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Stimulation of Sphingosine-1-phosphate Formation by the $P2Y_2$ Receptor in HL-60 Cells: Ca^{2+} Requirement and Implication in Receptor-Mediated Ca^{2+} Mobilization, but Not MAP Kinase Activation

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ABSTRACT

Sphingosine-1-phosphate (SPP), produced by sphingosine kinase, has recently been reported to act as an intracellular second messenger for Ca^{2+} and mitogenic responses triggered by membrane receptors and as an extracellular ligand for specific SPP receptors. Here, we investigated the signaling pathway leading to SPP production by the G protein-coupled $P2Y_2$ receptor and its functional implication in human leukemia (HL-60) cells, which do not respond to extracellular SPP. $P2Y_2$ receptor activation by UTP or ATP resulted in rapid and transient production of SPP, which was insensitive to pertussis toxin and blocked by the sphingosine kinase inhibitor, DL-*threo*-dihydrosphingosine. Treatment of HL-60 cells with this inhibitor did not affect activation of mitogen-activated protein kinases, but suppressed Ca^{2+} mobilization by the $P2Y_2$ receptor. However, receptor-induced SPP production apparently required an increase in intracellular Ca^{2+} concentration, but not Ca^{2+} influx, and was mimicked by exposure of cells to Ca^{2+} ionophores. Taken together, activation of the $P2Y_2$ receptor stimulates SPP production in HL-60 cells, a process apparently not required for mitogen-activated protein kinase activation, but most likely representing an amplification system for receptor-mediated Ca^{2+} signaling.

Recent studies indicate that sphingolipid metabolites function as a new class of intra- and intercellular second messengers, involved in a large variety of cellular processes. Besides ceramide and sphingosine, sphingosine-1-phosphate (SPP), which results from the phosphorylation of sphingosine by sphingosine kinase, has been in the focus of recent interest. Two distinct cellular actions of SPP have been proposed, namely, as agonist ligand for plasma membrane receptors and as intracellular second messenger (Meyer zu Heringdorf et al., 1997). Specific G protein-coupled SPP receptors, first characterized by functional studies (van Koppen et al., 1996a), were recently identified as members of the Edg receptor family (Goetzl and An, 1998; Lee et al., 1998; Okamoto et al., 1998; Zondag et al., 1998; Ancellin and Hla, 1999). The evidence for an intracellular action of SPP is based on the following major findings. First, activation of various plasma membrane receptors, such as the platelet-derived growth factor receptor (Olivera and Spiegel, 1993), the FcεRI and FcγRI antigen receptors (Choi et al., 1996; Melendez et al., 1998a,b), and the tumor necrosis factor-α receptor (Xia et al., 1998), was found to rapidly increase intracellular SPP production by sphingosine kinase. Second, inhibition of sphingosine kinase with the competitive inhibitor DL-*threo*-dihydrosphingosine (tDHS) strongly reduced or even prevented cellular events triggered by these tyrosine kinase-linked receptors, such as activation of mitogen-activated protein (MAP) kinases, specifically of the extracellular signal-regulated kinases (Erks), stimulation of DNA synthesis, Ca^{2+} mobilization, and vesicular trafficking (Olivera and Spiegel, 1993; Choi et al., 1996; Pyne et al., 1996; Rani et al., 1997; Melendez et al., 1998a,b; Xia et al., 1998). Finally, intracellular SPP was found to mimic some of the receptor responses, i.e., it was shown to mobilize Ca^{2+} from internal stores and induce activation of Erks and DNA synthesis (Ghosh et al., 1994; Mattie et al., 1994; Qiao et al., 1998; Xia et al., 1998; van Brocklyn et al., 1998).

Recently, we have provided evidence that intracellular

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ABBREVIATIONS: SPP, sphingosine-1-phosphate; tDHS, DL-*threo*-dihydrosphingosine; MAP kinase, mitogen-activated protein kinase; Erk, extracellular signal-regulated kinase; PTX, pertussis toxin; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HBSS, Hanks' balanced salt solution; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; PMA, phorbol-12-myristate-13-acetate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine.

SPP production is apparently involved in Ca^{2+} signaling of some G protein-coupled receptors. Specifically, activation of M_2 and M_3 muscarinic acetylcholine receptors expressed in HEK-293 cells was found to rapidly increase intracellular SPP formation. Moreover, intracellular injection of SPP specifically and rapidly mobilized Ca^{2+} in intact HEK-293 cells, and inhibition of sphingosine kinase markedly inhibited Ca^{2+} mobilization by these and other G protein-coupled receptors (Meyer zu Heringdorf et al., 1998). In a comparable manner, we have recently reported that intracellular SPP formation apparently participates in Ca^{2+} signaling and Ca^{2+}-dependent enzyme release, but not superoxide production, triggered by the G_i-coupled formyl peptide receptor in human leukemia (HL-60) granulocytes (Alemany et al., 1999). Most important, the Ca^{2+}-mobilizing action of intracellular SPP was independent of plasma membrane SPP receptors. In HEK-293 cells, their action was prevented by pertussis toxin (PTX) treatment (Meyer zu Heringdorf et al., 1998), whereas in HL-60 cells extracellular SPP was inactive (van Koppen et al., 1996b; Alemany et al., 1999; present work). Furthermore, inhibition of Ca^{2+} signaling by the sphingosine kinase inhibitors did not affect phospholipase C stimulation by the G protein-coupled receptors or inositol 1,4,5-trisphosphate-induced Ca^{2+} release, and was not due to inhibition of protein kinase C (Meyer zu Heringdorf et al., 1998; Alemany et al., 1999).

