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Short communication

Effect of dimethylsphingosine on muscarinic M_3 receptor signalling in SH-SY5Y cells

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Abstract

The sphingosine kinase inhibitor, dimethylsphingosine, is an important tool for investigating intracellular effects of the putative second messenger compound, sphingosine 1-phosphate. However, the specificity of action of dimethylsphingosine has not been fully investigated. In human SH-SY5Y neuroblastoma cells, dimethylsphingosine (30 μM), produced a 25-fold increase in the EC_{50} for methacholine-induced Ca^{2+} mobilisation, and reduced the maximum response by 57 ± 5%, suggesting the involvement of sphingosine 1-phosphate production in the Ca^{2+} signal. However, dimethylsphingosine also inhibited [^{3}H]*N*-methylscopolamine binding to whole SH-SY5Y cells and reduced methacholine-induced phosphoinositide turnover. Thus, this compound must be used with caution when investigating the role of sphingosine kinase in G-protein coupled receptor-mediated Ca^{2+} mobilisation responses.

Keywords: Muscarinic receptor; Sphingosine 1-phosphate; Dimethylsphingosine; Ins(1,4,5)P_3; Ca^{2+} mobilisation; SH-SY5Y neuroblastoma cell

1. Introduction

Muscarinic M_3 receptor stimulation of the phosphoinositide/Ca^{2+} signalling cascade in the human neuroblastoma SH-SY5Y cell line has been well documented. Addition of the muscarinic receptor agonists carbachol or methacholine have been shown to stimulate inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) production, raise intracellular free Ca^{2+} ($[Ca^{2+}]_i$) (Lambert and Nahorski, 1990; Willars and Nahorski, 1995), and produce a concomitant depletion of phosphoinositide 4,5-bisphosphate (PIP_2) (Willars et al., 1998). However, there is now growing interest in the ability of various sphingolipid compounds to act as intracellular messengers for a number of processes, including Ca^{2+} mobilisation (reviewed in: Meyer zu Heringdorf et al., 1997; Hla et al., 1999). Specifically, sphingosine 1-phosphate and the related compound sphingosylphosphorylcholine, have been shown to mobilise intracellular Ca^{2+} stores in a variety of cells (Ghosh et al., 1990, 1994; Meyer zu Heringdorf et al., 1998), and as a number of cell surface receptors (including receptors for platelet-derived growth factor, immunoglobulin E, and formyl–methionyl–leucyl–phenylalanine (FMLP)) stimulate increases in intracellular levels of sphingosine 1-phosphate, most probably via activation of sphingosine kinase (Olivera and Spiegel, 1993; Choi et al., 1996; Alemany et al., 1999), parallels have been drawn with the Ins(1,4,5)P_3/Ca^{2+} mobilisation pathway.

Investigations into the role of sphingosine 1-phosphate production in raising $[Ca^{2+}]_i$ have been aided by the use of cell permeant, sphingolipid-based, inhibitors of sphingosine kinase such as dihydrosphingosine and dimethylsphingosine (Edsall et al., 1998; Meyer zu Heringdorf et al., 1998). Based on the actions of these sphingosine kinase inhibitors, it has been suggested that a number of G-protein coupled receptors, such as muscarinic M_2 and M_3 receptors, utilise sphingosine 1-phosphate to augment the more recognised Ins(1,4,5)P_3-mediated Ca^{2+} release pathway (Meyer zu Heringdorf et al., 1998). In support of this, Ca^{2+} mobilisation in SH-SY5Y cells, in response to maximal muscarinic M_3 receptor stimulation was partially inhibited by pretreatment with dimethylsphingosine (Young et al., 1999). Although the use of sphingosine kinase inhibitors is becoming more routine, their specificity of action remains to be completely established. In this current

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study, we demonstrate that, unlike previous reports using recombinant systems, dimethylsphingosine lowers the number of M_3 receptor binding sites, and inhibits Ins (1,4,5)P_3 production. This lack of specificity of block necessitates careful examination of dimethylsphingosine-mediated inhibition of responses in order to determine the role of sphingosine 1-phosphate production in G-protein coupled receptor-mediated Ca^{2+} signalling.

2. Methods

2.1. Cell culture

SH-SY5Y neuroblastoma cells were grown to near confluence in minimal essential medium with Earle's salts, 5% (v/v) foetal bovine serum, 5% (v/v) newborn bovine serum, 2 mM L-glutamine, 100 units ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 2.5 $\mu g\ ml^{-1}$ fungisone (GibcoBRL, UK), in a humidified atmosphere of 5% CO_2/95% air.

