

## Full Paper

## Dimethylsphingosine Regulates Intracellular pH and Ca<sup>2+</sup> in Human Monocytes

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**Abstract.** Dimethylsphingosine (DMS) was first reported as an inhibitor of protein kinase C and later has been used as a specific inhibitor of sphingosine kinase. Furthermore, its anti-cancer effect has become a basis for development of chemotherapy. Nevertheless, its anti-neoplastic mechanism has poorly been understood. In the present study, we observed that DMS increased intracellular pH and Ca<sup>2+</sup> concentration in U937 human monocytes. To further characterize these DMS-induced actions, we employed structurally-related sphingolipids and specific pharmacological tools such as inhibitors of protein kinase C and Na<sup>+</sup>/H<sup>+</sup> exchanger and found that the two responses of DMS were mimicked by four stereoisomers of sphingosine and two isomers to dihydrosphingosine, but not with sphingosine 1-phosphate, sphingosyl-phosphorylcholine, and C2-ceramide. Furthermore, DMS-induced pH increase was independent of Na<sup>+</sup>/H<sup>+</sup> exchanger activity. We also characterized the interrelationship between DMS-induced pH increase and DMS-induced Ca<sup>2+</sup> increase. Since DMS is considered to be a good anti-cancer candidate, our characterization of DMS actions provides useful information for development of DMS chemotherapy.

**Keywords:** dimethylsphingosine, pH, sphingosine, calcium, signal transduction

### Introduction

Sphingolipids, including sphingosine, sphingosine 1-phosphate (S1P), and ceramide, have intensively been studied during the last two decades (1, 2). Especially, sphingosine and ceramide have drawn a great deal of attention because of their second messenger roles in programmed cell death, apoptosis (3, 4). In contrast, S1P is known as a survival or cell proliferating factor (5) since it has been shown to rescue cells from apoptotic stimuli. Sphingosine kinase converts sphingosine to S1P, thus resulting in decreased sphingosine content and increased S1P content in the cells (5, 6). Therefore, sphingosine kinase is a central enzyme to regulate the intracellular ratio of sphingosine/ceramide versus S1P (6), and overexpression of sphingosine kinase results in

an increase of intracellular S1P content, making cells resistant to the pro-apoptotic environment (5, 7).

*N,N*-Dimethyl-D-erythro-sphingosine (DMS) is a natural sphingolipid metabolite in mammalian cells (8, 9). It was first reported as an inhibitor of protein kinase C (PKC) along with D-erythro sphingosine (D-Sph) (10–12). Later, Spiegel and her coworkers reported its specific inhibitory action on sphingosine kinase, but no inhibitory action on PKC in certain cell types (13, 14). Since then, it has been used as a specific inhibitor of sphingosine kinase. Furthermore, its anti-cancer effects such as inhibitions of tumor cell migration and cancer cell growth have been reported and provided a fundamental base for development of chemotherapy (15–17). However, its anti-neoplastic mechanism has poorly been understood.

Changes of intracellular pH have been implicated in a variety of cellular responses, including cell cycle regulation and tumor cell formation (18, 19). Increase of intra-

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cellular  $\text{Ca}^{2+}$  concentration also plays an important role in cell functions such as cell proliferation and insulin secretion (20, 21). Therefore, in order to study the basic mechanism of DMS action, we measured changes of intracellular pH and cytosolic  $\text{Ca}^{2+}$  concentration induced by DMS. We observed that DMS induced increases of intracellular pH and  $\text{Ca}^{2+}$  concentration in U937 human monocyte cells, and we further characterized several pharmacological aspects of its action.

## Materials and Methods

### Materials

*N,N*-Dimethyl-D-erythro-sphingosine (DMS), D-erythro- and L-threo-4,5-dihydrosphingosine (H2Sph), sphingosine 1-phosphate (S1P), and sphingosylphosphorylcholine (SPC) were purchased from Avanti Polar lipids (Alabaster, AL, USA); D-erythro-sphingosine (D-Sph), D-threo-sphingosine, L-threo-sphingosine (L-Sph), L-erythro-sphingosine were from Matreya, Inc. (Pleasant Gap, PA, USA); phytosphingosine (Phyto) was kindly provided from Doosan Biotech (Yongin, Korea). BCECF-AM was purchased from Biotium (Hayward, CA, USA); Fura 2-AM and Go6976 were from Calbiochem (Darmstadt, Germany); U73122, U73343, GF109203X, and Ro-31-8221 were from Biomol (Plymouth Meeting, PA, USA); staurosporin, phorbol 12-myristate 13-acetate (PMA) and H7 were from Tocris Cookson Ltd. (Avonmouth, Bristol, UK). All other materials were purchased from Sigma-Aldrich Korea (Seoul, Korea).

