Miller, L., Kalnoski, M., Yunossi, Z., Bulinsky, J. C., & Reisler, E. (1987) *Biochemistry 26*, 6064-6070.
Moir, A. J. G., & Levine, B. A. (1986) *J. Inorg. Chem. 27*, 271-278.
Moir, A. J. G., Levine, B. A., Goodeart, A. J., & Trayer, J. P. (1987) *J. Muscle Res. Cell Motil. 8*, 68.
Moss, R. L., Giulian, G. G., & Greaser, M. L. (1985) *J. Gen. Physiol. 86*, 585-600.
Moss, R. L., Giulian, G. G., & Greaser, M. L. (1986) *J. Gen. Physiol. 87*, 761-774.
Mossakowska, M., & Strzelecka-Golaszewska, H. (1985) *Eur. J. Biochem. 53*, 373-381.
Parker, J. M. R., Mant, C. T., & Hodges, R. S. (1987) *Chromatographia 24*, 832-838.
Rouayrenc, J., Bertrand, R., Kasab, R., Walzhöny, D., Bähler, M., & Walliman, T. (1985) *Eur. J. Biochem. 146*, 391-401.
Segal, I. H. (1975) in *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Systems*, pp 227-240, Wiley-Interscience Publication, John Wiley and Sons, New York.
Shaw, G., Hodges, R. S., & Sykes, B. (1990) *Science 249*, 280-283.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866-4871.
Stein, L. A., Schwarz, R. P., Chock, P. B., & Eisenberg, E. (1979) *Biochemistry 18*, 3895-3909.
Stein, L. A., Chock, P. B., & Eisenberg, E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1346-1350.
Sutoh, K. (1982) *Biochemistry 21*, 3654-3661.
Sutoh, K. (1983) *Biochemistry 22*, 1579-1585.
Suzuki, R., Nishi, N., Tokura, S., & Morita, F. (1987) *J. Biol. Chem. 262*, 11410-11412.
Talbot, J. A., & Hodges, R. S. (1979) *J. Biol. Chem. 254*, 3720-3723.
Talbot, J. A., & Hodges, R. S. (1981) *J. Biol. Chem. 256*, 2798-2802.
Van Eyk, J. E., & Hodges, R. S. (1988) *J. Biol. Chem. 263*, 1726-1732.
Van Eyk, J. E., Cachia, P. J., Ingraham, R. H., & Hodges, R. S. (1986) *J. Protein Chem. 5*, 335-354.
Weeds, A. G., & Taylor, R. S. (1975) *Nature 257*, 54-56.
Weeds, A. G., & Pope, B. (1977) *J. Mol. Biol. 111*, 129-157.
Yagi, K., Yazawa, Y., & Yasui, T. (1967) *Biochem. Biophys. Res. Commun. 29*, 331-336.
Yanagida, T., Nakase, M., Nishiyama, K., & Oosawa, F. (1984) *Nature (London) 307*, 58-60.
Young, D. M., Himmelfarb, S., & Harrington, W. F. (1964) *J. Biol. Chem. 239*, 2822-2827.

Downregulation of GMP-140 (CD62 or PADGEM) Expression on Platelets by *N,N*-Dimethyl and *N,N,N*-Trimethyl Derivatives of Sphingosine†

Kazuko Handa, Yasuyuki Igarashi, Mohammad Nisar, and Sen-itiroh Hakomori*
The Biomembrane Institute, 201 Elliott Avenue West, Seattle, Washington 98119, and Department of Pathobiology, University of Washington, Seattle, Washington 98195

