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Stimulation of intracellular sphingosine-1-phosphate production by G-protein-coupled sphingosine-1-phosphate receptors

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Abstract

Recently, a family of G-protein-coupled receptors named endothelial differentiation gene (Edg) receptor family has been identified, which are specifically activated by the two serum lipids, sphingosine-1-phosphate and lysophosphatidic acid. Sphingosine-1-phosphate can also act intracellularly to release Ca^{2+} from intracellular stores. Since in several cell types, G-protein-coupled lysophosphatidic acid or sphingosine-1-phosphate receptors mobilize Ca^{2+} in the absence of a measurable phospholipase C stimulation, it was analysed here whether intracellular sphingosine-1-phosphate production was the signalling mechanism used by extracellular sphingosine-1-phosphate for mobilization of stored Ca^{2+}. Sphingosine-1-phosphate and the low affinity sphingosine-1-phosphate receptor agonist, sphingosylphosphorylcholine, induced a rapid, transient and nearly complete pertussis toxin-sensitive Ca^{2+} mobilization in human embryonic kidney (HEK-293) cells. The G-protein-coupled sphingosine-1-phosphate receptors, Edg-1, Edg-3 and Edg-5, were found to be endogenously expressed in these cells. Most interestingly, sphingosine-1-phosphate and sphingosylphosphorylcholine did not induce a measurable production of inositol-1,4,5-trisphosphate or accumulation of inositol phosphates. Instead, sphingosine-1-phosphate and sphingosylphosphorylcholine induced a rapid and transient increase in production of intracellular sphingosine-1-phosphate with a maximum of about 1.4-fold at 30 s. Stimulation of sphingosine-1-phosphate formation by sphingosine-1-phosphate and sphingosylphosphorylcholine was fully blocked by pertussis toxin, indicating that extracellular sphingosine-1-phosphate via endogenously expressed G_i-coupled receptors induces a stimulation of intracellular sphingosine-1-phosphate production. As sphingosine-1-phosphate- and sphingosylphosphorylcholine-induced increases in intracellular Ca^{2+} were blunted by sphingosine kinase inhibitors, this sphingosine-1-phosphate production appears to mediate Ca^{2+} signalling by extracellular sphingosine-1-phosphate and sphingosylphosphorylcholine in HEK-293 cells.

Keywords: Sphingosine-1-phosphate; Edg receptor; Sphingosine kinase; Ca^{2+} signalling

1. Introduction

The sphingolipid metabolite, sphingosine-1-phosphate, can apparently act as intracellular second messenger and as ligand for specific G-protein-coupled receptors. First identified by functional studies (Meyer zu Heringdorf et al., 1996; Postma et al., 1996; Van Koppen et al., 1996; Yamamura et al., 1997), specific receptors for sphingosine-1-phosphate have recently been shown to be members of the endothelial differentiation gene (Edg) receptor family, namely Edg-1, Edg-3, Edg-5 and Edg-6 (An et al., 1997; Lee et al., 1998; Zondag et al., 1998; Ancellin and Hla, 1999; Kon et al., 1999; Van Brocklyn et al., 2000; for review, see Spiegel and Milstien, 2000; Pyne and Pyne, 2000). Other Edg family members serve as receptors for lysophosphatidic acid (Goetzl and An, 1998). On the other hand, activation of various membrane receptors, including receptor tyrosine kinases (Olivera and Spiegel, 1993; Meyer zu Heringdorf et al., 1999a), antigen receptors (Choi et al., 1996; Melendez et al., 1998), muscarinic acetylcholine receptors (Meyer zu Heringdorf et al., 1998a) and formyl peptide receptors (Alemany et al., 1999), has been reported to stimulate intracellular sphingosine-1-phosphate formation by sphingosine kinase. There are two major lines of evidence suggesting an important role for intracellular sphingosine-1-phosphate production in Ca^{2+} mobilization by these receptors. First, inhibition of sphingosine kinase

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with DL-*threo*-dihydrosphingosine and/or *N*,*N*-dimethylsphingosine reduced or suppressed the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by these receptors, e.g., by antigen receptors in rat basophilic leukemia (RBL) mast cells and U937 monocytes (Choi et al., 1996; Melendez et al., 1998), by muscarinic M_2 and M_3 receptors expressed in human embryonic kidney (HEK-293) cells (Meyer zu Heringdorf et al., 1998a) and by formyl peptide receptors in HL-60 leukemia cells (Alemany et al., 1999). Second, intracellular sphingosine-1-phosphate has been demonstrated to cause release of Ca^{2+} from intracellular stores, for example in permeabilized DDT_1MF-2 smooth muscle cells (Ghosh et al., 1994) or after microinjection into HEK-293 cells (Meyer zu Heringdorf et al., 1998a).

