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Molecular identification of nicotinic acid receptor☆

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Abstract

Nicotinic acid and its derivative, Acipimox, have been widely used in the treatment of hyperlipidemia. Pharmacological studies have demonstrated that they exert the beneficial effect through the activation of a Gi-protein-coupled receptor on adipocyte, which has remained elusive to date. Here we show that a novel GPCR, designated HM74b because of its high similarity to HM74, is a receptor for nicotinic acid. HM74b mRNA is found in human, murine, and rat adipose tissues. Nicotinic acid and Acipimox inhibit forskolin-stimulated intracellular cAMP accumulation in human HM74b-expressing cells and activate GTPγS binding in a dose-dependent manner. [^{3}H]Nicotinic acid specifically binds to HM74b-expressing membrane and its binding is replaced by Acipimox. This finding will open a new phase of research on the physiological role of nicotinic acid and will be a clue to develop novel antihyperlipidemic drugs.

Keywords: G-protein-coupled receptor; Nicotinic acid; Acipimox; Adipose

Nicotinic acid (niacin) is a member of water-soluble vitamins. Based on its well-known plasma lipid-lowering effect, nicotinic acid and its derivative, e.g., Acipimox [1], have been used clinically in the treatment for hyperlipidemia. These substances are known to lower elevated plasma concentration of low-density lipoprotein (LDL), intermediate-density lipoprotein, very low-density lipoprotein (VLDL), triglycerides (TG), and lipoprotein Lp(a), and also increase plasma high density lipoprotein (HDL) concentrations, resulting in an improvement of mortality rate against coronary artery disease [2]. Although the exact mechanism of the action of nicotinic acid on lipoprotein metabolism is still unknown, its inhibitory effect on fat-mobilizing lipolysis in adipose tissue is considered as the most plausible explanation of this beneficial mechanism. Plasma free fatty acid is known as a substance of VLDL synthesis in the liver. The decrease of the free fatty acid lowers the circulating levels of both plasma TG and LDL cholesterol [3]. In adipocytes, nicotinic acid is reported to inhibit lipolysis by inhibiting adenylyl cyclase [4], which inactivates protein kinase A, resulting in the suppression of hormone-sensitive lipase [5]. Recently, a target molecule for nicotinic acid was reported as a G-protein-coupled receptor existing in the rat spleen, adipose, and macrophages [6,7], which has remained elusive to date.

The recent progress in genetic analysis technologies enables us to identify and characterize numbers of novel orphan GPCRs, and we have succeeded in clarifying novel ligand-receptor systems on several orphan GPCRs by demonstrating their endogenous ligands, such as vasopressin V1b receptor [8], leukotriene B_4 BLT2 receptor [9], cysteinyl leukotriene $CysLT_2$ receptor [10], histamine H4 receptor [11], ADP P_2T_{AC} receptor [12], and prokineticin PK-R receptors [13]. In line with this course of research, we managed to identify an

☆ *Abbreviations:* GPCR, G-protein-coupled receptor; RT-PCR, reverse transcription-polymerase chain reaction; GTPγS, guanosine 5′-(γ-thio)triphosphate; EC_{50}, median effective concentration; G3PDH, glycerol-3-phosphate dehydrogenase; SE, standard error; IBMX, 3-isobutyl-1-methylxanthine; PBL, peripheral blood leucocyte; Pertussis toxin, PTX.

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endogenous receptor for nicotinic acid, which is a Gi/o-coupled receptor expressing in the adipose tissue.

Materials and methods

Materials and cell. Nicotinic acid, 3-isobutyl-1-methylxanthine (IBMX), Pertussis toxin (PTX), and guanosine 5′-(γ-thio)triphosphate (GTPγS) were purchased from Sigma. Forskolin was obtained from Wako. [^{35}S]GTPγS (1250 Ci/mmol) and [5,6-^{3}H]nicotinic acid (50 Ci/mmol) were purchased from Perkin–Elmer and American Radiolabeled Chemicals, respectively. Acipimox [1] was synthesized by chemists in Yamanouchi Pharmaceutical. 293EBNA cells were purchased from Invitrogen.