In this study, we examined intracellular SPP formation by the G protein-coupled $P2Y_2$ receptor in HL-60 cells and whether this reaction is involved in Ca^{2+} mobilization and MAP kinase activation by the purinergic receptor. The $P2Y_2$ receptor, formerly termed P_{2U} receptor, is expressed in promyelocytic and myeloid differentiated HL-60 cells and mediates its effects largely via PTX-insensitive G proteins, most likely G_{16} (Klinker et al., 1996; Baltensberger and Porzig, 1997). We report herein that $P2Y_2$ receptor activation induces rapid SPP production in HL-60 cells. Moreover, evidence is provided suggesting that intracellular SPP formation apparently represents an amplification system for receptor-mediated Ca^{2+} mobilization, but is not required for MAP kinase activation in HL-60 cells.

Experimental Procedures

Materials. 1,2-bis(2-Aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA)/AM, ionomycin, and A-23187 were from Calbiochem (Schwalbach, Germany). D-*erythro*-[^{3}H]Sphingosine (18 Ci/mmol) was from New England Nuclear (Brussels, Belgium). Rabbit anti-phosphospecific MAP kinase antibodies and rabbit anti-Erk antibodies were purchased from New England Biolabs (Schwalbach, Germany) and Santa Cruz (Heidelberg, Germany), respectively. All other materials were from previously described sources (Meyer zu Heringdorf et al., 1998; Alemany et al., 1999). Before use, tDHS was directly diluted in Hanks' balanced salt solution (HBSS) (118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose, and 15 mM HEPES, pH 7.4), containing in addition 1 mg/ml fatty-acid-free BSA. The respective solvent was used as vehicle control.

Cell Culture. Human promyelocytic HL-60 cells, provided by Dr. T. Wieland (Institut für Pharmakologie, Universität Hamburg), were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 150 U/ml penicillin, and 150 μg/ml streptomycin in 5% CO_2. For differentiation into neutrophil-like cells, HL-60 cells were cultured for 48 h in the presence of 0.5 mM dibutyryl cAMP. For PTX treatment, cells were incubated for 20 h with 100 ng/ml of the toxin. HEK-293 cells stably expressing the M_3 muscarinic acetylcholine receptor were cultured as reported in Meyer zu Heringdorf et al. (1998).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined with the fluorescent Ca^{2+} indicator dye Fura-2 in a Hitachi spectrofluorimeter as described in Alemany et al. (1999). Briefly, cells were loaded with 1 μM Fura-2/AM for 1 h at room temperature in HBSS. Thereafter, cells were washed twice, resuspended at a density of 1×10^6 cells/ml, and used for fluorescence measurements. In some experiments, $[Ca^{2+}]_i$ was determined in the absence of extracellular Ca^{2+} and/or after treatment of cells for 1 min with 10 μM tDHS.

Assay of SPP Formation. Formation of SPP in HEK-293 cells and HL-60 cells was determined as reported previously (Alemany et al., 1999; Meyer zu Heringdorf et al., 1999). In brief, HL-60 cells (1.8×10^6 cells) equilibrated in HBSS/BSA for 5 min at 37°C were incubated with 0.1 μCi [^{3}H]sphingosine (~10^5 cpm/tube; ~30 nM final concentration) and the agents indicated for the indicated periods of time at 37°C in a total volume of 200 μl. The reactions were stopped by addition of 2 ml of ice-cold methanol, followed by 1 ml of chloroform. After pelleting particulate material, the supernatant was evaporated to dryness and redissolved in 20 μl of methanol. Then, the samples were spotted onto silica gel 60 thin layer chromatography plates, together with authentic unlabeled sphingosine and SPP. Separation of the products was achieved with 1-butanol:acetic acid:water (3:1:1) as solvent system. Sphingosine and SPP spots

