# Depolarisation induces rapid and transient formation of intracellular sphingosine-1-phosphate

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Received 30 July 2001; revised 26 October 2001; accepted 5 November 2001

First published online 21 November 2001

Edited by Felix Wieland

**Abstract Formation of sphingosine-1-phosphate (SPP) by sphingosine kinase serves as a signalling pathway for various membrane receptors. Here, we show that membrane depolarisation is another mechanism by which this pathway can be activated. Formation of [$^3$H]SPP as well as levels of endogenous SPP were rapidly and transiently increased in PC12 pheochromocytoma cells depolarised with high KCl. Time course and maximum were similar to those induced by bradykinin. Depolarisation-induced SPP production was also observed in RINm5F insulinoma cells, dependent on extracellular $Ca^{2+}$ and fully suppressed by verapamil, thus apparently caused by $Ca^{2+}$ influx via voltage-gated $Ca^{2+}$ channels. Studies with sphingosine kinase inhibitors and overexpression of sphingosine kinase revealed a partial contribution of this pathway to depolarisation-induced noradrenaline release and $Ca^{2+}$ increase. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Sphingosine kinase; Sphingosine-1-phosphate; KCl depolarisation; Bradykinin; Noradrenaline release; PC12 cell

## 1. Introduction

Sphingosine-1-phosphate (SPP), produced from sphingosine by sphingosine kinase, acts as extracellular lipid mediator binding to and activating specific G-protein-coupled receptors (GPCRs) of the EDG receptor family [1,2]. There is, however, also ample evidence that SPP can serve as intracellular signalling molecule involved in $Ca^{2+}$ mobilisation and mitogenesis [3–6]. Several plasma membrane receptors, such as antigen receptors, receptor tyrosine kinases and various GPCRs, have been reported to stimulate sphingosine kinase activity and to increase intracellular SPP accumulation in diverse cell types [7]. Inhibition of sphingosine kinase by DL-*threo*-dihydrosphingosine (tDHS) or *N,N*-dimethylsphingosine (DMS) attenuated or blocked some cellular responses to these receptors, while others were not affected [4,8–10]. Specifically, these data and additional experimental approaches, including overexpression of sphingosine kinases, provided evidence for an involvement of intracellular SPP in stimulation of mitogenesis and inhibition of apoptosis [3,11]. Furthermore, microinjected SPP has been shown to cause $Ca^{2+}$ release from intracellular stores, in a manner similar to inositol-1,4,5-trisphosphate and independent of SPP receptors [4]. Together with the findings that receptor-induced intracellular SPP production is very fast and sphingosine kinase inhibitors attenuate $Ca^{2+}$ signalling by these receptors, it was proposed that intracellular SPP production can serve as a pathway for intracellular $Ca^{2+}$ release [4,6–8,10,12].

Although two mammalian sphingosine kinases have been cloned recently [13–17], very little is known how these enzymes are activated by membrane receptors. Protein kinase C (PKC) and cyclic AMP have been reported to regulate sphingosine kinase activity in certain cell types [7]. Interestingly, there are independent reports on the $Ca^{2+}$ dependence of sphingosine kinase activation [18,19]. In HL-60 cells, intracellular SPP production was stimulated by $Ca^{2+}$ ionophores, and GPCR agonist (fMLP or ATP)-induced SPP production was inhibited by chelation of intracellular $Ca^{2+}$ [18]. Similarly, in TRMP cells, sphingosine kinase activity was enhanced by intracellular $Ca^{2+}$ release induced by thapsigargin, and chelation of intracellular $Ca^{2+}$ blocked the stimulatory effect of platelet-derived growth factor [19]. These findings led us to investigate whether intracellular SPP production can be induced by $Ca^{2+}$ influx via voltage-gated $Ca^{2+}$ channels, a major mechanism for intracellular $Ca^{2+}$ increase in excitable cells. We demonstrate here that, similar to the GPCR agonist bradykinin, KCl depolarisation of PC12 pheochromocytoma cells leads to rapid and transient SPP production, apparently caused by $Ca^{2+}$ influx via voltage-gated $Ca^{2+}$ channels. Furthermore, evidence is provided suggesting that the sphingosine kinase/SPP pathway contributes, at least in part, to the depolarisation-induced neurotransmitter release in these cells.