Cloning of human, murine, and rat HM74b. During our attempt to amplify a transcriptional open reading frame (ORF) of HM74 [14] from human spleen cDNA (Clontech), one novelly predicted GPCR was found. Since its predicted amino acid sequence is very similar to that of HM74, we termed this GPCR as HM74b (hHM74b). The transcriptional ORF of HM74b was amplified using the following *Xba*I restriction-site-containing primers: 5′-GGTCTAGAATGAA TCGGCACCATCT-3′ and 5′-GGTCTAGATTAAGGAGAGGTT GGGC-3′. This amplified product was subcloned into the mammalian expression vector pEF-BOS (pEF-h.HM74b) [15]. TBLASTN search provided that an ortholog to human HM74 and HM74b corresponds to *Mus musculus* PUMA-G gene [16], which we termed mHM74b. Homology-based PCR cloning using rat spleen cDNA as template provided the rat HM74b, which we termed rHM74b. The ORFs of mHM74b and rHM74b cDNAs were amplified and subcloned into pEF-BOS. pEF-hHM74b, and pcDNA3.1/Zeo (Invitrogen) were co-transfected into 293EBNA cells. 293EBNA cells stably expressing hHM74b were established by resistance to 80 μg/ml zeocin.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Poly(A)$^{+}$ RNAs from various human, murine, and rat tissues were purchased (Clontech and Biochain) and cDNAs were synthesized as described previously [17]. One microliter of first-strand cDNA from this cDNA panel was used as a template for PCR. Specific primers for human HM74 (sense 5′-ATCTGCCTGCCGTTCG-3′, antisense 5′-ATGGCAAACATGAAGAGCAC-3′), human HM74b (sense 5′-CATCTGCCTGCCCTTCC-3′, antisense 5′-TCATAGCCAACATG AAGAGCAT-3′), and murine and rat HM74b (sense 5′-TTGACGG ACAACTATGTCCA-3′, antisense 5′-CTGTCCATCTGTCTCTGC CT-3′) were used to amplify the fragments of hHM74, hHM74b, mHM74b, and rHM74b, respectively. Human, murine, and rat G3PDH mRNA was also amplified as an internal control. The PCR was performed for 30 cycles (hHM74 and hHM74b: 94 °C, 15 s and 69 °C, 60 s; mHM74b and rHM74b: 94 °C, 15 s and 60 °C, 60 s; and hG3PDH, mG3PDH, and rG3PDH: 94 °C, 15 s and 55 °C, 60 s) with Pyrobest DNA polymerase (Takara), and the amplified products were detected by ethidium-bromide staining.

Assay for inhibition of forskolin-stimulated intracellular accumulation of cAMP. 293EBNA cells stably expressing hHM74b were plated in 96-well plates at 5×10^3 cells/well and incubated for 36 h. The cells were washed in DMEM containing 1 mM IBMX. Cells were treated with appropriate ligands and 1 μM forskolin. After incubation for 15 min, the cells were harvested with 0.2% Triton X-100 in phosphate-buffered saline. Intracellular cAMP was measured using with a cAMP homogeneous time-resolved fluorescence kit (Sheering). For pertussis toxin sensitivity, cells were pretreated for 24 h with 100 ng/ml pertussis toxin. The data were analyzed using Prism (Graphpad Software).

[^{35}S]GTPγS binding assay. Membranes were prepared from 293EBNA cells transiently expressing hHM74b, mHM74b, or rHM74b for use in radioligand and [^{35}S]GTPγS binding studies. pEF-BOS-hHM74b, pEF-BOS-mHM74b, or pEF-BOS-rHM74b was transfected into 293EBNA cells and incubated for 36 h. The cells were harvested and the membranes were prepared as described previously [10]. Five microgram of membranes was incubated in 96-well plates with test compounds in 200 μl of assay buffer containing 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.1% bovine serum albumin, 5 μM GDP, and 250 pM [^{35}S]GTPγS (1250 Ci/mmol). After a 60 min incubation at room temperature, membranes were harvested on GF/B filter plates and bound radionuclides were measured using TopCount (Packard). The data were analyzed using Prism.

