Sphingosine kinase-mediated Ca^{2+} signalling by G-protein-coupled receptors

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Formation of inositol 1,4,5-trisphosphate (IP_3) by phospholipase C (PLC) with subsequent release of Ca^{2+} from intracellular stores, is one of the major Ca^{2+} signalling pathways triggered by G-protein-coupled receptors (GPCRs). However, in a large number of cellular systems, Ca^{2+} mobilization by GPCRs apparently occurs independently of the PLC- IP_3 pathway, mediated by an as yet unknown mechanism. The present study investigated whether sphingosine kinase activation, leading to production of sphingosine-1-phosphate (SPP), is involved in GPCR-mediated Ca^{2+} signalling as proposed for platelet-derived growth factor and Fc ϵ RI antigen receptors. Inhibition of sphingosine kinase by DL-threo-dihydrosphingosine and N,N-dimethylsphingosine markedly inhibited $[\text{Ca}^{2+}]_i$ increases elicited by m2 and m3 muscarinic acetylcholine receptors (mAChRs) expressed in HEK-293 cells without affecting mAChR-induced PLC stimulation. Activation of mAChRs rapidly and transiently stimulated production of SPP in HEK-293 cells. Finally, intracellular injection of SPP induced a rapid and transient Ca^{2+} mobilization in HEK-293 cells which was not antagonized by heparin. We conclude that mAChRs utilize the sphingosine kinase-SPP pathway in addition to PLC- IP_3 to mediate Ca^{2+} mobilization. As Ca^{2+} signalling by various, but not all, GPCRs in different cell types was likewise attenuated by the sphingosine kinase inhibitors, we suggest a general role for sphingosine kinase, besides PLC, in mediation of GPCR-induced Ca^{2+} signalling.

Keywords: calcium mobilization/G-proteins/muscarinic acetylcholine receptor/sphingosine kinase/sphingosine-1-phosphate

Introduction

Plasma membrane receptors coupled to heterotrimeric G-proteins (GPCRs), a protein family with ~1000 members, mediate a large variety of important physiological actions induced by hormones, neurotransmitters and sensory signals. At the cellular level, one of the major early events triggered by many GPCRs is the increase in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Since an

increase in $[\text{Ca}^{2+}]_i$ is crucial for numerous cellular responses, it is of paramount importance to understand how GPCRs induce this early response. At present, the best-known Ca^{2+} signalling pathway triggered by GPCRs, and also by tyrosine kinase receptors, is the activation of the phosphatidylinositol 4,5-bisphosphate-hydrolysing phospholipase C (PLC) which produces the Ca^{2+} release second messenger, inositol 1,4,5-trisphosphate (IP_3) (Berridge, 1993; Clapham, 1995; Mikoshiba, 1997). Different PLC isozymes have been identified which are activated by either tyrosine kinase receptors or GPCRs, apparently involving distinct G-protein α or $\beta\gamma$ subunits (Cockcroft and Thomas, 1992; Lee and Rhee, 1995; Exton, 1996). However, PLC- IP_3 -independent Ca^{2+} mobilization by GPCRs has also been demonstrated. Examples include Ca^{2+} signalling by α_2 -adrenoceptors (Michel *et al.*, 1989), neuropeptide Y receptors (Motulsky and Michel, 1988), purinergic receptors (Frelin *et al.*, 1993), parathyroid hormone receptors (Seuwen and Boddeke, 1995) and thyrotropin-releasing hormone receptors (Tashjian *et al.*, 1987). Although there has been a long-standing search, the identity of the signalling pathway(s) and the second messenger(s) involved have remained elusive.

We have recently reported that Ca^{2+} mobilization by the m2 muscarinic acetylcholine receptor (mAChR), a prototypical GPCR expressed in HEK-293 cells, is ~50% pertussis toxin (PTX)-sensitive, whereas PLC stimulation is fully PTX-insensitive (Schmidt *et al.*, 1995). Furthermore, m2 mAChR-mediated Ca^{2+} mobilization was not affected by *Clostridium difficile* toxin B, which, however, decreased receptor-mediated PLC stimulation markedly (Schmidt *et al.*, 1996). These observations suggested a PLC-independent Ca^{2+} signalling by m2 mAChRs in HEK-293 cells. In search of the putative mechanism involved, we examined whether a Ca^{2+} signalling pathway recently described for tyrosine kinase-linked receptors may also be used by GPCRs. Compared with the PLC- IP_3 pathway, this alternative Ca^{2+} signalling pathway is thought to be triggered by activation of sphingosine kinase, resulting in the production of the Ca^{2+} release second messenger, sphingosine-1-phosphate (SPP). The main evidence for this pathway is of three types (Beaven, 1996). First, direct application of SPP to permeabilized DDT₁MF-2 cells or microsomal preparations (Ghosh *et al.*, 1990, 1994) and Swiss-3T3 cells (Mattie *et al.*, 1994) causes a rapid release of Ca^{2+} from internal stores in an IP_3 receptor-independent manner. Secondly, stimulation of platelet-derived growth factor (PDGF) receptors in a number of cell lines (Olivera and Spiegel, 1993; Bornfeldt *et al.*, 1995), as well as stimulation of Fc ϵ RI antigen receptors in RBL-2H3 mast cells (Choi *et al.*, 1996), activates sphingosine kinase, resulting in enhanced SPP production. Finally, micromolar concentrations of the sphingosine kinase inhibitor, DL-threo-dihydrosphingosine

