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ScienceDirect
Biochemical and Biophysical Research Communications 362 (2007) 928–934
BBRC
www.elsevier.com/locate/ybbrc

Identification of GPR55 as a lysophosphatidylinositol receptor

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Received 9 August 2007
Available online 24 August 2007

Abstract

GPR55 is an orphan G protein-coupled receptor. In this study, we explored a possible endogenous ligand for GPR55 using HEK293 cells which expressed GPR55. We found that lysophosphatidylinositol induced rapid phosphorylation of the extracellular signal-regulated kinase in transiently or stably GPR55-expressing cells. On the other hand, lysophosphatidylinositol did not induce phosphorylation of the extracellular signal-regulated kinase in vector-transfected cells. Lysophosphatidic acid and sphingosine 1-phosphate also induced phosphorylation of the extracellular signal-regulated kinase in GPR55-expressing cells. However, these lipid phosphoric acids elicited similar responses in vector-transfected cells. Various types of other lysolipids as well as the cannabinoid receptor ligands did not induce phosphorylation of the extracellular signal-regulated kinase. We also found that lysophosphatidylinositol elicited a rapid Ca^{2+} transient in GPR55-expressing cells. Lysophosphatidylinositol also stimulated the binding of GTPγS to the GPR55-expressing cell membranes. These results strongly suggest that GPR55 is a specific and functional receptor for lysophosphatidylinositol.

Keywords: Lysophosphatidylinositol; G protein-coupled receptor; GPR55; Lipid mediator; Bioactive lipid; Extracellular signal-regulated kinase; Ca^{2+} transient; HEK293 cell

GPR55 is an orphan G protein-coupled receptor [1]. The human GPR55 gene is mapped to chromosome 2 at q37 and encodes a protein of 319 amino acids. GPR55 is expressed in several mammalian tissues such as the breast adipose tissues, testis, spleen, and several regions of the brain [1,2], although its physiological and/or pathophysiological significance remains elusive. Recently, two independent groups proposed in patents that GPR55 may be a novel receptor for cannabinoids [2,3]. However, no subsequent report has appeared to date which demonstrates that GPR55 is a specific receptor for cannabinoids based on precise experimental data [4,5]. Of note, GPR55 has low sequence identity with the cannabinoid receptors; i.e., 13.5% for the CB1 receptor and 14.4% for the CB2 receptor. Moreover, GPR55 does not have a classical 'cannabinoid binding pocket' which is present in both the CB1 and CB2 receptors [4]. Thus, whether or not GPR55 is a cannabinoid receptor is an open question.

What then is GPR55 the receptor for? Devane et al. [6] demonstrated that anandamide (*N*-arachidonoylethanolamine) is an endogenous ligand for the cannabinoid receptors. We [7] and Mechoulam et al. [8] demonstrated that 2-arachidonoylglycerol (2-AG) is another endogenous ligand for the cannabinoid receptors. Based on studies of the structure–activity relationship, we concluded that 2-AG, rather than anandamide, is the true natural ligand for the cannabinoid receptors (CB1 and CB2) [9,10]. Meanwhile, 2-AG is assumed to be a member of the bioactive lysolipid super-family which includes various types of lipid mediators. These lipid mediators are known to interact with specific G protein-coupled receptors [11]. Considering that a number of G protein-coupled receptors have been identified as lysolipid receptors and the number is still increasing, it seems possible that a significant proportion of the remaining orphan receptors as well are the receptors for bioactive lysolipids known or unknown.

In this study, we explored in detail the effects of various lysolipids in addition to various synthetic cannabinoid receptor ligands and related molecules on human embry-

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doi:10.1016/j.bbrc.2007.08.078

onic kidney 293 cells (HEK293 cells) which expressed GPR55. We obtained evidence that GPR55 is an intrinsic functional receptor for lysophosphatidylinositol (LPI). To the best of our knowledge, this is the first report showing evidence for the occurrence of an LPI receptor in mammalian tissues.

Materials and methods

Chemicals. LPI sodium salt (derived from soybean), lysophosphatidylcholine (LPC) (derived from egg yolk), lysophosphatidylethanolamine (LPE) (derived from egg yolk), platelet-activating factor (PAF) (1-*O*-hexadecyl), lysophosphatidic acid (LPA) (1-oleoyl) sodium salt, sphingosine 1-phosphate (S1P), sphingosylphosphorylcholine (SPC), and psychosine were purchased from Sigma (St. Louis, MO). Lysophosphatidylserine (lysoPS) sodium salt (derived from porcine brain) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 2-AG was purchased from Cayman (Ann Arbor, MI). pcDNA4/TO, Lipofectamine™ 2000 and Stealth™ RNAi were purchased from Invitrogen Life Technologies (Carlsbad, CA). [^{35}S]GTPγS (46.25 TBq/mmol) was obtained from Perkin-Elmer Japan Co., Ltd. (Kanagawa, Japan).