Radioligand binding assay. Twenty μg of membranes was incubated with several concentrations (0.5–200 nM) of [5,6-^{3}H]nicotinic acid (50 Ci/mmol) for 2 h at room temperature in assay buffer (50 mM Tris–HCl (pH 7.4), 1 mM $MgCl_2$, and 0.02% CHAPS). After incubation, the reaction was terminated by collecting the membranes onto GF/B filter, followed by washing three times with ice-cold assay buffer. The radioactivity retained on each filter was measured with a liquid scintillation counter. The nicotinic acid-specific binding was calculated by subtracting the nonspecific binding which was defined with 400 μM unlabeled nicotinic acid. Specific binding of [^{3}H]nicotinic acid for HM74b-expressing membranes accounted for 80–90% of total binding. For the competition studies, 10 nM [^{3}H]nicotinic acid was added to aliquots of HM74b membrane preparation, which were then incubated with one of the several concentrations of compounds. The data were analyzed using Prism.

Results

Nicotinic acid and Acipimox inhibited forskolin-evoked intracellular cAMP accumulation in human HM74b stably expressing cells

Nicotinic acid inhibited forskolin-stimulated intracellular cAMP accumulation in human HM74b (hHM74b) stably expressing cells in a dose-dependent manner. Acipimox, which has a similar pharmacological profile to that of nicotinic acid, also inhibited cAMP accumulation in a similar fashion (Fig. 1). The values of EC_{50} of nicotinic acid and Acipimox were 128 ± 18 and

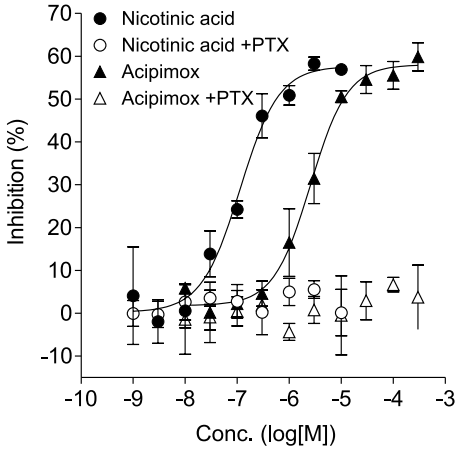

Fig. 1. HM74b is a Gi/o-coupled receptor for nicotinic acid. Nicotinic acid (●) and Acipimox (▲) inhibited forskolin-evoked intracellular cAMP accumulation in human HM74b stably expressing cells. The cAMP concentrations in the presence of IBMX with or without forskolin (1 μM) were normalized to 0% and 100%, respectively. The effect of pertussis toxin (100 ng/ml, 24 h) on cAMP inhibition by nicotinic acid (○) and Acipimox (△) in hHM74b-expressing 293EBNA cells was also measured. The data represent means ± SE in $n = 4$. This graph shown is representative of at least three experiments.

4350 ± 937 nM, respectively. They did not affect parent cells on cAMP accumulation (data not shown). The activity of adenylyl cyclase inhibition of hHM74b by nicotinic acid or Acipimox was completely blocked by 100 ng/ml pertussis toxin (Fig. 1). These results clearly demonstrated that hHM74b is an endogenous receptor for nicotinic acid involving Gi/o-activation.