2.2. [^{3}H]inositol phosphate accumulation

SH-SY5Y neuroblastoma cells were grown on 24-well plates and loaded to equilibrium with [^{3}H]inositol (3 $\mu Ci\ ml^{-1}$, Amersham) for 48 h. Incubations were carried out in Krebs–Henseleit buffer (KHB in mM: NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 1.3; KH_2PO_4, 1.2; $NaHCO_3$, 4.2; Hepes, 10, pH 7.4, supplemented with 10 $\mu g\ ml^{-1}$ fatty acid-free bovine serum albumin which acts as a low affinity carrier for sphingolipids). Cells were pre-incubated at 37°C for 10 min in KHB lacking in added $CaCl_2$, but containing 10 mM LiCl (± 30 μM dimethylsphingosine or *N*-acetylsphingosine if appropriate), before being challenged with agonists or KHB. $CaCl_2$ was omitted from these studies to minimise potential effects of dimethylsphingosine on Ca^{2+} entry. Dimethylsphingosine was purchased from Calbiochem (UK), and *N*-acetylsphingosine was from Sigma (UK). Both compounds were made up as aliquots of sock solutions in DMSO, which were defrosted immediately prior to use. Reactions were terminated after 5 min by addition of trichloroacetic acid (final concentration 0.5 M), followed by addition of EDTA (final concentration 2 mM). [^{3}H]inositol phosphates were extracted by the addition of tri-*n*-octylamine/1,1,2,-trichlorotrifluoroethane (1:1 v/v), and the tritium content was estimated by liquid scintillation counting (see Martin et al., 1999).

2.3. Measurement of Ins(1,4,5)P_3 mass

SH-SY5Y cells on 24 well plates at 37°C were pre-incubated for 10 min with KHB ± 30 μM dimethylsphingosine where appropriate. Incubation media was removed from each well, before the addition of methacholine ± 30 μM dimethylsphingosine. The reaction was stopped with trichloroacetic acid, and Ins(1,4,5)P_3 was extracted as described above. Ins(1,4,5)P_3 mass measurements were determined using a radioreceptor assay (Challiss et al., 1990).

2.4. [^{3}H]N-methyl scopolamine binding to SH-SY5Y cells

For saturation binding studies, SH-SY5Y neuroblastoma cells were grown on 24 well plates and incubated in KHB supplemented with increasing concentrations of [^{3}H]*N*-methyl scopolamine (Amersham). Non-specific binding was determined using 2 μM atropine. Each well also contained 30 μM dimethylsphingosine, 50 μM dimethylsphingosine, or KHB alone, and incubations were for 20 min at 37°C. After washing twice with ice-cold KHB, the well contents were extracted in 500 mM NaOH, then neutralised with 500 mM HCl. Tritium content was estimated by liquid scintillation counting. Protein content was determined using a Lowry protein assay.

2.5. Ca^{2+} imaging

Confluent monolayers of SH-SY5Y cells, grown on 22-mm diameter coverslips, were incubated in KHB supplemented with 2 μM fura-2 AM for 60 min at 22°C before being mounted on the stage of Nikon Diaphot inverted epifluorescene microscope and heated to 37°C. Images at wavelengths above 510 nm were collected, after excitation at 340 and 380 nm, with an intensified charge-coupled device camera (Photonic Science). Cells were pre-incubated in KHB to which no Ca^{2+} had been added, plus dimethylsphingosine (where appropriate) for 10 min before agonist addition. Each condition was directly compared with control responses measured in parallel.

2.6. Analysis of data

Concentration response curves were fitted by non-linear regression to a logistic equation (GraphPad Prism). Statistical differences were examined using Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of dimethylsphingosine on methacholine-induced intracellular Ca^{2+} mobilisation in SH-SY5Y cells

After a 10-min pre-incubation period in the absence of extracellular Ca^{2+} (to minimise potential effects of dimethylsphingosine on Ca^{2+} entry) methacholine, acting on endogenous muscarinic M_3 receptors produced a rapid transient increase in $[Ca^{2+}]_i$ in SH-SY5Y cells. The maximum peak Ca^{2+} response occurred between 10^{-5} and 10^{-3} M methacholine, at which point the increase in $[Ca^{2+}]_i$ was 511 ± 23 nM (Fig. 1). Basal $[Ca^{2+}]_i$ was 73 ± 3 nM ($n = 14$). The best-fit log EC_{50} was calculated