A contribution of intracellular sphingosine-1-phosphate production to Ca^{2+} signalling has been reported so far mainly for receptors, which besides sphingosine kinase also stimulate phospholipase C and thus, induce production of the Ca^{2+}-releasing second messenger, inositol 1,4,5-trisphosphate (IP_3). Therefore, it remains uncertain which Ca^{2+} signalling pathway is preferentially used by a specific receptor or whether possibly activation of both pathways is required for Ca^{2+} mobilization. However, receptor-mediated Ca^{2+} mobilization can also occur in the complete absence of IP_3 production. For example, lysophosphatidic acid increased $[Ca^{2+}]_i$ in SH-SY5Y neuroblastoma cells without detectable IP_3 formation or inositol phosphate accumulation (Young et al., 1999). As the lysophosphatidic acid-induced Ca^{2+} mobilization was blocked by *N*,*N*-dimethylsphingosine, an involvement of sphingosine kinase was suggested. Similarly, rapid Ca^{2+} mobilization induced by extracellular sphingosine-1-phosphate can occur without concomitant phospholipase C activation. For example, in bovine aortic endothelial cells (Meyer zu Heringdorf et al., 1996), thyroid FRTL-5 cells (Törnquist et al., 1997) and Chinese hamster ovary (CHO) cells (Sato et al., 1999), sphingosine-1-phosphate induced rapid $[Ca^{2+}]_i$ increases, but failed to activate phospholipase C. Therefore, we studied whether G-protein-coupled sphingosine-1-phosphate receptors may mobilize Ca^{2+} by inducing intracellular sphingosine-1-phosphate production. For this, we chose HEK-293 cells since $[Ca^{2+}]_i$ increase induced by extracellular sphingosine-1-phosphate is almost completely blocked by pertussis toxin, indicating that is mediated by a G_i-coupled sphingosine-1-phosphate receptor (Van Koppen et al., 1996). Furthermore, intracellular sphingosine-1-phosphate has previously been shown to mobilize Ca^{2+} from intracellular stores in these cells (Meyer zu Heringdorf et al., 1998a). In addition to sphingosine-1-phosphate, we analysed signalling by sphingosylphosphorylcholine, which appears to be a low-affinity agonist at sphingosine-1-phosphate/Edg receptors (Ancellin and Hla, 1999, and many other studies), including in HEK-293 cells (Van Koppen et al., 1996), and which is not the product of sphingosine kinase. It should be noted that sphingosylphosphorylcholine, similar to sphingosine-1-phosphate, can induce Ca^{2+} release from intracellular stores when applied to permeabilized cells (Meyer zu Heringdorf et al., 1998b). However, in contrast to the intracellular action, Ca^{2+} mobilization by extracellularly applied sphingosylphosphorylcholine is fully pertussis toxin-sensitive, thus, receptor-mediated (Van Koppen et al., 1996; Meyer zu Heringdorf et al., 1998b). Our results demonstrate that G_i-coupled sphingosine-1-phosphate receptors can stimulate intracellular sphingosine-1-phosphate production to induce Ca^{2+} mobilization.

2. Materials and methods

2.1. Materials

Sphingosine-1-phosphate, sphingosylphosphorylcholine (D-*erythro*), DL-*threo*-dihydro-sphingosine and *N*,*N*-dimethylsphingosine were obtained from Biomol (Hamburg, Germany); *N*-acetylsphingosine (C2-ceramide) was from Matreya (Biotrend, Köln, Germany). Gö 6976 and phorbol-12-myristate-13-acetate were from Calbiochem-Novabiochem (Schwalbach, Germany). D-*Erythro*-[3-^{3}H]sphingosine (18 Ci/mmol) and [^{3}H]IP_3 (21 Ci/mmol) were purchased from NEN Life Science Products (Bruxelles, Belgium). All other materials were from previously described sources (Meyer zu Heringdorf et al., 1996, 1998a; Schmidt et al., 1994). Stock solutions of the sphingolipids were made in methanol. Before use, sphingosine-1-phosphate and sphingosylphosphorylcholine were dried down and redissolved in 1 mg/ml fatty acid-free bovine serum albumin (Sigma, Deisenhofen, Germany). DL-*threo*-dihydrosphingosine, *N*,*N*-dimethylsphingosine and *N*-acetylsphingosine were directly diluted into the albumin-containing solution. The respective solvents were used as control.

2.2. Cell culture

HEK-293 cells expressing muscarinic M_3 receptors were cultured in Dulbecco's modified Eagle's medium (DMEM/F12), containing 10% fetal calf serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin, as reported in Schmidt et al. (1994). Treatment with pertussis toxin was for 16–20 h with 100 ng/ml of the toxin.

2.3. Ca^{2+} mobilization

$[Ca^{2+}]_i$ was determined with the fluorescent calcium indicator dye fura-2 in a Hitachi spectrofluorimeter as described before (Meyer zu Heringdorf et al., 1996). Briefly, cell monolayers were washed with Hank's balanced salt solution (HBSS) (118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 15 mM HEPES, pH 7.4), and cells were detached by a buffer stream.

Suspended cells were loaded with 1 μM fura-2/AM for 1 h at 37°C in HBSS. Thereafter, cells were washed twice, resuspended at a density of 1×10^6 cells/ml and used for fluorescence measurements in a continuously stirred cell suspension at room temperature within the next hour (Meyer zu Heringdorf et al., 1996). In some experiments, Ca^{2+} was omitted from the extracellular medium.

2.4. IP_3 mass determination

Suspensions of HEK-293 cells in HBSS (200 μl, 2×10^5 cells) were incubated for 20 s at 37°C with the indicated agonists. The reactions were stopped by addition of 800 μl of 0.625 M trichloroacetic acid. IP_3 mass was determined as described (Chilvers et al., 1991). Briefly, the cells were pelleted by centrifugation (10 min, $800 \times g$), and the acid supernatants were extracted three times with 3 ml of water-saturated diethyl ether. The neutralized samples (200 μl) were complemented with 50 μl EDTA (30 mM) and 50 μl $NaHCO_3$ (60 mM). IP_3 mass was measured by competition with [^{3}H]IP_3, using IP_3 binding protein prepared from bovine adrenal cortex (Chilvers et al., 1991).

2.5. Inositol phosphate formation

Phospholipase C-catalysed production of inositol phosphates was determined in *myo*-[^{3}H]inositol-labeled cells in the presence of LiCl. HEK-293 cells were labeled for 48 h with 2 μCi/ml of *myo*-[^{3}H]inositol. Then, cell monolayers were washed twice with HBSS, and cells were detached by a buffer stream. Cell suspensions (200 μl, 2×10^5 cells) were incubated for 10 min at 37°C in HBSS containing 10 mM LiCl, before stimulation with the indicated agonists for 30 min at 37°C. Reactions were stopped by addition of methanol. Analysis of [^{3}H]inositol phosphates was performed as reported before (Schmidt et al., 1994).