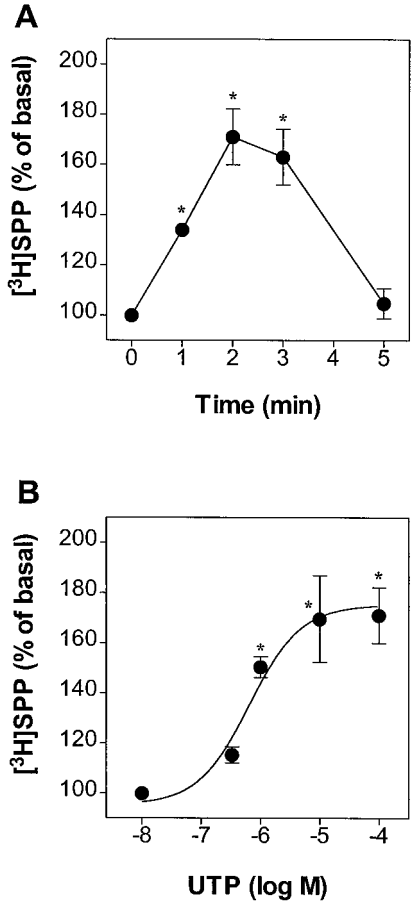

Fig. 1. UTP-stimulated SPP production in HL-60 cells. Formation of [^{3}H]SPP from [^{3}H]sphingosine was measured in promyelocytic HL-60 cells for the indicated periods of time in the absence and presence of 100 μM UTP (A) or for 2 min with UTP at the indicated concentrations (B) as described under *Experimental Procedures*. Values are expressed as percentage of [^{3}H]SPP production relative to unstimulated cells, amounting to 3400 to 3900 cpm/1.8×10^6 cells at 1 to 5 min of incubation. *, significantly different from basal SPP formation ($P < .05$, 4 to 12 experiments).

visualized by staining with ninhydrin spray were scraped off, and radioactivity was measured by liquid scintillation counting. Formation of [^{3}H]SPP is expressed as counts per minute per 1.8 $\times$ 10^6 cells and corrected for time 0 values, amounting to ~100 cpm. The assay of [^{3}H]SPP formation in HEK-293 cells was as in HL-60 cells, except that 0.7 $\times$ 10^6 cells were present in the assay and that lipid extraction was performed on cells filtered over glass fiber filters to stop the reaction (Meyer zu Heringdorf et al., 1999).

Assay of MAP Kinase Activation. HL-60 cells (5 $\times$ 10^6 cells) serum-starved overnight in growth medium were incubated for 5 min at 37°C in HBSS without and with 30 μM tDHS, followed by stimulation for 1 min with the indicated agonists. The reactions were stopped by addition of 0.5 ml of lysis buffer, containing 1% SDS and 10 mM Tris-HCl, pH 7.4, and heating of the lysates for 5 min at 95°C. After five passages through a 25-gauge needle, insoluble material was pelleted and the supernatant diluted with lysis buffer. Aliquots of the diluted samples (100 μl) were mixed with 200 μl of 3-fold concentrated electrophoresis sample buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromphenol blue, and 2% 2-mercaptoethanol) and boiled for another 10 min. Equal amounts of protein (~20 μg, determined by the bicinchoninic acid method) of each sample were subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels and then blotted onto nitrocellulose filters. Nitrocellulose was then blocked with 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 5% BSA (fraction V; Sigma, Deisenhofen, Germany). After washing three times for 5 min in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.2% Tween 20, phosphorylated Erk1, Erk2, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p38 MAP kinase were detected on the blots by incubating with rabbit anti-phosphospecific Erk1/Erk2 antibodies (1 h at room temperature), anti-phosphospecific SAPK/JKN antibodies, and anti-phosphospecific p38 MAP kinase antibodies (each overnight at 4°C), respectively (dilution of the antibodies 1:1000). After three washes for 5 min, the blots were incubated with goat peroxidase-conjugated anti-rabbit antibodies (Sigma). After 1 h, the blots were washed again and immunoreactivity was visualized by enhanced chemiluminescence (Amersham, Freiburg, Germany). Total amount of Erk was detected with a rabbit anti-ERK1 antibody (0.1 μg/ml).

Data Presentation and Analysis. Unless otherwise stated, results are presented as mean $\pm$ S.E. of at least three independent experiments, each performed in duplicate or triplicate. Curve fitting was done by using iterative nonlinear regression analysis with the Prism program (GraphPad, San Diego, CA). Statistical analysis was performed by Student's two-tailed t test for unpaired data.

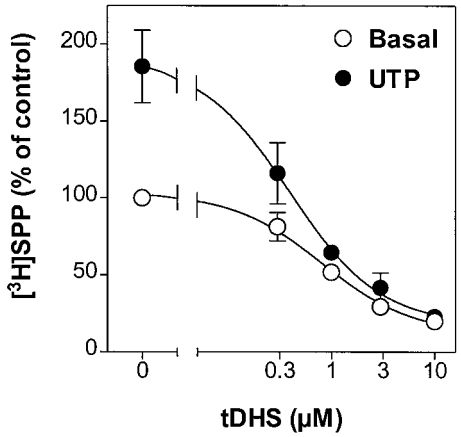

Fig. 2. Inhibition of basal and UTP-stimulated SPP production by tDHS. Basal and UTP (100 μM)-stimulated [^{3}H]SPP formation was measured for 2 min in HL-60 cells in the absence and presence of tDHS at the indicated concentrations. Values are expressed as percentage of [^{3}H]SPP production relative to unstimulated control cells, amounting to 2480 $\pm$ 194 cpm/1.8 $\times$ 10^6 cells (three to five experiments).