[^{35}S]GTPγS binding study and [^{3}H]nicotinic acid binding study of human, murine, and rat HM74b

BLASTN search provided that an ortholog to human HM74b corresponds to a *Mus musculus* PUMA-G gene, which we termed mHM74b [16]. A homology-based PCR cloning identified a rat counterpart, termed rHM74b (Fig. 2). Human HM74b was 82% and 83% identical to murine and rat HM74b, respectively, and mHM74b was 95% identical to rHM74b. To examine whether rodent HM74bs react to nicotinic acid and Acipimox, [^{35}S]GTPγS binding activity was measured. Human, murine, and rat HM74b responded to nicotinic acid and Acipimox in a dose-dependent manner (Figs. 3A–C). As shown in Table 1, the EC_{50} values of nicotinic acid were almost the same among human and rodent (hHM74b, 61.8 ± 9.16 nM; mHM74b, 40.7± 10.9 nM; and rHM74b, 74.9 ± 6.20 nM). Interestingly, the EC_{50} ratio (Acipimox/nicotinic acid) between human and rodent HM74b was different. The ratio of hHM74b is 42, on the other hand, that of mHM74b and rHM74b was 8.4 and 12, respectively. These results indicated that Acipimox is more potent to rodent HM74b than the human counterpart.

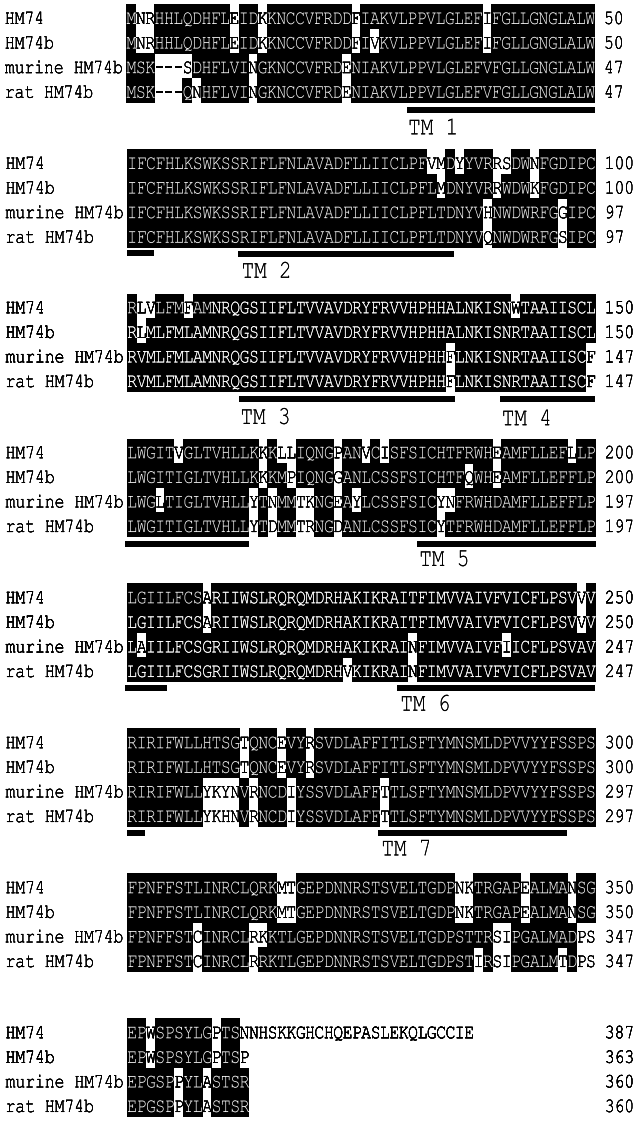

Fig. 2. Alignment of human, murine, rat HM74b, and human HM74 polypeptide sequences. The predicted seven transmembrane domains (TM1-7) are underlined. The Accession Nos. in GenBank of human, murine, HM74b, and human HM74 are AB083632, NM_030701, and NM_006018, respectively. The nucleotide sequence data of rat HM74b have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence database as Accession Nos., AB103062.

