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Lysophospholipid Receptor-Dependent and -Independent Calcium Signaling

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Abstract Changes in cellular Ca^{2+} concentrations form a ubiquitous signal regulating numerous processes such as fertilization, differentiation, proliferation, contraction, and secretion. The Ca^{2+} signal, highly organized in space and time, is generated by the cellular Ca^{2+} signaling toolkit. Lysophospholipids, such as sphingosine-1-phosphate (S1P), sphingosylphosphorylcholine (SPC), or lysophosphatidic acid (LPA) use this toolkit in a specific manner to initiate their cellular responses. Acting as agonists at G protein-coupled receptors, S1P, SPC, and LPA increase the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) by using the classical, phospholipase C (PLC)-dependent pathway as well as PLC-independent pathways such as sphingosine kinase (SphK)/S1P. The $S1P_1$ receptor, via protein kinase C, inhibits the $[Ca^{2+}]_i$ transients caused by other receptors. Both S1P and SPC also act intracellularly to regulate $[Ca^{2+}]_i$. Intracellular S1P mobilizes Ca^{2+} in intact cells independently of G protein-coupled S1P receptors, and Ca^{2+} signaling by many agonists requires SphK-mediated S1P production. As shown for the FcεRI receptor, PLC and SphK may contribute specific components to the overall $[Ca^{2+}]_i$ transient. Of the many open questions, identification of the intracellular S1P target site(s) appears to be of particular importance. J. Cell. Biochem. 92: 937–948, 2004.

Key words: calcium; phospholipase C; sphingosine kinase; sphingosine-1-phosphate; sphingosylphosphorylcholine; lysophosphatidic acid; G protein-coupled receptors

Ca^{2+} is an ubiquitous signal regulating cellular functions from fertilization to cell death (for review see [Berridge et al., 2000]). A comprehensive collection of proteins sense local changes in cellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) and transduce these signals into specific responses. Besides the classical Ca^{2+} sensitive processes, for example, contraction of muscle cells, secretion of hormones or neurotransmitters, and regulation of metabolic functions, $[Ca^{2+}]_i$ elevations also modulate other signaling pathways. The differential signaling by free Ca^{2+} ions is achieved by encoding the Ca^{2+} signal in a spatio-temporal manner. For this, the cells use a toolkit of Ca^{2+} channels, mechanisms for $[Ca^{2+}]_i$ increase and mechanisms for shut down [Berridge et al., 2000]. Lysophospholipids such as sphingosine-1-phosphate (S1P), sphingosylphosphorylcholine (SPC), or lysophosphatidic acid (LPA) use this toolkit in a specific manner to initiate their respective cellular responses. S1P in particular appears to be an evolutionary conserved Ca^{2+} signaling molecule in yeast, plant, and mammals. S1P is formed from sphingosine by sphingosine kinase (SphK) and degraded by S1P lyase or phosphatases, all of which are evolutionary conserved enzymes (for review see [Spiegel and Milstien, 2003]).

In yeast, addition of sphingosine to cells that lack S1P lyase induces a Ca^{2+} influx that is not observed in SphK-deficient cells and thus caused by accumulation of S1P [Birchwood et al., 2001]. The Ca^{2+} channel that mediates the influx, Cch1, is stimulated upon depletion of Ca^{2+} from secretory organelles in yeast, and its function can thus be compared to mammalian store-operated channels. Interestingly, the rise in $[Ca^{2+}]_i$ had a lag phase of ~1 h and required the synthesis of a protein [Birchwood et al., 2001]. Therefore, the primary action of S1P in yeast might be to induce the transcription of a

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protein crucial for Ca^{2+} influx, but whether this protein is then regulated by S1P remains an open question.

In plants, Ca^{2+} signaling by S1P appears to be more complex. In guard cells of *Commelina communis*, exogenous S1P (6–50 μM) induced oscillations in $[Ca^{2+}]_i$ [Ng et al., 2001]. Furthermore, in the leaves of *Commelina Communis* [Ng et al., 2001] and *Arabidopsis thaliana* [Coursol et al., 2003], exogenous S1P elicited a closure of stomatal pores that was dependent on extracellular Ca^{2+} [Ng et al., 2001]. While $[Ca^{2+}]_i$ oscillations in response to S1P were immediate, the stomatal closure appeared with a time lag that was strongly dependent on S1P concentration and amounted to 120 min at 6 μM S1P and 4 min at 50 μM S1P [Ng et al., 2001]. Both effects required relatively high concentrations of S1P, and dihydro-S1P was inactive, suggesting an intracellular site of action [Ng et al., 2001]. In agreement with a putative intracellular second messenger role of S1P in plants, stomatal closure stimulated by the plant hormone, abscisic acid, was dependent on SphK activation and S1P production [Ng et al., 2001; Coursol et al., 2003]. Furthermore, abscisic acid stimulated S1P production with a rapid time course, very similar to that observed in mammals after stimulation with diverse agonists [Coursol et al., 2003]. On the other hand, the heterotrimeric G protein of Arabidopsis, GPA1, was required for extracellular S1P to induce a stomatal closure or inhibit the opening of closed stomata [Coursol et al., 2003]. How GPA1 is activated by S1P remains an open question presently. Interestingly, abscisic acid still induced stomatal closure in GPA1-deficient mutants, although this effect was attenuated by SphK inhibition [Coursol et al., 2003], suggesting that either the SphK inhibitors or S1P had a second mechanism of action. Taken together, S1P signaling in plants appears to be complex and might have similarities to S1P signaling in animals.