## 2. Materials and methods

### 2.1. Materials

D-*erythro*-[3-$^3$H]Sphingosine (20 Ci/mmol) and L-[*ring*-2,5,6-$^3$H]-noradrenaline (55 Ci/mmol) were purchased from NEN Life Science Products (Köln, Germany). SPP, tDHS and DMS were from Biomol (Hamburg, Germany), ionomycin, Gö 6976 and staurosporine from Calbiochem-Novabiochem (Bad Soden, Germany), and bradykinin,

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*Abbreviations:* BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentration; DMS, *N,N*-dimethylsphingosine; GPCR, G-protein-coupled receptor; HBSS, Hanks' balanced salt solution; PKC, protein kinase C; SPK1, sphingosine kinase-1; SPP, sphingosine-1-phosphate; tDHS, DL-*threo*-dihydrosphingosine

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PII: S0014-5793(01)03168-4

verapamil and fatty acid-free bovine serum albumin (BSA) from Sigma (Deisenhofen, Germany). All other materials were from previously described sources [4,10,20]. Stock solutions of tDHS and DMS were made in methanol and further diluted in 1 mg/ml fatty acid-free BSA. The respective methanol/BSA solution was used as control.

### 2.2. Cell culture and transfection

PC12 cells and RINm5F cells were cultured in DMEM/F12 and RPMI-1640 medium, respectively, each supplemented with 10% (v/v) foetal bovine serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin, in a humidified atmosphere of 5% $CO_2$/95% air at 37°C. Transient transfection of PC12 cells was performed with 100 μg DNA of pcDNA3.1 or murine sphingosine kinase-1 (SPK1, [13]) per 14.5-cm dish, respectively, using the calcium phosphate precipitation method. Overexpression of SPK1 was confirmed by immunofluorescence microscopy using an antibody against the N-terminal myc-tag [21].

### 2.3. Measurement of [$^3$H]SPP formation

Formation of [$^3$H]SPP from [$^3$H]sphingosine was measured as reported before [4,10]. Cells were washed with Hanks' balanced salt solution (HBSS), containing 118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 15 mM HEPES, pH 7.4, and detached by a buffer stream. The reactions were started by addition of 100 μl of cell suspension ($0.5\times10^5$ cells) to 100 μl of a reaction mixture containing 2 mg/ml BSA, 1 μCi/ml [$^3$H]sphingosine and the indicated agonists in HBSS. Incubation was for the indicated periods of time at 37°C. Reactions were stopped by addition of 3 ml methanol/chloroform (2/1), and lipids were extracted for 60 min at 37°C. After centrifugation for 10 min at $2000\times g$, the lipids were dried down in a SpeedVac vacuum centrifuge, dissolved in a small volume of methanol and applied to Silica gel 60 thin layer chromatography (TLC) plates together with sphingosine and SPP standards. The lipids were separated in 1-butanol/acetic acid/water (3/1/1), visualised with ninhydrin, spots were scraped off, and radioactivity was measured by liquid scintillation counting.