### Cell culture

U937 human monocytes were maintained in RPMI medium containing 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin, 2 mM glutamine, and 1 mM pyruvate sodium at 37°C in a humidified 5%  $\text{CO}_2$  incubator. Cells were treated with pertussis toxin (PTX) by adding the toxin (100 ng/ml) to medium 24 h before the experiments.

### Measurement of intracellular pH or $\text{Ca}^{2+}$ concentration

The cells were sedimented, resuspended in a Hepes-buffered medium (HBM) consisting of 20 mM Hepes (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 0.5 mM  $\text{CaCl}_2$ , 25 mM  $\text{NaHCO}_3$ , 15 mM glucose, and 0.1% bovine serum albumin (fatty acid free); and then they were incubated for 40 min with 5  $\mu\text{M}$  BCECF-AM for pH measurement or fura 2-AM for  $\text{Ca}^{2+}$  measurement. The BCECF fluorescence was recorded at excitation wavelengths of 440 and 490 nm and an emission wavelength of 530 nm (F4500; Hitachi, Tokyo). The 490/440 fluorescence ratios were calibrated

by nigericin and FCCP (22, 23). The amount of  $[\text{Ca}^{2+}]_i$  was estimated from the change in the fluorescence of the fura 2-loaded cells (24). Fluorescence emission at 510 nm wavelength from two excitation wavelengths (340 and 380 nm) were measured every 0.1 s (F4500, Hitachi), and the ratio of fluorescence intensities from the two wavelengths was monitored as an estimate of intracellular  $\text{Ca}^{2+}$  concentration.

### Data presentation

Representative traces for intracellular pH or  $\text{Ca}^{2+}$  concentration were chosen out of 3–5 separate experiments and shown in Figs. 1, 3, 4, and 7. In Figs. 5, 6, and 8, the results of 3 independent experiments in the presence of inhibitors are shown as % of control to DMS-induced responses. Student's *t*-test was conducted to analyze significant differences in pH increases between the control group and inhibitor-treated group.

## Results

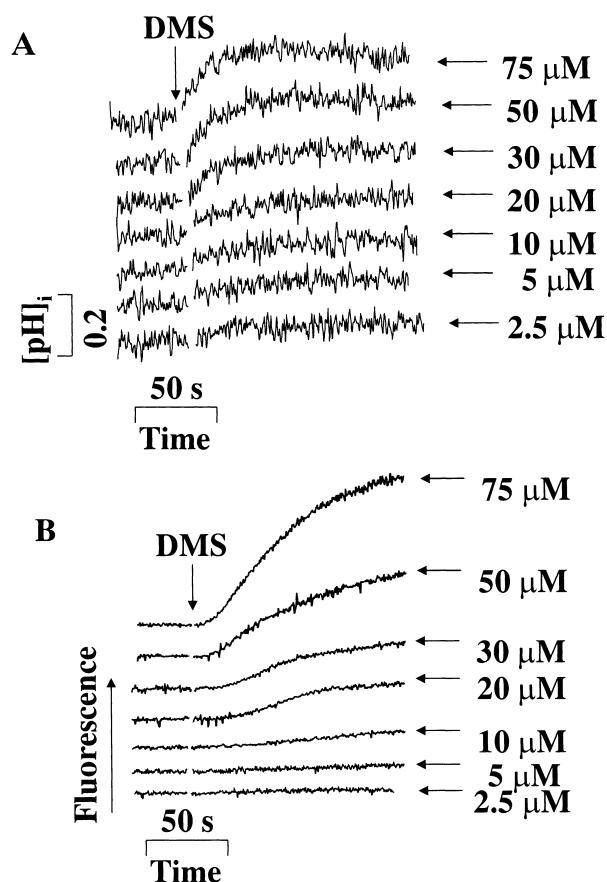
### DMS increases intracellular pH and $\text{Ca}^{2+}$ concentration in human monocytes

As shown in Fig. 1A, DMS was found to dose-dependently increase intracellular pH in U937 human monocytes. At high concentrations, the response became stronger. Furthermore, DMS-induced pH increase was saturated within 60 s and lasted for several minutes. The response to 30  $\mu\text{M}$  DMS was chosen for further study because it achieved almost the maximum response and a higher concentration could induce cell toxicity.

Next, we measured changes of intracellular  $\text{Ca}^{2+}$  concentration with DMS in U937 human monocytes. As shown in Fig. 1B, DMS dose-dependently induced increase of  $\text{Ca}^{2+}$  concentration very slowly and progressively.