Results

Stimulation of SPP Production by the P2Y$_2$ Receptor in HL-60 Cells. To study whether the P2Y$_2$ receptor stimulates intracellular SPP production in HL-60 cells, formation of [^{3}H]SPP from [^{3}H]sphingosine was determined in the presence of the P2Y$_2$ receptor agonists UTP and ATP. Basal conversion of [^{3}H]sphingosine to [^{3}H]SPP in promyelocytic HL-60 cells was rapid, and within 3 to 5 min a plateau of [^{3}H]SPP accumulation was reached (data not shown). Activation of P2Y$_2$ receptors by UTP (100 μM), which was applied simultaneously with [^{3}H]sphingosine and which did not affect its cellular uptake, induced a rapid and transient increase in [^{3}H]SPP production (Fig. 1A). After 2 min, the increase in [^{3}H]SPP accumulation reached a maximum of 60 to 80% above basal values and then declined, approaching basal values after 5 min. Half-maximal and maximal stimulation of [^{3}H]SPP production was observed at 0.6 $\pm$ 0.2 μM and 10 to 100 μM UTP, respectively (Fig. 1B). P2Y$_2$ receptor activation with ATP induced a similar rapid and transient increase in [^{3}H]SPP accumulation, reaching 75% above basal values after a 2-min stimulation with 100 μM ATP. Furthermore, [^{3}H]SPP production induced by UTP and ATP was rather similar in promyelocytic and myeloid differentiated HL-60 cells (data not shown and see below). When the measurements were performed in the presence of the sphingosine

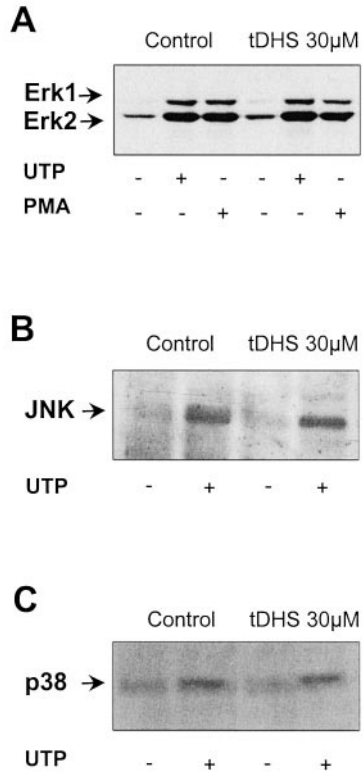

Fig. 3. tDHS does not inhibit activation of MAP kinases in HL-60 cells. Promyelocytic (A and B) and myeloid differentiated HL-60 cells (C) (5 $\times$ 10^6 cells each) were incubated for 5 min at 37°C in the absence (Control) and presence of 30 μM tDHS and then stimulated for 1 min with 100 μM UTP or 1 μM PMA as indicated. After SDS gel electrophoresis and transfer to nitrocellulose membranes, phosphorylation of Erk1 and Erk2 (A), JNK/SAPK (B), and p38 MAP kinase (C) was detected by immunoblotting with antiphosphospecific MAP kinase antibodies. The phosphorylated Erk1 (p44), Erk2 (p42), JNK/SAPK (p46), and p38 MAP kinase are marked by the arrows. Shown is a representative experiment repeated twice.

kinase inhibitor tDHS (Buehrer and Bell, 1992), basal and UTP-stimulated [^{3}H]SPP formation was strongly reduced (Fig. 2). At 10 μM tDHS, basal [^{3}H]SPP formation was reduced by about 80%, and that stimulated by UTP was completely suppressed, indicating that sphingosine kinase is responsible for $P2Y_2$ receptor-mediated SPP production in HL-60 cells.

Cellular responses to $P2Y_2$ receptors in HL-60 cells are largely mediated by PTX-insensitive G proteins (Klinker et al., 1996). In agreement, we observed that treatment of HL-60 cells with PTX (100 ng/ml, 20 h) had only a minor effect on UTP (100 μM)-stimulated Ca^{2+} mobilization (about 20% inhibition) and neither altered basal nor UTP-stimulated [^{3}H]SPP formation (data not shown). Thus, in contrast to the formyl peptide receptor response (Alemany et al., 1999), stimulation of SPP production by the $P2Y_2$ receptor in HL-60 cells is not mediated by PTX-sensitive G proteins.

SPP Production and MAP Kinase Activation. To study whether intracellular SPP formation participates in MAP kinase activation, the effect of the sphingosine kinase inhibitor tDHS on $P2Y_2$ receptor-mediated activation of various MAP kinases was examined. In the absence of tDHS,

