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, besides PLC, in mediation of GPCR-induced Ca\(^{2+}\) mobilization by GPCRs has also been demonstrated. Examples include Ca\(^{2+}\) signalling by \(\alpha_2\)-adrenoceptors (Michel et al., 1989), neuropeptide Y receptors (Motulsky and Michel, 1988), purinergic receptors (Frelin et al., 1993), parathyroid hormone receptors (Souwen and Boddeke, 1995) and thryrotropin-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 DDT, MF-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 FcεRI 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 increase in [Ca\(^{2+}\)], 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 \(\alpha\) 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 \(\alpha_2\)-adrenoceptors (Michel et al., 1989), neuropeptide Y receptors (Motulsky and Michel, 1988), purinergic receptors (Frelin et al., 1993), parathyroid hormone receptors (Souwen and Boddeke, 1995) and thryrotropin-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.
sphingosine kinase inhibitors applied [3H]sphingosine into [3H]SPP by 70–80% (Table 1). Pretreatment of HEK-293 cells. Pretreatment of the cells with 30 μM DHS (30 μM) effectively blocked sphingosine kinase activity in HEK-293 cells. Pretreatment of the cells for 10 min with 30 μM DHS (30 μM) reduced [Ca\textsuperscript{2+}]\textsubscript{i} by 44–74% and 79±4%, respectively (Figure 1B and C). In contrast, N-acetylsphingosine (30 μM), which differs from DHS in having an -NHCOCH\textsubscript{3} group instead of an -N(CH\textsubscript{2})\textsubscript{3} 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\textsuperscript{2+}]\textsubscript{i} increase (Figure 1D). Also, sphingosine (30 μM) did not alter m2 mAChR-induced [Ca\textsuperscript{2+}]\textsubscript{i} elevations (data not shown).

The inhibitory effect of DHS and DMS on m2 mAChR-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase was concentration dependent, with pEC\textsubscript{50} values of 4.70±0.04 and 5.15±0.03, respectively (Figure 2, upper panel). The corresponding IC\textsubscript{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\textsuperscript{2+}]\textsubscript{i} increase elicited by 100 μM carbachol was observed in cells pretreated with 60 μM DHS or 30 μM DMS. Since Ca\textsuperscript{2+}\textsubscript{i} 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\textsuperscript{2+}\textsubscript{i} mobilization. Indeed, DHS (30 μM) reduced [Ca\textsuperscript{2+}]\textsubscript{i} increase by carbachol (100 μM) in PTX-treated m2 mAChR-expressing cells by a magnitude (58±8%, n=3) similar to that seen in control cells.

For comparison, the effects of the sphingosine kinase inhibitors on Ca\textsuperscript{2+}\textsubscript{i} signalling were studied in HEK-293 cells expressing the highly efficient PLC stimulatory m3 mAChR. The m3 mAChR-mediated [Ca\textsuperscript{2+}]\textsubscript{i} elevations in m3 and m2 mAChR-expressing cells are of comparable magnitude. However, m3 mAChR-mediated [Ca\textsuperscript{2+}]\textsubscript{i} increases are fully PTX-insensitive and mainly (almost 90%) due to Ca\textsuperscript{2+}\textsubscript{i} influx (Schmidt et al., 1995). As the EC\textsubscript{50} values for carbachol for rising [Ca\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{i} in m3 mAChR-expressing HEK-293

### Results

**Inhibition of mAChR-mediated Ca\textsuperscript{2+}\textsubscript{i} signalling by sphingosine kinase inhibitors**

To test the hypothesis that m2 mAChR-induced Ca\textsuperscript{2+}\textsubscript{i} 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\textsuperscript{2+}]\textsubscript{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\textsuperscript{2+}]\textsubscript{i}, by 447±187 nM (n=132) (Figure 1A). Pretreatment of the cells for 10 min with 30 μM DHS or 15 μM DMS inhibited the agonist-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase by 74±4% and 79±9%, respectively (Figure 1B and C). In contrast, N-acetylsphingosine (30 μM), which differs from DHS in having an -NHCOCH\textsubscript{3} group instead of an -N(CH\textsubscript{2})\textsubscript{3} 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\textsuperscript{2+}]\textsubscript{i} increase (Figure 1D). Also, sphingosine (30 μM) did not alter m2 mAChR-induced [Ca\textsuperscript{2+}]\textsubscript{i} elevations (data not shown).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[3H]SPP formed (c.p.m./10\textsuperscript{6} cells)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>223±12</td>
</tr>
<tr>
<td>DHS (30 μM)</td>
<td>46±3</td>
</tr>
<tr>
<td>DMS (15 μM)</td>
<td>61±10</td>
</tr>
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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\textsubscript{0} and basal formation of [3H]SPP was determined for either 30 s or 2 min. Values shown are mean±SD of two independent experiments, each performed in triplicate, and corrected for t\textsubscript{0} count rates of 39±5 c.p.m./10\textsuperscript{6} cells. Pretreatment with DHS or DMS did not alter the cellular uptake of [3H]sphingosine.
cells by 401±82 nM (n=16). Pretreatment of the cells with DHS (30 μM) or DMS (15 μM) inhibited this m3 mAChR-induced \([\text{Ca}^{2+}]_i\) with similar pEC\textsubscript{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 μM carbachol (Figure 2, lower panel). Inhibition of the m3 mAChR-mediated \([\text{Ca}^{2+}]_i\) increased by 0.3 μM carbachol was essentially complete at 100 μM DHS and 30 μM DMS.