### 2.4. SPP mass determination

Endogenous SPP levels were determined as described before [20]. Briefly, PC12 cells were suspended in 1 ml HBSS ($10^7$ cells/ml) and stimulated with bradykinin or KCl for the indicated periods of time at 37°C. Reactions were stopped by the addition of 1 ml methanol containing 2.5 μl concentrated HCl. Dihydro-SPP (50 pmol) was added and lipids were extracted by addition of 1 ml chloroform and 200 μl 4 M NaCl. For alkalisation, 100 μl 3 N NaOH were added. After centrifugation ($300\times g$, 5 min), the alkaline aqueous phase was transferred into a siliconised glass tube, and the organic phase was re-extracted with 0.5 ml methanol, 0.5 ml 1 M NaCl and 50 μl 3 N NaOH. The aqueous phases were combined, acidified with 100 μl concentrated HCl and extracted twice with 1.5 ml chloroform. The combined organic phases were evaporated, and the dried lipids were dissolved in 275 μl methanol/0.07 M $K_2HPO_4$ (9:1) by rigorous vortexing and sonication on ice for 5 min. A derivatisation mixture of 10 mg *o*-phthaldialdehyde, 200 μl ethanol, 10 μl 2-mercaptoethanol and 10 ml 3% boric acid was prepared and adjusted to pH 10.5 with potassium hydroxide. 25 μl of this derivatisation mixture was added to the resolved lipids for 15 min at room temperature. The derivatives were analysed by a Merck Hitachi LaChrom HPLC system (Merck Hitachi, Darmstadt, Germany) using a RP 18 Kromasil column (Chromatographie Service, Langerwehe, Germany) kept at 35°C. Separation was done with a gradient of methanol and 0.07 M $K_2HPO_4$ [20] and the *o*-phthaldialdehyde derivatives were detected by fluorescence. Resulting profiles were evaluated using the Merck system manager software. The recovery of SPP was calculated using dihydro-SPP as standard [20].

### 2.5. Noradrenaline release

PC12 cells grown on 24-well plates or 35-mm dishes were incubated with 0.1 μCi/ml [$^3$H]noradrenaline in HBSS or serum-free medium containing 1 mM ascorbic acid for 90–120 min at 37°C and washed twice. Thereafter, the cells were pretreated with or without tDHS, DMS or PKC inhibitors dissolved in HBSS for the indicated periods of time. Stimulation with KCl or other stimuli followed for 10 min at 37°C. Released [$^3$H]noradrenaline was measured in the supernatant, while for measurement of cellular [$^3$H]noradrenaline content, the remaining cell pellet was lysed in 1% (w/v) sodium dodecyl sulphate, followed by liquid scintillation counting.

### 2.6. Measurement of intracellular $Ca^{2+}$ concentration

Intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) was determined with the fluorescent $Ca^{2+}$ indicator dye fura-2 in a Hitachi spectrofluorimeter as described before [4]. Briefly, cell monolayers were washed with HBSS, 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 $1\times10^6$ cells/ml and used for fluorescence measurements at room temperature within the next hour.

### 2.7. Data analysis and presentation

Data are mean ± S.E.M. from the indicated number ($n$) of experiments or mean ± S.D. from a representative experiment with the indicated number ($n$) of replicates. Statistical significance was calculated using the Prism program (GraphPad Software). Two groups of values were compared by two-tailed $t$-test, while more than two groups of values were compared by one-way analysis of variance followed by Dunnett's multiple comparison test.

## 3. Results

To study whether depolarisation-induced $Ca^{2+}$ influx can stimulate sphingosine kinase activity and SPP formation in excitable cells, PC12 pheochromocytoma cells were treated with high extracellular $K^+$ in the presence of 1 mM extracel-