In radioligand binding study, [^{3}H]nicotinic acid specifically bound to human, murine or rat HM74b-expressing 293EBNA cell membranes. There was no specific binding observed for parental 293EBNA cells (data not shown). As summarized in Table 1, K_d values of nicotinic acid were almost the same among the three species (hHM74b, 63.1 ± 3.89 nM; mHM74b, 45.3± 2.33 nM; and rHM74b, 60.8 ± 11.2 nM). K_i values were also analyzed to study the affinity order of nicotinic acid and Acipimox to each HM74b by competitive binding assay (Figs. 3D–F). In consistent with the result obtained with the GTPγS binding study, the analysis of K_i values indicated that Acipimox is more potent to rodent HM74bs than the human counterpart (Table 1).

Despite human HM74 being closely similar to HM74b (Fig. 2), nicotinic acid (up to 100 μM) and Acipimox (up to 1 mM) showed no significant agonistic activity at human HM74 in GTPγS binding assay (data not shown). And also in the radioligand binding assay, the specific binding for nicotinic acid (up to 200 nM) was not detected (data not shown).

Tissue distribution of human, murine, and rat HM74b

Tissue distribution of human HM74 and HM74b of the three species mRNA was studied by RT-PCR analysis. Because the homology between HM74 and HM74b is extremely high in human, the primer set and the condition of PCR were confirmed using each cloned cDNA as template in order to discriminate between these two transcripts (data not shown). The expression of hHM74b was detected in the adipose, lung, trachea, and spleen, on the other hand, that of hHM74 was detected in the lung, spleen, PBL, and adipose (Fig. 4A). In murine and rat tissues, the expression of HM74b was also detectable in the adipose, lung, and spleen (Figs. 4B and C). These

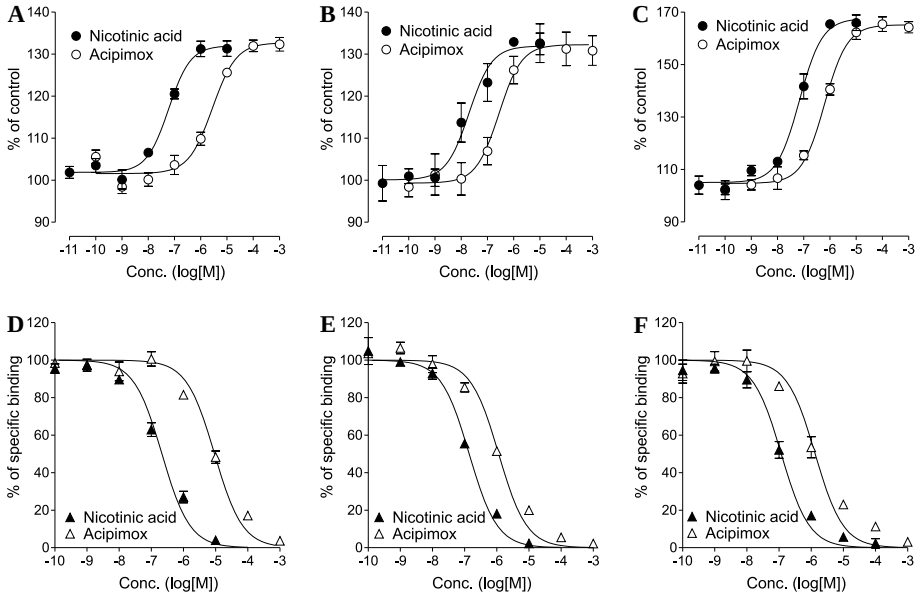

Fig. 3. [^{35}S]GTPγS binding study and radioligand binding study of human, murine, and rat HM74b. Nicotinic acid (●) and Acipimox (○) stimulate GTPγS binding in human HM74b- (A), murine HM74b- (B), or rat HM74b- (C) expressing 293EBNA cell membranes. The basal radioactivity was normalized to 100%. Competitive binding assays for human HM74b (D), murine HM74b (E), and rat HM74b (F) were performed. Various concentrations of nicotinic acid (▲) and Acipimox (△) were challenged to each HM74b in the presence of 10 nM [^{3}H]nicotinic acid. The data represent means ± SE in $n = 4$ (A–C) or duplicate (D–F). These graphs shown are representative of at least three experiments.

