Role of sphingosine kinase in Ca^{2+} signalling by epidermal growth factor receptor

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Abstract Contribution of sphingosine kinase (SPK)-catalyzed production of sphingosine-1-phosphate (SPP), in comparison to phospholipase C (PLC), to Ca^{2+} signalling by epidermal growth factor (EGF) was studied in two HEK-293 cell clones (HEK2 and HEK3), expressing functional EGF receptors and exhibiting release of stored Ca^{2+} by intracellular SPP. In HEK3 cells, EGF increased $[Ca^{2+}]_i$ and stimulated both, SPK and PLC. $[Ca^{2+}]_i$ increase, but not PLC stimulation, was strongly reduced by SPK inhibition. In HEK2 cells, EGF similarly stimulated PLC, but did not increase $[Ca^{2+}]_i$ or stimulate SPK, suggesting that intracellular SPP production plays a major role for Ca^{2+} signalling by EGF in HEK-293 cells.

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Key words: Epidermal growth factor; Sphingosine kinase; Ca^{2+} signalling; Phospholipase C

1. Introduction

In the past few years, a novel Ca^{2+} mobilization pathway has been proposed which involves receptor activation of sphingosine kinase (SPK) and subsequent production of sphingosine-1-phosphate (SPP), which can release intracellularly stored Ca^{2+}. Olivera and Spiegel [1] first reported a rapid formation of SPP upon stimulation of Swiss 3T3 cells with platelet-derived growth factor (PDGF) or serum. Inhibition of SPK with DL-*threo*-dihydrosphingosine (DHS) suppressed PDGF- and serum-stimulated DNA synthesis, suggesting an important role for SPK and its product SPP in growth factor-stimulated mitogenesis. Further experiments with DHS suggested that PDGF-stimulated activation of several signal transduction components, such as mitogen-activated protein kinase, activator protein-1 and Crk, was apparently mediated by SPK, whereas activation of others, including receptor autophosphorylation, Shc phosphorylation, Shc/Grb2 association and activation of phosphoinositide 3-kinase, was not [2].

A specific participation of SPK stimulation in receptor-mediated Ca^{2+} signalling was first reported for the FcεRI antigen receptor in RBL-2H3 mast cells [3], followed by similar reports for the FcγRI receptor in U937 macrophages [4,5]. We have recently provided evidence that G protein-coupled receptors (GPCRs) can use the SPK/SPP pathway as well for Ca^{2+} mobilization. In HEK-293 cells stably transfected with M2 and M3 muscarinic acetylcholine receptors (mAChRs), the increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by these mAChRs was markedly inhibited by DHS and *N*,*N*-dimethylsphingosine, another SPK inhibitor [6]. Furthermore, M2 and M3 mAChRs rapidly stimulated intracellular SPP formation. Finally, microinjection of SPP into intact HEK-293 cells caused rapid release of Ca^{2+} from intracellular stores, in a manner similar to microinjected IP_3 but apparently independent of IP_3 receptors, and also independent of the previously described plasma membrane sphingolipid receptors [6]. In a comparable manner, SPK-mediated intracellular SPP formation is apparently involved in formyl peptide receptor-mediated Ca^{2+} signalling and Ca^{2+}-dependent enzyme release in HL-60 granulocytes [7]. As $[Ca^{2+}]_i$ increases by several distinct GPCRs in various cell types were attenuated by DHS [6], the SPK/SPP pathway seems to play a widespread function for Ca^{2+} signalling by GPCRs.

Although it is now established that plasma membrane receptors can stimulate SPK with subsequent production of intracellular SPP, there are several open questions on the role of this pathway, in comparison to the ubiquitous phospholipase C-inositol 1,4,5-trisphosphate (PLC/IP_3) pathway, for Ca^{2+} signalling by individual membrane receptors. The present study analyses the Ca^{2+} signalling pathways of epidermal growth factor (EGF) receptor in HEK-293 cells, specifically in two HEK-293 cell clones which were established by Peralta and coworkers in 1988 [8]. HEK2 cells (stably expressing the M2 mAChR) and HEK3 cells (stably expressing the M3 mAChR) have been and are widely used by us and others to study signal transduction by mAChRs (see e.g. [6,9–12]). As previous studies demonstrated that the SPK/SPP pathway plays an important role in Ca^{2+} signalling by the overexpressed mAChRs in these cells [6], we used these two HEK-293 cell clones to analyze the Ca^{2+} signalling pathway of the endogenously expressed EGF receptor.

