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Selective activation of G alpha i mediated signalling of $S1P_3$ by FTY720-phosphate

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Abstract

The immune modulator FTY720 is phosphorylated *in vivo* to FTY720 phosphate (FTY-P), which activates four sphingosine 1-phosphate (S1P) receptors including $S1P_3$. Upon activation with S1P, $S1P_3$ couples to G_i- and G_q-protein-dependent signalling pathways. Here we show that FTY-P selectively activates the $S1P_3$-mediated and G_i-coupled inhibition of adenylyl cyclase. Contemporaneously, it antagonizes the S1P-induced activation of G_q via $S1P_3$ in intracellular calcium flux measurements, GTP-binding experiments, and flow cytometric analyses of activation-induced receptor down-regulation. In contrast to S1P, pre-treatment with FTY-P did not desensitize S1P-induced calcium flux or chemotaxis via $S1P_3$. The lack of receptor desensitization prevented $S1P_3$-mediated migration to FTY-P. Human umbilical vein endothelial cells express $S1P_1$ and $S1P_3$, and respond to S1P and FTY-P by ERK1/2 phosphorylation and by intracellular calcium release in a pertussis toxin-sensitive manner. But whereas a mixture of S1P and FTY-P was not affecting ERK1/2 phosphorylation, the intracellular calcium flux was hampered with increasing amounts of FTY-P, which points to a cross-talk between $S1P_1$ and $S1P_3$. FTY-P is therefore one of the rare ligands which bind to a receptor that couples multiple G-proteins but selectively activates only one signalling pathway.

Keywords: G-protein coupling; Adenylyl cyclase; Pertussis toxin; Calcium; GTP-binding; Chemotaxis; Sphingosine 1-phosphate

1. Introduction

The immune modulator Fingolimod (2-amino-2-(2-[4-octylphenyl]ethyl]-1,3-propanediol, FTY720) is phosphorylated *in vivo* by sphingosine kinase type 2 to FTY-P [1,2], which activates, except of $S1P_2$, all known sphingosine 1-phosphate (S1P) receptors [3,4]. Its beneficial effect was attributed to the onset of lymphopenia by blocking S1P and $S1P_1$-mediated lymphocyte egress from lymphoid tissues [5–7]. One of its reported side-effects is the incidence of bradycardia, which was linked to activation of $S1P_3$ [8,9]. The compound was shown to have clinical efficacy in phase II clinical studies in multiple sclerosis (MS) patients [10] and is currently tested in phase III clinical studies for treatment of MS.

FTY-P mimics S1P which is constitutively present in blood [3,4,11]. Blood-borne S1P and $S1P_1$ expression on lymphocytes are critical for lymphocyte circulation [6,7]. FTY-P is thought to

Abbreviations: AC, adenylyl cyclase; PLC-β, phospholipase C-beta; β2-AR, β2 adrenergic receptors; EAE, experimental autoimmune encephalomyelitis; ERK, extracellular signal-regulated protein kinase; FTY720, Fingolimod (2-amino-2-(2-[4-octylphenyl]ethyl]-1,3-propanediol); FTY-P, FTY-Phosphate; GPCRs, G-protein-coupled receptors; HUVEC, human umbilical vein endothelial cells; MAP kinase, mitogen-activated protein kinase; MS, multiple sclerosis; PKA, protein kinase A; PTx, pertussis toxin; S1P, sphingosine 1-phosphate; cAMP, cyclic adenosine monophosphate; S.E., standard error; IP, inositol phosphate.

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be a superagonist for $S1P_1$ which induces receptor down-regulation leading to its inhibition [10]. On the other hand it was shown that $S1P_1$ agonists activate $S1P_1$ on sinus lining endothelial cells of lymphoid tissues [12,13]. Activation of $S1P_1$ leads to the closure of postulated endothelial cell portals, which consequently generate a barrier for exiting lymphocytes [12]. The contribution of $S1P_1$ activation on endothelial cells and its down-regulation and subsequent inhibition on lymphocytes for FTY-P induced lymphopenia is unclear. One of the major questions that need to be answered is why $S1P_1$ deficiency on lymphocytes and administration of $S1P_1$ agonists like FTY-P share the same phenotype of lymphopenia, whereas $S1P_1$ antagonists are ineffective [14–16].

FTY720′s mode of action is complex. It was shown to inhibit cytosolic phospholipase A2 independently from S1P receptors by an intracellular mechanism [17]. After phosphorylation it acts as an extracellular agonist on four S1P receptors [3,4]. But FTY-P also induces receptor internalization and degradation [5], which has opposite effects to its agonistic activity. S1P receptor activation by FTY-P induces bradycardia and protects capillary integrity amongst others [8,9]. The internalization and degradation of S1P receptors after FTY-P treatment mediate distinct phenotypes of S1P receptor deficiency, like the observed block of lymphocyte emigration in lymphocyte-specific conditional $S1P_1$ knockout mice [6,18]. Some of its effects are considered to be beneficial. For example, the down-regulation of $S1P_1$ cell surface expression on lymphocytes is important for the onset of lymphopenia and for the immunosuppressive activity [6]. Others however are unintentional side-effects. Bradycardia induced by $S1P_3$ activation is only one example of an unwanted activity of FTY-P [8,9]. The quest for compounds with similar efficacy and lesser side-effects than FTY720 requires detailed information about its interplay with receptors and their signalling pathways.

Here we report that FTY-P activates G_i proteins via $S1P_3$, and simultaneously blocks G_q-mediated signalling pathways of $S1P_3$ after S1P-stimulation. Thus FTY-P does not mimic S1P as the natural ligand for $S1P_3$. It stabilizes signalling events via G_i proteins and contemporaneously antagonizes S1P-mediated G_q coupling at $S1P_3$.