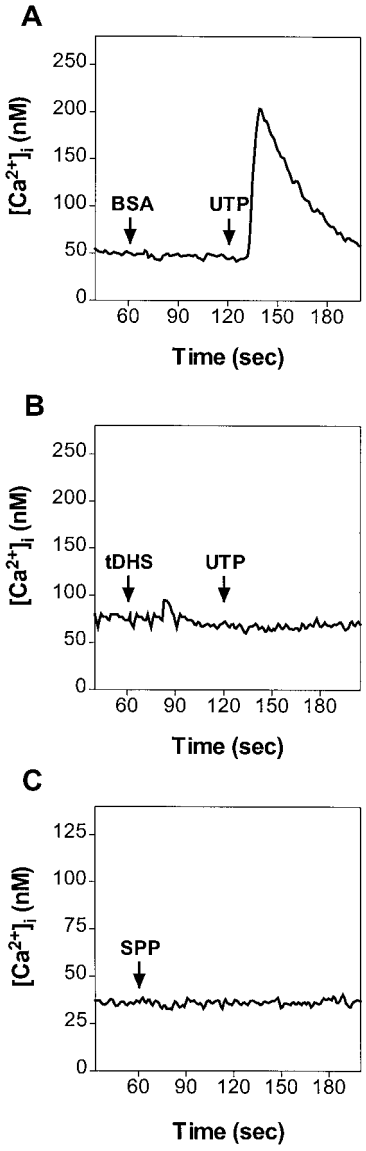

Fig. 4. Inhibition of UTP-induced Ca^{2+} mobilization by tDHS and lack of effect of extracellular SPP on $[Ca^{2+}]_i$. Shown are typical traces of UTP (10 μM)-induced $[Ca^{2+}]_i$ increase determined in HL-60 cells pretreated for 1 min with vehicle (A) or 10 μM tDHS (B). Ca^{2+} was omitted from the extracellular medium, and 50 μM EGTA was added 2 min before stimulus exposure. C, typical trace of $[Ca^{2+}]_i$ after the extracellular application of SPP (10 μM) in the presence of 1 mM extracellular Ca^{2+}. Addition of vehicle (BSA), tDHS, UTP, and SPP is indicated by the arrows. Shown are representative experiments repeated five times.

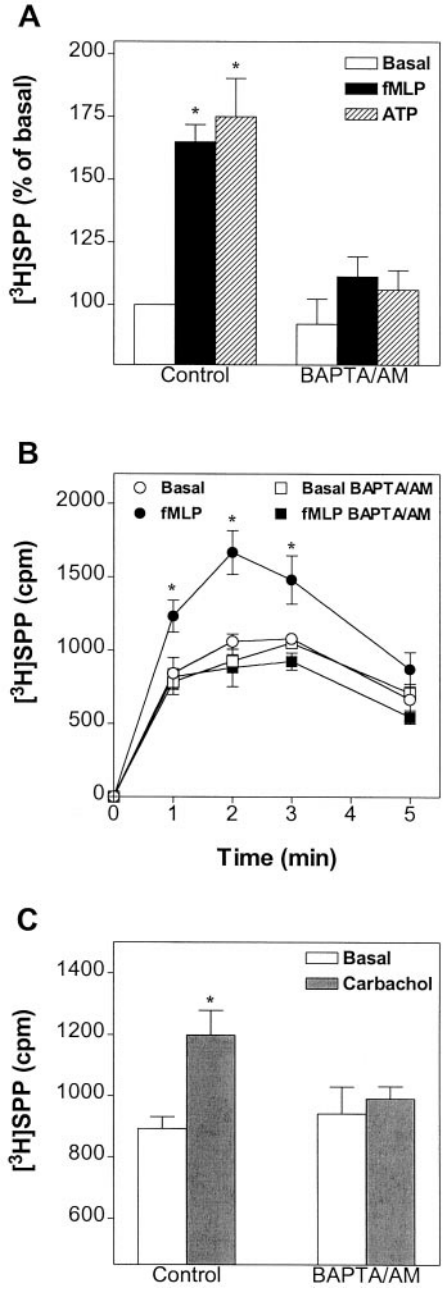

Fig. 5. Involvement of intracellular Ca^{2+} in receptor-mediated [^{3}H]SPP formation. Myeloid differentiated HL-60 cells (A and B) or HEK-293 cells (C) were pretreated for 30 min without (Control) and with 20 μM BAPTA/AM, followed by measurement of [^{3}H]SPP formation in the absence (Basal) and presence of 10 μM fMLP or 100 μM ATP for 2 min (A), 10 μM fMLP for the indicated periods of time (B) or 100 μM carbachol for 30 s (C). In A, values are expressed as percentage of [^{3}H]SPP production relative to unstimulated control cells, amounting to 1642 $\pm$ 320 cpm/1.8×10^6 cells. In C, values are mean $\pm$ S.D. from a representative experiment performed three times. $*$, significantly different from basal SPP formation ($P < .05$, three to four experiments).

addition of 100 μM UTP induced a strong and rapid (maximum at 1 min) increase in phosphorylation states of Erk1 and Erk2 in promyelocytic HL-60 cells, as detected with phosphospecific antibodies against these MAP kinases (Fig. 3A). Pretreatment of the cells for 5 min with 30 μM tDHS, fully blocking UTP-induced SPP formation, did not alter basal Erk phosphorylation and had no effect on phosphorylation of Erk1 and Erk2 induced by UTP. Total amount of Erk, measured with an anti-Erk1 antibody, was not affected by either UTP or tDHS (data not shown). tDHS also did not inhibit phosphorylation of Erk1 and Erk2 by the phorbol ester phorbol-12-myristate-13-acetate (PMA, 1 μM), inducing after 1-min stimulation an increase in ERK phosphorylation equivalent to that seen with 100 μM UTP (Fig. 3A). Similar to promyelocytic cells, treatment of myeloid differentiated HL-60 cells with tDHS (30 μM, 5 min) had no effect on phosphorylation of Erk1 and Erk2 induced by UTP (100 μM), PMA (1 μM), or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, 10 μM) (data not shown). P2Y$_2$ receptor activation by UTP (100 μM, 1 min) also induced distinct increases in phosphorylation states of SAPK/JNK (p46) and p38 MAP kinase in promyelocytic and differentiated HL-60 cells, respectively (Fig. 3, B and C). Similar to receptor-induced Erk phosphorylation, pretreatment of the cells with tDHS (30 μM, 5 min) did not affect UTP-induced phosphorylation of SAPK/JNK or p38 MAP kinase.

