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Enhanced Ca^{2+} storage in sphingosine-1-phosphate lyase-deficient fibroblasts

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ABSTRACT

Sphingosine-1-phosphate (S1P) regulates cell growth and survival, migration and adhesion in many cell types. S1P is generated by sphingosine kinases (SphKs), and dephosphorylated by phosphatases or cleaved by S1P lyase. Extracellular S1P activates specific G protein-coupled receptors while intracellular S1P can mobilize Ca^{2+} from thapsigargin-sensitive stores. Here, we have studied Ca^{2+} signalling in mouse embryonic fibroblasts (MEFs) deficient in S1P lyase. In these cells, S1P and sphingosine concentrations were elevated about 6-fold and 2-fold, respectively, as measured by liquid chromatography/tandem mass spectrometry. Measurements with fura-2-loaded cells in suspension revealed that resting $[Ca^{2+}]_i$ was elevated and agonist-induced $[Ca^{2+}]_i$ increases were augmented in S1P lyase-deficient MEFs both in the presence and absence of extracellular Ca^{2+}. Importantly, $[Ca^{2+}]_i$ increases and Ca^{2+} mobilization induced by the SERCA inhibitor, thapsigargin, were augmented, indicating enhanced Ca^{2+} storage in S1P lyase-deficient MEFs. Measurements with single cells expressing the calmodulin-based Ca^{2+} sensor, cameleon, revealed that at least two cell types could be distinguished in both MEF cell populations, one with a rapid and transient $[Ca^{2+}]_i$ increase and the other with a slower and prolonged $[Ca^{2+}]_i$ elevation upon stimulation with thapsigargin. The area under the time course of thapsigargin-induced $[Ca^{2+}]_i$ increases, reflecting overall Ca^{2+} release, was significantly increased by more than 50% in both rapidly and slowly responding S1P lyase-deficient cells. It is concluded that elevated concentrations of S1P and/or sphingosine lead to enhanced Ca^{2+} storage and elevated basal $[Ca^{2+}]_i$. S1P metabolism thus plays a role not only in acute Ca^{2+} mobilization but also in long-term regulation of Ca^{2+} homeostasis.

1. Introduction

The lipid mediator, sphingosine-1-phosphate (S1P), plays a role in cell proliferation, survival, migration and adhesion [1]. S1P is formed by sphingosine kinase (SphK)-catalysed phosphorylation of sphingosine, and degraded either by dephosphorylation or by irreversible cleavage catalysed by S1P lyase [2–4]. Initially, S1P was regarded as a second messenger, formed for example after stimulation with serum or platelet-derived growth factor, mediating cell growth and Ca^{2+} mobilization [5,6]. However, since that time it has become clear that important activities of extracellular S1P such as vasculogenesis, neurite outgrowth, regulation of vascular permeability, or lymphocyte trafficking, are mediated by specific G protein-coupled receptors, termed $S1P_{1-5}$ [4,7,8]. These receptors are nearly ubiquitously expressed and have emerged as important pharmacological targets [9]. Furthermore, S1P that is formed inside cells can be extruded and act on the G protein-coupled S1P receptors [10]. Nevertheless, there is also substantial evidence for intracellular target sites and activities of S1P. Thus, S1P receptors evolved with the vertebrates although enzymes of S1P metabolism such as SphKs and S1P lyase play important roles also in primitive organisms such as yeast, worms and flies [2]. Furthermore, the subcellular localization of SphKs underlies a complex regulation that involves translocation not only to the plasma membrane but also to intracellular vesicles as well as nuclear-cytoplasmic shuttling, supporting a role for local S1P specifically formed also at intracellular sites (reviewed in [11]). Very recently, one of the long-searched intracellular targets of S1P has been identified

Abbreviations: BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; ECFP, enhanced cyan fluorescent protein; FRET, fluorescence resonance energy transfer; HBSS, Hank's balanced salt solution; IP_3, inositol-1,4,5-trisphosphate; LPA, lysophosphatidic acid; MEFs, mouse embryonic fibroblasts; S1P, sphingosine-1-phosphate; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SKI-II, sphingosine kinase inhibitor compound II (2-[p-hydroxyanilino]-4-[p-chlorophenyl]thiazole); SphK, sphingosine kinase.