2.6. Sphingosine-1-phosphate production

Formation of [^{3}H]sphingosine-1-phosphate was measured in cell suspensions essentially as reported before (Meyer zu Heringdorf et al., 1998a, 1999b). Cells were washed with HBSS and detached by a buffer stream. The reactions were started by addition of 100 μl of cell suspension ($0.5–1 \times 10^5$ cells) to 100 μl of a reaction mixture containing 2 mg/ml bovine serum albumin, 1 μCi/ml [^{3}H]sphingosine (~100,000 cpm/tube) and the indicated agonists in HBSS. Incubation was for the indicated periods of time at 37°C. Reactions were stopped by addition of 1 ml of ice-cold HBSS, rapid filtration over glass fiber filters (Whatman GF/C; Whatman, Maidstone, England) and a wash with 1 ml of ice-cold HBSS. Filters were extracted twice with 3 ml methanol/chloroform (2:1) for 30 min at 37°C. Extracts were combined and centrifuged for 10 min at $2000 \times g$, and supernatants were dried down in a SpeedVac vacuum centrifuge. Dried lipids were dissolved in a small volume of methanol and applied to Silica gel 60 TLC plates, together with sphingosine-1-phosphate and sphingosine standards for identification of [^{3}H]sphingosine-1-phosphate and [^{3}H]sphingosine, respectively. After separation in 1-butanol/acetic acid/water (3:1:1), lipids were visualized with ninhydrin, spots were scraped off, and radioactivity was measured by liquid scintillation counting.

2.7. Analysis of Edg receptor expression

HEK-293 cells were analysed for expression of Edg receptors by reverse transcriptase polymerase chain reaction (PCR). Total cellular RNA was prepared by guanidinium isothiocyanate-phenol-chloroform extraction according to Chomczynski and Sacchi (1987). Reverse transcription was performed with a kit from Superscript (Life Technologies, Eggenstein, Germany), using 2 μg RNA and 200 ng random hexanucleotide primers. PCR was performed with a *Taq* polymerase kit (MBI Fermentas, St. Leon-Rot, Germany). Oligonucleotide primers were chosen as follows: Edg-1, 5′-GCAATCTGGCCCTCTCAGAC-3′ and 5′-CGCCACATTCTCAGAGCTGC-3′; Edg-2, 5′-CATGGTGGCAATCTATGTCAAC-3′ and 5′-TTCAGAAGACTCATCATGGTATC-3′; Edg-3, 5′-TGCAGCTTACTGGCCATCGC-3′ and 5′-AACACGATGAACCACTGAGCC-3′; Edg-4, 5′-TGGTCATCATGGGCCAGTGC-3′ and 5′-GGTGCCATGCGTGAGCAGC-3′; Edg-5, 5′-ACGCCTGTGCAGTGGTTTGC-3′ and 5′-GACGATAAAGACGCCTAGCAC-3′; Edg-6, 5′-GCTGGAGAACTTGCTGGTGC-3′ and 5′-TAGAGGCCCATGATGGTGGC-3′. Thermocycler steps were 3 min at 95°C, then 35 cycles of 1 min at 95°C, 1 min at 59°C and 1 min at 72°C. PCR products were separated by electrophoresis in 1% agarose with *Alu*I-digested pBR322 plasmid as marker. Control reactions were performed using RNA that had not been reverse transcribed to check for contaminating genomic DNA. The suitability of the primers for Edg-4 and Edg-6 was tested using Edg-4 plasmid DNA and human genomic DNA, respectively. The identity of the amplified fragments was confirmed by restriction enzyme analysis using *Pvu*II, *Sph*I, *Nco*I and *Xho*I (Life Technologies) for Edg-1, Edg-2, Edg-3 and Edg-5, respectively.

2.8. Overexpression of sphingosine-1-phosphate/Edg receptors

DNA constructs encoding human Edg-1, Edg-3 and Edg-5 in pCR3.1 or pcDNA3 (Invitrogen) were kindly provided by Dr. Udo Stropp, Bayer, Leverkusen. For myc-tagging of Edg-3 and Edg-5, these constructs were subcloned into pCMV-Tag3 (Stratagene). Cells grown to near confluence on 145-mm culture dishes were transfected with 50 μg of plasmid DNA by the calcium phos-

phate method. Experiments were performed 48 h after transfection. Overexpression of Edg-3 and Edg-5 was confirmed by immunofluorescence microscopy. For this, aliquots of the transfected cells were seeded onto polylysine-coated culture slides (Falcon), fixed for 30 min with 3.7% formaldehyde, washed with HBSS and permeabilized by incubation for 2 min in 0.05% (v/v) Triton X-100. After incubation for 60 min in 5% milkpowder, the cells were stained for 60 min with anti-c-Myc antibody (OP10, Calbiochem; diluted 1/200 in HBSS containing 0.5% bovine serum albumin). Then, the cells were washed with HBSS and incubated for another 60 min with a fluorescein