Table 1. Inhibition of basal [^3H]SPP formation by DHS and DMS in HEK-293 cells

Treatment	[^3H]SPP formed (c.p.m./ 10^6 cells)	
	30 s	2 min
Control	223 $\pm$ 12	709 $\pm$ 145
DHS (30 μM)	46 $\pm$ 3	187 $\pm$ 46
DMS (15 μM)	61 $\pm$ 10	207 $\pm$ 47

Following pretreatment of HEK-293 cells for 10 min with vehicle (Control), 30 μM DHS or 15 μM DMS, [^3H]sphingosine was added to the cells at t_0 and basal formation of [^3H]SPP was determined for either 30 s or 2 min. Values shown are mean $\pm$ SD of two independent experiments, each performed in triplicate, and corrected for t_0 count rates of 39 ± 5 c.p.m./ 10^6 cells. Pretreatment with DHS or DMS did not alter the cellular uptake of [^3H]sphingosine.

(DHS), markedly inhibit Ca^{2+} signalling by these receptors, which either exhibit intrinsic tyrosine kinase activity (PDGF receptor) or are coupled to cytosolic protein tyrosine kinases (Fc ϵ R1 antigen receptor).

Although previous data suggested that Ca^{2+} mobilization by GPCRs, for example by the m1 mAChR, is independent of sphingosine kinase (Beaven, 1996; Choi *et al.*, 1996), we re-examined this issue, applying the criteria mentioned above, to establish the alternative Ca^{2+} signalling pathway. Here we demonstrate that inhibition of sphingosine kinase markedly inhibits Ca^{2+} signalling by mAChRs and other GPCRs in various cell types, that mAChR activation results in rapid production of SPP by sphingosine kinase, and that SPP acts as an intracellular Ca^{2+} release agent in HEK-293 cells, thus strongly suggesting that the sphingosine kinase–SPP pathway represents an alternative Ca^{2+} signalling pathway for mAChRs and other GPCRs.

Results

Inhibition of mAChR-mediated Ca^{2+} signalling by sphingosine kinase inhibitors

To test the hypothesis that m2 mAChR-induced Ca^{2+} signalling involves the generation of SPP by sphingosine kinase, we first determined the effects of DHS and *N,N*-dimethylsphingosine (DMS), two membrane-permeable sphingosine kinase inhibitors (Buehrer and Bell, 1992; Olivera *et al.*, 1994; Yatomi *et al.*, 1996), on m2 mAChR-induced [Ca^{2+}] $_i$ increase. The inhibitors effectively blocked sphingosine kinase activity in HEK-293 cells. Pretreatment of the cells with 30 μM DHS or 15 μM DMS inhibited the conversion of exogenously applied [^3H]sphingosine into [^3H]SPP by 70–80% (Table 1). Stimulation of m2 mAChR-expressing HEK-293 cells with a submaximally effective concentration of carbachol (100 μM) increased [Ca^{2+}] $_i$ by 447 ± 132 nM ($n=15$) (Figure 1A). Pretreatment of the cells for 10 min with 30 μM DHS or 15 μM DMS inhibited the agonist-induced [Ca^{2+}] $_i$ increase by $74\pm 4\%$ and $79\pm 9\%$, respectively (Figure 1B and C). In contrast, *N*-acetylsphingosine (30 μM), which differs from DMS in having an -NHCOCH $_3$ group instead of an -N(CH $_3$) $_2$ group at C-2 and which does not inhibit sphingosine kinase at micromolar concentrations (Yatomi *et al.*, 1996), did not inhibit the carbachol-induced [Ca^{2+}] $_i$ increase (Figure 1D). Also,

Fig. 1. Inhibition of m2 mAChR-mediated [Ca^{2+}] $_i$ increase by sphingosine kinase inhibitors. Carbachol (100 μM)-induced [Ca^{2+}] $_i$ increases were determined in m2 mAChR-expressing HEK-293 cells pretreated for 10 min, without (A) (Control) and with (B) 30 μM DHS, (C) 15 μM DMS or (D) 30 μM *N*-acetylsphingosine (*N*-Ac-SP). The addition of carbachol is indicated by arrows.

sphingosine (30 μM) did not alter m2 mAChR-induced [Ca^{2+}] $_i$ elevations (data not shown).

The inhibitory effect of DHS and DMS on m2 mAChR-induced [Ca^{2+}] $_i$ increase was concentration dependent, with pEC $_{50}$ values of 4.70 ± 0.04 and 5.15 ± 0.03 , respectively (Figure 2, upper panel). The corresponding IC $_{50}$ values of 20 and 7 μM are well within the ranges described (i.e. 5–50 μM for DHS and 5–10 μM for DMS) for inhibition of sphingosine kinase activity (Buehrer and Bell, 1992; Olivera and Spiegel, 1993; Olivera *et al.*, 1994; Choi *et al.*, 1996; Yatomi *et al.*, 1996). Complete inhibition of [Ca^{2+}] $_i$ increase elicited by 100 μM carbachol was observed in cells pretreated with 60 μM DHS or 30 μM DMS. Since Ca^{2+} signalling by m2 mAChRs in HEK-293 cells is ~50% PTX-insensitive (Schmidt *et al.*, 1995), these observations suggested that DHS and DMS inhibit PTX-sensitive, as well as PTX-insensitive, Ca^{2+} mobilization. Indeed, DHS (30 μM) reduced [Ca^{2+}] $_i$ increase by carbachol (100 μM) in PTX-treated m2 mAChR-expressing cells by a magnitude ($58\pm 8\%$, $n=3$) similar to that seen in control cells.