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

The inhibitory action of DHS and DMS on \(\text{Ca}^{2+}\) signalling in HEK-293 cells was not due to inhibition of carbachol–receptor interaction, as measured by \(\text{N}-\text{[\text{H}]methylscopolamine}-\text{carbachol}\) 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 μM), for 30 min did not affect either m2 or m3 mAChR-induced \([\text{Ca}^{2+}]_i\) elevations (n=3) (data not shown). Importantly, inhibition of \(\text{Ca}^{2+}\) signalling by DHS and DMS was not caused by diminished production of inositol phosphates. Pretreatment of HEK-293 cells for 10 min with 30 μM DHS or 15 μM DMS, causing a marked inhibition of \(\text{Ca}^{2+}\) mobilization, did not inhibit production of IP\(_3\) or total inositol phosphates in m2 and m3 mAChR-expressing cells stimulated with 100 μM and 0.3 μM carbachol, respectively (Figure 3).

Recently, enhanced formation of cyclic ADP-ribose, apparently activating ryanodine-sensitive \(\text{Ca}^{2+}\) channels, has been implicated as an alternative \(\text{Ca}^{2+}\) 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 \(\text{Ca}^{2+}\) signalling. No increase in \([\text{Ca}^{2+}]_i\) was observed upon the addition of caffeine (up to 40 mM) to activate ryanodine receptors. Likewise, pretreatment of
elicted 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 \(\text{Ca}^{2+}\) 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 \(^{[\text{H}]\text{SPP}}\) from \(^{[\text{H}]\text{sphingosine}}\) 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 \(^{[\text{H}]\text{SPP}}\) production (Figure 5). After 30 s, the increase in \(^{[\text{H}]\text{SPP}}\) reached a maximum of 44±8% relative to unstimulated cells. Likewise, stimulation of m3 mAChR-expressing HEK-293 cells with 0.3 μM carbachol rapidly and transiently increased \(^{[\text{H}]\text{SPP}}\) formation, reaching its maximum of 99±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 \(^{[\text{H}]\text{SPP}}\) 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 \(\text{Ca}^{2+}\) from intracellular stores in HEK-293 cells. To ensure that \(\text{Ca}^{2+}\) 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 IP3 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-acetylphosphatidylcholine (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...
with 5 mM extracellular EGTA to eliminate Ca\(^{2+}\) influx diminished and shortened SPP-induced Ca\(^{2+}\) spikes (Figure 6E). However, [Ca\(^{2+}\)], increases were still present in nine of 11 cells (82% positive), suggesting that the SPP-induced [Ca\(^{2+}\)], 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\(^{2+}\)], 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 amplitude, width and reversibility (Figure 6F). Coinjection of heparin (100 \(\mu\)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 \(\mu\)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\(^{2+}\)], 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 internal 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\(^{2+}\)], elevations. The small difference

Table II. Effect of DHS on [Ca\(^{2+}\)], increases by various G-protein-coupled receptors

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Thrombin</th>
<th>LPA</th>
<th>Bradykinin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (nM)</td>
<td>DHS (%)</td>
<td>Control (nM)</td>
</tr>
<tr>
<td>HEK-293</td>
<td>469±35</td>
<td>56±9(a)</td>
<td>337±55</td>
</tr>
<tr>
<td>J82</td>
<td>67±9</td>
<td>82±12</td>
<td>615±23</td>
</tr>
<tr>
<td>OK</td>
<td>n.d.</td>
<td>469±36</td>
<td>41±8(a)</td>
</tr>
</tbody>
</table>

Following pretreatment with vehicle (Control) or 30 \(\mu\)M DHS for 10 min, agonist-induced [Ca\(^{2+}\)], 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\(^{2+}\)], 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 \(\mu\)M lysophosphatidic acid (LPA) and 1 \(\mu\)M bradykinin.