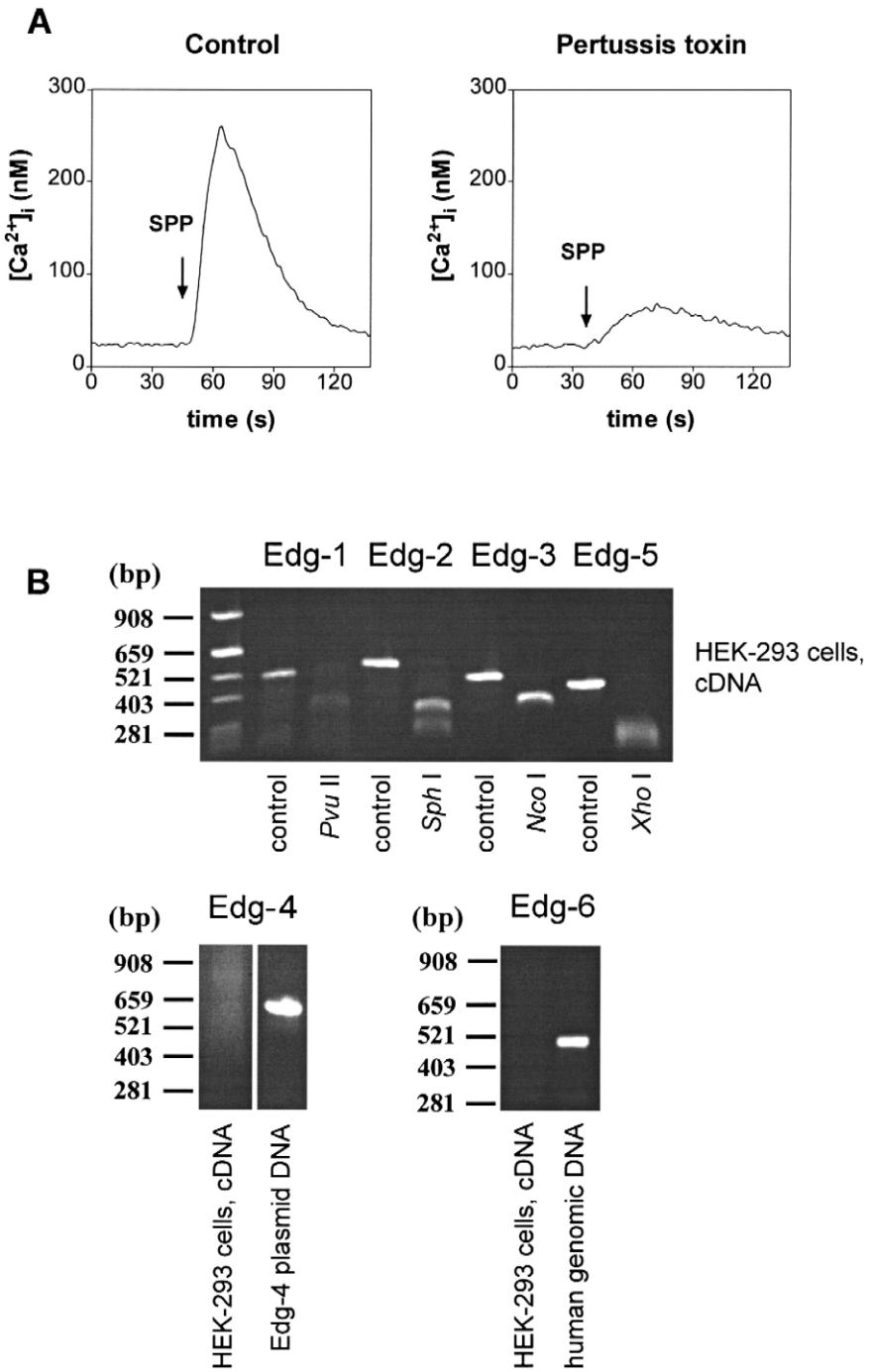

Fig. 1. Pertussis toxin-sensitive Ca^{2+} mobilization by sphingosine-1-phosphate and endogenous expression of sphingosine-1-phosphate/Edg receptor subtypes in HEK-293 cells. (A) Sphingosine-1-phosphate (SPP, 100 nM)-induced Ca^{2+} mobilization was determined in Ca^{2+}-free buffer in fura-2-loaded cells, pretreated or not (control) with pertussis toxin. Shown are typical traces of $[Ca^{2+}]_i$. (B) Reverse transcriptase PCR was performed with cDNA prepared from HEK-293 cells, with human genomic DNA or with Edg-4 plasmid DNA. Identity of the fragments was confirmed by restriction enzyme analysis as indicated. Control reactions with RNA that had not been reverse transcribed were negative (data not shown).

isothiocyanate-conjugated anti-mouse IgG antibody (F2012, Sigma, diluted 1:200 in HBSS containing 0.5% bovine serum albumin). Finally, the cells were washed three times in HBSS, mounted with Moviol (Calbiochem) and examined using a Zeiss Axiovert microscope. Transfection efficiency was about 40%.

3. Results

As reported before (Van Koppen et al., 1996), application of sphingosine-1-phosphate at nanomolar concentrations to HEK-293 cells induced rapid and transient increases in $[Ca^{2+}]_i$, which are due to Ca^{2+} mobilization from intracellular stores (Fig. 1A). Similar results were obtained with sphingosylphosphorylcholine, albeit at higher concentrations (Van Koppen et al., 1996). The $[Ca^{2+}]_i$ increases induced by sphingosine-1-phosphate and sphingosylphosphorylcholine were nearly completely blocked by pretreatment of the cells with pertussis toxin, indicating that Ca^{2+} mobilization is mediated by G_i-coupled receptors. Reverse transcriptase PCR with Edg receptor subtype-specific oligonucleotide primers indicated that HEK-293 cells express specific mRNA transcripts for the sphingosine-1-phosphate/Edg receptors, Edg-1, Edg-3 and Edg-5, as well as for the lysophosphatidic acid receptor, Edg-2 (Fig. 1B). As specific agonists or antagonists of these sphingosine-1-phosphate/Edg receptor subtypes are not available, it remains unknown which of these sphingosine-1-phosphate receptors mediate(s) the Ca^{2+} signalling in HEK-293 cells. Overexpression of Edg-3 or Edg-5 did not lead to a further increase in sphingosine-1-phosphate-induced Ca^{2+} signalling (Fig. 2A), although the receptors were located at the plasma membrane (Fig. 2B). Overexpression of Edg-1 even decreased sphingosine-1-phosphate-induced $[Ca^{2+}]_i$ increase (Fig. 2A). In contrast, overexpression of Edg-3 and Edg-5 in CHO cells clearly augmented sphingosine-1-phosphate-induced $[Ca^{2+}]_i$ increase, while transfection efficiency was equal to or even less than in HEK-293 cells (data not shown). This differential response may be explained by a different number of endogenous sphingolipid receptors in CHO (probably low) and HEK-293 (probably high) cells. A further increase in receptor number in HEK-293 cells did not increase efficacy or potency of sphingosine-1-phosphate to increase $[Ca^{2+}]_i$, either because the number of receptor-activated G proteins and/or the magnitude of the receptor-releasable Ca^{2+} pool were limited. However, it is also possible that another not studied or as yet unidentified receptor mediates sphingosine-1-phosphate-induced Ca^{2+} signalling in HEK-293 cells. Taken together, the overexpression experiments did not lead to identification of the receptor(s) mediating sphingosine-1-phosphate-induced Ca^{2+} signalling in HEK-293 cells.