For comparison, the effects of the sphingosine kinase inhibitors on Ca^{2+} signalling were studied in HEK-293 cells expressing the highly efficient PLC stimulatory m3 mAChR. The mAChR-mediated [Ca^{2+}] $_i$ elevations in m3 and m2 mAChR-expressing cells are of comparable magnitude. However, m3 mAChR-mediated [Ca^{2+}] $_i$ increases are fully PTX-insensitive and mainly (almost 90%) due to Ca^{2+} influx (Schmidt *et al.*, 1995). As the EC $_{50}$ values for carbachol for rising [Ca^{2+}] $_i$ in m3 mAChR-expressing HEK-293 cells are ~100-fold lower than in m2 mAChR-expressing cells, we challenged m3 mAChR-expressing cells with 0.3 μM carbachol which, like 100 μM carbachol for m2 mAChRs, is a submaximally effective concentration (see below). Incubation with 0.3 μM carbachol increased [Ca^{2+}] $_i$ in m3 mAChR-expressing HEK-293

Fig. 2. Concentration-dependent inhibition of m2 and m3 mAChR-induced $[Ca^{2+}]_i$ increases by DHS and DMS. $[Ca^{2+}]_i$ increases induced by 100 and 0.3 μ M carbachol were determined in m2 (upper panel) and m3 (lower panel) mAChR-expressing HEK-293 cells, pretreated for 10 min with the indicated concentrations of DMS ($\square$) or DHS ($\blacksquare$). Mean $\pm$ SEM of three independent experiments, each carried out at least in duplicate.

cells by 401 ± 82 nM ($n=16$). Pretreatment of the cells with DHS (30 μ M) or DMS (15 μ M) inhibited this m3 mAChR-induced $[Ca^{2+}]_i$ increase with similar pEC_{50} values (i.e. 4.58 ± 0.06 and 5.18 ± 0.01 , respectively) as in m2 mAChR-expressing HEK-293 cells stimulated by 100 μ M carbachol (Figure 2, lower panel). Inhibition of the m3 mAChR-mediated $[Ca^{2+}]_i$ increase induced by 0.3 μ M carbachol was essentially complete at 100 μ M DHS and 30 μ M DMS.

Recently, Putney and colleagues (Ribeiro *et al.*, 1997) reported that disruption of the cytoskeleton abolishes Ca^{2+} mobilization by ATP and PDGF in NIH 3T3 cells. However, treatment of m2 and m3 mAChR-expressing HEK-293 cells with cytochalasin B (10 μ M, 1 h at 37°C) did not affect either the control carbachol-induced $[Ca^{2+}]_i$ increase or its inhibition by DHS (data not shown). Furthermore, a number of experiments were carried out to confirm that DHS and DMS did not deplete intracellular Ca^{2+} stores. First, addition of DHS (30 μ M) or DMS (15 μ M) did not cause release of Ca^{2+} from internal stores during a 10 min incubation period, as measured by fura-2 fluorescence. Secondly, a 10 min treatment with DHS (30 μ M) or DMS (15 μ M) did not reduce $[Ca^{2+}]_i$ elevations induced by the endoplasmic reticulum Ca^{2+} -ATPase inhibitor, thapsigargin (2 μ M) ($n=3$, data not shown). Thirdly, incubation of HEK-293 cells for 10 min with DHS (30 μ M) did not reduce the cellular $^{45}Ca^{2+}$ content of $^{45}Ca^{2+}$ -preloaded cells (i.e. $98 \pm 2\%$ of control cells), whereas the Ca^{2+} ionophore, ionomycin (1 μ M), decreased cellular $^{45}Ca^{2+}$ by $76 \pm 3\%$ ($n=5$) in the same period.

Fig. 3. Influence of DHS and DMS on m2 and m3 mAChR-mediated PLC stimulation. Following 10 min treatment without (Control) and with 30 μ M DHS or 15 μ M DMS, formation of $[^3H]IP_3$ (upper panels) and total $[^3H]$ inositol phosphates ($[^3H]IPs$, lower panels) was determined in m2 and m3 mAChR-expressing HEK-293 cells in the absence (open bars) and presence (solid bars) of 100 μ M (m2 mAChR) or 0.3 μ M carbachol (m3 mAChR). Mean $\pm$ SEM ($n=4$) from one experiment, representative of at least two similar experiments.