SPP Production and Ca^{2+} Mobilization. Previous studies with sphingosine kinase inhibitors in myeloid differentiated HL-60 cells suggested that intracellular SPP formation plays a major role in Ca^{2+} mobilization by the G_i-coupled formyl peptide receptor (Alemany et al., 1999). Therefore, P2Y$_2$ receptor-induced $[Ca^{2+}]_i$ increases and inhibition of this response by tDHS were studied. Half-maximal and maximal $[Ca^{2+}]_i$ increases were observed at ~0.5 μM and 10 to 100 μM UTP, respectively (data not shown), thus, at concentrations very similar to those required for stimulation of SPP formation (Fig. 1B). To study specifically the effect of tDHS on Ca^{2+} release from intracellular stores, we measured $[Ca^{2+}]_i$ transients in the absence of extracellular Ca^{2+}. Figure 4A illustrates typical changes in $[Ca^{2+}]_i$ in HL-60 cells stimulated with 10 μM UTP under this condition. There was a rapid $[Ca^{2+}]_i$ increase, by about 200 nM, which declined to basal levels within 1 to 2 min. Pretreatment of HL-60 cells for 1 min with 10 μM tDHS, which by itself did not alter $[Ca^{2+}]_i$, suppressed the UTP-induced $[Ca^{2+}]_i$ increase (Fig. 4B). Under the same condition, tDHS did not inhibit the increase in $[Ca^{2+}]_i$ induced by thapsigargin (1 μM), amounting to 149 $\pm$ 38 and 146 $\pm$ 12 nM above basal in the presence of vehicle and 10 μM tDHS, respectively. Most important, extracellularly applied SPP (10 μM) had no effect on $[Ca^{2+}]_i$ in HL-60 cells (Fig. 4C). tDHS (10–30 μM) also strongly inhibited (by 80–90%) UTP- or ATP-induced $[Ca^{2+}]_i$ increases measured in the presence of extracellular Ca^{2+} (data not shown). These data, thus, suggested that sphingosine kinase-catalyzed SPP formation plays a major role in Ca^{2+} mobilization by the P2Y$_2$ receptor in HL-60 cells, similarly as reported for the formyl peptide receptor (Alemany et al., 1999).

Finally, by using several approaches, we studied whether Ca^{2+} is involved in receptor-mediated SPP production. First, HL-60 cells were loaded with the intracellular Ca^{2+} chelator, BAPTA/AM (20 μM, 30 min), which prevented receptor-mediated $[Ca^{2+}]_i$ increases without affecting phospholipase C stimulation (data not shown). In cells (myeloid differentiated) pretreated with BAPTA/AM, stimulation of [^{3}H]SPP formation induced by either ATP (100 μM) or fMLP (10 μM) was fully blunted (Fig. 5A), and this was observed over the full time course of receptor-stimulated [^{3}H]SPP formation (Fig. 5B). Similarly, treatment of HEK-293 cells stably expressing the M_3 muscarinic acetylcholine receptor with BAPTA/AM (20 μM, 30 min) suppressed the carbachol (100 μM)-induced [^{3}H]SPP formation (Fig. 5C). Second, depletion of intracellular Ca^{2+} stores by pretreatment of HL-60 cells with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (1 μM, 30 min, in the absence of extracellular Ca^{2+}), which abrogated receptor-mediated Ca^{2+} mobilization, completely prevented UTP (100 μM)-induced [^{3}H]SPP formation (data not shown). Third, to study whether Ca^{2+} influx is required for receptor-mediated SPP production, $[Ca^{2+}]_i$ and [^{3}H]SPP production were measured in cells pretreated for 2 min with 5 mM EGTA (in the absence of extracellular Ca^{2+}). Under this condition, UTP (100 μM) increased $[Ca^{2+}]_i$ by 352 $\pm$ 36 nM compared with 536 $\pm$ 48 nM in Ca^{2+} (1 mM)-containing medium. Despite these different $[Ca^{2+}]_i$ increases, UTP (100 μM) increased [^{3}H]SPP formation to a