The sphingosine-1-phosphate-induced $[Ca^{2+}]_i$ increase in HEK-293 cells occurred without detectable phospholipase C activation. As shown in Fig. 3, neither sphingosine-1-phosphate (1 μM) nor sphingosylphosphorylcholine (5 or 10 μM) increased IP_3 production, measured 20 s after agonist exposure, or accumulation of inositol phosphates, measured over 30 min in the presence of 10 mM LiCl. In contrast, carbachol activating muscarinic M_3 receptors expressed in these cells strongly activated both parameters of phospholipase C activity.

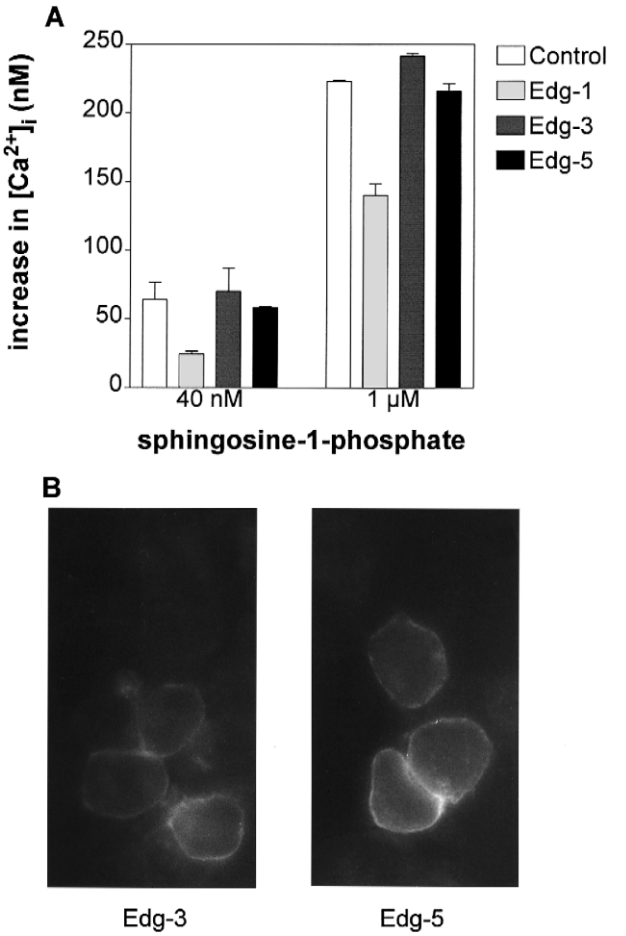

Fig. 2. Influence of sphingosine-1-phosphate/Edg receptor overexpression on sphingosine-1-phosphate-induced $[Ca^{2+}]_i$ increase. (A) $[Ca^{2+}]_i$ was measured in HEK-293 cells transfected with Edg-1, Edg-3 (myc-tagged), Edg-5 (myc-tagged) or control vector. The cells were stimulated with the indicated concentrations of sphingosine-1-phosphate. Values are mean ± S.D. of a representative experiment performed in duplicate. (B) Overexpression of Edg-3 and Edg-5 was confirmed by immunofluorescence microscopy using an anti-c-Myc antibody and a fluorescein isothiocyanate-conjugated second antibody.

Several recent reports indicate that Ca^{2+} mobilization induced by G-protein-coupled receptors is dependent on sphingosine kinase activation (Meyer zu Heringdorf et al., 1998a; Alemany et al., 1999; Young et al., 1999). To study whether this pathway contributes to Ca^{2+} signalling by G_i-coupled sphingosine-1-phosphate receptors in HEK-293 cells, it was first examined whether intracellular sphingosine-1-phosphate production by sphingosine kinase is stimulated by sphingosine-1-phosphate receptors in HEK-293 cells. As shown in Fig. 4, both sphingolipid receptor