The inhibitory action of DHS and DMS on Ca^{2+} signalling in HEK-293 cells was not due to inhibition of carbachol-receptor interaction, as measured by *N*- $[^3H]$ methylscopolamine-carbachol competition-binding experiments to m2 mAChR-expressing cells, or G-protein function, as determined by m2 mAChR-mediated inhibition of adenylyl cyclase. Furthermore, although DHS and DMS may act as protein kinase C inhibitors (Igarashi *et al.*, 1989), pretreatment of HEK-293 cells with the protein kinase C inhibitor, staurosporine (2 μ M), for 30 min did not affect either m2 or m3 mAChR-induced $[Ca^{2+}]_i$ elevations ($n=3$) (data not shown). Importantly, inhibition of Ca^{2+} signalling by DHS and DMS was not caused by diminished production of inositol phosphates. Pretreatment of HEK-293 cells for 10 min with 30 μ M DHS or 15 μ M DMS, causing a marked inhibition of Ca^{2+} mobilization, did not inhibit production of IP_3 or total inositol phosphates in m2 and m3 mAChR-expressing cells stimulated with 100 μ M and 0.3 μ M carbachol, respectively (Figure 3).

Recently, enhanced formation of cyclic ADP-ribose, apparently activating ryanodine-sensitive Ca^{2+} channels, has been implicated as an alternative Ca^{2+} signalling pathway for G-protein-coupled receptors. In membranes of NG108-15 neuroblastoma $\times$ glioma hybrid cells, formation of cyclic ADP-ribose by ADP-ribosyl cyclase is activated by m1 and m3 mAChRs, while m2 and m4 mAChRs are inhibitory (Higashida *et al.*, 1997). In HEK-293 cells, however, this pathway most likely does not contribute to Ca^{2+} signalling. No increase in $[Ca^{2+}]_i$ was observed upon the addition of caffeine (up to 40 mM) to activate ryanodine receptors. Likewise, pretreatment of

Fig. 4. Influence of receptor stimulation on DHS-induced inhibition of m2 and m3 mAChR-mediated $[\text{Ca}^{2+}]_i$ increases. Increase in $[\text{Ca}^{2+}]_i$ by the indicated concentrations of carbachol was determined in m2 (upper panel) and m3 (lower panel) mAChR-expressing HEK-293 cells pretreated for 10 min with (●) and without (○) 30 μM DHS. Mean $\pm$ SEM of three (m2 mAChR) or eight (m3 mAChR) independent experiments, each carried out at least in duplicate. *Significantly different from cells not treated with DHS ($p < 0.05$, two-tailed paired t -test).

HEK-293 cells with 100 μM ryanodine for 30 min to block ryanodine receptors had no influence on m2 or m3 mAChR-stimulated $[\text{Ca}^{2+}]_i$ increases (data not shown).

To determine whether the contribution of the sphingosine kinase pathway in Ca^{2+} signalling was dependent on the level of receptor stimulation, we studied the effect of DHS on m2 and m3 mAChR-mediated $[\text{Ca}^{2+}]_i$ rises at increasing carbachol concentrations (Figure 4). In m2 mAChR-expressing HEK-293 cells, pretreatment with DHS (30 μM) reduced $[\text{Ca}^{2+}]_i$ increases by 50–70% at all carbachol concentrations. In contrast, inhibition of $[\text{Ca}^{2+}]_i$ elevation in m3 mAChR-expressing cells was clearly dependent on the degree of receptor stimulation. While DHS suppressed $[\text{Ca}^{2+}]_i$ increase in response to 0.1 μM carbachol by 50%, the inhibitory effect of DHS on $[\text{Ca}^{2+}]_i$ increase was reduced to only 15% at 10 μM carbachol and was lost completely at ≥ 100 μM carbachol in m3 mAChR-expressing cells.

Sphingosine kinase-mediated Ca^{2+} signalling by various GPCRs

Besides mAChRs, other members of the superfamily of GPCRs were also found to utilize the sphingosine kinase pathway in a cell type-dependent manner. These included receptors which are endogenously expressed. As shown in Table II, pretreatment of HEK-293 cells for 10 min with 30 μM DHS significantly inhibited $[\text{Ca}^{2+}]_i$ increases

elicited by thrombin (1 U/ml) and lysophosphatidic acid (LPA, 1 μM). However, DHS (30 μM) did not affect the thrombin-induced $[\text{Ca}^{2+}]_i$ increase in J82 cells, a transitional-cell carcinoma cell line, whereas $[\text{Ca}^{2+}]_i$ responses to LPA and bradykinin (1 μM) were largely blunted. Similarly, DHS (30 μM) strongly inhibited $[\text{Ca}^{2+}]_i$ elevations induced by bradykinin and LPA receptors in opossum kidney cells. In contrast, in bovine aortic endothelial cells, concentration–response curves of bradykinin (0.1 nM to 1 μM) in rising $[\text{Ca}^{2+}]_i$ were not different in control cells and cells pretreated with 30 μM DHS (Table II). In conclusion, the contribution of sphingosine kinase to GPCR-mediated Ca^{2+} signalling is apparently dependent both on the respective receptor and the respective cell type.