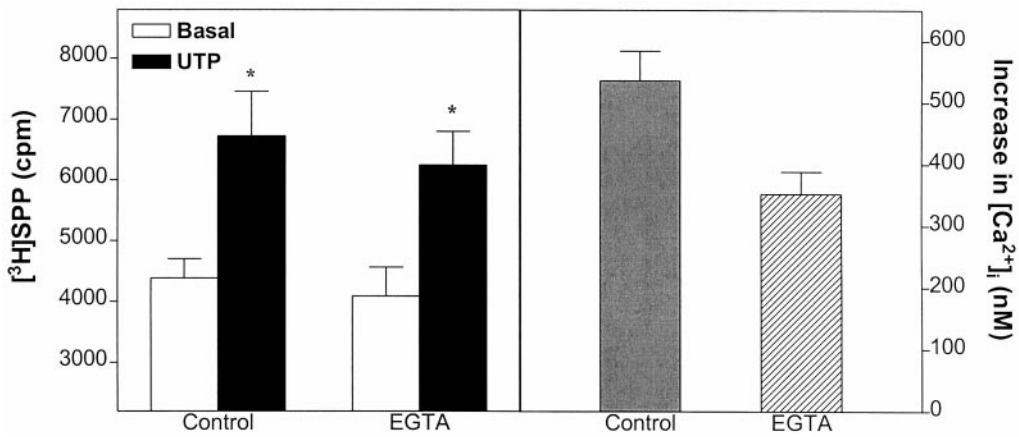

Fig. 6. Ca^{2+} influx is not required for UTP-induced SPP production. UTP (100 μM)-induced increases in $[Ca^{2+}]_i$ and [^{3}H]SPP production were measured in HL-60 cells either in the presence of extracellular Ca^{2+} (Control) or in cells treated for 2 min with 5 mM EGTA in the absence of extracellular Ca^{2+}. Shown are basal and UTP-stimulated [^{3}H]SPP production measured for 2 min (left) and maximal UTP-induced $[Ca^{2+}]_i$ increases (right). *, significantly different from basal SPP formation ($P < .05$, four to five experiments).

similar extent in the absence and presence of extracellular Ca^{2+} (Fig. 6). Similarly, fMLP (10 μM)-induced [^{3}H]SPP production in myeloid differentiated HL-60 cells was identical in the absence and presence of extracellular Ca^{2+} (increase by 67 ± 5 and 70 ± 11%, respectively, $n = 2$). Finally, the effect on SPP formation of increasing $[Ca^{2+}]_i$ by Ca^{2+} ionophores was examined. Exposure of HL-60 cells for 2 min to ionomycin and A-23187 (10 μM each) increased [^{3}H]SPP formation to a similar extent as the formyl peptide fMLP (10 μM) (Fig. 7). There was no further increase when fMLP was combined with either ionomycin or A-23187. Thus, an increase in $[Ca^{2+}]_i$, but not Ca^{2+} influx, is apparently required for receptor-mediated SPP production in HL-60 cells.

Discussion

Exposure of HL-60 cells to UTP and ATP caused a rapid and transient production of SPP from sphingosine, with a time course and magnitude similar as reported for various membrane receptors in different cell types (Olivera and Spiegel, 1993; Melendez et al., 1998b; Meyer zu Heringdorf et al., 1998; Alemany et al., 1999). The potency and specificity of the nucleotides as well as the finding that UTP and ATP increased SPP production in both promyelocytic and myeloid differentiated HL-60 cells, which was blocked by the sphingosine kinase inhibitor tDHS, strongly suggest that SPP production by sphingosine kinase is stimulated by $P2Y_2$ receptors endogenously expressed in HL-60 cells (Klinker et al., 1996). Similar to other $P2Y_2$ receptor responses in HL-60 cells, UTP-induced SPP accumulation was not affected by PTX, in contrast to the formyl peptide receptor response, which was fully PTX sensitive (Alemany et al., 1999). As direct G protein activation by AlF_4^- can stimulate SPP production in HL-60 cells (Alemany et al., 1999), it is thus feasible to assume that PTX-insensitive G proteins mediate this $P2Y_2$ receptor action.

Stimulation of SPP formation by G protein-coupled receptors in HL-60 and HEK-293 cells is apparently a Ca^{2+}-dependent process. First, chelation of intracellular Ca^{2+} with BAPTA/AM eliminated SPP formation by $P2Y_2$ and formyl peptide receptors in HL-60 cells as well by the M_3 muscarinic receptor in HEK-293 cells. Second, depletion of intracellular Ca^{2+} stores by pretreatment of HL-60 cells with thapsigargin also prevented UTP-induced SPP formation. In contrast, removal of extracellular Ca^{2+} did not affect the $P2Y_2$ and formyl peptide receptor-mediated SPP accumulation. Thus, an increase in $[Ca^{2+}]_i$ caused by mobilization of intracellular Ca^{2+}, but not Ca^{2+} influx, is apparently required for stimulation of SPP formation by G protein-coupled receptors in HL-60 cells. Finally, SPP formation also was increased by exposure of HL-60 cells and HEK-293 cells (our unpublished observations) to the Ca^{2+} ionophores, ionomycin, and A-23187. While this manuscript was in preparation, a study performed in TRMP canine kidney epithelial cells transfected with the platelet-derived growth factor receptor similarly concluded that sphingosine kinase activation by this tyrosine kinase receptor is a Ca^{2+}-dependent event (Olivera et al., 1999). The mechanism of the Ca^{2+}-dependent SPP formation is presently not clear. The sequence of the recently cloned murine sphingosine kinase enzymes contains potential phosphorylation and Ca^{2+}/calmodulin-binding sites (Kohama et al., 1998). It is thus possible that enzyme activity is regulated by Ca^{2+}/calmodulin and/or Ca^{2+}-dependent phosphorylation, which however was not reported with the expressed enzyme.