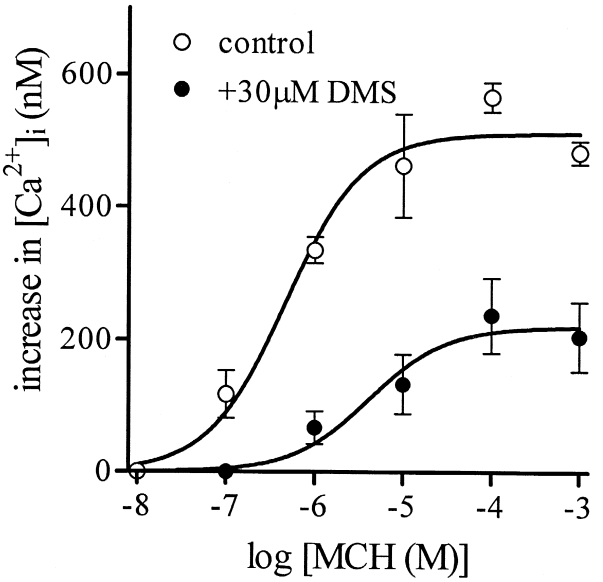

Fig. 1. Confluent monolayers of SH-SY5Y cells, loaded with fura-2, were incubated in 0 Ca^{2+} KHB for 10 min ±30 μM DMS as appropriate. Values represent the mean±S.E.M. peak increase in $[Ca^{2+}]_i$, after subtraction of basal levels (three paired experiments for each condition). The best-fit maximal responses and $-\log EC_{50}$ values were 511±23 nM and 6.33±0.14 (0.16 μM) for MCH alone, and 220±26 nM and 5.38±0.30 (4.1 μM), for MCH plus DMS.

to be -6.33 ± 0.14 (0.16 μM, Fig. 1). Pretreatment with 30 μM dimethylsphingosine in 0 Ca^{2+} (10 min) caused a slight increase in basal $[Ca^{2+}]_i$ (14 ± 5 nM increase, $n = 14$). Dimethylsphingosine pretreatment also produced a rightward-shift in the concentration–response curve for methacholine, and reduced the maximum Ca^{2+} response when compared with paired controls ($P < 0.05$; Fig. 1). Thus, the maximum increase in $[Ca^{2+}]_i$ was reduced to 220 ± 26 nM (57 ± 5% reduction), and the log EC_{50} increased to -5.38 ± 0.30 (4.1 μM, $n = 3$ for each concentration). This concentration of dimethylsphingosine was the lowest concentration in our system to cause reproducible inhibitions (data not shown).

3.2. Effect of dimethylsphingosine on [^{3}H]N-methylscopolamine binding in SH-SY5Y cells

Effects of dimethylsphingosine on the binding of [^{3}H]*N*-methylscopolamine to whole SH-SY5Y cells was investigated to determine possible direct interactions with the endogenous muscarinic M_3 receptor population. Concentrations of dimethylsphingosine of 30 and 50 μM, but not 1 and 5 μM, were observed to reduce the specific binding of 0.5 nM [^{3}H]*N*-methylscopolamine to SH-SY5Y cells (data not shown). Further investigation demonstrated that dimethylsphingosine produced a concentration-dependent reduction in the B_{max} for muscarinic M_3 receptor binding with only a modest increase in the K_D value. At 30 μM dimethylsphingosine, the maximum [^{3}H]*N*-methylscopolamine binding was reduced by 21 ± 2%, whilst the K_D increased from 0.14 ± 0.01 to 0.39 ± 0.04 nM ($n = 5$). At 50 μM dimethylsphingosine, the maximum was reduced by 60 ± 2% and the K_D was 0.37 ± 0.03 nM ($n = 5$).

3.3. Effect of dimethylsphingosine and N-acetylsphingosine on methacholine-induced Ins(1,4,5)P_3 production in SH-SY5Y cells

The ability of dimethylsphingosine to inhibit methacholine-induced Ca^{2+} mobilisation responses could either be due to an action on sphingosine kinase or due to a reduction in the ability of methacholine to stimulate Ins(1,4,5)P_3 production via blockade of muscarinic M_3 receptors, as demonstrated for [^{3}H]*N*-methylscopolamine binding. To answer this question directly, the effect of dimethylsphingosine on methacholine-induced Ins(1,4,5)P_3