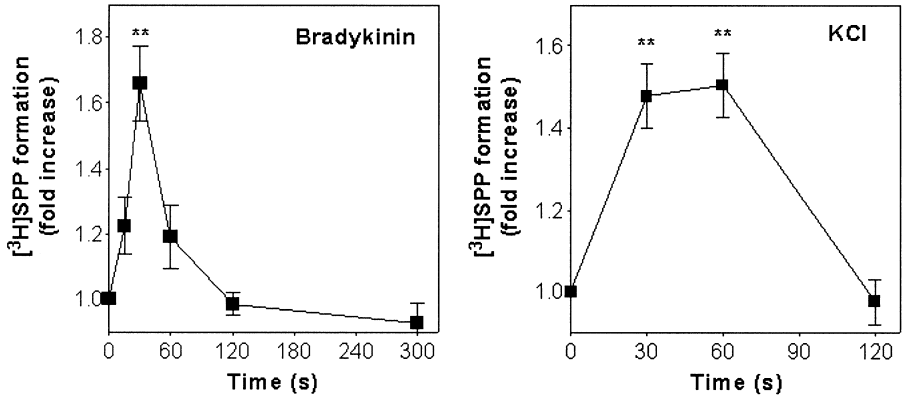


Fig. 1. Stimulation of [$^3$H]SPP formation by bradykinin and KCl depolarisation. [$^3$H]Sphingosine was added to PC12 cells together with 10 μM bradykinin or 60 mM KCl, and formation of [$^3$H]SPP was determined at the indicated periods of time. Data are mean ± S.E.M. of 4–9 (bradykinin) or 3–5 experiments (KCl) and given as fold increases over basal [$^3$H]SPP formation, amounting to 150 ± 24 cpm (bradykinin, $n=8$) or 241 ± 21 cpm (KCl, $n=4$) at 30 s. Please note the different time scales in left and right panel. $^{**}P<0.01$.

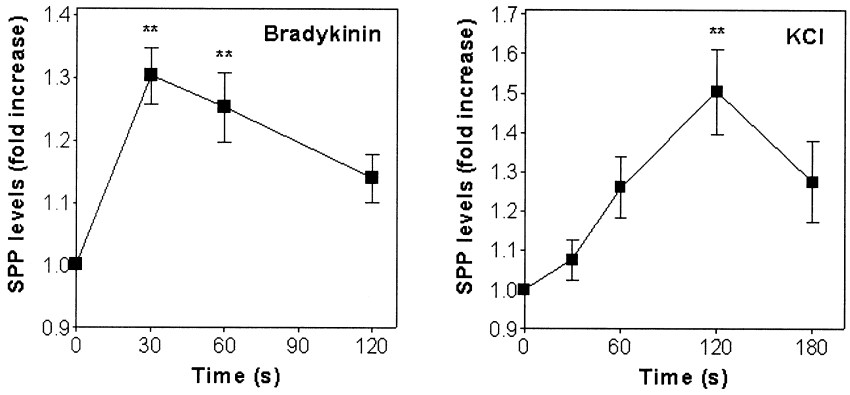


Fig. 2. Elevation of endogenous SPP levels by bradykinin and KCl depolarisation. PC12 cells were stimulated with 10 μM bradykinin or 60 mM KCl for the indicated periods of time. Intracellular SPP mass was determined as described in Section 2. Data are mean ± S.E.M. of three experiments each. Basal SPP levels were 50 ± 9 pmol/$10^8$ cells. $^{**}P < 0.01$.

lular $Ca^{2+}$. As shown in Fig. 1 (right panel), the addition of KCl (60 mM) rapidly and transiently increased formation of [$^3$H]SPP from [$^3$H]sphingosine. The maximum increase was observed at 30–60 s and amounted to ~1.5-fold above basal values. Addition of the GPCR agonist, bradykinin (10 μM), caused a similar increase (by ~1.7-fold) in [$^3$H]SPP formation, with a maximum at 30 s (Fig. 1, left panel). With either stimulus, high KCl and bradykinin, basal values of [$^3$H]SPP were reached again after 120 s. Since measurement of [$^3$H]SPP formation from added [$^3$H]sphingosine provides no information about the actual intracellular SPP levels, SPP mass determinations were performed. Basal SPP levels in PC12 cells were ~50 pmol/$10^8$ cells, in agreement with own previous results [20] and with data obtained by enzymatic measurement of SPP (90 pmol/$10^8$ cells) [22]. Addition of bradykinin (10 μM) and high KCl (60 mM) rapidly increased SPP levels, by maximally ~1.3- and ~1.5-fold, respectively (Fig. 2). As observed with [$^3$H]SPP formation, the increase in SPP levels induced by either bradykinin or high KCl were transient, although with slightly different kinetics for time to peak and decline to basal values.