Received July 17, 1991; Revised Manuscript Received October 2, 1991

ABSTRACT: GMP-140 (CD62 or PADGEM), a member of the selectin family, is a membrane glycoprotein in secretory granules of platelets and endothelial cells. When these cells are activated by agonists such as thrombin or AMP, GMP-140 is rapidly redistributed to the cell surface. The carbohydrate epitope defined by GMP-140 was identified as sialosyl-Le^x (as for ELAM-1), which may play an essential role in adhesion of leukocytes or tumor cells on endothelial cells, through aggregation with platelets. Redistribution of GMP-140 from α-granules of platelets to the cell surface, induced by thrombin and PMA, was strongly inhibited by preincubation of platelets with *N,N*-dimethylsphingosine (DMS) or *N,N,N*-trimethylsphingosine (TMS) at 10-20 μM concentration for a brief period (5 min). Inhibition of GMP-140 redistribution to the cell surface by DMS or TMS was also detected by a cell adhesion assay using HL60 cells, which highly express sialosyl-Le^x; i.e., HL60 cells adhered on platelets activated by thrombin or PMA but not on platelets which were briefly preincubated with DMS or TMS followed by activation. The inhibitory effect of DMS or TMS on GMP-140 redistribution is not due to cytotoxicity, since the TMS-treated platelets were fully capable of aggregating in the presence of ristocetin. Sphingosine (SPN) and protein kinase C inhibitors such as H-7 and calphostin C showed weaker inhibitory activity than DMS and TMS. Our results indicate that both DMS and TMS could be useful reagents to inhibit cell surface expression of crucial selectins which promote adhesion of Le^x- or sialosyl-Le^x-expressing cells with platelets and endothelial cells. They may therefore display effective inhibition of a variety of biological processes (e.g., inflammation and tumor metastasis) based on the expression and function of selectins.

Interaction of leukocytes with activated platelets and endothelial cells is an initial step in inflammatory processes and is mediated by various adhesion molecules including selectins. Selectins, which include LECCAM-1 (LAM-1 in man, MEL-14 in mice), ELAM-1, and GMP-140 (CD62/PADGEM), are all characterized by a similar structural motif consisting of a lectin domain at the N-terminal region, followed by an EGF sequence, a complement-regulatory domain, a transmembrane region, and a C-terminal domain (Stoolman, 1989; Osborn, 1990; Bevilacqua et al., 1989; Springer, 1990). A number of recent reports have focused on the identification of carbohydrate ligands recognized by members of the selectin family [e.g., Brandley et al. (1990), Lowe et al. (1990), Walz

† This paper is dedicated to Professor Saul Roseman (Johns Hopkins University) in honor of his 70th birthday. This study was supported by funds from The Biomembrane Institute, and S.H. was supported by National Cancer Institute Outstanding Investigator Grant CA-42505.
* Address correspondence to this author at the Biomembrane Institute.

0006-2960/91/0430-11682$02.50/0 © 1991 American Chemical Society

et al. (1990), Goelz et al. (1990), Larsen et al. (1990), and Corral et al. (1990)]. On the basis of inhibition studies using a variety of GSL[1] liposomes, binding epitopes of both ELAM-1 and GMP-140 expressed on HL60 cells were identified as sialosyl-Le^x (Phillips et al., 1990; Polley et al., 1991). Expression of these selectins is upregulated by the inductive effect of lymphokine, IL-1β, TNFα, bacterial lipopolysaccharides (for ELAM-1), thrombin, ADP and PMA (for GMP-140), and perhaps many other compounds. Leukocytes, together with platelets, are thereby recruited to the inflammatory site. Since tumor cells are capable of activating platelets (Ugen et al., 1988; Watanabe et al., 1988; Cavanaugh et al., 1988; Grignani & Jamieson, 1988; Tohgo et al., 1990; Karpatkin et al., 1988), a similar process is expected to occur during tumor cell adhesion, together with platelets, on microvascular endothelia. Thus, the process of tumor cell metastasis might be initiated by selectin-dependent tumor cell adhesion. Although there is no evidence of direct activation of endothelial cells by tumor cells, IL-1- or TNFα-activated endothelial cells have been shown to adhere to a variety of tumor cells (Walz et al., 1990; T. Irimura et al., unpublished results).