### Structure-activity relationship of DMS on pH increase and $\text{Ca}^{2+}$ increase

DMS-induced increases of intracellular pH and  $\text{Ca}^{2+}$  concentration were further characterized by employing structurally-related sphingolipids. Four stereoisomers of sphingosine, including D-erythro-, D-threo-, L-erythro-, and L-threo-, induced similar responses of pH increase, suggesting that the configuration of the C2 amino group and C3 hydroxy moiety might not be critical for the response (Fig. 3, chemical structures in Fig. 2). Low stereoselectivity was further confirmed with two isomers of dihydrosphingosine (also known as sphinganine) that lack an unsaturation between C4 and C5 (Fig. 3). The result also suggests that the unsaturation is not important for the pH response. The pH increase was not observed with S1P, SPC, or C2-ceramide, suggesting



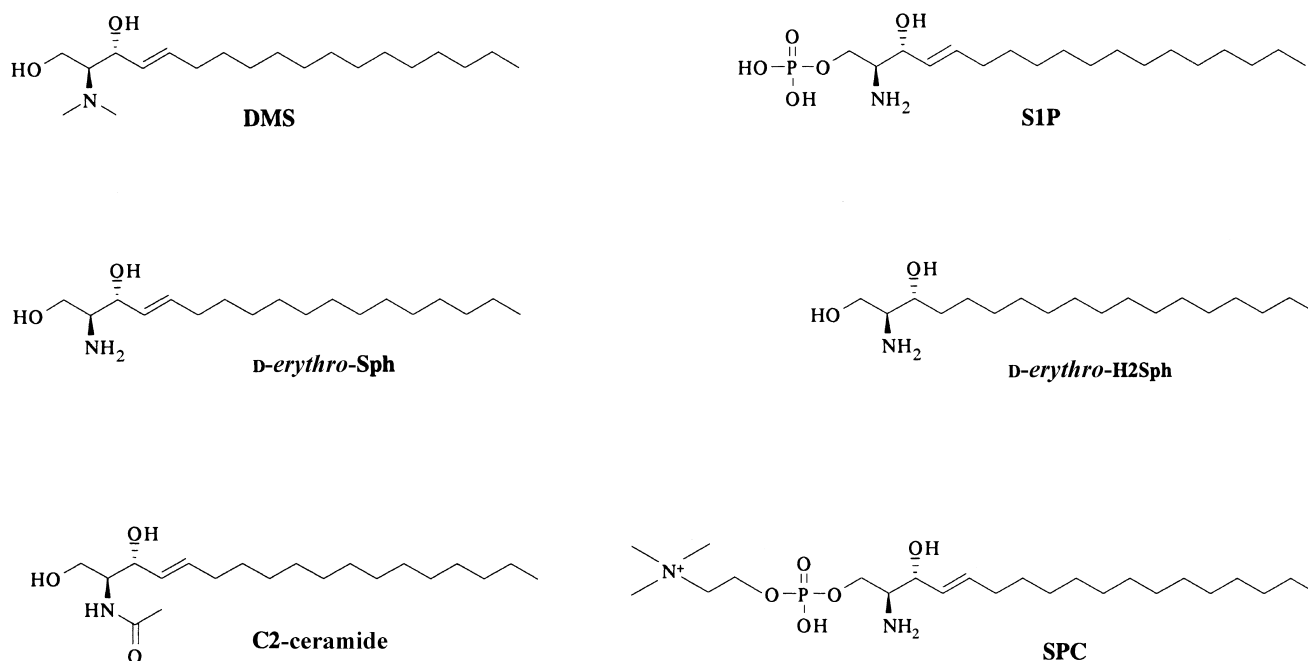
**Fig. 1.** Dose-dependence of DMS-induced intracellular pH increase and cytosolic  $\text{Ca}^{2+}$  increase. Representative pH traces (A) or  $\text{Ca}^{2+}$  traces (B) with each concentration of DMS in U937 cells.

that sphingosine structure without modification on the phosphate or without acyl modification on the C2 amine is critical for the pH response (Fig. 3).