In mammals, S1P, LPA, and SPC activate specific G protein-coupled receptors, as described in greater detail elsewhere in this volume (for review see also [Fukushima et al., 2001; Pyne and Pyne, 2002; Spiegel and Milstien, 2003]). The presently best characterized lysophospholipid receptors are those of the EDG family, of which $S1P_{1-5}$ are activated by S1P and LPA_{1-3} by LPA [Fukushima et al., 2001]. Besides activating many other signaling pathways, these receptors regulate the cellular Ca^{2+} homeostasis [Fukushima et al., 2001; Kluk and Hla, 2002; Siehler and Manning, 2002]. The specific features of lysophospholipid receptor-mediated Ca^{2+} signaling will be discussed below. Interestingly, both S1P and SPC also act intracellularly to regulate $[Ca^{2+}]_i$. Furthermore, there is compelling evidence that SphK and S1P form a second messenger pathway used by many receptors for Ca^{2+} mobilization, although the target site by which S1P mediates this action has not yet been identified. The current data and open questions related to S1P's second messenger role will be in the focus of the present review.

CALCIUM SIGNALING BY G PROTEIN-COUPLED LYSOPHOSPHOLIPID RECEPTORS

Extracellular S1P, SPC, and LPA induce $[Ca^{2+}]_i$ increases in many cell types, for example, in endothelial cells, smooth muscle cells, fibroblasts, or diverse tumor cell lines (for review see [Tigyi, 2001; Spiegel and Milstien, 2003]). The identification of the individual lysophospholipid receptors that mediate the response in a certain cell type has been hampered by the fact that most cells express more than one S1P or LPA receptor, and that receptor subtype-specific pharmacological tools are largely missing. Therefore, most conclusions about Ca^{2+} signaling by individual lysophospholipid receptors have been drawn from overexpression studies, in which however a high receptor number can cause artificial coupling to G proteins and downstream signaling. Furthermore, the research has so far mainly focused on Ca^{2+} mobilization (Fig. 1), while little is known about the regulation of plasma membrane Ca^{2+} channels by lysophospholipid receptors.

Of the recombinant LPA receptors, particularly LPA_2 and LPA_3 have been shown to mediate strong increases in $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores [An et al., 1998; Bandoh et al., 1999; Im et al., 2000]. $[Ca^{2+}]_i$ increases by human LPA_2 and LPA_3 receptors expressed in zero-background cells such as HTC4, Sf9, or RH7777 cells were mediated by pertussis toxin (PTX)-insensitive G proteins and phospholipase C (PLC) [An et al., 1998; Bandoh et al., 1999; Im et al., 2000]. In comparison, human LPA_1 mediated relatively

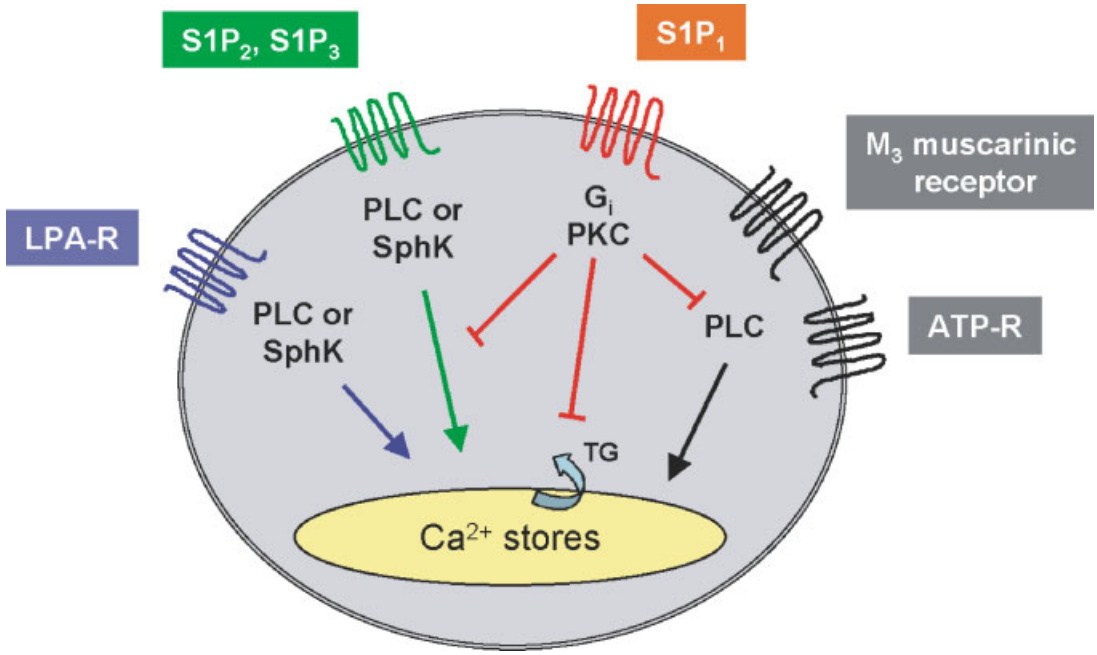

Fig. 1. Ca^{2+} signaling by G protein-coupled lysophospholipid receptors. LPA and S1P receptors activate PLC via PTX-sensitive or -insensitive G proteins and thereby stimulate $[Ca^{2+}]_i$ increases in diverse expression systems. However, they can also induce $[Ca^{2+}]_i$ increases in the absence of a measurable PLC activation or IP_3 production. $[Ca^{2+}]_i$ transients induced by S1P receptors endogenously expressed in HEK-293 cells, and by the LPA_2 receptor endogenously expressed in SH-SY5Y cells, were mediated by the SphK pathway and not by PLC. The $S1P_1$ receptor appears to be unique so far because it inhibited $[Ca^{2+}]_i$ increases caused by other receptors or thapsigargin (TG) in a number of cell types without inducing $[Ca^{2+}]_i$ increases by itself. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

small $[Ca^{2+}]_i$ elevations when expressed in TAg-Jurkat, HTC4, or RH7777 cells [An et al., 1998; Im et al., 2000; Yokoyama et al., 2002]. $[Ca^{2+}]_i$ increases by the LPA_1 receptor were caused by PLC-mediated Ca^{2+} mobilization that was fully sensitive to PTX and thus mediated by G_i proteins [An et al., 1998]. Of the mouse LPA receptors, LPA_1, LPA_2, and LPA_3 activated PLC to roughly the same extent when expressed in B103 cells [Ishii et al., 2000]. Considering this and the effective Ca^{2+} signaling of the endogenously expressed LPA_1 receptor in diverse mouse cells (see below), it might be possible that the mouse LPA_1 receptor is more efficiently coupled to the PLC/Ca^{2+} mobilization pathway than the human receptor.