Elevation of intracellular SPP formation induced by high KCl was completely dependent on influx of extracellular $Ca^{2+}$. First, in the absence of extracellular $Ca^{2+}$, KCl (60 mM) failed to enhance [$^3$H]SPP formation (0.99 ± 0.04-fold compared to unstimulated cells at 30 s of stimulation, mean ± S.D., $n = 4$, data not shown). Second, pretreatment of PC12 cells with the $Ca^{2+}$ channel blocker, verapamil (50 μM, 10 min), fully suppressed the KCl-induced [$^3$H]SPP production (Fig. 3, left panel), indicating that it is caused by $Ca^{2+}$ influx via voltage-gated $Ca^{2+}$ channels. The $Ca^{2+}$ dependence of SPP formation in PC12 cells was furthermore corroborated by the finding that treatment of the cells with the $Ca^{2+}$ ionophore, ionomycin (1 μM), for 30 s increased [$^3$H]SPP formation by 1.82 ± 0.12-fold (mean ± S.D., $n = 3$, data not shown). A first hint that depolarisation-induced SPP production is a rather general phenomenon in excitable cells was obtained with the insulinoma cells RINm5F. Similar as in PC12 cells, addition of KCl (60 mM) for 30 s to RINm5F cells increased [$^3$H]SPP formation by ~1.3-fold (Fig. 3, right panel).

Since elevation of intracellular SPP by KCl depolarisation was rapid, we examined whether it contributes to the rapid process of depolarisation-induced noradrenaline release. As

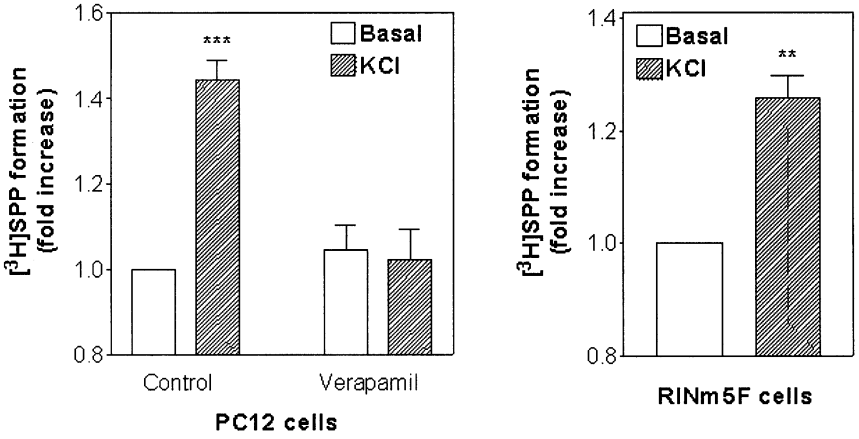


Fig. 3. Left panel: Inhibition of depolarisation-induced SPP production by verapamil. PC12 cells were incubated for 10 min without (control) and with 50 μM verapamil before stimulation of the cells for 30 s without (basal) and with 60 mM KCl and measurement of [$^3$H]SPP formation. Data are mean ± S.E.M. of four experiments. Right panel: Depolarisation-induced SPP production in RINm5F cells. Formation of [$^3$H]SPP was measured in RINm5F cells treated for 30 s without (basal) and with 60 mM KCl. Data are mean ± S.E.M. of three experiments. $^{***}P < 0.001$; $^{**}P < 0.01$.