Activation of sphingosine kinase by mAChRs in HEK-293 cells

To demonstrate that mAChRs activate sphingosine kinase, formation of $[\text{H}^3]\text{SPP}$ from $[\text{H}^3]\text{sphingosine}$ was determined in m2 and m3 mAChR-expressing HEK-293 cells. Activation of m2 mAChRs by 100 μM carbachol induced a rapid and transient increase in $[\text{H}^3]\text{SPP}$ production (Figure 5). After 30 s, the increase in $[\text{H}^3]\text{SPP}$ reached a maximum of $44 \pm 8\%$ relative to unstimulated cells. Likewise, stimulation of m3 mAChR-expressing HEK-293 cells with 0.3 μM carbachol rapidly and transiently increased $[\text{H}^3]\text{SPP}$ formation, reaching its maximum of $99 \pm 20\%$ after 30 s of receptor stimulation (Figure 5). Pretreatment of the cells with DHS (30 μM) or DMS (15 μM) blocked the mAChR-induced increases in $[\text{H}^3]\text{SPP}$ formation (Table III). These results indicate that sphingosine kinase is stimulated by m2 and m3 mAChRs in HEK-293 cells.

$[\text{Ca}^{2+}]_i$ increase by microinjected SPP in HEK-293 cells

Finally, we investigated whether microinjected SPP was able to release Ca^{2+} from intracellular stores in HEK-293 cells. To ensure that Ca^{2+} mobilization did not occur via activation of the SPP receptor in the plasma membrane of HEK-293 cells as described previously (van Koppen *et al.*, 1996), cells were pretreated with PTX which completely abolished the $[\text{Ca}^{2+}]_i$ increase by extracellularly applied SPP (data not shown). Typical traces obtained after injection of SPP and IP_3 in HEK-293 cells are shown in Figure 6. Injection of intracellular buffer solution containing 1 mM SPP rapidly and transiently increased $[\text{Ca}^{2+}]_i$ in 82 of 99 cells (83% positive), whereas the vehicle-containing solution could evoke $[\text{Ca}^{2+}]_i$ increases in only 10 of 61 cells (16% positive) (Figure 6A and B). Injection of *N*-acetylsphingosine (1 mM) elevated $[\text{Ca}^{2+}]_i$ in only four of 20 cells (20% positive, i.e. not different from vehicle-containing solution), strongly suggesting that SPP did not act through a non-specific mechanism (data not shown). The ability of intracellular SPP to raise $[\text{Ca}^{2+}]_i$ was concentration dependent, with 100 μM SPP in the pipette solution increasing $[\text{Ca}^{2+}]_i$ in 18 of 19 cells (95% positive) and 10 μM SPP being effective in only three of 15 cells (20% positive, i.e. not different from vehicle-containing solution). Thus, the threshold concentration of SPP in the pipette for increasing $[\text{Ca}^{2+}]_i$ lies between 10 and 100 μM (Figure 6C and D). Pretreatment of the cells

Table II. Effect of DHS on $[Ca^{2+}]_i$ increases by various G-protein-coupled receptors

Cell type	Thrombin		LPA		Bradykinin	
	Control (nM)	DHS (%)	Control (nM)	DHS (%)	Control (nM)	DHS (%)
HEK-293	469±35	56±9 ^a	337±55	38±14 ^a	n.d.	
J82	67±9	82±12	615±23	34±6 ^a	430±36	40±7 ^a
OK	n.d.		469±36	41±8 ^a	299±7	35±5 ^a
BAEC	n.d.		n.d.		291±124	102±2 ^b

Following pretreatment with vehicle (Control) or 30 μ M DHS for 10 min, agonist-induced $[Ca^{2+}]_i$ increases were determined by measuring fura-2 fluorescence in HEK-293 cells, J82 transitional-cell carcinoma cells, opossum kidney cells (OK) and bovine aortic endothelial cells (BAEC). Peak $[Ca^{2+}]_i$ increases in control cells (in nM) and DHS-treated cells (as a percentage of those in control cells) are the mean±SEM of 4–13 experiments. Concentrations applied were 1 U/ml thrombin, 1 μ M lysophosphatidic acid (LPA) and 1 μ M bradykinin.

^a $[Ca^{2+}]_i$ increase is significantly inhibited by DHS pretreatment ($p < 0.05$, t -test).

^bEven at lower concentrations of bradykinin (0.1–100 nM), no influence of DHS was apparent. n.d., not determined.

with 5 mM extracellular EGTA to eliminate Ca^{2+} influx diminished and shortened SPP-induced Ca^{2+} spikes (Figure 6E). However, $[Ca^{2+}]_i$ increases were still present in nine of 11 cells (82% positive), suggesting that the SPP-induced $[Ca^{2+}]_i$ increases are due to intracellular Ca^{2+} mobilization leading to a secondary Ca^{2+} influx. For comparison, IP_3 (1 mM) was also microinjected, causing $[Ca^{2+}]_i$ increases in 47 of 56 cells (84% positive). Ca^{2+} spikes induced by microinjected IP_3 were similar to SPP-induced Ca^{2+} spikes in terms of rapidity, magnitude and reversibility (Figure 6F). Coinjection of heparin (100 μ g/ml) largely blocked IP_3 -induced Ca^{2+} spikes in 16 of 26 cells (38% positive), whereas SPP-induced Ca^{2+} spikes were unaffected in 28 of 42 cells (67% positive). Conversely, pretreatment of HEK-293 cells with DHS (30 μ M) did not alter IP_3 -induced Ca^{2+} release in 16 of 17 cells (94% positive) (data not shown). Thus, it seems likely that SPP and IP_3 act via distinct intracellular Ca^{2+} release channels in HEK-293 cells, as has been shown by others in other cellular systems.