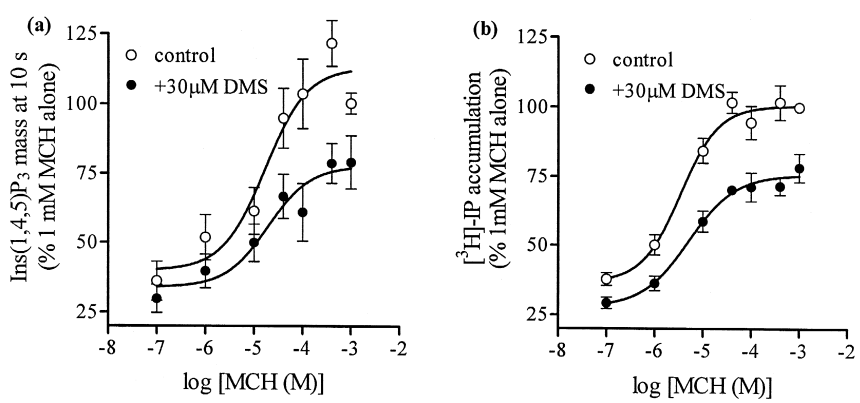

Fig. 2. (a) MCH-induced Ins(1,4,5)P_3 production in SH-SY5Y cells pre-incubated with KHB ±30 μM DMS for 10 min. The best-fit $-\log EC_{50}$ values were 4.78 ± 0.23 (16.5 μM) for MCH alone and 4.71 ± 0.34 (19.4 μM) for MCH plus DMS. DMS reduced the maximal response to MCH by 31 ± 7%. Each point represents the mean ± S.E.M. data from four paired experiments. (b) SH-SY5Y cells were labelled with [^{3}H]-inositol for 48 h, and then incubated in 0 Ca^{2+} KHB containing 10 mM Li^+ ±30 μM DMS, for 10 min before addition of MCH. Incubations were terminated after 5 min. The best-fit $-\log EC_{50}$ values were 5.47 ± 0.15 (3.4 μM) for MCH alone, and 5.29 ± 0.20 (5.1 μM) for MCH plus DMS. DMS treatment reduced the best-fit maximal response to MCH by 24 ± 10% ($n = 4$).

production, and [^{3}H]inositol phosphate accumulation in the presence of 10 mM Li^+, was investigated. Methacholine-induced Ins(1,4,5)P_3 production in SH-SY5Y cells is biphasic, with a peak response occurring around 10 s (Lambert and Nahorski, 1990). A 10-min pretreatment with 30 μM dimethylsphingosine reduced the maximum response without altering the EC_{50} value for methacholine-induced Ins(1,4,5)P_3 production, as measured at a 10-s time point (31 ± 7% reduction, log EC_{50} -4.78 ± 0.23 (16.5 μM) and -4.71 ± 0.34 (19.4 μM) in the absence and presence of dimethylsphingosine, respectively, $n = 4$) (Fig. 2a). A similar effect of dimethylsphingosine was observed on methacholine-induced [^{3}H]inositol phosphate production in the absence of extracellular Ca^{2+}, measured over a 5-min accumulation period (24 ± 10% reduction, $-\log EC_{50}$ -5.47 ± 0.15 (3.4 μM) in the absence, and -5.29 ± 0.20 (5.1 μM) in the presence of 30 μM dimethylsphingosine, $n = 4$) (Fig. 2b). In contrast, the related compound *N*-acetylsphingosine which does not block sphingosine kinase activity, and is without effect of methacholine-induced Ca^{2+} responses in SH-SY5Y cells (Young et al., 1999), was without significant effect on methacholine-induced [^{3}H]inositol production (data not shown).

4. Discussion

The role of sphingolipids as intracellular signalling molecules remains controversial, mainly because sphingosine 1-phosphate is most likely the extracellular ligand for certain members of the Edg family of G-protein coupled receptors (Hla et al., 1999). However, intracellular levels of sphingosine 1-phosphate are regulated by a number of G-protein coupled receptors and intracellular sphingosine 1-phosphate does activate cellular processes, most notably Ca^{2+} mobilisation (Choi et al., 1996; Meyer zu Heringdorf et al., 1998; Alemany et al., 1999), and so sphingosine 1-phosphate may function as both intra- and extra-cellular messenger. The possible involvement of sphingosine 1-phosphate in G-protein coupled receptor-mediated Ca^{2+} responses in SH-SY5Y cells is of particular interest as previous work from this laboratory has demonstrated a lack of correlation between Ins(1,4,5)P_3 production and Ca^{2+} increases stimulated by methacholine and bradykinin in this cell line (Martin et al., 1999). Indeed, it was suggested that selective activation of other Ca^{2+} mobilisation pathways, perhaps one involving sphingosine 1-phosphate, could account for this complex relationship.