2. Materials and methods

2.1. Materials

EGF and DHS were obtained from Biomol (Hamburg, Germany). D-*erythro*-[3-^{3}H]sphingosine (18 Ci/mmol) and [^{3}H]IP_3 (21 Ci/mmol) were purchased from New England Nuclear (Bad Homburg, Germany). All other materials were from previously described sources [6,13,14]. Stock solutions of DHS were made in methanol and diluted in 1 mg/ml fatty acid-free bovine serum albumin.

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Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; DHS, DL-*threo*-dihydrosphingosine; EGF, epidermal growth factor; GPCR, G-protein-coupled receptor; HBSS, Hanks' balanced salt solution; IP_3, *myo*-inositol 1,4,5-trisphospate; mAChR, muscarinic acetylcholine receptor; MAP kinase, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; PLC, phospholipase C; PTX, pertussis toxin; SPK, sphingosine kinase; SPP, sphingosine-1-phosphate

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2.2. Cell culture

HEK2 cells (overexpressing the M2 mAChR) and HEK3 cells (overexpressing the M3 mAChR) were cultured in Dulbecco's modified Eagle's medium (DMEM/F12), containing 10% fetal calf serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin. Two days prior to experiments, cells were washed and cultured in the absence of serum. Pre-treatment with pertussis toxin (PTX) was performed for the last 16–20 h of cultivation with 100 ng/ml of the toxin.

2.3. Ca^{2+} mobilization

$[Ca^{2+}]_i$ was determined with the fluorescent calcium indicator dye fura-2 in a Hitachi spectrofluorimeter as described before [14]. Briefly, cell monolayers were washed with Hanks' balanced salt solution (HBSS) (118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 15 mM HEPES, pH 7.4) and the cells were detached by a buffer stream. Suspended cells were loaded with 1 μM fura-2/AM for 1 h at 37°C in HBSS. Thereafter, cells were washed twice, resuspended at a density of 1×10^6 cells/ml and used for fluorescence measurements within the next hour. $[Ca^{2+}]_i$ was measured in a continuously stirred cell suspension at room temperature as described [14].

2.4. IP_3 mass determination

Suspensions of HEK2 and HEK3 cells in HBSS (200 μl, 2×10^6 cells) were incubated for 20 s at 37°C. The reactions were stopped by addition of 800 μl of 0.625 M trichloroacetic acid. IP_3 mass was determined as described previously [15]. In short, the cells were pelleted by centrifugation (10 min, $800\times g$) and the acid supernatants were extracted three times with 3 ml of water-saturated diethyl ether. The neutralized samples (200 μl) were complemented with 50 μl EDTA (30 mM) and 50 μl $NaHCO_3$ (60 mM). IP_3 mass was measured by competition with [^{3}H]IP_3 using IP_3 binding protein prepared from bovine adrenal cortex as described [15].

2.5. Inositol phosphate formation

PLC-catalyzed production of inositol phosphates was determined in *myo*-[^{3}H]inositol-labeled cells in the presence of LiCl. HEK2 and HEK3 cells were labeled for 48 h with 2 μCi/ml of *myo*-[^{3}H]inositol. Then, cell monolayers were washed twice with HBSS and cells were detached by a buffer stream. Cell suspensions (200 μl, 2×10^6 cells) were incubated for 10 min at 37°C in HBSS containing 10 mM LiCl before stimulation with the respective agonists for 60 min at 37°C. Reactions were stopped by addition of methanol. Analysis of [^{3}H]inositol phosphates was performed as reported before [13].