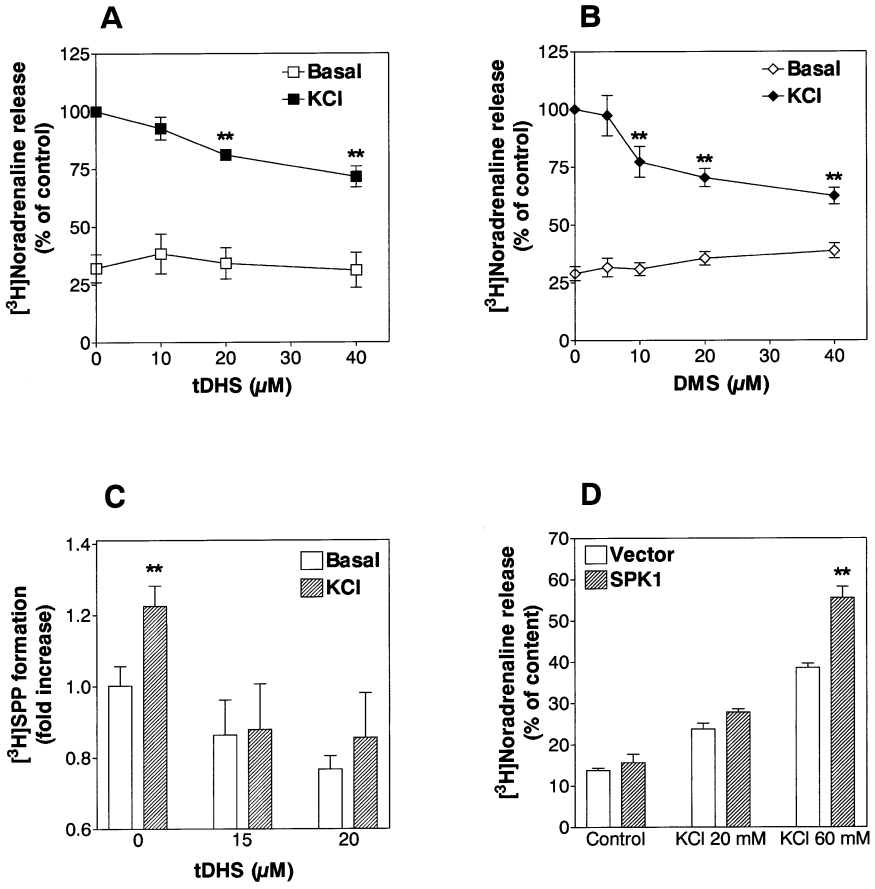


Fig. 4. Role of sphingosine kinase in depolarisation-induced noradrenaline release. A and B: Inhibition of KCl-induced [$^3$H]noradrenaline release by sphingosine kinase inhibitors. PC12 cells loaded with [$^3$H]noradrenaline were treated for 10 min with the indicated concentrations of tDHS or DMS before stimulation of the cells for 10 min without (basal) and with 30 mM KCl. Data are mean ± S.E.M. of 3–5 experiments and expressed as % of [$^3$H]noradrenaline release induced by KCl in the absence of inhibitor, which varied between 1000 and 5000 cpm/dish. C: Inhibition of KCl-induced [$^3$H]SPP formation by the sphingosine kinase inhibitor, tDHS. PC12 cells pretreated for 10 min with tDHS or the respective solvent were stimulated for 30 s with 60 mM KCl, and production of [$^3$H]SPP was determined as described above. Data are mean ± S.D. of a representative experiment performed in quadruplicate. D: Enhancement of KCl-induced [$^3$H]noradrenaline release by overexpression of SPK1. PC12 cells, transiently transfected with SPK1 or control vector, respectively, and loaded with [$^3$H]noradrenaline, were stimulated for 10 min with the indicated concentrations of KCl. Data are mean ± S.D. of a representative experiment performed in triplicate and expressed as % of cellular [$^3$H]noradrenaline content. $^{**}P < 0.01$.

shown in Fig. 4A,B, pretreatment of PC12 cells with the sphingosine kinase inhibitors, tDHS and DMS, concentration-dependently reduced KCl-induced [$^3$H]noradrenaline release. Inhibition by tDHS was significant at 20 μM and amounted to maximally 30–40% (after subtraction of basal release). DMS, which by itself slightly elevated [$^3$H]noradrenaline release at 20–40 μM, also significantly reduced KCl-stimulated [$^3$H]noradrenaline release, at 10 μM by ~35%. As sphingolipids, including sphingosine and tDHS, can inhibit PKC, we studied whether prototypical PKC inhibitors mimicked the effects of the sphingosine kinase inhibitors. However, pretreatment of the cells for 30 min with 100 nM Gö 6976 or 100 nM staurosporine did not affect KCl-induced [$^3$H]noradrenaline release, amounting to 97 ± 5% and 116 ± 3% of untreated cells, respectively (mean ± S.D., $n = 3$, data not shown). tDHS completely inhibited KCl-stimulated [$^3$H]SPP formation (Fig. 4C). Thus, the only partial inhibition of KCl-induced [$^3$H]noradrenaline release was not due to an incomplete inhibition of sphingosine kinase but rather reflects the partial contribution of intracellular SPP to the overall transmitter release. Further evidence for a role of sphingosine kinase in [$^3$H]noradrenaline release was finally obtained by overexpression of murine SPK1. KCl-stimulated [$^3$H]noradrenaline release was clearly enhanced in PC12 cells overexpressing SPK1 (Fig. 4D).