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

\(^{b}\)Even at lower concentrations of bradykinin (0.1–100 nM), no influence of DHS was apparent. n.d., not determined.

Fig. 5. Stimulation of [\(^{3}\)H]SPP formation by m2 and m3 mACHRs.

Formation of [\(^{3}\)H]SPP from [\(^{3}\)H]sphingosine was determined in m2 and m3 mACHR-expressing HEK-293 cells for the indicated periods in the absence and presence of 100 \(\mu\)M (m2 mACHR) or 0.3 \(\mu\)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 [\(^{3}\)H]sphingosine. *Significantly different from unstimulated cells (\(p<0.05\), two-tailed paired t-test).

Table III. Inhibition of receptor-stimulated [\(^{3}\)H]SPP formation by DHS and DMS

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>[(^{3})H]SPP formed (c.p.m./10(^6) cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Control</td>
<td>179±27</td>
</tr>
<tr>
<td>DHS (30 (\mu)M)</td>
<td>26±1</td>
</tr>
<tr>
<td>DMS (15 (\mu)M)</td>
<td>41±4</td>
</tr>
</tbody>
</table>

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

between peak [\(^{3}\)H]SPP formation and [Ca\(^{2+}\)], elevation most likely results from the time span required for extracellularly applied [\(^{3}\)H]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
mAChRs in HEK-293 cells, and Ca$^{2+}$ after pretreatment for 30–120 s with 5 mM EGTA, or (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$_3$.

Fig. 6. Fura-2 imaging of single HEK-293 cells injected with SPP and IP$_3$. [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 FcεRI 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; Lümmen 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 FcεRI 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, [Ca$^{2+}$], 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 signalling 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 (Bünemann 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<sup>2+</sup> 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<sub>3</sub> and SPP are generated upon GPCR stimulation within a specific cellular environment by increasing [Ca<sup>2+</sup>]<sub>i</sub>, the present findings are of far-reaching and immediate significance.

**Materials and methods**

**Materials**

SPP, N-acetyl-sphingosine, DHS and IP<sub>3</sub> were from Biomol, 3-epathydro- and 3-erythro-sphingosine were from Calbiochem and DMS from Matrex. [H<sup>3</sup>]Sphingosine, 3-erythro (15 Ci/mmol) was purchased from ARC. Stock solutions of the lipids were made in mehtanol 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.

**[Ca<sup>2+</sup>]<sub>i</sub> 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). [Ca<sup>2+</sup>]<sub>i</sub> 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<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 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-[H<sup>3</sup>]inositol (2.5 μCi/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<sub>2</sub>, 1 mM MgCl<sub>2</sub> 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 carbochal at 37°C for either 15 s to measure formation of [HJIP<sub>3</sub>], or 30 min in the presence of 10 mM LiCl to measure formation of total [H<sup>3</sup>]inositol phosphates. The reactions were stopped and labelled phospholipids and inositol phosphates were analysed as described previously (Schmidt et al., 1995).

**Measurement of [H<sup>3</sup>]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 μM medium containing [H<sup>3</sup>]sphingosine (~200 000 c.p.m., final concentration ~15 μM), 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 (EMerck). Authentic sphingosine and SPP were applied with the samples for identification of [H<sup>3</sup>]SPP and [H<sup>3</sup>]sphingosine. Separation of the products was achieved by TLC in l-butanol:acetic acid:water (3:1:1) as solvent system. Sphingosine (R<sub>t</sub> 0.67) and SPP (R<sub>t</sub> 0.51) spots were visualized with ninhydrin (staining sphingosine and SPP) and molybdenium blue (staining SPP). After scraping off the spots, radioactivity was measured by liquid scintillation counting. Authenticity of [H<sup>3</sup>]SPP and [H<sup>3</sup>]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 [Ca<sup>2+</sup>]<sub>i</sub> imaging**

Following pretreatment with 75 mg/ml PTFX 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<sup>2+</sup> 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 [Ca<sup>2+</sup>]<sub>i</sub> was determined using the 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 were injected within 30 s. After injection of 27 mM K<sub>2</sub>HPO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 26 mM KH<sub>2</sub>PO<sub>4</sub> and 1 mg/ml BSA pH 7.4. Microinjection of samples in Femtotips II (Eppendorf) was performed with an Eppendorf micromanipulator type 5171 system with a transrector type 5246. The system was run in the semiautomatic mode with the following instrumental set-up conditions: pipette angle 45°, injection pressure 80 kPa, injection time 0.1 s and velocity of the pipette 700 μm/s.

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**References**


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