Relatively little is known about receptor subtype-specific Ca^{2+} signaling of endogenously expressed LPA receptors. In mouse embryonic fibroblasts, it has been shown that both LPA_1 and LPA_2 contribute to the $[Ca^{2+}]_i$ transient stimulated by LPA [Contos et al., 2002]. In fibroblasts from mice lacking either the LPA_1 or LPA_2 receptor, LPA-induced $[Ca^{2+}]_i$ increase as well as inositol phosphate production were reduced, while depletion of both LPA_1 and LPA_2 fully abrogated $[Ca^{2+}]_i$ increase and inositol phosphate production stimulated by LPA [Contos et al., 2002]. Another study in microglial cells revealed that LPA_1 was the predominant LPA receptor in mouse microglial cells, mediating PTX-sensitive $[Ca^{2+}]_i$ increases that were caused by Ca^{2+} mobilization from thapsigargin-sensitive stores [Möller et al., 2001]. In contrast, LPA_3 was the predominant LPA receptor in rat microglial cells, inducing Ca^{2+} increases that were not affected by thapsigargin pretreatment but blocked by removal of extracellular Ca^{2+} and thus due to a Ca^{2+} influx. This Ca^{2+} influx was activated in a PTX-insensitive manner, however, the identity of the involved channel is not known [Möller et al., 2001].

Of the G protein-coupled S1P receptors, $S1P_1$, $S1P_2$, and $S1P_3$ are widely expressed and often found co-expressed in a single cell type [Fukushima et al., 2001; Pyne and Pyne, 2002]. Studies comparing the effectiveness by which these receptors couple to $[Ca^{2+}]_i$ increase revealed that particularly $S1P_3$ appears to

mediate strong increases in $[Ca^{2+}]_i$, followed by $S1P_2$ [An et al., 1999; Kon et al., 1999; Okamoto et al., 1999]. $[Ca^{2+}]_i$ increases induced by $S1P_3$ or $S1P_2$ expressed in HTC4 or CHO cells were due to a mobilization of Ca^{2+} from thapsigargin-sensitive stores that was accompanied by activation of PLC and inhibited by the PLC inhibitor, U73122, and thus mediated by PLC [An et al., 1999; Kon et al., 1999; Okamoto et al., 1999; Sato et al., 1999]. PLC activation as well as $[Ca^{2+}]_i$ increases induced by $S1P_3$ and $S1P_2$ were more or less insensitive to PTX and thus probably mediated predominantly by G_q and to a lesser extent by G_i, to which both receptors have been shown to couple [An et al., 1999; Okamoto et al., 1999; Sato et al., 1999]. Furthermore, the $S1P_2$-like receptor from zebrafish mediated $[Ca^{2+}]_i$ increases in response to S1P, while the mutants that affected cardiac development were deficient in Ca^{2+} signaling when expressed in Jurkat T-cells [Kupperman et al., 2000]. In mouse embryonic fibroblasts that endogenously express $S1P_{1-3}$ but not $S1P_4$ or $S1P_5$, PLC activation and $[Ca^{2+}]_i$ increases induced by S1P were nearly completely due to the activity of $S1P_3$, while deletion of the $S1P_2$ receptor had no major influence on these parameters [Ishii et al., 2001, 2002]. In $S1P_2$/$S1P_3$ double knock-out cells, S1P still induced a very small PLC activation but no significant increases in $[Ca^{2+}]_i$, indicating that the remaining $S1P_1$ receptor did not couple to $[Ca^{2+}]_i$ increase in these cells [Ishii et al., 2001, 2002].

Although S1P induced small $[Ca^{2+}]_i$ increases in HEL or CHO cells expressing $S1P_1$ [Okamoto et al., 1998; Kon et al., 1999], there are several reports demonstrating that $S1P_1$ did not couple to increases in $[Ca^{2+}]_i$ in diverse cell types (see discussion in [Siehler and Manning, 2002]). For example, S1P did not induce $[Ca^{2+}]_i$ increases in HEK-293, COS-7, or RH7777 cells expressing $S1P_1$ [Van Brocklyn et al., 1998; Zondag et al., 1998; Meyer zu Heringdorf et al., 2003b]. Furthermore, artifical coupling of $S1P_1$ to G_q was necessary to link the otherwise inactive receptor to $[Ca^{2+}]_i$ increase in TAg-Jurkat cells or *Xenopus* oocytes [An et al., 1999; Ancellin and Hla, 1999]. Recently, it was shown that the $S1P_1$ receptor on the contrary inhibited $[Ca^{2+}]_i$ increases induced by other receptors in HEK-293, CHO, and RH7777 cells [Meyer zu Heringdorf et al., 2003b]. In HEK-293 cells, endogenously expressing $S1P_{1-3}$ (high background), the overexpressed $S1P_1$ receptor was constitutively active, stimulating extracellular signal-regulated kinases (ERK), and attenuating $[Ca^{2+}]_i$ increases and PLC activation by the muscarinic M_3 receptor as well as $[Ca^{2+}]_i$ increases induced by LPA receptors or the SERCA ATPase inhibitor, thapsigargin. In RH7777 cells lacking endogenous S1P receptors (zero background), activation of the recombinant $S1P_1$ receptor attenuated $[Ca^{2+}]_i$ increases induced by the purinergic agonist, ATP. This activity of $S1P_1$ was mediated by a PTX-sensitive G protein and protein kinase C [Meyer zu Heringdorf et al., 2003b]. Protein kinase C is well known to negatively regulate Ca^{2+} signaling by targeting receptors, G proteins, PLC isoforms, or Ca^{2+} channels (see discussion in [Meyer zu Heringdorf et al., 2003b]). This shutdown mechanism is often activated by receptors that are coupled to PLC, with the PLC products, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol, mediating Ca^{2+} mobilization and PKC activation, respectively, the latter leading to negative feedback and heterologous desensitization. Interestingly, the $S1P_1$ receptor did not couple to PLC and $[Ca^{2+}]_i$ increase by itself, but nevertheless used the protein kinase C pathway for desensitizing Ca^{2+} signaling by other receptors in diverse cell types [Meyer zu Heringdorf et al., 2003b].