2.6. SPP production and sphingosine uptake

Uptake of [^{3}H]sphingosine and formation of [^{3}H]SPP was measured in cell suspensions essentially as reported before [6]. Cells were washed with HBSS and detached by a buffer stream. The reactions were started by addition of 100 μl of cell suspension ($0.5–1\times10^6$ cells) to 100 μl of a reaction mixture containing 2 mg/ml bovine serum albumin, 1 μCi/ml [^{3}H]sphingosine (~100 000 cpm/tube) and the respective agonists in HBSS. Incubation was for the indicated periods of time at 37°C. Reactions were stopped by addition of 1 ml of ice-cold HBSS, rapid filtration over glass fiber filters (Whatman GF/C; Whatman, Maidstone, England) and a wash with 1 ml of ice-cold HBSS. Filters were extracted twice with 3 ml methanol/chloroform (2/1) for 30 min at 37°C. Extracts were combined and centrifuged for 10 min at $2000\times g$ and supernatants were dried down in a SpeedVac vacuum centrifuge. Dried lipids were dissolved in a small volume of methanol and applied to silica gel 60 TLC plates. Sphingosine and SPP standards were applied with the samples for identification of [^{3}H]SPP and [^{3}H]sphingosine, respectively. The lipids were separated by TLC in 1-butanol/acetic acid/water (3/1/1). Standard lipids were visualized with ninhydrin, spots were scraped off, and radioactivity was measured by liquid scintillation counting.

2.7. Activation of mitogen-activated protein kinase (MAP kinase)

HEK2 and HEK3 cells were detached, washed with HBSS and resuspended at a density of 5×10^7 cells/ml. Aliquots of the cell suspension (90 μl) were incubated with EGF (10 μl) for 5 min at 37°C. The reactions were stopped by addition of 500 μl of lysis buffer (1% SDS, 10 mM Tris-HCl, pH 7.4), followed by heating for 5 min at 95°C and five passages through a 25-gauge needle. After removal of insoluble material by centrifugation, the lysates were diluted with lysis buffer to an equal amount of protein measured by the BCA method (Pierce). Aliquots of the samples (30 μg protein) were subjected to SDS polyacrylamide gel electrophoresis using 10% acrylamide. After blotting onto nitrocellulose membranes, the phosphorylated MAP kinases, ERK1 and ERK2, were detected with an anti-phospho-p42/p44 MAP kinase antibody (New England Biolabs) and visualized using an enhanced chemiluminescence detection system (Amersham, Pharmacia Biotech Europe GmbH).

2.8. Data presentation and analysis

Data are mean ± S.E.M. from the indicated number (n) of experiments performed in replicates, if not stated otherwise. The concentration-response curve in Fig. 4 was analyzed by fitting a sigmoidal function to the experimental data, using iterative non-linear regression analysis with the Prism program (GraphPad Software).

3. Results

EGF caused transient $[Ca^{2+}]_i$ elevations in HEK3 cells (Fig. 1). $[Ca^{2+}]_i$ elevations were detectable at≥3 ng/ml EGF and amounted to 71 ± 5.7 nM ($n=4$) and 90 ± 6.4 nM ($n=10$) at 30 ng/ml and 100 ng/ml EGF, respectively. There was a time lag between EGF addition and peak $[Ca^{2+}]_i$ increase, which was concentration-dependent and amounted to about 60 s at 100 ng/ml EGF. In contrast, virtually no $[Ca^{2+}]_i$ increase by