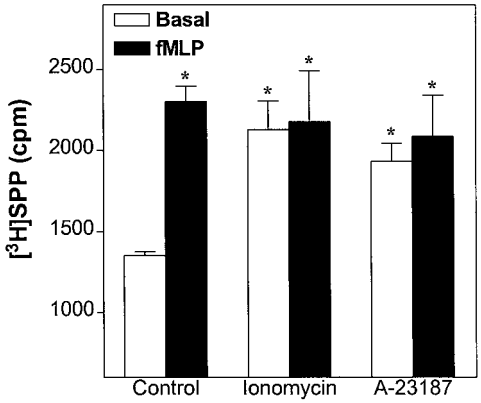

Fig. 7. Increase in SPP production by Ca^{2+} ionophores. [^{3}H]SPP formation was measured for 2 min in myeloid differentiated HL-60 cells in the presence of 1 mM extracellular Ca^{2+} without (Basal) and with fMLP, ionomycin, A-23187 (10 μM each) or combinations of fMLP with ionomycin or A-23187 as indicated. *, significantly different from basal SPP formation ($P < .05$, three to four experiments).

In agreement with our previous findings on formyl peptide receptor action in myeloid differentiated HL-60 cells (Alemany et al., 1999), we report herein that short-term pretreatment of the cells with the sphingosine kinase inhibitor tDHS suppresses the $P2Y_2$ receptor-mediated Ca^{2+} mobilization, thus arguing for a major role of SPP production in Ca^{2+} signaling by the $P2Y_2$ receptor. tDHS by itself did not increase $[Ca^{2+}]_i$ and did not deplete internal Ca^{2+} stores. Moreover, we reported before that suppression of Ca^{2+} signaling in HL-60 cells and HEK-293 cells by tDHS is not caused by inhibition of protein kinase C or perturbation of receptor-mediated phospholipase C stimulation (Meyer zu Heringdorf et al., 1998; Alemany et al., 1999). Because extracellularly applied SPP did not increase $[Ca^{2+}]_i$ in HL-60 cells, it is highly unlikely that intracellularly formed SPP induces Ca^{2+} mobilization by activating cell surface sphingolipids receptors after being released from the cells. Thus, SPP production appears to be essential for receptor-mediated Ca^{2+} mobilization, but an increase in $[Ca^{2+}]_i$ is apparently required for stimulation of sphingosine kinase (see above). We therefore propose that the activated $P2Y_2$ and formyl peptide receptors induce via increased formation of inositol 1,4,5-trisphosphate by phospholipase C a local discrete $[Ca^{2+}]_i$ increase, not detectable by the methodology used, that then activates SPP production by sphingosine kinase, ultimately leading to full Ca^{2+} mobilization. The difference between peak [^{3}H]SPP formation and $[Ca^{2+}]_i$ 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 to be converted to [^{3}H]SPP. Thus, as also suggested for Ca^{2+} signaling by the platelet-derived growth factor receptor in TRMP cells (Olivera et al., 1999), stimulation of intracellular SPP formation by G protein-coupled receptors in HL-60 cells may represent an amplification system for Ca^{2+} signaling by these receptors that is primarily initiated by phospholipase C stimulation. In line with this hypothesis, Li et al. (2000) recently reported that peritoneal neutrophils from mice lacking both phospho-

lipase C-β2 and C-β3 do not respond to fMLP with $[Ca^{2+}]_i$ increases.

Based on inhibitory effects observed with sphingosine kinase inhibitors and/or addition of extracellular SPP, SPP has been postulated to act as a second messenger of receptor-mediated mitogenic responses, including activation of MAP kinases (Su et al., 1994; Wu et al., 1995; Cuvillier et al., 1996; Pyne et al., 1996; Blakesley et al., 1997; Kozawa et al., 1997; Rani et al., 1997; van Brocklyn et al., 1998). However, treatment of HL-60 cells with the sphingosine kinase inhibitor tDHS, causing full inhibition of receptor-mediated SPP production, had no effect on P2Y$_2$ or formyl peptide receptor-induced activation of MAP kinases, including Erk1, Erk2, SAPK/JNK, and p38 MAP kinase. These data, thus, strongly argue against an essential role of sphingosine kinase stimulation and intracellular SPP in activation of MAP kinases by G protein-coupled receptors in HL-60 cells. In agreement with our results, several recent studies strongly suggest that some of the cellular effects formerly attributed to intracellular SPP, including Erk activation, are probably caused by an action of SPP at cell surface receptors (Sato et al., 1999; Tolan et al., 1999).

In conclusion, this study demonstrates that purinergic P2Y$_2$ receptors endogenously expressed in HL-60 cells stimulate SPP production by sphingosine kinase, a process apparently not required for activation of MAP kinases. However, the Ca^{2+} requirement of receptor-mediated SPP production and the inhibition of Ca^{2+} mobilization by blockade of this response suggest that intracellular SPP formation may represent an amplification system for receptor-mediated Ca^{2+} signaling and Ca^{2+}-regulated cellular processes in HL-60 granulocytes.

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