It is now required to show the inhibitory Ca^{2+} signaling also for the endogenous $S1P_1$ receptor. Since the constitutive activity of endogenously expressed $S1P_1$ might be much lower than that of the overexpressed receptor, it is not clear whether a deletion of $S1P_1$ will lead to an enhanced Ca^{2+} signaling. Therefore, cells expressing $S1P_1$ as the only or the predominant S1P receptor will be a better model system. Inhibitory Ca^{2+} signaling by the endogenous $S1P_1$ receptor will implicate that cells can regulate their responsiveness to $[Ca^{2+}]_i$ increasing receptors by upregulating their $S1P_1$ receptor expression. Interestingly, not only Ca^{2+} signaling, but also Rac/cell migration and ERK are regulated by different S1P receptors in an opposite manner (for review see [Kluk and Hla, 2002]), and, for example, cell migration of vascular smooth muscle cells in response to S1P is stimulated or inhibited depending on the S1P receptor expression profile [Kluk and Hla, 2001].

Of the more specifically expressed S1P receptors, $S1P_4$, which is found in lymphoid tissues and couples to $G\alpha_i$ and $G\alpha_{12/13}$ proteins, but not

to $G\alpha_q$ or $G\alpha_{15/16}$, stimulates PLC and $[Ca^{2+}]_i$ increases via PTX-sensitive G proteins when expressed in CHO or K562 cells [Yamazaki et al., 2000; Gräler et al., 2003]. The $S1P_5$ receptor, specifically expressed in brain and skin and coupled to $G\alpha_i$ and $G\alpha_{12}$ proteins, has not been reported to cause $[Ca^{2+}]_i$ increases and did not stimulate PLC when expressed in CHO cells [Malek et al., 2001; Kluk and Hla, 2002].

The PLC isoforms activated by lysophospholipid receptors have not yet been comprehensively studied. However, inositol phosphate production stimulated by LPA or S1P was sometimes sensitive to PTX, sometimes not, and thus probably due to the activity of PLCβ1 (activated by $G\alpha_q$) as well as PLCβ2 (activated by βγ subunits of G_i proteins). Recently, it has been demonstrated that LPA and S1P receptors can also activate PLCε [Kelley et al., 2003]. Of diverse PLC isoforms overexpressed in COS-7 cells, endogenous LPA and S1P receptors activated PLCε to a greater extent than PLCβ1 or PLCβ2 [Kelley et al., 2003]. Stimulation of PLCε by LPA and S1P was partially dependent on the Ras-associated domain of the enzyme, mimicked by known effectors of the lysophospholipid receptors such as $G\alpha_{12}$, $G\alpha_{13}$, RhoA, and Rac and other small GTPases, and largely inhibited by a scavenger of $G_{12/13}$ proteins, the RGS domain of p115RhoGEF [Kelley et al., 2003]. Thus, PLCε activation by LPA and S1P receptors apparently involves $G\alpha_{12/13}$ and some not yet identified small GTPases. PLCε might therefore account for part of the PTX-insensitive inositol phosphate production initiated by the lysophospholipid receptors, however, the involved receptor subtypes, the precise signaling pathway(s) and the cells that use this pathway endogenously still have to be determined.

Interestingly, Ca^{2+} mobilization by G protein-coupled lysophospholipid receptors is not always dependent on PLC. For example, LPA generated $[Ca^{2+}]_i$ transients in SH-SY5Y neuroblastoma cells without inducing a measurable IP_3 production [Young et al., 1999, 2000]. The same was true for G protein-coupled S1P receptors in bovine aortic endothelial cells, thyroid FRTL-5 cells, or HEK-293 cells [Meyer zu Heringdorf et al., 1996, 2001; Törnquist et al., 1997]. Furthermore, the $S1P_2$ receptor, endogenously expressed in CHO cells, mediated $[Ca^{2+}]_i$ increases but no PLC activation in response to S1P [Sato et al., 1999]. Similarly, increases in $[Ca^{2+}]_i$ induced by SPC in HL-60 cells, that were mediated by a so far not identified G protein-coupled SPC receptor (S1P was inactive in these cells), were not accompanied by an activation of PLC [van Koppen et al., 1996]. The LPA_2 receptor, endogenously expressed in SH-SY5Y cells, has been shown to stimulate intracellular S1P production in a rapid and transient manner, and $[Ca^{2+}]_i$ increases elicited by this receptor were dependent on SphK activation [Young et al., 2000]. The same was true for S1P receptors, probably $S1P_3$ and $S1P_2$, endogenously expressed in HEK-293 cells [Meyer zu Heringdorf et al., 2001]. The SphK/S1P Ca^{2+} mobilization pathway can obviously be addressed by both PTX-sensitive (S1P receptors) as well as -insensitive (LPA_2 receptor) G proteins [Young et al., 1999; Meyer zu Heringdorf et al., 2001]. Interestingly, recombinant LPA_2 as well as $S1P_2$ and $S1P_3$ receptors have been shown to cause a clear stimulation of PLC (see above). Therefore, the conditions in which a specific lysophospholipid receptor uses a certain Ca^{2+} mobilization pathway have to be studied in future work. Particularly, the cellular background in terms of availability and spatial organization of signal transduction components, and the co-expressed lysophospholipid receptors might play a role.