Discussion

Here, we provide compelling evidence that Ca^{2+} signalling by mAChRs in HEK-293 cells involves the activation of sphingosine kinase and the formation of SPP as second messenger. First, pretreatment of HEK-293 cells with two distinct inhibitors of sphingosine kinase, DHS and DMS, potently reduced m2 and m3 mAChR-induced increases in $[Ca^{2+}]_i$, with IC_{50} values in the ranges of those described for the inhibition of sphingosine kinase activity (Buehrer and Bell, 1992; Olivera and Spiegel, 1993; Olivera *et al.*, 1994; Choi *et al.*, 1996; Yatomi *et al.*, 1996). Control experiments excluded the possibility that suppression of Ca^{2+} signalling by DHS and DMS was caused by a reduction in agonist–mAChR binding, G-protein activation, depletion of internal Ca^{2+} stores, disruption of the cytoskeleton or inhibition of protein kinase C. Furthermore, the sphingosine kinase inhibitors did not perturb the PLC– IP_3 pathway. Pretreatment of the cells with DHS or DMS did not reduce mAChR-induced IP_3 production or alter Ca^{2+} spikes induced by microinjected IP_3 . In addition, m2 and m3 mAChRs rapidly and transiently increased the conversion of sphingosine into SPP in intact HEK-293 cells, with a time course similar to that for mAChR-induced $[Ca^{2+}]_i$ elevations. The small difference

Fig. 5. Stimulation of $[^3H]$ SPP formation by m2 and m3 mAChRs. Formation of $[^3H]$ SPP from $[^3H]$ sphingosine was determined in m2 and m3 mAChR-expressing HEK-293 cells for the indicated periods in the absence and presence of 100 μ M (m2 mAChR) or 0.3 μ M (m3 mAChR) carbachol. Values are expressed as percentage increase relative to the radioactivity in unstimulated cells and are mean±SEM of three or more independent experiments, each carried out at least in triplicate. Incubation with carbachol did not alter the uptake of $[^3H]$ sphingosine. *Significantly different from unstimulated cells ($p < 0.05$, two-tailed paired t -test).

Table III. Inhibition of receptor-stimulated $[^3H]$ SPP formation by DHS and DMS

Pretreatment	$[^3H]$ SPP formed (c.p.m./ 10^6 cells)	
	Basal	Carbachol
Control	179±27	305±63
DHS (30 μ M)	26±1	22±29
DMS (15 μ M)	41±4	58±11

Following pretreatment of HEK-293 cells expressing m2 mAChRs for 10 min with vehicle (Control), 30 μ M DHS or 15 μ M DMS, basal and receptor-stimulated formation of $[^3H]$ SPP from $[^3H]$ sphingosine was determined for 30 s in the absence and presence of 100 μ M carbachol, respectively. Data shown are mean±SD, and corrected for t_0 values as in Table I. This experiment was performed twice in triplicate, yielding equivalent results.

between peak $[^3H]$ SPP formation and $[Ca^{2+}]_i$ elevation most likely results from the time span required for extracellularly applied $[^3H]$ sphingosine to cross the plasma membrane and reach intracellular sphingosine kinase. The magnitude of mAChR-mediated SPP production in HEK-293 cells (40–120% over basal) was comparable with that reported for PDGF receptors in Swiss-3T3 cells and

Fig. 6. Fura-2 imaging of single HEK-293 cells injected with SPP and IP_3 . $[\text{Ca}^{2+}]_i$ was measured in adherent m2 mAChR-expressing HEK-293 cells pretreated with PTX (75 ng/ml, 16 h). In the panels, several individual fluorescence traces representative of between 8 and 40 recordings are shown. Arrows indicate time points of injection. Injection buffer solution contained (A) vehicle (control), (B) 1 mM SPP, (C) 100 μM SPP, (D) 10 μM SPP, (E) 1 mM SPP after pretreatment for 30–120 s with 5 mM EGTA, or (F) 1 mM IP_3 .

cultured arterial smooth muscle cells (Olivera *et al.*, 1993; Bornfeldt *et al.*, 1995), as well as Fc ϵ RI receptors in RBL-2H3 mast cells (Choi *et al.*, 1996), i.e. 40–120% and 60–100%, respectively. Furthermore, mAChR-induced sphingosine kinase activation was abolished by pretreatment of the cells with DHS or DMS. Finally, injection of SPP into HEK-293 cells caused release of Ca^{2+} from internal stores in a heparin-insensitive manner, with a time course and magnitude comparable with that following injection of IP_3 .