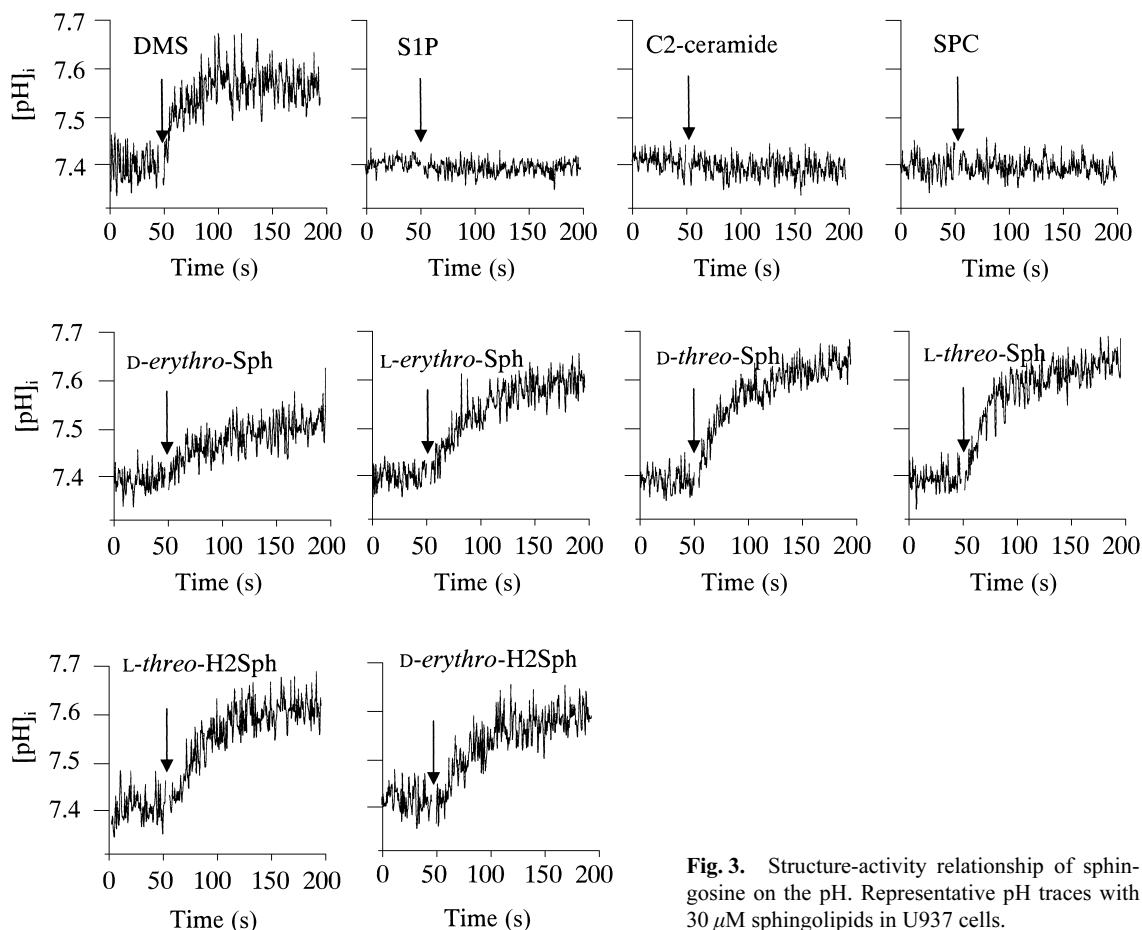
DMS-induced increase of intracellular  $\text{Ca}^{2+}$  concentration was also compared with structurally-related sphingolipids. Four stereoisomers of sphingosine and two isomers of dihydrosphingosine induced an increase of intracellular  $\text{Ca}^{2+}$  concentration, although the magnitude of increase varied (Fig. 4).  $\text{Ca}^{2+}$  increase induced by L-Sph or L-threo-dihydrosphingosine was similar to that of DMS, but the other isomers evoked a weaker response than DMS, indicating that there is stereoselectivity in the  $\text{Ca}^{2+}$  response (Fig. 4). DMS used in our present study was D-erythro configuration. Therefore, if available, L-threo-DMS is expected to induce response stronger than that observed with D-erythro-DMS. The stereoselectivity difference between the pH response and the  $\text{Ca}^{2+}$  response may suggest that the molecular targets of DMS for both responses may be different (Figs. 3 and 4). However, S1P, C2-ceramide, or SPC could not elicit any response in both pH and  $\text{Ca}^{2+}$  concentration (Figs. 3 and 4).

#### *DMS activated $\text{Na}^+/\text{H}^+$ exchanger*

Intracellular pH is tightly regulated by pH regulator proteins such as  $\text{Na}^+/\text{H}^+$  exchanger (NHE), bicarbonate transporter (BCT), and proton pump (18). To find out if there is any involvement of such regulators, the effect of DMS on intracellular pH was measured in  $\text{Na}^+$ -free or  $\text{HCO}_3^-$ -free medium. Depletion of  $\text{Na}^+$  in the extra-



**Fig. 2.** Chemical structures of sphingolipids.



**Fig. 3.** Structure-activity relationship of sphingosine on the pH. Representative pH traces with 30  $\mu$ M sphingolipids in U937 cells.

cellular medium did not abrogate the DMS-induced pH increase, and also the pH response in  $\text{HCO}_3^-$ -free medium was intact (Fig. 5). To further confirm that NHE was not involved in the DMS-induced pH increase, EIPA {5-(*N*-ethyl-*N*-isopropyl)-amiloride}, a specific inhibitor of NHE, was used. As shown in Fig. 5, EIPA did not inhibit the DMS-induced pH increase even at a high concentration such as over 100  $\mu$ M. Application of SITS (4-acetoamino-4'-isothiocyanostilbene-2,2'-disulfonic acid), a specific inhibitor of BCT, or omeprazol, a specific inhibitor of the proton pump, did not influence the DMS activity (Fig. 5), excluding the possibility of involvement of BCT and the proton pump in the response and supporting the result obtained in  $\text{HCO}_3^-$ -free medium. Therefore, the above results excluded the involvement of NHE, BCT, or proton pump in DMS-induced pH increase.