The involvement of sphingosine 1-phosphate in G-protein coupled receptor-mediated Ca^{2+} responses is often investigated using inhibitors of sphingosine kinase, such as dimethylsphingosine or dihydrosphingosine. However, these compounds are extremely lipid soluble and can inhibit Ca^{2+} release activated Ca^{2+} current in RBH-2H3 cells (Mathes et al., 1998), suggesting possible complications with their specificity of action. The K_i for dimethylsphingosine in whole cell systems is approximately 5 μM (Edsall et al., 1998). In recombinant systems, dimethylsphingosine reduces muscarinic M_3 receptor-mediated Ca^{2+} signalling, with a maximum response occurring at 30 μM (Meyer zu Heringdorf et al., 1998). At this concentration of dimethylsphingosine, muscarinic M_3 receptor-mediated Ca^{2+} responses are completely inhibited, which raises the question as to the role of Ins(1,4,5)P_3 in the Ca^{2+} response. It is for this reason 30 μM dimethylsphingosine was examined on muscarinic M_3 receptors endogenously expressed in SH-SY5Y cells. The inhibition of specific [^{3}H]*N*-methyl scopolamine binding in SH-SY5Y cells by dimethylsphingosine as observed in this current study clearly indicates an effect on muscarinic M_3 receptors. This contrasts with a reported lack of effect of dimethylsphingosine on muscarinic M_3 receptors in recombinant systems (Meyer zu Heringdorf et al., 1998). Whether this is due to a higher level of receptor expression in the recombinant studies is not clear. The reason for the reduction in B_{max} has not been further investigated, but may be due to an accumulation of dimethylsphingosine in the cell membrane, causing a reduction in the available number of muscarinic M_3 receptor binding sites. This reduction in muscarinic M_3 receptor binding capacity in SH-SY5Y cells translates to a reduced stimulation of Ins(1,4,5)P_3 levels, and this clearly complicates interpretation of effects on the Ca^{2+} signal. Thus, the inhibition of Ca^{2+} release may reflect a reduced Ins(1,4,5)P_3 response, or indeed inhibition of some other pathway, rather than a specific reduction in agonist-induced sphingosine 1-phosphate production. This is not a general effect of all sphingolipids, as the related compound *N*-acetylsphingosine was without effect.

Therefore, in a given system, all potential effects of dimethylsphingosine need to be examined. It is important to note that 30 μM dimethylsphingosine is the lowest concentration of inhibitor which would be expected to completely inhibit sphingosine kinase activity, and that complete inhibition would be required to dissect out the relative contributions of sphingosine 1-phosphate and Ins(1,4,5)P_3 to the Ca^{2+} response. However, as described above, this concentration of dimethylsphingosine also has effects on the muscarinic M_3 receptor. In SH-SY5Y cells, there is a marked amplification between Ins(1,4,5)P_3 production and Ca^{2+} mobilisation in methacholine-induced responses (EC_{50} values of 16.5 and 0.16 μM, respectively). Thus, the Ca^{2+} response should be less sensitive than the Ins(1,4,5)P_3 response to dimethylsphingosine, if this was solely due to muscarinic M_3 receptor blockade. This is clearly not the case. Furthermore, if the response to 10 μM methacholine, which produces a maximal Ca^{2+} response in control cells, is compared with 1 mM methacholine in the presence of dimethylsphingosine, it can be seen that although the same amount of Ins(1,4,5)P_3 is being produced under both conditions, the Ins(1,4,5)P_3 produced in

sphingosine kinase inhibited cells is less effective at releasing Ca^{2+} (compare Figs. 1 and 2a). This is not due to depletion of intracellular Ca^{2+} stores as demonstrated using the Ca^{2+} ionophore ionomycin (Young et al., 1999). Similarly, dimethylsphingosine does not alter Ins(1,4,5)P_3 receptor binding (unpublished observations), or the direct ability of Ins(1,4,5)P_3 to release Ca^{2+} (Meyer zu Heringdorf et al., 1998). Therefore, in this specific example, the inhibition of methacholine-induced Ca^{2+} mobilisation by dimethylsphingosine cannot be solely attributed to a reduced Ins(1,4,5)P_3 production resulting from muscarinic M_3 receptor blockade. However, importantly, these results indicate that inhibition of G-protein coupled receptor-mediated Ca^{2+} signals via treatment with dimethylsphingosine by itself does not necessarily imply the involvement of sphingosine 1-phosphate in the response.

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