Besides the abovementioned signaling pathways, there are other possibilities for lysophospholipid receptors to regulate Ca^{2+} signaling. For example, voltage-gated Ca^{2+} channels can be regulated both positively and negatively by G protein-coupled receptors, for example, via G_s/cyclic AMP/protein kinase A, via protein kinase C, or via βγ subunits of G_i proteins [Catterall, 2000], all of which are downstream effectors of lysophospholipid receptors. Preliminary data suggest that for example, SPC and glucopsychosine receptors can inhibit voltage-gated Ca^{2+} channels (reviewed in [Meyer zu Heringdorf et al., 2002]), however, more work has to be done in this area.

CALCIUM SIGNALING BY INTRACELLULAR S1P AND SPC

The intracellular Ca^{2+} mobilizing activities of S1P and SPC have been first described by Ghosh and co-workers [Ghosh et al., 1990, 1994]. These authors observed that sphingosine, S1P and SPC released stored $^{45}Ca^{2+}$ from permeabilized DDT_1MF-2 smooth muscle cells

and endoplasmic reticulum (ER) microsomes of these cells. The activity of sphingosine was dependent on ATP and temperature, and sphingosine was converted by the microsomes to S1P, leading to the conclusion that S1P and not sphingosine was the active molecule and that the required SphK was located at the ER. The activity of SPC, on the other hand, was rapid and could be observed at 4°C, suggesting that SPC had a direct action on a Ca^{2+} channel. SPC released more than 80% of stored Ca^{2+} with an EC_{50} of ~3 μM, and its action was not inhibited by heparin, indicating that it did not involve the IP_3 binding site of the IP_3-gated Ca^{2+} channel [Ghosh et al., 1990, 1994].

Later, it was found that SPC was active in many preparations of permeabilized cells. SPC released Ca^{2+} from permeabilized pancreatic acinar cells, thyroid FRTL-5 cells, rat basophilic leukemia cells, human endothelial EA.hy926 cells, HEK-293 cells, and SH-SY5Y cells (for review see [Meyer zu Heringdorf et al., 2002]). Ca^{2+} release by intracellular SPC was not stereospecific, however, it was not due to a non-specific membrane perturbation because the SPC concentrations required for this activity (EC_{50} ~3–6 μM) were below those that damage intracellular membrane integrity. Furthermore, Ca^{2+} release by intracellular SPC was not inhibited by heparin, and not mediated by ryanodine receptors. However, SPC can also modulate the activity of ryanodine receptors, probably by sensitizing them for Ca^{2+}-induced Ca^{2+} release (reviewed in [Meyer zu Heringdorf et al., 2002]). Nevertheless, Ca^{2+} release by SPC could also be observed in cells that do not express ryanodine receptors, such as HEK-293 cells, and thus another mechanism must also be involved. Finally, in permeabilized rat basophilic leukemia cells and microsomes thereof, a Ca^{2+} channel gated by SPC was characterized that had a conductance of 160 pS with Ba^{2+} as charge carrier [Kindman et al., 1994].

S1P, on the other hand, did not release Ca^{2+} in most of the abovementioned preparations in which SPC was active. Only about 18% of stored Ca^{2+} was released by 50 μM S1P in SH-SY5Y cells, one of the few cells in which this activity could be observed, while SPC released 34% [Young et al., 2000]. It is not clear why the early work of Ghosh et al. on the intracellular Ca^{2+} releasing activity of S1P could not be reproduced later. Instead, it had to be proven that intracellular S1P can indeed mobilize Ca^{2+} independently of G protein-coupled S1P receptors, which are widely expressed and mediate $[Ca^{2+}]_i$ increases in many cell types. This work was done in HEK-293 cells, in which $[Ca^{2+}]_i$ increases by G protein-coupled S1P receptors are fully blocked by PTX, and in cells such as SKNMC or HepG2 cells, that physiologically do not respond to extracellular S1P with $[Ca^{2+}]_i$ increases [Meyer zu Heringdorf et al., 1998, 2003a]. For inducing a Ca^{2+} response, intracellular S1P had to be applied in a rapid manner imitating the formation of a Ca^{2+} mobilizing second messenger. Microinjection of S1P into PTX-treated HEK-293 cells caused rapid and transient increases in $[Ca^{2+}]_i$, while extracellular S1P was inactive [Meyer zu Heringdorf et al., 1998]. However, the microinjection experiments required relatively high concentrations of S1P in the injection solution that may disturb the integrity of cellular membranes. Therefore, in a recent study, S1P was generated intracellularly by photolysis of caged S1P [Meyer zu Heringdorf et al., 2003a]. Release of S1P by photolysis caused $[Ca^{2+}]_i$ increases in PTX-treated HEK-293 cells that were due to an intracellular action of S1P for the following reasons: (1) extracellular S1P up to 10 μM did not induce $[Ca^{2+}]_i$ increases while photolysis of 10–40 μM caged S1P elicited strong elevations of $[Ca^{2+}]_i$ in the PTX-treated cells; (2) the caged S1P had to be loaded into the cells for a couple of minutes to generate the intracellular response, while G protein-coupled S1P receptors responded to photolysis immediately after addition of the caged compound; and (3) photolysis of caged S1P elicited $[Ca^{2+}]_i$ increases after washing of the loaded cells. Furthermore, S1P generation by photolysis elicited $[Ca^{2+}]_i$ increases in SKNMC and HepG2 cells that were non-responsive to extracellular S1P. Finally, the intracellular action of S1P was not caused by a non-specific membrane perturbation because it was observed upon photolysis of concentrations of caged S1P as low as 10 μM. Therefore, in addition to acting via G protein-coupled receptors, S1P also acts intracellularly to increase $[Ca^{2+}]_i$ [Meyer zu Heringdorf et al., 2003a], and this activity can be observed in intact cells but is hardly detectable in permeabilized cells.