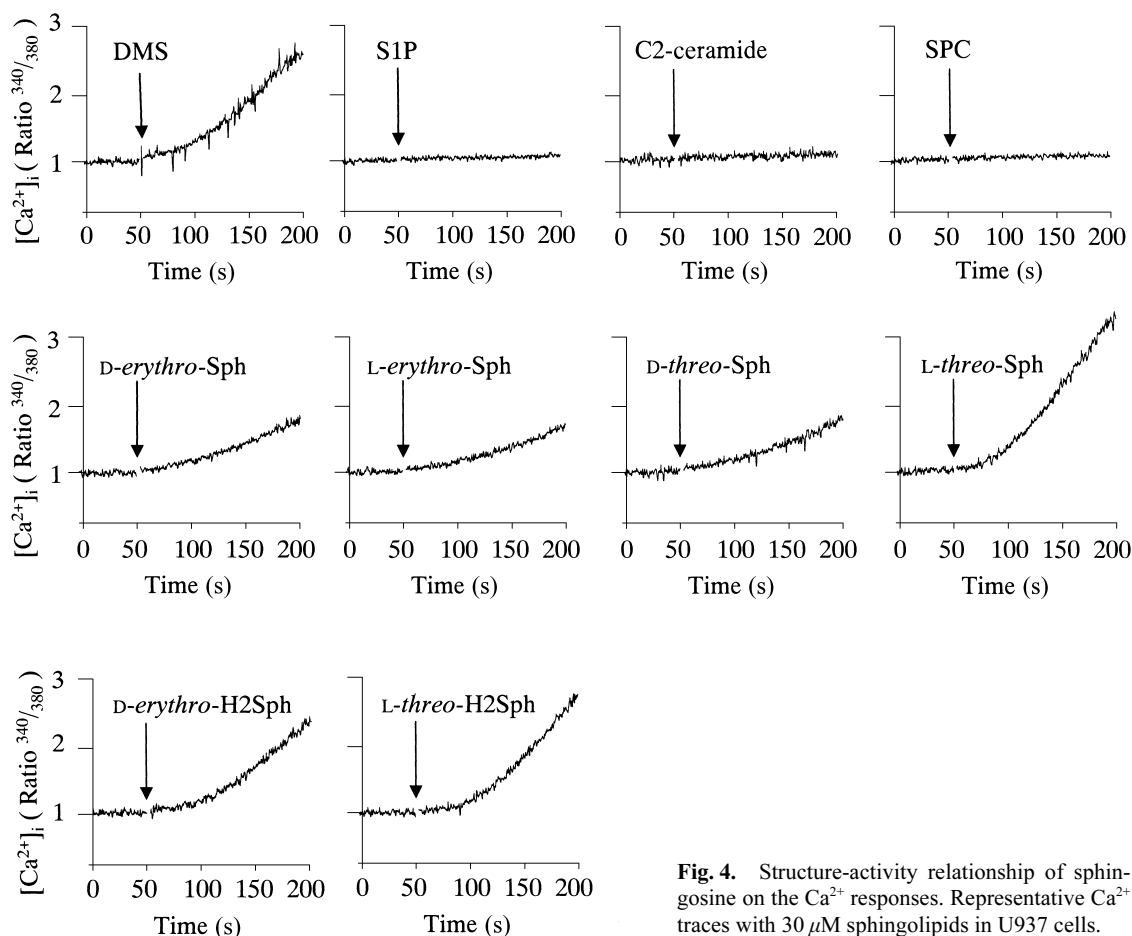
#### *Involvement of internal $\text{Ca}^{2+}$ stores, G proteins, and phospholipase C*

Increase of intracellular  $\text{Ca}^{2+}$  concentration could be induced by either release of  $\text{Ca}^{2+}$  from intracellular  $\text{Ca}^{2+}$  stores or influx of  $\text{Ca}^{2+}$  across the plasma membrane.