Most important, extracellularly applied SPP (1 μM) or sphingosylphosphorylcholine (SPC; 10 μM) had no effect on noradrenaline release (Fig. 5), indicating that intracellularly produced SPP does not act via plasma membrane SPP receptors after being released from the cells. Furthermore, extracellularly applied SPP (1 μM) had no effect on $[Ca^{2+}]_i$ in PC12 cells (data not shown). Since intracellular SPP formation apparently plays a role in cellular $Ca^{2+}$ homeostasis [4,6–8,10,12], we also examined the effect of sphingosine kinase inhibitors on KCl-induced $[Ca^{2+}]_i$ increase and found that it was inhibited (Fig. 5). As in KCl-induced [$^3$H]noradrenaline release, staurosporine rather enhanced than inhibited KCl-induced $[Ca^{2+}]_i$ increase, which amounted to 126 ± 12% of control values after pretreatment with 100 nM staurosporine for 30 min (mean ± S.E.M., $n = 3$, data not shown), indicating that the inhibitory effect of the sphingosine kinase inhibitors was not due to PKC inhibition.

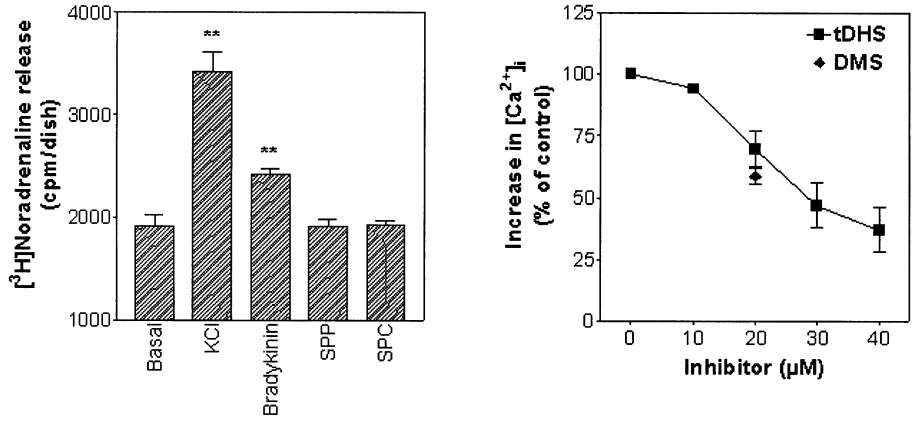


Fig. 5. Left panel: Extracellular SPP and SPC have no effect on noradrenaline release in PC12 cells. PC12 cells loaded with [$^3$H]noradrenaline were challenged for 10 min without (basal) and with 30 mM KCl, 10 μM bradykinin, 1 μM SPP or 10 μM SPC. Data are mean ± S.D. of a representative experiment ($n = 3$). $^{**}P < 0.01$. Right panel: Inhibition of depolarisation-induced $[Ca^{2+}]_i$ increase by sphingosine kinase inhibitors. Fura-2-loaded PC12 cells were pretreated for 1 min with the indicated concentrations of tDHS or DMS before stimulation with 30 mM KCl. Data are mean ± S.E.M. of three experiments each and expressed as % of KCl-induced $[Ca^{2+}]_i$ increase in the absence of inhibitor.