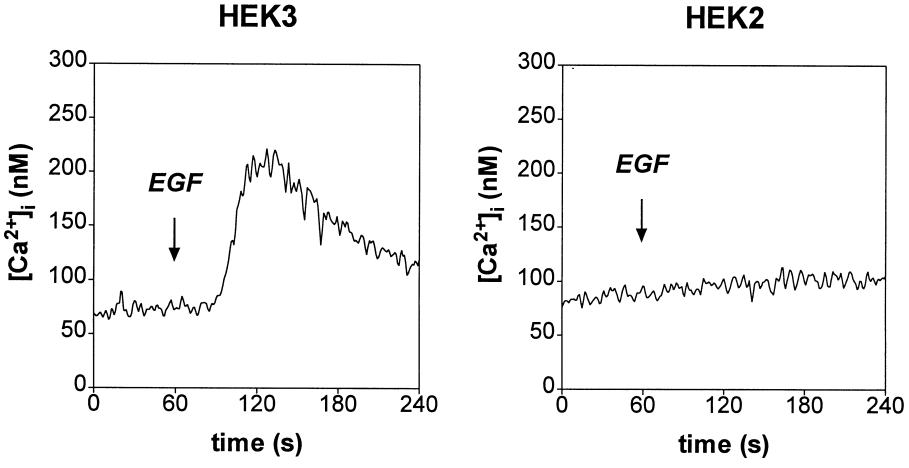

Fig. 1. Ca^{2+} signalling by EGF in HEK3 and HEK2 cells. Shown are typical traces of $[Ca^{2+}]_i$ in suspensions of fura-2-loaded cells after stimulation with 100 ng/ml EGF.

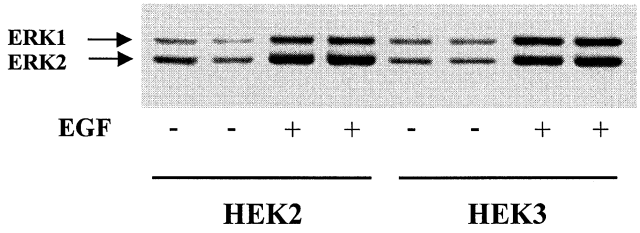

Fig. 2. EGF-induced MAP kinase activation in HEK3 and HEK2 cells. The cells were incubated in the absence (−) and presence (+) of 100 ng/ml EGF for 5 min. Proteins were separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and phosphorylated ERK1 and ERK2 were detected by immunoblotting with an anti-phospho-MAP kinase antibody.

EGF could be observed in HEK2 cells (Fig. 1). In these cells, $[Ca^{2+}]_i$ elevations were 2.4 ± 0.9 nM ($n = 16$) and 3.7 ± 1.6 nM ($n = 9$) at 30 ng/ml and 100 ng/ml EGF, respectively. This difference in Ca^{2+} signalling was not due to the absence of EGF receptors in HEK2 cells. First, addition of EGF induced tyrosine phosphorylation of various proteins (data not shown) and caused a similar activation of the MAP kinases, ERK1 and ERK2, in both cell lines (Fig. 2). Second, EGF stimulated PLC in both HEK3 and HEK2 cells. Specifically, EGF (30 ng/ml) increased IP_3 production by 1.79 ± 0.18-fold in HEK3 cells and by 2.21 ± 0.08-fold in HEK2 cells ($n = 3$, Fig. 3). Thus, in HEK2 cells lacking EGF-induced Ca^{2+} mobilization, EGF increased IP_3 levels even slightly, although not significantly, more than in HEK3 cells. Similarly, EGF-induced production of [^{3}H]inositol phosphates was identical in HEK3 and HEK2 cells (Fig. 3). Maximal stimulation was observed at 30 ng/ml EGF and amounted to 1.32 ± 0.04-fold in HEK3 cells and 1.32 ± 0.03-fold in HEK2 cells ($n = 4$). Taken together, there was no difference in EGF-induced PLC activation between HEK3 and HEK2 cells which could account for the distinct difference in EGF-stimulated Ca^{2+} mobilization. Furthermore, the difference in EGF-induced Ca^{2+} mobilization between HEK2 and HEK3 cells was apparently not caused by the overexpressed mAChRs. Pretreatment of the cells with the mAChR antagonist, atropine (50 μM), did not alter the differential Ca^{2+} signalling by EGF in HEK3 and HEK2 cells (data not shown). Thus, PLC activation was obviously not sufficient for Ca^{2+} signalling by the EGF receptor. Therefore, we examined whether HEK3 cells expressed another EGF-regulated Ca^{2+} signalling pathway which could explain the differential Ca^{2+} signalling pattern observed in the two HEK-293 cell clones.