Sphingosine kinase was stimulated by both m2 and m3 mAChRs in HEK-293 cells, and Ca^{2+} signalling by both mAChR subtypes was sensitive to inhibition by DHS and DMS. Moreover, Ca^{2+} signalling by bradykinin receptors, which strongly activate PLC (Meyer zu Heringdorf *et al.*, 1996; Lmmen *et al.*, 1997), was inhibited significantly by DHS, at least in J82 transitional-cell carcinoma and opossum kidney cells. Thus, the sphingosine kinase pathway is apparently utilized, not only by GPCRs which couple inefficiently to PLC, but also by those which strongly activate PLC. However, the contribution of the sphingosine kinase pathway to overall Ca^{2+} signalling was larger for the m2 mAChR, which weakly activates PLC, and smaller for the m3 mAChR, which strongly stimulates formation of IP_3 to trigger Ca^{2+} release. As

illustrated in Figure 4, the level of m3 mAChR agonist activation apparently determined the Ca^{2+} signalling pathway used, i.e. the sphingosine kinase–SPP pathway at low agonist occupancy, and the PLC– IP_3 pathway at high agonist occupancy. This may help to explain the discrepancy often observed with GPCRs, that at low agonist concentrations, even in the absence of measurable inositol phosphate production, Ca^{2+} mobilization is nevertheless stimulated. Recently, Choi *et al.* (1996) reported that in m1 mAChR-transfected RBL-2H3 mast cells, Ca^{2+} signals induced by 100 μM carbachol were unaffected by pretreatment with DHS, while Ca^{2+} responses following Fc ϵ RI antigen receptor stimulation were blunted significantly. This observation led the authors to propose that Ca^{2+} signalling by m1 mAChRs is sphingosine kinase-independent. However, similar to m3 mAChRs in HEK-293 cells, m1 mAChRs in mast cells couple efficiently to PLC. Thus, the carbachol concentration of 100 μM was apparently too high to detect involvement of sphingosine kinase in m1 mAChR-mediated Ca^{2+} signalling.

We also demonstrate that Ca^{2+} signalling by GPCRs can be independent of sphingosine kinase activation. For example, $[\text{Ca}^{2+}]_i$ increases induced by thrombin in J82 cells and by bradykinin in bovine aortic endothelial cells were not inhibited by DHS. Recently, Sugawara *et al.* (1997) demonstrated in B-cell-derived DT40 cells, in which all three types of IP_3 receptors had been deleted, that Ca^{2+} mobilization by transfected m1 mAChRs was abolished completely. Thus, a second messenger function of SPP in Ca^{2+} signalling by GPCRs may be not only receptor-specific but also dependent on the cell type, for example absent in some cells such as DT40 B cells and bovine aortic endothelial cells. However, the study by Sugawara *et al.* (1997) may indicate that an intact IP_3 signalling pathway is required for sphingosine kinase-mediated Ca^{2+} mobilization. At present, it is not clear whether the PLC– IP_3 and the sphingosine kinase–SPP pathways are fully independent Ca^{2+} signalling pathways. As IP_3 and SPP are generated in a similar time-frame, there may be a concerted action of SPP and IP_3 in releasing Ca^{2+} from internal stores.

Other open questions to be addressed in future studies are the mechanisms and signalling components by which plasma membrane receptors, tyrosine kinase receptors and GPCRs, activate sphingosine kinase. There may be multiple forms of sphingosine kinase, as sphingosine kinase activity has been shown to exist in both cytosolic (Louie *et al.*, 1974; Olivera and Spiegel, 1994) and membrane-associated fractions (Buehrer and Bell, 1992; Ghosh *et al.*, 1994). Also, the exact intracellular target of SPP in HEK-293 cells is currently unknown. It is likely that SPP operates by activating the putative sphingolipid-gated Ca^{2+} channel in the endoplasmic reticulum (Ghosh *et al.*, 1990, 1994; Mattie *et al.*, 1994). Our data add further evidence for the notion that SPP is a versatile molecule, acting as a primary messenger stimulating high-affinity plasma membrane receptors coupled to heterotrimeric G-proteins (Bnemann *et al.*, 1996; Meyer zu Heringdorf *et al.*, 1996; Postma *et al.*, 1996; van Koppen *et al.*, 1996), as well as fulfilling the role of a second messenger that stimulates a putative Ca^{2+} channel in the endoplasmic reticulum.

In conclusion, this study demonstrates for the first time

that the Ca^{2+} signalling pathway based on sphingosine kinase activation is not restricted to tyrosine kinase-linked receptors but is also used by members of the GPCR superfamily in a cell type-dependent manner. As both IP_3 and SPP are generated upon GPCR stimulation within a similar time frame, cells can apparently utilize both pathways to mobilize Ca^{2+} from internal stores. Contribution of sphingosine kinase to overall Ca^{2+} signalling is apparently dependent on the coupling selectivity of the respective receptor, which in turn is dependent on the level of receptor activation. Since GPCRs constitute the largest family of membrane receptors and regulate numerous cellular processes by increasing $[\text{Ca}^{2+}]_i$, the present findings are of far-reaching and immediate significance.

Materials and methods

Materials

SPP, *N*-acetylsphingosine, DHS and IP_3 were from Biomol. *D*-erythro-sphingosine was from Calbiochem and DMS from Matreya. $[\text{^3H}]$ Sphingosine, *D*-erythro (15 Ci/mmol) was purchased from ARC. Stock solutions of the lipids were made in methanol or isopropanol. Immediately before use, sphingolipid solutions were dried and dissolved in phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS) or 25 mM HEPES-buffered DME-F12 medium, containing 1 mg/ml bovine serum albumin (BSA). Control tubes or plates received the same buffer solution without added sphingolipid.