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[12]. It was reported that in agreement with the occurrence of SphK2 in the nucleus of many cell types, nuclear S1P directly binds to histone deacetylases and thereby regulates gene transcription [12]. While this important finding is a break-through in our understanding of the complex cellular activities of S1P, the mechanism by which intracellular S1P within seconds releases stored Ca^{2+} still remains unclear.

A role for S1P in Ca^{2+} mobilization has been based on the finding that it was able to release Ca^{2+} from permeabilized cells [13] and to mobilize Ca^{2+} from thapsigargin-sensitive stores when injected or generated by photolysis in intact cells [14,15]. Furthermore, Ca^{2+} mobilization induced by various agonists that also caused a rapid and transient increase in S1P formation was sensitive to SphK inhibitors, and thus S1P was regarded as Ca^{2+} mobilizing second messenger (reviewed in [16]). Since not only formation but also rapid clearance is crucial for a second messenger, we set out to analyse the role of S1P degrading enzymes in Ca^{2+} signalling. Interestingly, both the specific S1P phosphatases and S1P lyase are localized at the endoplasmic reticulum. The catalytic site of S1P phosphatases is predicted to be directed towards the lumen of the endoplasmic reticulum, while that of S1P lyase is facing the cytosol [17–19]. Thus, S1P lyase should be able to regulate cytosolic S1P pools in the close vicinity of the Ca^{2+} stores. Mammalian S1P lyase is a pyridoxal phosphate-dependent enzyme with a molecular weight of 63 kDa [20–22]. Its transmembrane domain close to its N-terminus attaches the enzyme to the endoplasmic reticulum [19,23]. While the lack of one of the two SphK isoenzymes in mice can largely be compensated [24–27], S1P lyase deficiency can not. Mice lacking S1P lyase have a reduced growth rate and usually die within 8 weeks [28]. They have blood vessel abnormalities such as microaneurysms, anaemia and lymphopenia, malformations of the skeleton, and a kidney dysfunction [28]. In the present study we have analysed the Ca^{2+} homoeostasis in embryonic fibroblasts (MEFs) from S1P lyase-deficient (*Sgpl1*$^{-/-}$) and control mice (*Sgpl1*$^{+/+}$). We show that in S1P lyase-deficient MEFs, in which S1P and sphingosine accumulate, basal $[Ca^{2+}]_i$ is elevated, agonist-induced $[Ca^{2+}]_i$ increases are augmented, and thapsigargin-sensitive Ca^{2+} store size is enhanced. Our data thus link S1P metabolism with long-term regulation of Ca^{2+} homoeostasis.

2. Materials and methods

2.1. Materials

Lysophosphatidic acid (LPA), bradykinin and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). Fura-2/AM was from Molecular Probes/Invitrogen (Invitrogen GmbH, Karlsruhe, Germany). Thapsigargin, ionomycin and sphingosine kinase inhibitor compound II (SKI-II; 2-[p-hydroxyanilino]-4-[p-chlorophenyl]thiazole) [29] were from Calbiochem/Merck Biosciences (Merck Biosciences GmbH, Schwalbach, Germany). S1P was obtained from Biomol (Biomol GmbH, Hamburg, Germany). Stock solutions of S1P were made in methanol, and aliquots thereof were dried down and dissolved in 1 mg/ml fatty acid-free BSA.