SPK stimulation has been shown to play an important role in PDGF signalling [1,2,16] and is implicated in Ca^{2+} signalling by various receptors in different cell types [3–7]. To assess a possible involvement of SPK in EGF-induced Ca^{2+} signalling, first, the effect of the SPK inhibitor DHS [17] on EGF-induced $[Ca^{2+}]_i$ increase in HEK3 cells was studied. Pretreatment of HEK3 cells with DHS for 1 min concentration dependently inhibited the EGF-induced $[Ca^{2+}]_i$ increase, reaching about 65% inhibition at 40 μM DHS (Fig. 4). In contrast, EGF (30 ng/ml)-induced [^{3}H]inositol phosphate accumulation was not altered by DHS. It amounted to 101% and 100% of control values at 40 μM DHS in HEK3 and HEK2 cells, respectively (data not shown). Next, it was studied whether EGF activated SPK in HEK3 and HEK2 cells, by measuring the production of [^{3}H]SPP from [^{3}H]sphingosine. Addition of EGF (30 ng/ml) rapidly and transiently stimulated formation of [^{3}H]SPP in HEK3 cells, which was maximal at 1–2 min and disappeared thereafter (Fig. 5B). Under the same conditions, EGF had no effect on the cellular uptake of [^{3}H]sphingosine (data not shown). Maximal SPK stimulation by EGF was observed at 30 ng/ml and amounted to a 1.40 ± 0.04-fold stimulation above basal ($n = 7$, Fig. 5C). Pretreatment of HEK3 cells with DHS inhibited both basal and EGF-stimulated [^{3}H]SPP formation and at 40 μM DHS the EGF receptor stimulation was completely abolished (Fig. 5D). In contrast to HEK3 cells, addition of EGF (30 ng/ml) did not promote [^{3}H]SPP formation in HEK2 cells, which was 1.03 ± 0.05-fold above basal values ($n = 4$, Fig. 5A). Also at other concentrations (3–100 ng/ml), EGF did not stimulate SPK in HEK2 cells (data not shown). To examine the possibility that SPP formed after SPK stimulation with EGF might be released and act in an autocrine manner via plasma membrane sphingolipid receptors, EGF-induced $[Ca^{2+}]_i$ increase was measured in PTX-treated cells. The PTX treatment (100 ng/ml, 16–20 h) effectively blocked the $[Ca^{2+}]_i$ increase induced by extracellular SPP (1 μM), however, Ca^{2+} signalling induced by EGF (100 ng/ml) was not inhibited by PTX (99 ± 7.3% of control values, mean ± S.D., $n = 6$). Taken together, these data suggest that enhanced formation of intracellular SPP by SPK is re-

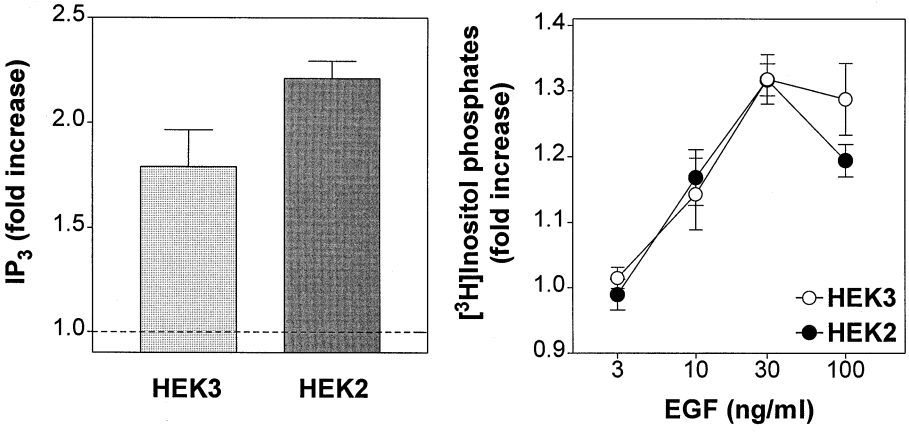

Fig. 3. EGF-induced PLC activation in HEK3 and HEK2 cells. Left panel, IP_3 production was measured after stimulation with 30 ng/ml EGF. Basal IP_3 production was 10–20 pmol/mg protein. Right panel, [^{3}H]inositol phosphate accumulation was measured after stimulation with the indicated concentrations of EGF. Basal values were 300–600 cpm/mg protein. Data are from three or four experiments.