$[\text{Ca}^{2+}]_i$ measurements

HEK-293 cells stably expressing human m2 or m3 mAChRs were cultured in DME-F12 medium containing 10% fetal calf serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin (Schmidt *et al.*, 1995). $[\text{Ca}^{2+}]_i$ was determined using the fluorescent calcium indicator dye fura-2 (Molecular Probes) in a Hitachi spectrofluorimeter as described previously (Meyer zu Heringdorf *et al.*, 1996). Briefly, cells were loaded with 3 μM fura-2-AM for 1 h at 37°C in PBS (137 mM NaCl, 3 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 7 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 1 mg/ml BSA and 5 mM glucose pH 7.4). Thereafter, cells were washed twice, resuspended in fresh buffer and used for fluorescence measurements within the next hour.

PLC assay

PLC activity in HEK-293 cells was determined as described previously (Schmidt *et al.*, 1995). In brief, after labelling for 48 h with *myo*- $[\text{^3H}]$ inositol (2.5 $\mu\text{Ci}/\text{ml}$), cells were equilibrated for 10 min at 37°C in 1 ml HBSS (118 mM NaCl, 15 mM HEPES, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 and 5 mM glucose pH 7.4) containing 1 mg/ml BSA with or without DHS (30 μM) or DMS (15 μM). Cells were then incubated with carbachol at 37°C for either 15 s, to measure formation of $[\text{^3H}]\text{IP}_3$, or 30 min in the presence of 10 mM LiCl, to measure formation of total $[\text{^3H}]$ inositol phosphates. The reactions were stopped and labelled phospholipids and inositol phosphates were analysed as described previously (Schmidt *et al.*, 1995).

Measurement of $[\text{^3H}]$ SPP formation

HEK-293 cells, on 35 mm plates, were equilibrated in 1 ml of 25 mM HEPES-buffered DME-F12 medium pH 7.4, containing 1 mg/ml fatty-acid-free BSA for 10 min at 37°C. The reactions were started by the addition of 100 μl medium containing $[\text{^3H}]$ sphingosine (~200 000 c.p.m., final concentration ~15 nM), carbachol (final concentration either 0.3 or 100 μM) and 1 mg/ml fatty-acid-free BSA. Following incubation at 37°C for the indicated times, the medium was rapidly removed and 0.5 ml of ice-cold methanol was added to the plates. The cells were scraped into polypropylene tubes, and the plates were rinsed a second time with 0.5 ml methanol. One millilitre of chloroform together with 1 ml methanol were added to the tubes. After vortexing at high speed for 10 s, samples were centrifuged at 2000 g for 10 min to remove any particulate matter. The pellets were re-extracted with 3 ml chloroform:methanol (1:2), and the pooled supernatants were evaporated to dryness in a Speedvac centrifuge. After redissolving in methanol, samples were spotted onto Silica gel 60 TLC plates (E.Merck). Authentic sphingosine and SPP were applied with the samples for identification of $[\text{^3H}]$ SPP and $[\text{^3H}]$ sphingosine. Separation of the products was achieved

by TLC in 1-butanol:acetic acid:water (3:1:1) as solvent system. Sphingosine (R_f 0.67) and SPP (R_f 0.51) spots were visualized with ninhydrin (staining sphingosine and SPP) and molybdenum blue (staining SPP). After scraping off the spots, radioactivity was measured by liquid scintillation counting. Authenticity of $[\text{^3H}]$ SPP and $[\text{^3H}]$ sphingosine was verified in two other solvent systems, i.e. chloroform:methanol:water (60:35:8) and chloroform:methanol:acetic acid:water (90:90:6:15).

Microinjection and $[\text{Ca}^{2+}]_i$ imaging

Following pretreatment with 75 ng/ml PTX for 16 h (to block G-protein-coupled SPP receptors in the plasma membrane), HEK-293 cells, on 35 mm dishes, were loaded for 60 min at 37°C with 3 μM fura-2-AM in 25 mM HEPES-buffered DME-F12 medium. After washing, cells were transferred for Ca^{2+} imaging to the thermostatted stage of an inverted Zeiss Axiovert 100 fluorescence microscope heated to 37°C. Fluorescence measurements were performed using the Attofluor digital fluorescence imaging system (Atto Instruments). Cytosolic $[\text{Ca}^{2+}]_i$ was determined by ratio imaging of fura-2 fluorescence, using excitation wavelengths of 340 and 380 nm and an emission wavelength of 520 nm. The data sampling rate was usually a single ratio measurement every 5 s. Regions of interest were defined to cover as much of the area of each single cell as possible, and mean ratios were calculated for each region using software supplied by the manufacturer. Extracellular SPP, EGTA or DHS was added in 25 mM HEPES-buffered DME-F12 medium. The compounds to be injected were diluted in 27 mM K_2HPO_4 , 8 mM Na_2HPO_4 , 26 mM KH_2PO_4 and 1 mg/ml BSA pH 7.2. Microinjection of samples in Femtotips II (Eppendorf) was performed with an Eppendorf micromanipulator type 5171 system with a transjector type 5246. The system was run in the semiautomatic mode with the following instrumental set-up conditions: pipette angle 45°, injection pressure 80 hPa, injection time 0.1 s and velocity of the pipette 700 $\mu\text{m}/\text{s}$.

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