## 4. Discussion

Rapid activation of sphingosine kinase and intracellular SPP formation has been reported during the last few years for a wide variety of plasma membrane receptors in diverse cell types [4,6–12,18,19,23]. We demonstrate here for the first time that SPP formation can also be triggered by depolarisation of excitable cells, using two distinct methods which provided very similar results. Depolarisation of PC12 cells with high $K^+$ caused an increase in [$^3$H]SPP formation from exogenous [$^3$H]sphingosine as well as an elevation of endogenous SPP levels, both occurring with a similar time course and magnitude. Comparison of the two methods revealed that the application of exogenous radioactive substrate apparently mimics the physiological situation quite well. The slightly delayed peak response and the slower decline of elevated SPP levels compared to [$^3$H]SPP formation is probably due to a more sustained availability of endogenous sphingosine. Similar observations were made with the GPCR agonist, bradykinin, known to act via $B_2$ receptors in PC12 cells [24] and shown before to stimulate sphingosine kinase in HEK-293 cells overexpressing this receptor [25]. Furthermore, KCl depolarisation of PC12 cells increased SPP formation as efficiently as bradykinin, and this stimulation was in the range of that reported for diverse plasma membrane receptors in other cell types [4,8,10,12,18,19,23].

Depolarisation-induced increase in SPP production in PC12 cells was dependent on extracellular $Ca^{2+}$ and fully suppressed by the $Ca^{2+}$ channel blocker, verapamil, thus apparently caused by $Ca^{2+}$ influx via voltage-gated $Ca^{2+}$ channels. KCl depolarisation increased SPP production also in RINm5F insulinoma cells, suggesting that this is a more general phenomenon in excitable cells. As receptor-induced sphingosine kinase stimulation and SPP production is apparently also dependent on intracellular $Ca^{2+}$ [18,19] and intracellular SPP can cause $Ca^{2+}$ release from intracellular stores [4,6], the data suggest that the sphingosine kinase pathway may serve as a $Ca^{2+}$-induced $Ca^{2+}$ release mechanism. In agreement with such a hypothesis, we show here that treatment of PC12 cells with the sphingosine kinase inhibitors, tDHS and DMS, attenuated the depolarisation-induced $[Ca^{2+}]_i$ increase. However, this reduction could also be due to inhibition of $Ca^{2+}$ influx. The $Ca^{2+}$ release-activated $Ca^{2+}$ current ($I_{CRAC}$) in RBL cells was shown to be inhibited by sphingosine, and it was proposed that activation of sphingosine kinase might, by metabolising sphingosine, lead to disinhibition of $I_{CRAC}$ channels [26]; this could also be true for voltage-gated $Ca^{2+}$ channels in PC12 cells.

Previous studies in PC12 cells suggested that sphingosine kinase and its product SPP are involved in cell differentiation and survival in these cells [27,28]. The results presented in this study suggest a role for the sphingosine kinase pathway in neurotransmitter release. Treatment of PC12 cells with the sphingosine kinase inhibitors, tDHS and DMS, which have been widely used to study the cellular function of sphingosine kinase [4,8,10,18,29], reduced the KCl-induced noradrenaline release by about 30%, suggesting that intracellular SPP formation contributes to a significant extent to overall catecholamine release. Furthermore, overexpression of SPK1 enhanced KCl-induced noradrenaline release. With regard to the mechanism by which intracellular SPP enhances transmitter release, it was excluded that it is secreted and acts as autocrine extracellular mediator, which however can occur in other contexts [30]. Instead, we assume that the intracellularly formed SPP acts by enhancing depolarisation-induced $[Ca^{2+}]_i$ increase (see above). Further studies are needed to define the exact roles of sphingosine kinase and intracellular SPP in neurotransmitter release and depolarisation-induced $[Ca^{2+}]_i$ increase.

*Acknowledgements:* We thank Ellen Schäfer for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft and the Interne Forschungsförderung Essen of the Universitätsklinikum Essen.

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