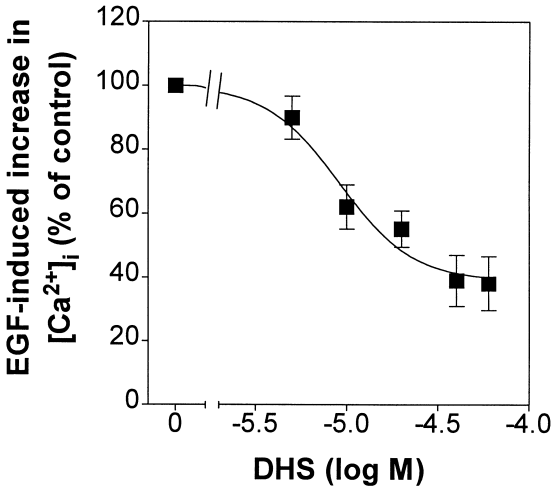

Fig. 4. Inhibition of EGF-induced $[Ca^{2+}]_i$ increase by DHS. Peak $[Ca^{2+}]_i$ increases were measured in HEK3 cells preincubated for 1 min with DHS at the indicated concentrations and stimulated with 100 ng/ml EGF. Average increase in $[Ca^{2+}]_i$ induced by 100 ng/ml EGF was 90 nM (see Section 3). Data are from six experiments.

quired for effective Ca^{2+} signalling by the EGF receptor in HEK-293 cells.

4. Discussion

SPP has recently gained much attention as first and second messenger [18,19]. One finding of the present study is that EGF belongs to the receptor agonists which can activate SPK and promote intracellular SPP production. Extent and time course of EGF-induced SPP production closely resembled that of PDGF reported by others [1,16]. However, activation of SPK by EGF was cell type specific. Comparing the two HEK-293 cell clones [8], EGF stimulated SPP production only in HEK3 but not in HEK2 cells, although HEK2 cells express a functional EGF receptor and a receptor (M2 mAChR)-regulated SPK [6]. Similarly, in Swiss 3T3 fibroblasts, only PDGF but not EGF receptor activation stimulated SPP formation, although both PDGF and EGF stimulated DNA synthesis in these cells [1], but apparently by distinct mechanisms [2]. So far, it is unclear how plasma membrane receptors such as GPCRs and receptor tyrosine kinases stimulate SPK. Recently, using various PDGF receptor mutants expressed in canine kidney epithelial cells, it was shown that SPK does not directly associate with the PDGF receptor, but that for SPK activation tyrosine-1021 of the receptor is required [20]. Since tyrosine-1021 mediates binding of PLCγ to the PDGF receptor, it was suggested that SPK activation occurs downstream of PLCγ stimulation [20]. Whether or not this is also the case for EGF receptor-mediated SPK activation remains to be studied. As EGF stimulated SPK in HEK3 but not in HEK2 cells, while mAChR stimulation of SPK was observed in either cell type [6], it may be speculated that in HEK2 cells a signalling component is missing, which is specifically required for SPK activation by EGF, but not by mAChRs, and which is present in HEK3 cells. The recent cloning of a SPK enzyme [21] will most likely also speed up our understanding of receptor coupling mechanisms to SPK stimulation, and thus help to resolve the question why the EGF receptor stimulates SPK in HEK3 cells, but not in others, i.e. HEK2 cells and Swiss 3T3 fibroblasts.