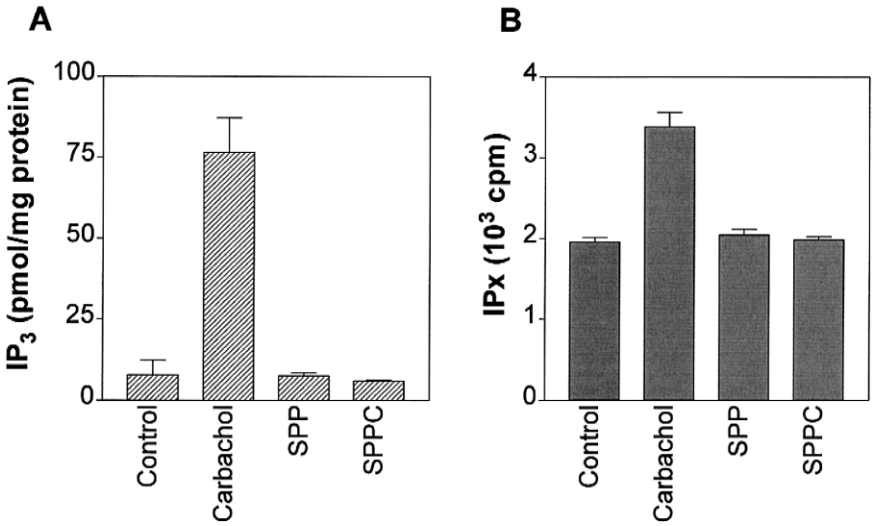

Fig. 3. Influence of sphingosine-1-phosphate, sphingosylphosphorylcholine and the muscarinic acetylcholine receptor agonist carbachol on phospholipase C activity. In (A), IP_3 production was determined in HEK-293 cells stimulated for 20 s without (control) and with 1 mM carbachol, 1 μM sphingosine-1-phosphate (SPP) or 5 μM sphingosylphosphorylcholine (SPPC). In (B), [^{3}H]inositol phosphate (IP_x) accumulation was determined over 30 min in [^{3}H]inositol-prelabeled cells in the presence of 10 mM LiCl without (control) and with 1 mM carbachol, 1 μM sphingosine-1-phosphate (SPP) or 10 μM sphingosylphosphorylcholine (SPPC). Values are mean ± S.D. of representative experiments performed in triplicate.

agonists rapidly stimulated sphingosine kinase activity. Formation of intracellular [^{3}H]sphingosine-1-phosphate from [^{3}H]sphingosine was 1.4- and 1.5-fold above basal values after stimulation of the cells for 30 s with 1 μM sphingosine-1-phosphate and 10 μM sphingosylphosphorylcholine, respectively. Time course experiments demonstrated that sphingosine kinase stimulation by sphingosylphosphorylcholine was rapid and transient, reaching maximum values after 30 s and again basal values after 5 min of incubation (Fig. 4A). Most importantly, stimulation of [^{3}H]sphingosine-1-phosphate production by sphingosine-1-phosphate and sphingosylphosphorylcholine was completely blocked by pertussis toxin (Fig. 4B), indicating that the G_i-coupled sphingolipid receptors were involved in this action. Activation of sphingosine kinase by antigen receptors in U937 monocytes was apparently dependent on phospholipase D stimulation (Melendez et al., 1998). Therefore, we determined whether sphingosine-1-phosphate stimulates phospholipase D activity in HEK-293 cells, by measuring formation of [^{3}H]phosphatidylethanol in [^{3}H]oleic acid-prelabeled cells in the presence of ethanol (Schmidt et al., 1994). However, while carbachol stimu-

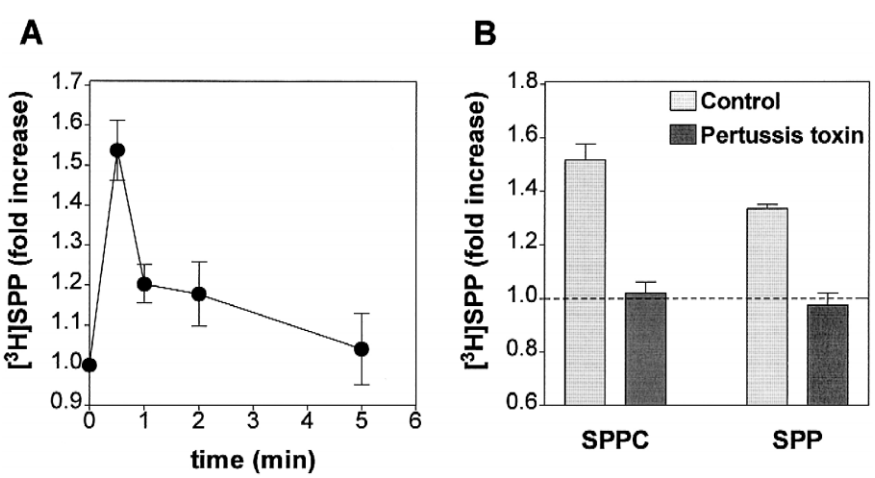

Fig. 4. Sphingolipid receptor-induced sphingosine-1-phosphate production. In (A), a time course of sphingosylphosphorylcholine (10 μM)-stimulated [^{3}H]sphingosine-1-phosphate ([^{3}H]SPP) formation in HEK-293 cells is shown. Values are mean ± S.D. from a representative experiment performed in triplicate. In (B), [^{3}H]sphingosine-1-phosphate production induced by 10 μM sphingosylphosphorylcholine (SPPC) or 1 μM sphingosine-1-phosphate (SPP) was measured for 30 s in control and pertussis toxin-treated cells. Values are mean ± S.E.M. from three experiments. Data are given as -fold increases in [^{3}H]sphingosine-1-phosphate formation observed in control untreated cells, amounting to 2300 ± 430 cpm$/10^5$ cells ($n = 7$).

lated phospholipase D activity, as reported before (Schmidt et al., 1994), production of [^{3}H]phosphatidylethanol was not affected in cells stimulated with 1 μM sphingosine-1-phosphate (data not shown). Thus, both agonists at G-protein-coupled sphingosine-1-phosphate receptors, sphingosine-1-phosphate and sphingosylphosphorylcholine, stimulated intracellular sphingosine-1-phosphate production in HEK-293 cells and this stimulation was mediated by G_i-proteins.

