the pellet (particulate fraction) were allowed to react with 100 μM *N*-palmitoylethanolamine either at pH 5 or at pH 9. MAFP at 1 μM or compound **5** at 100 μM was also included as indicated. Mean values ± SD are shown ($n = 3$). The activities in the absence of inhibitor (∗, 0.18 nmol/min/mg protein; #, 0.27 nmol/min/mg protein) were expressed as 100%, respectively.

tissues and macrophages, suggests a different role of the acid amidase. For example, the acid amidase may participate in the control of the levels of anandamide and other NAEs at the site of inflammation and tissue degeneration, where the formation of NAEs should increase (Hansen et al., 2000; Schmid, 2000). Further characterization of the enzyme including cDNA cloning will be necessary to elucidate its physiological and pathophysiological roles. Compound **5** appeared to be a useful tool to catalytically distinguish the acid amidase from FAAH. This compound or more potent inhibitors expected to be developed may contribute to in vivo studies on this enzyme.

5. Note added in proof

Recently, Tsuboi et al. (2005) reported cDNA cloning and functional expression of the acid amidase from human, rat, and mouse.

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