Cloning and cell culture. A DNA fragment containing the entire open reading frame of the human GPR55 (GenBank™ Accession No. NM_005683) was amplified from human spleen cDNA by PCR using Pyrobest® DNA Polymerase (Takara Bio Inc.) and oligonucleotides (forward primer containing a KpnI site, a Kozak sequence, and a FLAG sequence, 5′-AAAAAAGGTACCGCCACCATGGACTACAAGGACG ACGATGACAAGAGTCAGCAAAACACCAGTGGGGAC-3′; reverse primer containing a XbaI site, 5′-AAAAAATCTAGATTAGCCCCGG GAGATCGTGGTGTC-3′). The resultant DNA fragment was digested with KpnI and XbaI and subsequently cloned into the mammalian expression vector pcDNA4/TO between the KpnI and XbaI sites. The integrity of the plasmid was verified by DNA sequencing. HEK293 cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO). Cells were transfected with GPR55-pcDNA4/TO or an empty vector using Lipofectamine™ 2000 reagent. The stably transfected clones were selected in the presence of zeocin (100 ng/ml, Invitrogen).

RNA interference (RNAi) analysis. GPR55-expressing HEK293 cells were transfected with duplex small interfering RNA (siRNA) for GPR55 using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions. Following transfection, the cells were incubated at 37 °C for 24 h. The sequences of the siRNA used were as follows; sense, 5′-AGGUGUUUGGCUUCCUCCUUCCCAU-3′; antisense, 5′-AUG GGAAGGAGGAAGCCAAACACCU-3′. The stealth RNAi negative control duplex (Invitrogen), whose GC content is similar to that of the duplex siRNA, was used as a negative control.

Western blot analysis. The subconfluent cells were incubated in 1 ml of DMEM containing 5 mM Hepes–NaOH (pH 7.4) and 0.1% BSA in the presence of LPI (final concentration, 1 μM) or various ligands (final concentration, 1 μM) or the vehicle (final concentration of DMSO, 0.02%, v/v) in 35-mm dishes at 37 °C for 5 min. Following the incubation, the medium was aspirated and the cells were washed with ice-cold (4 °C) Tyrode's solution containing 5 mM Hepes–NaOH (pH 7.4). Western blot analysis was carried out according to the previously described method [12]. The membranes were analyzed using the ECL reagent. The intensity of the bands was quantified using NIH *Image*, and the ratio of phospho-ERK to total ERK was calculated. The data were expressed as fold-stimulation (compared with vehicle alone or time 0).

Measurement of the intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ was estimated using a CAF-100 Ca^{2+} analyzer (JASCO, Tokyo, Japan) as previously described [9,10]. LPI or various ligands were dissolved in DMSO and an aliquot (1 μl each) was added to the cuvette (final concentration of DMSO, 0.2%, v/v). DMSO (0.2%, v/v) *per se* did not markedly affect $[Ca^{2+}]_i$. In some experiments, the cells suspended in 500 μl of Hepes-buffered Tyrode's solution ($-Ca^{2+}$) containing 0.1% BSA were pretreated with LPI, LPA or S1P (final concentration, 10 μM) or the vehicle (1 μl) at 37 °C for 1 min. The cells were then sedimented by centrifugation and resuspended in Hepes-buffered Tyrode's solution ($-Ca^{2+}$) containing 0.1% BSA. After adding $CaCl_2$ (final concentration, 1 mM), LPI (final concentration, 3 μM) was added to the cuvette, and the changes in the $[Ca^{2+}]_i$ were analyzed.

GTPγS binding assay. The [^{35}S]GTPγS binding assay was carried out according to the method described by Breivogel et al. [13].

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) followed by Dunnett's test (Figs. 1C–F, 3A and B, and 4) or Tukey's test (Fig. 1G). $p < 0.05$ was considered to be a significant difference.

Results

Effects of LPI on ERK in GPR55-expressing HEK293 cells

We first examined the effects of LPI on ERK in HEK293 cells expressing GPR55, since ERK is a major protein-located downstream of various intracellular signaling pathways. We found that LPI (1 μM) induced a rapid phosphorylation of ERK in transiently GPR55-expressing cells (Fig. 1A), whereas LPI had no effect on ERK in the vector-transfected cells. The LPI-induced rapid phosphorylation was also observed with stably GPR55-expressing cells (Fig. 1B). In the subsequent experiments, we employed HEK293 cells which stably expressed GPR55 as GPR55-expressing cells.

Fig. 1C and D shows the time-dependent changes in the phosphorylation of ERK following stimulation with LPI (1 μM). LPI did not induce the phosphorylation of ERK in the vector-transfected cells. On the other hand, LPI evoked a rapid phosphorylation of ERK in the GPR55-expressing cells. The maximal response was observed at 5 min after which the phosphorylation declined.

Fig. 1E and F shows the dose-dependency. LPI did not affect the phosphorylation of ERK at least up to 10 μM in the vector-transfected cells. On the other hand, LPI dose-dependently provoked the phosphorylation of ERK in the GPR55-expressing cells. The response was detectable from 10 nM and reached a plateau at 1 μM. The EC_{50} was 200 nM.

The effect of siRNA directed toward GPR55 on the LPI-induced phosphorylation of ERK was examined next. As demonstrated in Fig. 1G, treatment of the cells with siRNA against GPR55 markedly reduced the response induced by LPI in the GPR55-expressing cells. On the other hand, the control siRNA did not markedly affect the response induced by LPI.

Effects of various cannabinoid receptor ligands and related molecules, LPI, and other lysolipids on ERK in GPR55-expressing HEK293 cells

We next examined the effects of various types of cannabinoid receptor ligands and related molecules and lysolipids on ERK in the GPR55-expressing cells and compared them with those of the vector-transfected cells. As demonstrated