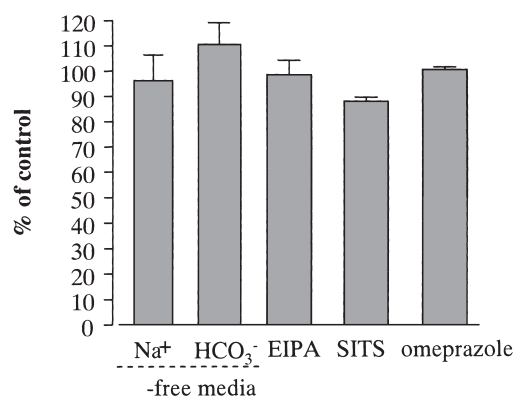
Application of EGTA, a calcium chelator, almost completely blocked the DMS-induced  $\text{Ca}^{2+}$  increase, suggesting that DMS mobilized  $\text{Ca}^{2+}$  by influx of  $\text{Ca}^{2+}$  across the plasma membrane (Fig. 6). In order to check further whether the DMS-induced  $\text{Ca}^{2+}$  increase involved G protein-coupled receptors and their signaling molecules such as G proteins and phospholipase C, we used PTX and U73122, specific inhibitors of  $\text{G}_{i/o}$  proteins and phospholipase C, respectively. However, treatment of PTX or U73122 did not inhibit the DMS-induced  $\text{Ca}^{2+}$  increase (Fig. 6). Increase of intracellular pH with DMS was also tested with the same pharmacological means, but none of them was found to be involved in the DMS-induced pH increase (Fig. 6).

#### *Interrelationship between DMS-induced pH increase and $\text{Ca}^{2+}$ increase*

Influence of DMS-induced  $\text{Ca}^{2+}$  increase on DMS-induced pH response was investigated by using BAPTA-AM, an intracellular  $\text{Ca}^{2+}$  chelator. BAPTA totally trapped intracellular  $\text{Ca}^{2+}$ , but did not influence the DMS-induced pH increase (Fig. 7). Conversely, to investigate whether the DMS-induced pH increase



**Fig. 4.** Structure-activity relationship of sphingosine on the  $\text{Ca}^{2+}$  responses. Representative  $\text{Ca}^{2+}$  traces with  $30 \mu\text{M}$  sphingolipids in U937 cells.



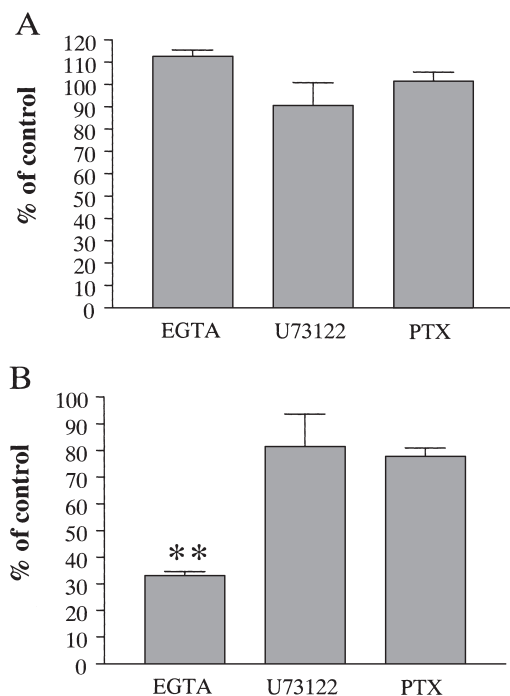
**Fig. 5.** No involvement of NHE, BCT, and proton pump on DMS-induced pH increase. Increases of pH with  $30 \mu\text{M}$  DMS in U937 cells in  $\text{Na}^+$ -free media,  $\text{HCO}_3^-$ -free media, or in the presence of EIPA ( $100 \mu\text{M}$ ), SITS ( $100 \mu\text{M}$ ), and omeprazole ( $20 \mu\text{M}$ ) are shown as % of control to DMS-induced response. Numbers of experiments for each condition or inhibitor were 3. No statistical significance was observed.

influenced the DMS-induced  $\text{Ca}^{2+}$  increase, two chemicals were used to increase intracellular pH; that is, monensin, an  $\text{H}^+$  ionophore, and  $\text{NH}_4\text{Cl}$ . Both chemicals

induced robust increase of intracellular pH, but their application induced only a slight  $\text{Ca}^{2+}$  increase or no response (Fig. 7), implying that the increase of intracellular pH might not be enough to induce  $\text{Ca}^{2+}$  increase in human monocytes.

#### Possibility of PKC as an upstream signaling molecule

As mentioned earlier, DMS was first reported as an inhibitor of PKC along with D-Sph (11). Thus, to test whether PKC is involved in DMS-induced pH increase, several PKC inhibitors were used: Go6976, Ro-31-8220, celastrol, calphostin C1, and GF109203X. However, as shown in Fig. 8, these PKC inhibitors did not increase intracellular pH. Furthermore, the presence of PKC inhibitors did not change the DMS-induced pH increase (Fig. 8), thus excluding PKC as a molecular target of DMS for the pH response. Furthermore, a PKC activator PMA was applied, but neither a short-term activation of PKC ( $10 \text{ nM}$  PMA,  $10 \text{ min}$ ) nor down-regulation of PKC ( $100 \text{ nM}$  PMA,  $48 \text{ h}$ ) influenced the DMS-induced pH increase (Fig. 8), confirming that PKC is not an upstream molecular target of DMS in the pH response.