$[Ca^{2+}]_i$ increases by microinjected S1P were comparable in magnitude to those induced by IP_3 and, like those by IP_3, due to a mobilization of Ca^{2+} from intracellular stores that was followed by a Ca^{2+} influx. Furthermore, $[Ca^{2+}]_i$

increases by microinjected S1P were not prevented by co-injection of heparin that blocked the action of IP_3 [Meyer zu Heringdorf et al., 1998]. $[Ca^{2+}]_i$ increases by photolysis of caged S1P were rapid and transient in PTX-treated HEK-293 and HepG2 cells, while they were rapid and prolonged in SKNMC cells. Again, the $[Ca^{2+}]_i$ increases by photolysis of caged S1P were caused by a mobilization of Ca^{2+} from the stores in HepG2 and PTX-treated HEK-293 cells; in the latter cells, it was shown that these Ca^{2+} stores were sensitive to thapsigargin [Meyer zu Heringdorf et al., 2003a]. Therefore, at least in HEK-293 cells, intracellular S1P caused a Ca^{2+} mobilization from the stores that are also addressed by IP_3.

Interestingly, intracellular S1P not only induces a Ca^{2+} mobilization, as outlined above, but also can stimulate a Ca^{2+} influx. This has been shown so far in neutrophils and HL-60 cells [Itagaki and Hauser, 2003]. Addition of exogenous S1P to the cells stimulated a Ca^{2+} influx that displayed characteristics of store-operated Ca^{2+} influx. Furthermore, S1P enhanced store-operated Ca^{2+} influx activated by formyl peptide, but did not cause a Ca^{2+} mobilization or store depletion by itself. S1P-induced Ca^{2+} entry was not sensitive to PTX and not dependent on PLC, and considering the concentrations that were required (5–10 μM), most likely due to an intracellular action of S1P [Itagaki and Hauser, 2003]. There is also evidence that agonists such as platelet-activating factor (PAF) activate store-operated Ca^{2+} influx in neutrophils via S1P production, because inhibition of SphK by *N*,*N*-dimethylsphingosine (DMS) blocked Ca^{2+} entry but not Ca^{2+} mobilization stimulated by PAF. For this, DMS had to be applied before PAF addition, while DMS after PAF addition, and probably after PAF-stimulated S1P production, had no effect, indicating that it did not directly inhibit the involved Ca^{2+} channel [Itagaki and Hauser, 2003].

At present, there are many open questions with regard to Ca^{2+} signaling by intracellular S1P and SPC (see Fig. 2). Firstly, do S1P and SPC act on the same target sites? Results from the work on G protein-coupled lysophospholipid receptors suggest that this might be possible since SPC appears to be a low-affinity agonist at the S1P receptors, $S1P_{1-4}$ [Meyer zu Heringdorf