2.2. Cell culture

Embryonic fibroblasts were prepared from embryos of S1P lyase-deficient and corresponding wild type mice obtained by crossing *Sgpl1*$^{+/-}$ mice inbred in a C57Bl6/J genetic background. The heterozygotic *Sgpl1*$^{+/-}$ non-inbred mouse was generated from a gene trapped ES cell (OST 58278; OmniBank®, Texas; P. P. Van Veldhoven, Chem Phys Lipids 136 (2005) 164; P. P. Van Veldhoven, unpublished data). MEFs were cultured in Dulbecco's modified Eagles medium (DMEM/F12) supplemented with 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 10% fetal calf serum in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Before experiments, the cells were kept in serum-free medium overnight.

2.3. Measurements of cellular S1P and sphingosine concentrations

The cells were seeded onto 3.5 cm-dishes and grown to near confluence. They were kept in serum-free medium with vehicle or 10 μM SKI-II for 16 h before extraction of the lipids. Liquid chromatography/tandem mass spectrometry was performed as previously described [30] using D-*erythro*-C17-S1P and D-*erythro*-C17-sphingosine (Avanti Polar Lipids Inc., Alabaster, AL, USA) as internal standards.

2.4. $[Ca^{2+}]_i$ measurements in cell suspensions

Measurements of $[Ca^{2+}]_i$ with the ratioable dye, fura-2, were performed in a Hitachi F2500 spectrofluorometer as described before [31]. Briefly, monolayers of MEFs were detached with trypsin and loaded with 1 μM fura-2/AM in Hank's balanced salt solution (HBSS; 118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose and 15 mM HEPES pH 7.4) for 1 h at 37 °C. Thereafter, the cells were washed with HBSS and resuspended at a density of $\sim 1 \times 10^6$ cells/ml. Fluorescence measurements were performed at room temperature. Excitation was altered between 340 nm and 380 nm while emission was recorded at 510 nm. $[Ca^{2+}]_i$ was calculated after determination of maximum and minimum fluorescence according to Grynkiewicz et al. [32]. For determination of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, the cells were suspended in Ca^{2+} free HBSS, 50 μM EGTA was added shortly before stimulation with agonists, and 1 mM $CaCl_2$ was re-added before determination of maximum and minimum fluorescence.

2.5. $[Ca^{2+}]_i$ measurements in single cells

The cells were seeded onto polylysine-coated 8-well slides (ibidi GmbH, Martinsried, Germany) and transfected with Premo™ cameleon Ca^{2+} sensor (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's instructions. $[Ca^{2+}]_i$ measurements were performed one day after transfection using a Zeiss LSM510 Meta inverted confocal laser scanning microscope and a Plan-Neofluar 40x/1.30 oil immersion objective. The cells were kept in HBSS at room temperature. Fluorescence resonance energy transfer (FRET) between enhanced cyan fluorescent protein (ECFP) and the yellow fluorescent protein variant, venus, was measured using a 405 nm laser line for excitation and band pass filters 465–510 nm and 530–600 nm for emission, respectively. Photomultiplier sensitivity was always the same for both emission channels. Fluorescence time courses were calculated by averaging regions of interest covering the single cells. $[Ca^{2+}]_i$ was calculated as ratio between yellow (emission at 530–600 nm) and cyan fluorescence (emission at 465–510 nm).

2.6. Data analysis and presentation

Individual traces of $[Ca^{2+}]_i$ are representative for at least three similar experiments. Averaged data are means ± SEM from the indicated number (n) of traces or measurements. Statistical tests, regression analyses and area under the curve measurements were performed using the Prism program (GraphPad Software, San Diego, California, USA). Area under the curve measurements were performed by integrating the area between basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ at all time points after stimulation until return to baseline or up to a defined end point. The velocity of $[Ca^{2+}]_i$ increases in single MEFs transfected with the cameleon Ca^{2+} sensor was determined by fitting linear functions to the linear part of the $[Ca^{2+}]_i$ increases. The decay of the curves was defined as difference between maximum $[Ca^{2+}]_i$ increase and $[Ca^{2+}]_i$ increase at 2 min after stimulation.