**Fig. 6.** Influence of DMS-induced Ca<sup>2+</sup> response on DMS-induced pH increase. Increases of pH (A) or Ca<sup>2+</sup> (B) with 30  $\mu$ M DMS in U937 cells in the presence of EGTA (5  $\mu$ M) or U73122 (5  $\mu$ M) or in U937 cells treated with PTX (100 ng/ml, 24 h) are shown as % of control to DMS-induced responses. Numbers of experiments for each treatment were 3. Statistical significance: \*\* $P < 0.01$  vs control.

## Discussion

In the present study, we characterized DMS-induced increases of intracellular pH and Ca<sup>2+</sup> concentration in human monocytes. We found 1) different stereo-selectivity of sphingosine on two responses: stereo-selectivity in the Ca<sup>2+</sup> response and low stereo-selectivity in the pH response; 2) no involvement of NHE, BCT, or proton pump in DMS-induced pH increase; 3) Ca<sup>2+</sup> influx across the plasma membrane as a source of DMS-induced Ca<sup>2+</sup> mobilization; 4) no interrelationship between DMS-induced pH increase and Ca<sup>2+</sup> response; and 5) no involvement of PKC, G proteins, or PLC in both responses.

### Cellular signaling of DMS

Recently, Alfonso et al. reported that DMS increases cytosolic calcium and intracellular pH in human T lymphocytes (25). In the present study, we also observed DMS-induced pH increase and Ca<sup>2+</sup> increase in human monocytes. However, there are many differences in both responses between T lymphocytes and monocytes. In T lymphocytes, Ca<sup>2+</sup> influx across the plasma membrane was observed and involvement of G<sub>i/o</sub> proteins was

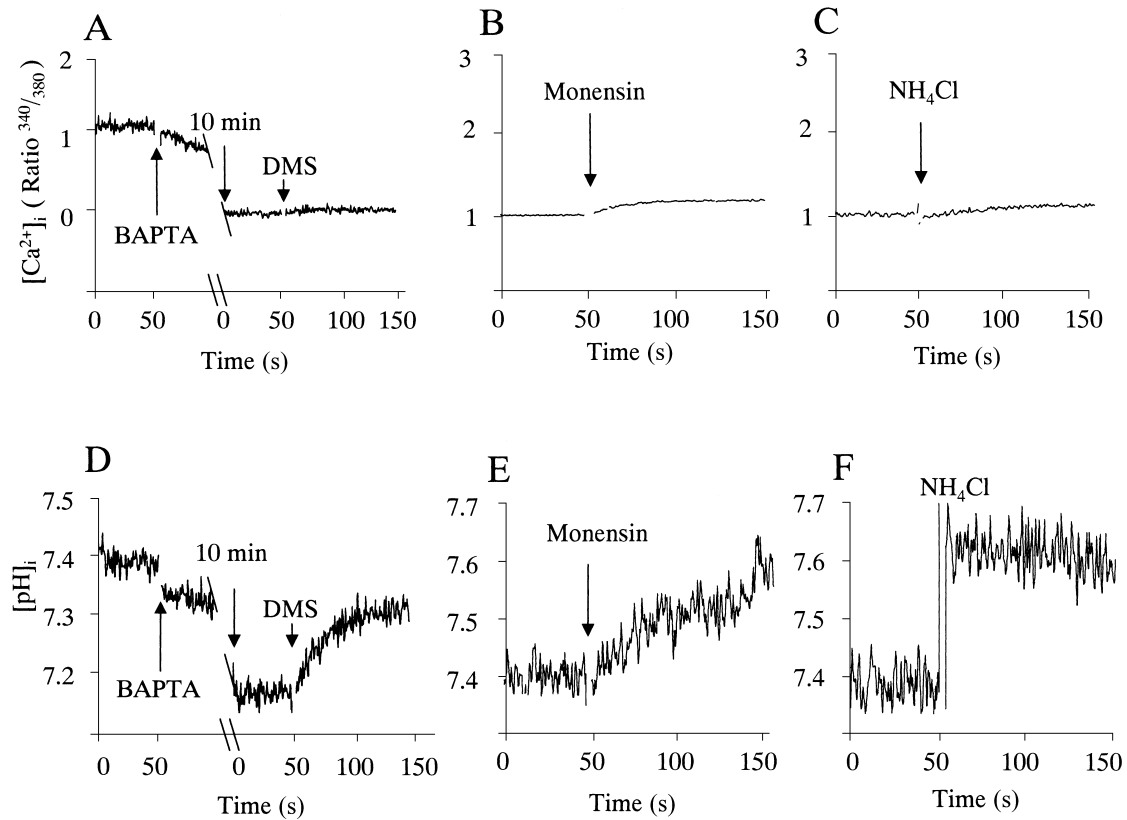
reported (25). However, in monocytes, Ca<sup>2+</sup> influx was not blocked by treatment of PTX. In T lymphocytes, genistein, an inhibitor of tyrosine kinase, modulated DMS-induced pH increase and Ca<sup>2+</sup> increase (25, 26). However, we were not able to observe any effect of genistein on DMS-induced pH increase (the authors, as an unpublished observation).