Finally, the effects of the sphingosine kinase inhibitors, DL-*threo*-dihydrosphingosine and *N,N*-dimethylsphingosine, on sphingosine-1-phosphate- and sphingosylphosphorylcholine-induced Ca^{2+} mobilization were examined. As shown in Fig. 5, DL-*threo*-dihydrosphingosine suppressed sphingosine-1-phosphate-as well as sphingosylphosphorylcholine-induced $[Ca^{2+}]_i$ elevations in a concentration-dependent manner, with almost 80% inhibition observed at 60 μM DL-*threo*-dihydrosphingosine. Half-maximal inhibition of sphingosine-1-phosphate (1 μM)-induced $[Ca^{2+}]_i$ increase was seen at 15 μM DL-*threo*-dihydrosphingosine (Fig. 5C). These concentrations of DL-*threo*-dihydrosphingosine also inhibit [^{3}H]sphingosine-1-phosphate production in HEK-293 cells (Meyer zu Heringdorf et al., 1999a). Analysis of the sphingosine-1-phosphate and sphingosylphosphorylcholine concentration–response curves revealed that inhibition by DL-*threo*-dihydrosphingosine was independent of the degree of receptor activation (Fig. 5A and B). While *N,N*-dimethylsphingosine, another sphingosine kinase inhibitor, also inhibited sphingosine-1-phosphate-induced $[Ca^{2+}]_i$ increase (Fig. 5D), a structurally related lipid, *N*-acetylsphingosine, was inactive. $[Ca^{2+}]_i$ increase induced by 1 μM sphingosine-1-phosphate was 92 ± 0.6% of control after pretreatment of the cells for 1 min with 30 μM *N*-acetylsphingosine (mean ± S.E.M., $n = 3$; data not shown). Finally, inhibition of protein kinase C by DL-*threo*-dihydrosphingosine or *N,N*-dimethylsphingosine was ruled out as the mechanism by which these lipids inhibit sphingosine-1-phosphate-induced $[Ca^{2+}]_i$ increase. The protein kinase C inhibitor, Gö 6976, had no influence on $[Ca^{2+}]_i$ increase induced by 1 μM sphingosine-1-phosphate (which was 101 ± 5% of control values after pretreatment with 100 nM Gö 6976 for 30 min, mean ± S.E.M., $n = 3$). Furthermore, activation of protein kinase C by phorbol-12-myristate-13-acetate did not augment but even reduced sphingosine-1-phosphate-induced $[Ca^{2+}]_i$ increase (data not shown).

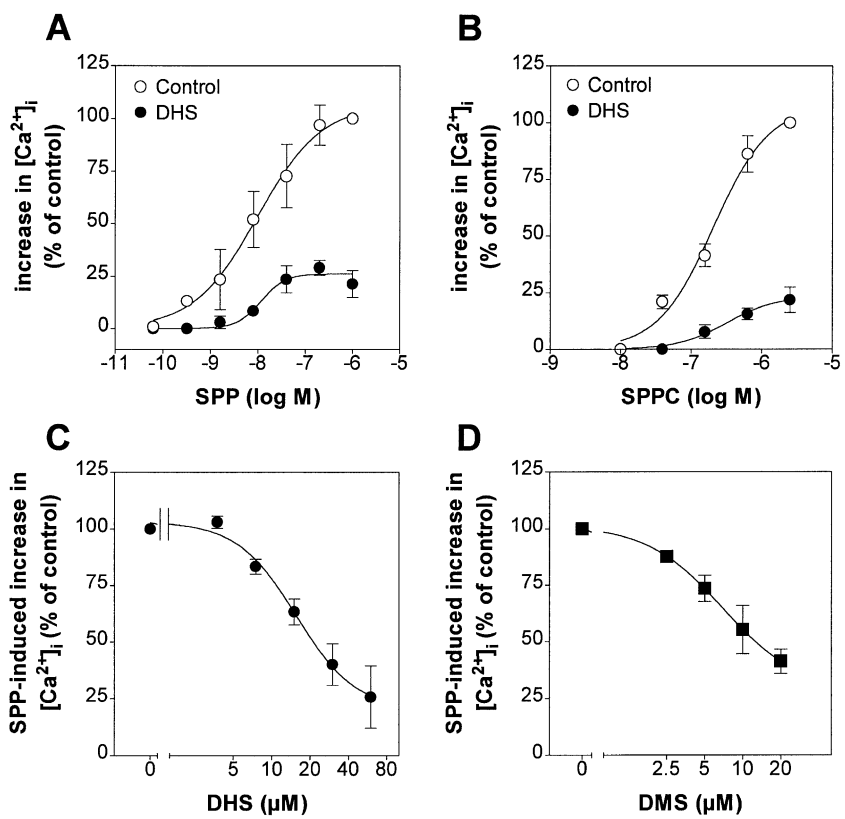

Fig. 5. Inhibition of sphingolipid receptor-mediated Ca^{2+} signalling by DL-*threo*-dihydrosphingosine (DHS) and *N,N*-dimethylsphingosine (DMS). Peak $[Ca^{2+}]_i$ increases induced by sphingosine-1-phosphate (SPP, A) and sphingosylphosphorylcholine (SPPC, B) at the indicated concentrations were measured in HEK-293 cells preincubated for 1 min without (control) and with 60 μM DL-*threo*-dihydrosphingosine (DHS). In C and D, cells were pretreated for 1 min with the indicated concentrations of DL-*threo*-dihydrosphingosine (DHS) or *N,N*-dimethylsphingosine (DMS) and then stimulated with 1 μM sphingosine-1-phosphate. Data are shown as % of maximal $[Ca^{2+}]_i$ increases in the absence of inhibitor, amounting to 300–400 nM and ~ 200 nM with 1 μM sphingosine-1-phosphate and 2.5 μM sphingosylphosphorylcholine, respectively. Values are mean ± S.E.M. from three experiments.