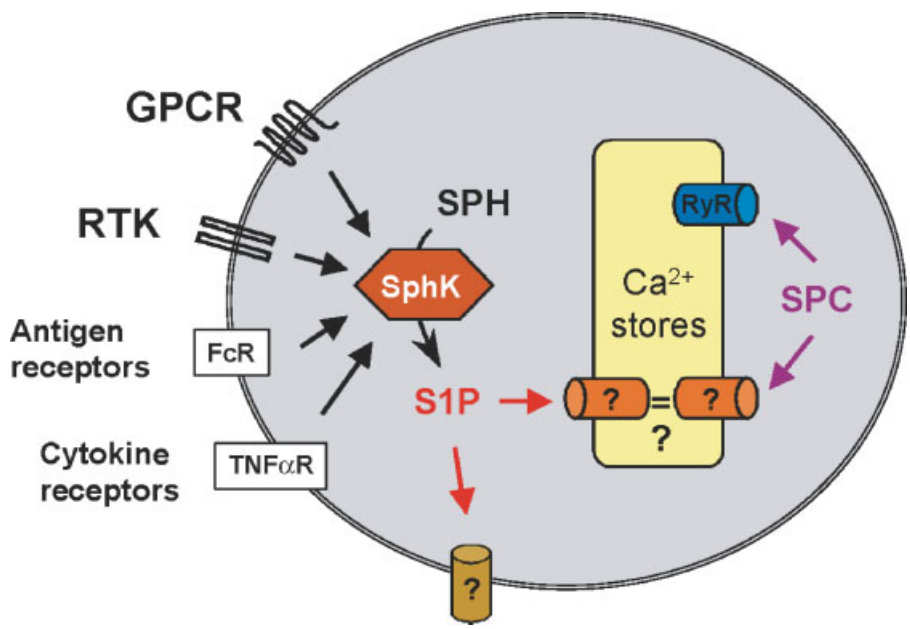

Fig. 2. Regulation of Ca^{2+} signaling by intracellular S1P and SPC. S1P, that is formed from sphingosine (SPH) by SphK after stimulation of G protein-coupled receptors (GPCR), receptor tyrosine kinases (RTK), antigen, and cytokine receptors (see Table I), mobilizes Ca^{2+} from intracellular stores by a so far unknown mechanism that might involve a novel Ca^{2+} channel. In neutrophils, intracellular S1P activates a Ca^{2+} influx. While SphK and S1P form a second messenger pathway, intracellular SPC seems not to be receptor-regulated. SPC releases Ca^{2+} by acting on ryanodine receptors (RyR) but also by acting independently of ryanodine receptors. Whether S1P and SPC address the same intracellular target sites remains an open question. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 2002], and both lipids act on the GPR12 receptor with SPC being more potent than S1P [Ignatov et al., 2003]. However, given the possibility that S1P and SPC address the same intracellular target site, why is SPC active in permeabilized cells and microsomes, while S1P apparently requires an intact cellular milieu? Furthermore, while intracellular S1P is formed rapidly and transiently after activation of diverse membrane receptors and has the features of a Ca^{2+} mobilizing second messenger (see below), the physiological significance of Ca^{2+} mobilization by SPC is unclear. A regulated SPC production that is used for intracellular signal transduction has yet to be identified.

Secondly, in which conditions does S1P induce a Ca^{2+} influx and in which a Ca^{2+} mobilization? Using the SphK inhibitors, DMS and DL-*threo*-dihydrosphingosine (tDHS), both activities have been shown in neutrophils and the related HL-60 granulocytes. Thus, while DMS blocked Ca^{2+} influx but had no major influence on Ca^{2+} mobilization triggered by PAF in human neutrophils [Itagaki and Hauser, 2003], DMS and tDHS nearly completely blocked Ca^{2+} mobilization triggered by formyl peptide in HL-60 granulocytes [Alemany et al., 1999]. It might be speculated that these specific cellular responses are dependent on the respective receptor and its signal transduction pathway, and thus the subcellular localization of S1P formation.

Finally, what are the target sites of S1P for inducing Ca^{2+} influx and Ca^{2+} mobilization? Candidates for a Ca^{2+} influx channel targeted by S1P are among the large family of transient receptor potential (TRP) channels, named after the *Drosophila* TRP protein. Some of these channels are regulated by lipids, for example, by diacylglycerol which is formed in parallel to IP_3 by PLC, the two messengers activating Ca^{2+} influx and mobilization, respectively [Berridge et al., 2000]. Much less is known about the Ca^{2+} channels on intracellular Ca^{2+} storage organelles. While IP_3 and ryanodine receptors are located at the ER, a receptor for nicotinic acid adenine dinucleotide phosphate (NAADP) was found on a lysosomal Ca^{2+} storage compartment that was insensitive to thapsigargin (see [Lee, 2003]). Ca^{2+} mobilization by intracellular S1P appears to involve thapsigargin-sensitive stores and is likely to be independent of ryanodine receptors since it was observed in cells that do not express these Ca^{2+} channels. The involvement of IP_3-gated Ca^{2+} channels in S1P's action has not been ruled out so far, because the insensitivity to heparin simply indicates that S1P does not act on the IP_3 binding site, which does not exclude an allosteric action on this big channel protein. On the other hand, it is likely that there are more channels besides ryanodine and IP_3 receptors that regulate Ca^{2+} fluxes at the ER. Still unclear is the role of SCaMPER that was cloned as a SPC-responsive Ca^{2+} channel from MDCK cells [Mao et al., 1996]. Later, the sequence of SCaMPER was revised, revealing that it consists of 110 amino acids and contains one putative transmembrane domain [Schnurbus et al., 2002]. While SCaMPER was not localized at ER membranes and did not confer responsiveness to SPC in one study [Schnurbus et al., 2002], it was found to be co-localized with the dihydropyridine receptor in cardiac myocytes, mediating $[Ca^{2+}]_i$ transients in response to extracellularly added SPC in another study [Cavalli et al., 2003]. However, HEK-293 cells, in which intracellular S1P effectively mobilized Ca^{2+}, do not express SCaMPER as measured by RT-PCR [Meyer zu Heringdorf et al., 2003a]. Taken together, it remains an intriguing possibility that S1P activates an as yet unknown intracellular Ca^{2+} channel.

CALCIUM MOBILIZATION BY THE SphK PATHWAY

Ca^{2+} mobilization by plasma membrane receptors is most often mediated by the ubiquitous PLC/IP_3 pathway. Although there are some molecules besides IP_3 that mediate intracellular Ca^{2+} release, only cyclic ADP ribose and NAADP are regarded to play a role in receptor-regulated Ca^{2+} mobilization and thus to act as second messengers [Berridge et al., 2000]. However, given that intracellular S1P can increase $[Ca^{2+}]_i$ (see above), there is further evidence that SphK/S1P forms a Ca^{2+} signaling pathway. Thus, the activity of SphK and the production of S1P are regulated by extracellular stimuli targeting plasma membrane receptors. In Table I, the receptors and agonists are listed that stimulate S1P production in a rapid and transient manner, which is required for Ca^{2+} signaling. S1P levels typically increase to only 1.5–2-fold of basal levels, however, it might be that S1P reaches higher concentrations in localized cellular compartments. Inhibition

TABLE I. Examples for Agonists That Rapidly Stimulate SphK Activity and/or S1P Production

Agonists	Role in Ca^{2+} signaling	References
Agonists at G protein-coupled receptors		
Carbachol	+	Meyer zu Heringdorf et al. [1998]
Formyl peptide	+	Alemany et al. [1999]
ATP, UTP	+	Alemany et al. [2000]
LPA	+	Young et al. [2000]
S1P	+	Meyer zu Heringdorf et al. [2001]
Bradykinin	?	Alemany et al. [2001]; Blaukat and Dikic [2001]
G protein activator		
AlF_4^-	?	Alemany et al. [1999]
Receptor tyrosine kinases		
Platelet-derived growth factor	?	Olivera and Spiegel [1993]; Olivera et al. [1999]
Epidermal growth factor	+	Meyer zu Heringdorf et al. [1999]
Antigen receptors—Crosslinking		Choi et al. [1996]; Melendez et al. [1998];
FcεRI	+ SphK1	Melendez and Khaw [2002]
FcγRI	+	
Cytokine receptors		
Tumor necrosis factor-α	?	Xia et al. [1998, 1999]
$[Ca^{2+}]_i$ increasing agents		
KCl depolarization	+ (Signal amplification)	Alemany et al. [2001]
Ca^{2+} ionophores	?	Alemany et al. [2000]
Thapsigargin	?	Olivera et al. [1999]