Table 1
Comparison of potencies of nicotinic acid and Acipimox at human, murine, and rat HM74b

	Nicotinic acid (nM)	Acipimox (nM)	AXA/NA ratio[c]
human HM74b			
K_d[a]	63.1 ± 3.89	—	
K_i[a]	113 ± 23.6	4390 ± 645	39
EC_{50} (GTPγS binding)[b]	61.8 ± 9.16	2580 ± 468	42
EC_{50} (cAMP assay)[b]	128 ± 18	4350 ± 937	33
murine HM74b			
K_d[a]	45.3 ± 2.33	—	
K_i[a]	122 ± 9.86	1100 ± 87.6	9.0
EC_{50} (GTPγS binding)[b]	40.7 ± 10.9	340 ± 87.4	8.4
rat HM74b			
K_d[a]	60.8 ± 11.2	—	
K_i[a]	100 ± 12.4	1340 ± 119	13
EC_{50} (GTPγS binding)[b]	74.9 ± 6.20	912 ± 165	12

[a] Values were expressed as means ± SE of at least three separated experiments performed in duplicate.
[b] Values were expressed as means ± SE of at least three separated experiments performed in $n = 4$–6.
[c] ACX: Acipimox, NA: nicotinic acid.

data indicated that HM74b mRNA was expressed in the adipose tissue, irrespective of species difference.

Discussion

This paper describes molecular identification and characterization of human, murine, and rat nicotinic acid receptors, designated HM74b. When the ORF of HM74 was amplified from human spleen cDNA as template, one closely similar GPCR was found, so we termed HM74b. GenBank-analysis revealed that these two receptors co-localized tandemly in the chromosome 12q24.31. BLASTN search provided the *M. musculus* PUMA-G gene as an ortholog to human HM74 and HM74b, and homology-based PCR cloning identified rat counterpart (Fig. 2). Our extensive search through genomic PCR and genomic database analysis failed to obtain a rodent second ortholog of human HM74 and HM74b. This suggests that HM74 and HM74b may be generated by a gene-duplication relatively recent in the process of molecular evolution. In spite of the high sequence homology, HM74 could not be activated by nicotinic acid (up to 100 μM) or Acipimox (up to 1 mM)