While the regulatory mechanism for expression of GMP-140 in platelets is poorly understood, it apparently involves a complex sequence of activation processes including structural transformation and translocation of α-granules and secretion of their contents. This process is triggered by transmembrane signaling transducers including agonist receptor associated G-proteins, phospholipases A_2 and C, PKC, and 47-kDa phosphoprotein (Hawiger, 1989; Lapetina, 1990). Some of these processes have been proposed to be modulated by SPN (Hannun et al., 1987), GSLs, and SPN derivatives (i.e., DMS and TMS) [Bremer et al., 1984; Bremer & Hakomori, 1984; Hannun & Bell, 1989; Igarashi et al., 1989, 1990; for reviews see Hakomori (1990) and Igarashi (1990)]. Platelet aggregation and associated ATP secretion were previously shown to be strongly inhibited by TMS; this phenomenon could be based on inhibition of 47-kDa protein phosphorylation or of phosphoinositide turnover as a membrane signaling pathway in platelets (Okoshi et al., 1991). We now report an inhibitory effect of SPN derivatives on GMP-140 expression in human platelets.

Materials and Methods

Reagents. Thrombin, PMA, ristocetin, and SPN were purchased from Sigma Chemical Co. (St. Louis, MO). SPN used throughout this study was in sulfate form. H-7 was purchased from Seikagaku America Inc. (St. Petersburg, FL). Calphostin C was kindly donated by Dr. H. Saitoh (Kyowa Hakko Co. Ltd., Tokyo, Japan) and is also available from Kamiya Biochemical Co., Thousand Oaks, CA. This compound *specifically* interacts with the regulatory domain of PKC and thereby specifically inhibits PKC activity. [*methyl*-^{3}H]Thymidine (6.7 Ci/mmol) was purchased from ICN Biomedicals Inc. (Irvine, CA). MAb AC1.2 was kindly donated by Dr. Bruce Furie (Tufts University School of Medicine, Boston, MA) and obtained through an arrangement with Becton-Dickinson Co. (San Jose, CA). DMS and TMS were synthesized as previously described (Igarashi et al., 1989). DMS and TMS used in this study were free form and in chloride form, respectively. SPN, TMS, and calphostin C were dissolved in 50% ethanol at concentrations of 2, 2, and 0.5 mM, respectively. DMS was dissolved in 100% ethanol at a concentration of 2 mM. These stock solutions were appropriately diluted with Tyrode's buffer and added to the platelet suspension in order to make various concentrations of these reagents (see Platelet Activation and Its Inhibition).

Preparation of HL60 Cells and Platelets. Human promyelocytic cell line HL60 was cultured and maintained in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS (Hyclone, Logan, UT). HL60 cells were labeled by incubating cells with 2 μCi/mL [^{3}H]-thymidine overnight. Platelets were isolated from "platelet-rich plasma" (Oregon Red Cross, Portland, OR). Contaminating erythrocytes were removed by centrifugation (80*g* for 10 min). Platelets were obtained by centrifugation (300*g* for 10 min), washed once in Tyrode's buffer (pH 6.5) containing 22 mM trisodium citrate and 0.35% BSA, and resuspended in the same buffer at a concentration of 1×10^9 platelets/mL. All procedures were performed at room temperature.

Platelet Activation and Its Inhibition. GMP-140 expression on the cell surface of platelets was determined by (i) flow cytometry with MAb AC1.2 (mouse IgG), which is directed to GMP-140, and (ii) adhesion of HL60 cells on the platelet-coated solid phase, as follows. A suspension of platelets (1×10^8/mL) in Tyrode's buffer as above was preincubated with inhibitor (see legends for Figures 1 and 2) at pH 7.2, 37 °C, for 5 min, supplemented with thrombin (final concentration 1 unit/mL) or PMA (final concentration 10^{-7} M), and the mixture was incubated at 37 °C for 10 min without stirring (Carmody et al., 1990). Subsequently, platelets were fixed with an equal volume of 2% paraformaldehyde in PBS, pH 7.2, washed twice with PBS containing 1% BSA, and subjected to flow cytometry as described below.