Recent studies performed on various cell types and different membrane receptors suggest that the SPK/SPP signal transduction pathway may significantly contribute to Ca^{2+} signalling processes [3–7]. Evidence for a SPK/SPP-mediated Ca^{2+} signalling pathway is generally based on the following three observations. First, receptor activation leads to stimulated production of the putative intracellular messenger. Second, inhibition of intracellular messenger production also inhibits Ca^{2+} mobilization by the activated receptor. Third, intracellularly applied second messenger causes release of intracellularly stored Ca^{2+}. Here, we demonstrate that EGF enhances SPP formation in HEK3 cells. Inhibition of SPK by DHS largely attenuated EGF-induced Ca^{2+} mobilization, while PLC stimulation by the EGF receptor was not affected. In a previous study, we already demonstrated that intracellularly applied SPP can release Ca^{2+} in HEK-293 cells [6]. Thus, the data strongly suggest that SPK contributes to EGF-stimulated Ca^{2+} signalling in HEK3 cells. The mechanism by which intracellular SPP mobilizes Ca^{2+} from intracellular stores is presently unknown. A specific SPP-gated Ca^{2+} channel on the endoplasmic reticulum has been proposed [22] but has not yet been identified. Another possibility could be that intracellularly produced SPP is secreted and activates plasma membrane SPP receptors, which also can induce Ca^{2+} mobilization in HEK-293 cells [23]. However, this was excluded as EGF-induced Ca^{2+} signalling in HEK3 cells was not affected by PTX, which, on the other hand, completely abolished Ca^{2+} mobilization induced by extracellular SPP [6,23].

Receptor-induced Ca^{2+} signalling is generally attributed to stimulation of the ubiquitously expressed PLC/IP_3 signal transduction pathway [24]. Since IP_3-sensitive Ca^{2+} stores are apparently found in any mammalian cell type [25], attribution of a Ca^{2+} response induced by a given membrane receptor to the PLC/IP_3 pathway relies, in many cases, simply on the fact that the activated receptor indeed stimulates PLC. However, our study suggests that there is not always such a simple one to one relationship, i.e. PLC stimulation always results in Ca^{2+} mobilization by the receptor. As demonstrated here for the EGF receptor expressed in HEK2 cells, receptor-stimulated IP_3 production can occur in the absence of measurable Ca^{2+} mobilization, although functional IP_3 receptors are expressed in these cells [6]. One likely explanation is that the level of IP_3 generated by EGF stimulation is not high enough to cause by itself a measurable $[Ca^{2+}]_i$ increase but requires intracellular SPP as a cofactor or amplification mechanism for efficient Ca^{2+} mobilization. In line with this assumption, we have previously demonstrated that SPK inhibition by DHS suppresses M3 mAChR-induced $[Ca^{2+}]_i$ increase only at low levels of receptor and PLC activation, whereas at high levels of IP_3 production DHS was rather ineffective [6]. Furthermore, as recently reported for PDGF-induced SPK stimulation in kidney epithelial cells [20], receptor-stimulated SPP formation may require some intracellular free Ca^{2+}, which is probably released by IP_3. Such a small and potentially localized $[Ca^{2+}]_i$ increase required for SPK stimulation might have escaped the fura-2 detection method used. Thus, we would like to suggest that for efficient Ca^{2+} mobilization by the EGF receptor in HEK-293 cells activation of both PLC and SPK is required.