The role of the SphK pathway in Ca^{2+} signaling by the diverse agonists was most often studied with the SphK inhibitors, tDHS and DMS. A role for SphK1 in augmentation of depolarization-induced Ca^{2+} increases was revealed by overexpression of SphK1 [Alemany et al., 2001]. The precise role of SphK1 besides PLCγ1 in generation of the overall Ca^{2+} transient after stimulation of the FcεRI receptor was demonstrated in an outstanding study with antisense oligonucleotides [Melendez and Khaw, 2002], see Figure 3.

of SphK activity specifically attenuated the $[Ca^{2+}]_i$ increases that were stimulated by many of these receptors, indicating that S1P mediated these $[Ca^{2+}]_i$ increases, working as a second messenger. It has to be noted that in most of these studies, tDHS and DMS have been used for SphK inhibition. Since these inhibitors raise concerns regarding their specificity, it was very

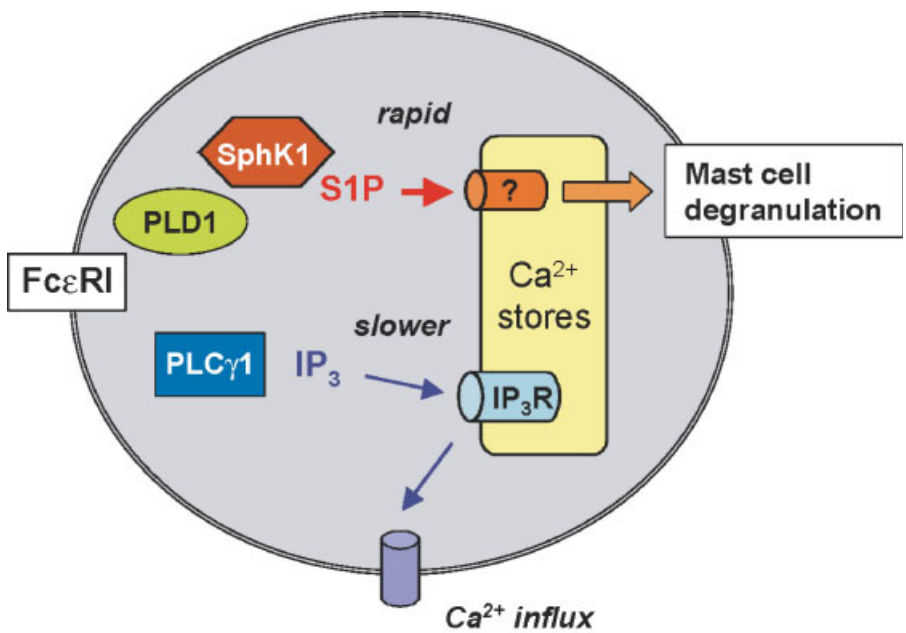

Fig. 3. Contribution of PLCγ1 and SphK1 to the overall $[Ca^{2+}]_i$ transient caused by the FcεRI antigen receptor in human bone marrow-derived mast cells, as shown with antisense oligonucleotides [Melendez and Khaw, 2002]. The FcεRI receptor, via phospholipase D1 (PLD1), activated SphK1, which mediated the initial $[Ca^{2+}]_i$ peak dependent on Ca^{2+} mobilization from intracellular stores. Parallel activation of PLCγ1 caused the generation of a second, slower Ca^{2+} wave and a Ca^{2+} influx into the cell. Both pathways appear to work independently of each other, and SphK1 was required for mast cell degranulation [Melendez and Khaw, 2002]. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

important to prove the involvement of SphK in Ca^{2+} signaling using SphK antisense oligonucleotides [Melendez and Khaw, 2002]. As depicted in more detail in Figure 3, SphK1 antisense inhibited Ca^{2+} mobilization by the FcεRI receptor in human mast cells [Melendez and Khaw, 2002]. In future studies, genetic approaches such as antisense or siRNA, or highly specific cell-permeable SphK inhibitors, will have to be used to define the role(s) of SphK isoforms in Ca^{2+} signaling as well as in other cellular functions.

We just begin to understand the role of the SphK/S1P pathway besides PLC/IP_3. Looking at the collection of receptors that have so far been shown to induce a rapid and transient S1P production (Table I), it appears that S1P signaling is widely used by G protein-coupled receptors, receptor tyrosine kinases as well as tyrosine kinase-linked receptors. Many receptors activate both, PLC and SphK, and the respective roles of the two pathways remain to be analyzed, as it has been done in case of the FcεRI antigen receptor in human mast cells (see Fig. 3). However, there are also, for example, the lysophospholipid receptors that increase $[Ca^{2+}]_i$ without causing a measurable IP_3 production, suggesting that SphK/S1P may fully substitute the PLC/IP_3 pathway in certain cases (see above).

Much work has still to be done to define the SphK isoforms that are involved in Ca^{2+} signaling, the pathways by which they are activated, the intracellular S1P targets mediating either Ca^{2+} influx or mobilization, and the interplay of the diverse Ca^{2+} signaling pathways in generation of the overall $[Ca^{2+}]_i$ transient that is induced by activation of a specific plasma membrane receptor.

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