4. Discussion

There is growing evidence that receptors can use other signalling pathways, besides the IP_3-producing phospholipase C, for mobilization of Ca^{2+} from intracellular Ca^{2+} stores. Of the rather well-characterized pathways, cyclic ADP-ribose, the product of ADP-ribosyl cyclase, selectively activates ryanodine receptor Ca^{2+} channels and this pathway can be involved in muscarinic acetylcholine receptor-induced Ca^{2+} mobilization (Morita et al., 1997). However, the HEK-293 cells used in the present study do not express ryanodine receptor Ca^{2+} channels (Meyer zu Heringdorf et al., 1998b). Instead, another Ca^{2+} signalling pathway has been described in these cells. In a previous study, we demonstrated the involvement of the sphingosine kinase/sphingosine-1-phosphate pathway in Ca^{2+} signalling by muscarinic M_2 and M_3 receptors in HEK-293 cells (Meyer zu Heringdorf et al., 1998a). The muscarinic acetylcholine receptors stimulated both, sphingosine kinase and phospholipase C, and inhibition of sphingosine kinase greatly reduced M_2 receptor-induced Ca^{2+} mobilization at all agonist concentrations, whereas M_3 receptor-induced Ca^{2+} mobilization was inhibited only at low agonist concentrations (Meyer zu Heringdorf et al., 1998a). This observation suggests a complex interplay between phospholipase C and sphingosine kinase and their products in Ca^{2+} mobilization by the muscarinic acetylcholine receptors. On the other hand, receptors can mobilize Ca^{2+} even after blockade of IP_3 receptors. Injection of heparin into CHO cells, completely inhibiting Ca^{2+} release after flash photolysis of caged IP_3, failed to block Ca^{2+} mobilization induced by thrombin and endothelin-1 receptors (Mathias et al., 1998). Thus, phospholipase C stimulation by thrombin and endothelin-1 is either redundant to another, unknown Ca^{2+} mobilization mechanism or needed for other purposes (Mathias et al., 1998). Another example that phospholipase C activation apparently not always leads to Ca^{2+} mobilization was recently reported in two subclones of HEK-293 cells. While epidermal growth factor (EGF) increased IP_3 production in both subclones, it increased $[Ca^{2+}]_i$ only in one of the two cell lines (Meyer zu Heringdorf et al., 1999a). In this case, the ability of EGF to mobilize Ca^{2+} was correlated with sphingosine kinase activation (Meyer zu Heringdorf et al., 1999a), but the exact role played by the phospholipase C remained unclear. It was speculated that phospholipase C is needed, in addition to sphingosine kinase, for Ca^{2+} mobilization by the EGF receptor.

There are, however, several reports of receptor-induced Ca^{2+} mobilization without detectable phospholipase C stimulation. For example, a truncated oxytocin receptor, capable of coupling to G_i-, but no longer to G_q-proteins, expressed in CHO cells increased $[Ca^{2+}]_i$ without stimulating inositol phosphate production (Hoare et al., 1999). Likewise, lysophosphatidic acid, acting at an endogenous G-protein-coupled receptor in SH-SY5Y cells, mobilized Ca^{2+} without detectable IP_3 production (Young et al., 1999). As similar results have been reported for Ca^{2+} mobilization by sphingosine-1-phosphate receptors in other cell types (Meyer zu Heringdorf et al., 1996; Törnquist et al., 1997), we asked whether G_i-coupled sphingosine-1-phosphate receptors, endogenously expressed in HEK-293 cells, can stimulate intracellular sphingosine-1-phosphate production by sphingosine kinase to induce Ca^{2+} mobilization. It is shown here that sphingosine-1-phosphate and sphingosylphosphorylcholine induce rapid intracellular sphingosine-1-phosphate formation, with a time course and magnitude similar to sphingosine kinase stimulation reported for other membrane receptors in various cell types (Olivera and Spiegel, 1993; Meyer zu Heringdorf et al., 1998a, 1999a; Choi et al., 1996; Melendez et al., 1998; Alemany et al., 1999). Furthermore, similar to Ca^{2+} mobilization, stimulation of sphingosine-1-phosphate production by extracellular sphingosine-1-phosphate and sphingosylphosphorylcholine was fully pertussis toxin-sensitive. Finally, inhibition of sphingosine kinase strongly suppressed sphingosine-1-phosphate receptor-induced Ca^{2+} mobilization. These data, thus, suggest that Ca^{2+} signalling by G_i-coupled sphingosine-1-phosphate receptors endogenously expressed in HEK-293 cells is dependent on sphingosine kinase activation. It can, however, not be completely excluded that a very small, localized IP_3 production and Ca^{2+} release precede or parallel Ca^{2+} mobilization by intracellular sphingosine-1-phosphate. Such localized events may have escaped the IP_3 and $[Ca^{2+}]_i$ measurements.

The type of Ca^{2+} signalling by sphingosine-1-phosphate receptors is apparently cell type-specific and/or restricted to a certain subset of receptors. Particularly, studies on recombinant sphingosine-1-phosphate/Edg receptors expressed in various cell types have shown that activation of Edg-1, Edg-3 and Edg-5 receptors can induce both Ca^{2+} mobilization and phospholipase C stimulation (Ancellin and Hla, 1999; Kon et al., 1999; Sato et al., 1999; Okamoto et al., 1998, 1999; Gonda et al., 1999). There is, however, distinct coupling of the different Edg receptors to G-protein subtypes. While Edg-3 and Edg-5 can apparently couple to both, G_i- and G_q-proteins, Edg-1 preferentially couples to G_i-proteins (Ancellin and Hla, 1999; Windh et al., 1999). At present, we cannot state which sphingosine-1-phosphate receptor subtype is responsible for Ca^{2+} signalling via the sphingosine kinase pathway in HEK-293 cells. The cells express at least three sphingosine-1-phosphate/Edg receptor subtypes, as revealed by reverse transcriptase PCR. Overexpression experiments did not lead to identification of the receptor subtype mediating the response. Therefore, specific receptor antagonists will be needed to resolve this question.

In conclusion, G_i-coupled sphingosine-1-phosphate receptors, endogenously expressed in HEK-293 cells, can stimulate intracellular sphingosine-1-phosphate production by sphingosine kinase, which apparently controls

receptor-induced Ca^{2+} mobilization. During the preparation of the present manuscript, it was reported by Young et al. (2000) that Ca^{2+} signalling by lysophosphatidic acid, potentially acting via the Edg-4 receptor, is dependent on sphingosine kinase activation in SH-SY5Y neuroblastoma cells. This result with a lipid closely related to sphingosine-1-phosphate, acting on receptors of the same superfamily, nicely supports the data presented here. Beyond that, we demonstrate the unique phenomenon that an extracellular agonist (sphingosine-1-phosphate) controls a cell function by stimulating its own intracellular production.

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