On the other hand, it is also possible that PLC stimulation

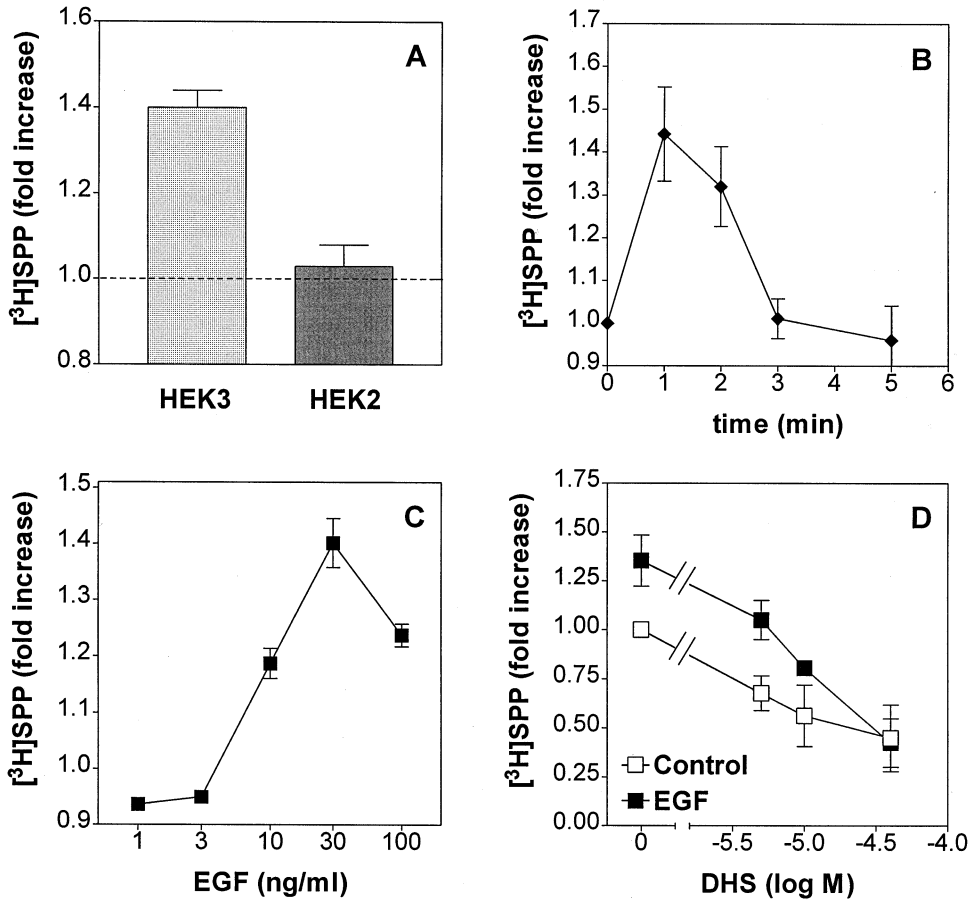

Fig. 5. EGF-induced stimulation of SPP formation. A: Production of [^{3}H]SPP from [^{3}H]sphingosine was measured after stimulation with 30 ng/ml EGF for 2 min in HEK3 and HEK2 cells, respectively. Data are from seven (HEK3) and four (HEK2) experiments. B: Time course of [^{3}H]SPP formation in HEK3 cells stimulated with 30 ng/ml EGF. Values are mean ± S.D. from a representative experiment performed in quadruplicates. C: Concentration dependence of EGF-induced [^{3}H]SPP formation in HEK3 cells. Stimulation was for 2 min. Data are from four experiments. D: Inhibition of basal and EGF-stimulated [^{3}H]SPP formation by DHS. Preincubation with DHS was for 1 min and stimulation with 30 ng/ml EGF for 2 min. Data are from two experiments performed in quadruplicates. Basal [^{3}H]SPP production was 200–800 cpm/mg protein in all experiments.

does not contribute to Ca^{2+} signalling by EGF but is needed primarily for other purposes, such as protein kinase C activation, as was recently suggested from studies in CHO cells. In these cells, α-thrombin and endothelin-1 stimulated PLC and induced Ca^{2+} mobilization. However, the $[Ca^{2+}]_i$ increase induced by these two receptor agonists was not inhibited by intracellularly applied heparin or IP_3 receptor antibody, which on the other hand efficiently blocked Ca^{2+} mobilization by PDGF and photolyzed caged IP_3 [26,27]. These data, thus, strongly suggest that other pathways, besides the established PLC/IP_3 pathway, can mediate Ca^{2+} signalling by membrane receptors and, furthermore, indicate that an involvement of the PLC/IP_3 pathway in Ca^{2+} signalling by a given receptor is not simply indicated by the fact that the receptor is capable to stimulate PLC.

In summary, evidence is provided that Ca^{2+} signalling by the EGF receptor, endogenously expressed in HEK-293 cells, is dependent on the SPK/SPP pathway, while the EGF-stimulated PLC/IP_3 pathway by itself is apparently not sufficient for efficient Ca^{2+} mobilization.

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