Low stereo-specificity observed in the pH response could raise doubt about the physiological significance of DMS. However, low stereo-selectivity of DMS and sphingosine on several biological effects, including inhibitions of PKC and sphingosine kinase, has earlier been shown (12, 14). Low stereo-selectivity may indicate that configuration of C2 amino moiety and C3 hydroxy group is not critical for DMS-induced pH increase and that only one of the groups is involved in interaction of sphingosine with target molecules. Different stereo-selectivity for the responses suggests that there might be different molecular targets for DMS in human monocytes, and such a contention was also supported by no interrelationship between the two responses (Fig. 7).

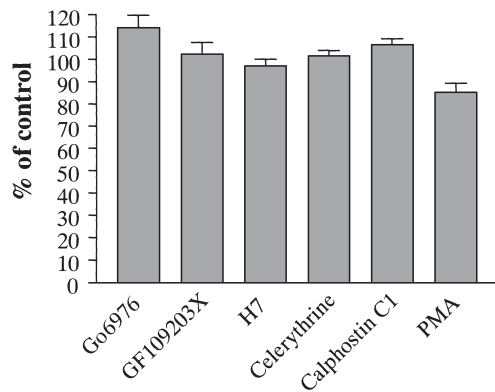
### Possible molecular targets of DMS

DMS has been widely used as a specific inhibitor of sphingosine kinase, and it has increasingly been employed due to expansion of sphingolipids biology to many pathophysiological studies (1, 5, 6). Based on the results described in the present study together with the fact that sphingosine, the substrate of sphingosine kinase, also induces similar responses, DMS-induced inhibition of sphingosine kinase appears to be likely the cause of the pH increase. However, we can not exclude other protein targets such as sphingosine-dependent kinases and phosphatases. It has been reported that DMS activates a serine kinase, called sphingosine-dependent protein kinase (SDK), different from other protein kinases (27, 28), and a fragment of PKC  $\delta$  by caspase 3 was recently identified as an SDK (29, 30). In our study, however, PKC, a historically-known target of DMS and sphingosine, was excluded in DMS-induced pH and Ca<sup>2+</sup> responses in human monocytes by using specific PKC inhibitors (10–12), supporting suggestion by Edsall et al. that PKC is not a target of DMS (13).

In other cells, PKC has been reported as regulators of NHE activity and Ca<sup>2+</sup> mobilization. Especially, sphingosine and other PKC inhibitors were shown to block PKC activity and subsequently inhibit NHE activity in platelets; therefore, thrombin-induced pH increase and Ca<sup>2+</sup> increase were inhibited by treatment with sphingosine (31). Inhibition of pH increase by sphingosine contradicts our present results obtained in monocytes (31). Different modulation of NHE by PKC



**Fig. 7.** Influence of DMS-induced pH increase on DMS-induced  $\text{Ca}^{2+}$  response. Representative  $\text{Ca}^{2+}$  (A, B, and C) and pH (D, E, and F) traces with 30  $\mu\text{M}$  DMS in U937 cells in the presence of BAPTA (A and D, 5  $\mu\text{M}$ ) or traces with monensin (B and E, 100  $\mu\text{M}$ ) or  $\text{NH}_4\text{Cl}$  (C and F, 10 mM).



**Fig. 8.** Effects of PKC inhibitors on intracellular pH and DMS-induced pH increase. Increases of pH with 30  $\mu\text{M}$  DMS in U937 cells in the presence of PKC inhibitors, Go6976 (20  $\mu\text{M}$ ), GF109203X (200 nM), H7 (10  $\mu\text{M}$ ), celerythrine (1  $\mu\text{M}$ ), calphostin C1 (1  $\mu\text{M}$ ), or after treatment of PMA (20 nM for 10 min) are shown as % of control to DMS-induced response. Numbers of experiments for each inhibitor were 3. Statistical significance was not observed in the presence of PKC inhibitors.

in different cell types might imply the presence of diverse signaling cascades.

#### Cell death and DMS actions

Regulation of intracellular pH is crucial in a variety of cellular responses (18, 19). Change of intracellular pH has been observed in response to cell growth, tumoral promoters, secretory processes, or changes in membrane permeability (18, 19, 25). Sustained increase of intracellular  $\text{Ca}^{2+}$  could induce cell proliferation and also cell death (20). Therefore, these increases of pH and  $\text{Ca}^{2+}$  induced by DMS might be the mechanism underlying DMS-induced inhibition of tumor cell growth. Nevertheless, further studies are in need to investigate the precise mechanism in DMS-induced cell death.

#### Acknowledgment

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