2. Experimental procedures

2.1. Chemicals

S1P and pertussis toxin (PTx) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Chemicals and solvents were purchased from Roth (Karlsruhe, Germany) if not stated otherwise. FTY720 and FTY-P were kindly provided by Dr. Volker Brinkmann (Novartis, Basel, Switzerland). FTY-P was dissolved in DMSO/50 mM HCl (stock concentration of 50 mM) and diluted with methanol to 1 mM FTY-P. FTY720 and S1P were dissolved directly in methanol to final concentrations of 1 mM.

2.2. Cell culture

Serum, cell culture media, antibiotics and supplements were purchased from PAA Laboratories (Coelbe, Germany) if not otherwise stated. Rat hepatoma HTC_4 and HTC_4 cells expressing human $S1P_1$ or $S1P_3$ were cultured in Eagle's minimum essential medium (MEM) with Earle's salts supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 2× non-essential amino acids, 100 U/ml penicillin G, 100 µg/ml streptomycin, 2 mM L-glutamine. HTC_4 cells that were stably transfected with an N-terminal hemagglutinin-epitope tagged human $S1P_1$ or $S1P_3$ construct ($S1P_1$-HA or $S1P_3$-HA) were cultured in the presence of G418 sulfate (0.4 mg/ml). Pooled human umbilical vein endothelial cells (HUVEC) were purchased from Cambrex (Verviers, Belgium) and cultured in EGM-2 medium (Lonza, Valais, Switzerland) with supplements according to the supplier's protocol. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified 7% CO_2 incubator. Lipofectamine™ 2000 (Invitrogen, Groningen, The Netherlands) was used for transient transfection of COS-7 cells. The G_{sq5} construct was generously provided by Dr. Evi Kostenis [19].

2.3. Calcium flux

HTC_4 cells and HUVEC were harvested with trypsin (PAA Laboratories) and washed three times with Ca^{2+}/Mg^{2+}-free Hank's BSS supplemented with 10% charcoal-adsorbed FBS. Cells (1×10^7 cells/ml) were loaded for 30 min at 37 °C with 2.5 µM FURA-2/AM (Cabiochem-Novabiochem, Bad Soden, Germany) in the same buffer. After three additional washing steps, 2×10^6 cells/ml were resuspended in Ca^{2+}-containing Hank's BSS supplemented with 10% charcoal-adsorbed FBS. Calcium release was determined at 37 °C in a 3 ml quartz cuvette (Thermo Fisher Scientific, Dreieich, Germany) using the spectrofluorophotometer Hyper RF-5301PC (Shimadzu, Duisburg, Germany) with 335 nm excitation wavelength and 505 nm emission wavelength. Relative calcium responses were normalized to the corresponding signal with 10 µM ATP (Sigma-Aldrich) in each single experiment, which was set to 100%.

2.4. Flow cytometry

Cell staining and analysis were performed according to standard protocols. Surface expression of $S1P_1$-HA and $S1P_3$-HA on HTC_4 cells was analyzed by fluorescence-activated cell sorting (FACS) using the FACS Calibur (Becton Dickinson, Heidelberg, Germany). The N-terminal HA-epitope (peptide sequence: MGYPYDVPDYAGGP) was detected with the rat anti-HA antibody 3F10, kindly provided by Dr. Elisabeth Kremmer (GSF Munich, Germany) and goat anti-rat Cy2 conjugated secondary antibody (Chemicon International, Inc., Temecula, USA). Cell staining was performed with 12.5 µg/ml rat anti-HA and 5 µg/ml goat anti-rat-Cy2 in phosphate-buffered saline (PBS) at 4 °C for 60 min.

2.5. GTP-Eu binding assay

Europium-labelled GTP (GTP-Eu) in combination with time-resolved fluorometry (TRF) technology was used to monitor $S1P_3$ activation in HTC_4 cell membranes. $S1P_3$ expressing HTC_4 cells were detached from culture flasks with trypsin, pelleted by centrifugation at 400 ×g for 5 min at 4 °C, washed once with PBS, and centrifuged again. The pellet was suspended in ice-cold lysis buffer (10 mM Tris-HCl, 0.1 mM EDTA, 0.32 mM sucrose, pH 7.5) and homogenized on ice with a Dounce homogenizer (Thermo Fisher Scientific). The homogenate was centrifuged at 400 ×g for 15 min at 4 °C. The supernatant was collected and centrifuged at 41,000 ×g for 30 min at 4 °C. The resulting pellet was washed with sucrose-free lysis buffer and centrifuged again (41,000 ×g, 4 °C, 30 min). The pellet was resuspended in sucrose-free lysis buffer to a concentration of 3 mg protein/ml. Membrane protein concentrations were determined with the BCA Protein Assay Kit (Merck, Darmstadt, Germany). The preparation was aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C until use. GTP-binding was determined using the DELFIA GTP Binding Assay Kit (PerkinElmer Life Sciences, Turku, Finland). The assay was optimized according to the manufacturer's protocol. The reaction was started by adding an aliquot of 10 µg membrane suspension to reaction buffer containing 50 mM HEPES buffer, pH 7.5, containing with 20 mM NaCl, 10 mM $MgCl_2$, 10 µM GDP, 30 µg/ml saponin, 10 nM europium-labeled GTP, and different agonist concentrations, to a total volume of 100 µl. Experiments were performed in triplicates and samples were incubated in AcroWell filter plates for 60 min at 30 °C and 55 rpm. The reaction was terminated by vacuum filtration (MultiScreen Vacuum Manifold, Millipore, Billerica, USA), followed by four