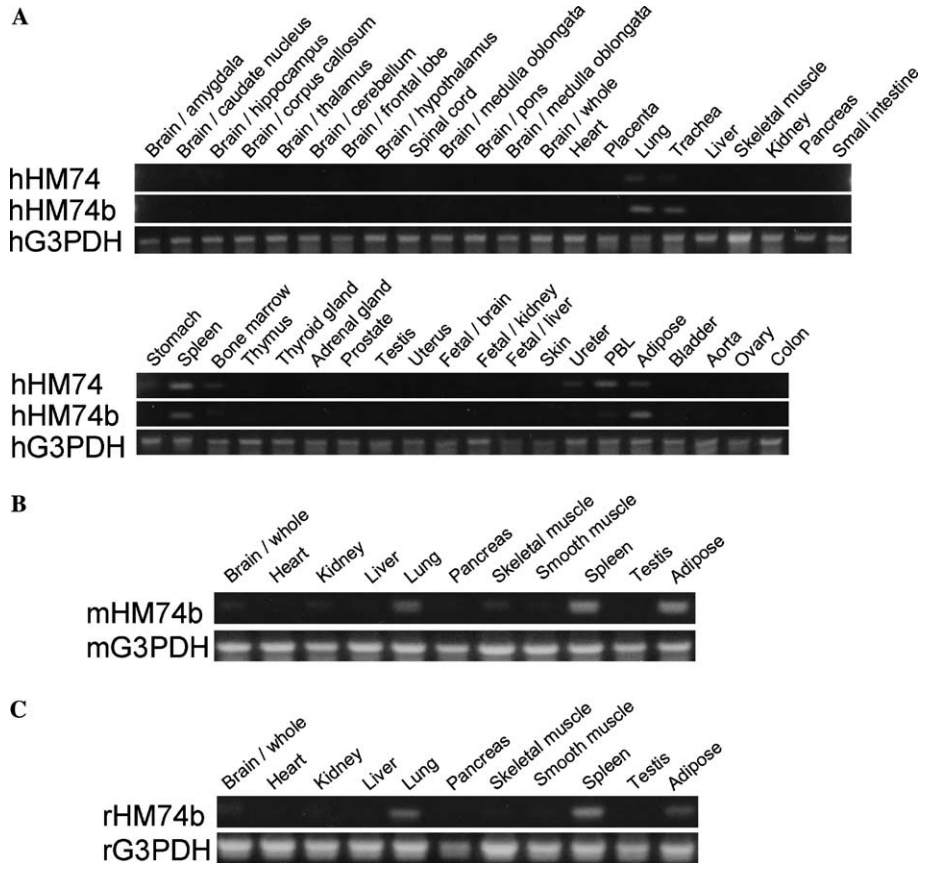

Fig. 4. Tissue distribution of HM74b mRNA in human, murine, and rat tissues, by RT-PCR. Amplification with each gene-specific primer set for human HM74 (A, top panel), human HM74b (A, middle panel), murine HM74b (B, top panel), and rat HM74b (C, top panel) is shown. G3PDH is also shown in each bottom panel as internal control.

in the GTPγS binding assay, and also the specific binding of [^{3}H]nicotinic acid was not detected for HM74-expressing membrane (data not shown). In view of a previous report on the agonistic activity of nicotinic acid [6], our results indicate that HM74 has little or no function as a nicotinic acid receptor in human.

Nicotinic acid and its analog, Acipimox, are believed to act on a Gi-protein-coupled receptor on the adipocytes [3,6]. In our present study, nicotinic acid and Acipimox inhibited forskolin-stimulated intracellular accumulation of cAMP in hHM74b stably expressing cells in a dose-dependent manner. The activity of adenylyl cyclase inhibition of hHM74b was sensitive to pertussis toxin (Fig. 1). These data indicate that HM74b is a Gi/o-protein-coupled receptor for nicotinic acid and the potency of nicotinic acid is in agreement with that of the previous report [6].

The expression of HM74b in the adipose, spleen, and lung was observed commonly among human, mouse, and rat. Our current expression profile data are consistent with the past finding that the pharmacological binding sites for nicotinic acid exist in the spleen and the adipose tissue in rat [6]. Since mHM74b (PUMA-G) was reported to be an interferon-γ-inducible gene in the macrophages [16], the expression of HM74b in the lung may be derived from alveolar macrophages.

Nicotinic acid and Acipimox specifically bound to human, murine, and rat HM74b-expressing membrane, and also activated GTPγS binding in a dose-dependent manner (Fig. 3). It is interesting to note that the K_d, K_i, and EC_{50} values of nicotinic acid were approximately equivalent among the three species, however, the K_i and EC_{50} values of Acipimox were different. The ratio (Acipimox/nicotinic acid) of human:murine:rat HM74b was about 40:9:13 (Table 1). Acipimox was discovered as an effective antilipolytic agent by performing rat in vivo screening [1]. In actual clinical situations, hyperlipidemic patients must take high dosage, which usually deteriorates patients' compliance. The difference of ratio between the species suggests that an improved drug, which can be used clinically at a less dosage, will be possibly discovered by using human HM74b as a target molecule.

During the preparation of this article, two other researchers reported *on line* articles for identification of receptor for nicotinic acid, which was identical to our current report [18,19]. The relative potencies of nicotinic acid and Acipimox are similar, but we further confirmed

the pharmacological characterization about ligand binding study and GTPγS binding study comparing among human, murine, and rat receptors.

In conclusion, we reported the molecular identification and characterization of human, murine, and rat receptors for antihyperlipidemic drug, nicotinic acid. We expect that this finding would facilitate the development of improved drugs for hyperlipidemia.

Acknowledgments

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