Determination of GMP-140 Expression at the Platelet Surface by Flow Cytometry. Paraformaldehyde-fixed platelets (2.5×10^7 cells) prepared as above were incubated with 50 μL of MAb AC1.2 (2.5 μg/mL) at room temperature for 30 min. Platelets were washed twice with PBS containing 1% BSA, supplemented with 50 μL of fluorescent isothiocyanate-labeled goat anti-mouse Ig (purchased from Tago Co., Burlingame, CA), incubated at room temperature for 30 min, again washed twice with PBS containing 1% BSA, and analyzed by Epics Profile (Coulter Corp., FL). As a negative control, the paraformaldehyde-fixed platelets were incubated with mouse IgG instead of MAb AC1.2 and treated as described above. For flow cytometric analysis of platelets, suitable gating was set (Carmody et al., 1990). To calculate the inhibitory effect of various reagents, the mean fluorescence intensity of resting platelets (obtained on incubation of platelets without activator) was subtracted from the value of each sample which was pretreated with inhibitors and subsequently activated.

Adhesion of HL60 Cells on the Platelet-Coated Solid Phase. HL60 cell adhesion on the platelet-coated solid phase was determined as follows. Each well of a 48-well plate (Costar Scientific, Cambridge, MA) was filled with poly(L-lysine) solution (100 μg/mL) in PBS and incubated for 1 h. Each well was then washed with PBS and supplemented with 150 μL of PBS containing 6×10^7 fixed platelets. Plates were centrifuged (300*g* for 7 min) and further incubated for 30 min at room temperature. Bound platelets were fixed by addition of 0.1% glutaraldehyde in PBS for 2 min at 4 °C. Each well

[1] Abbreviations: ADP, adenosine diphosphate; BSA, bovine serum albumin; DAG, diacylglycerol; DMS, *N,N*-dimethylsphingosine; FCS, fetal calf serum; GSL, glycosphingolipid; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; MAb, monoclonal antibody; PBS, Dulbecco's phosphate-buffered saline (2.7 mM KCl, 1.1 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4); PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SPN, sphingosine; TMS, *N,N,N*-trimethylsphingosine.

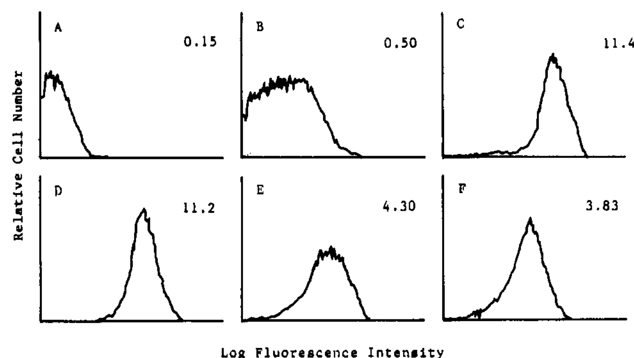

FIGURE 1: Cytofluorograph of the platelet expressing GMP-140 at the cell surface and inhibition of this expression by SPN derivatives. A total of 25 μL of the platelet suspension (2.5×10^7 cells) was mixed with 225 μL of Tyrode's buffer, pH 7.2, containing SPN, DMS, or TMS (final concentration 20 μM for each compound). Platelet suspensions were incubated at 37 °C for 5 min, then stimulated by addition of 10 μL of PMA (panels C–F) or not (panels A and B), and incubated for another 10 min. Cells were then fixed with paraformaldehyde, stained by normal mouse IgG (panel A) or MAb AC1.2 (panels B–F), and analyzed by flow cytometry. Numbers on each panel indicate mean fluorescence intensity. Panels D, E, and F include preincubation with 20 μM SPN, TMS, and DMS, respectively.

was washed with 10 mM glycine in PBS, and plates were incubated with 5% BSA containing 0.1% sodium azide and 10 mM glycine in PBS for 1 h at room temperature. After being washed with culture medium (RPMI 1640 containing 5% FCS), 1×10^6 HL60 cells labeled with [^{3}H]thymidine were added to each well. After incubation for 45 min at room temperature, unbound cells were aspirated and wells were washed once with medium (RPMI 1640 containing 5% FCS); bound cells were detached with 0.05% trypsin–0.02% EDTA (Irvine Scientific) in PBS and counted with a liquid scintillation counter.

Platelet Aggregation by Ristocetin. Platelets (3.5×10^8/mL) in Tyrode's buffer were preincubated with inhibitors, followed by addition of ristocetin (0.75 mg/mL). Platelet aggregation was evaluated by transmittance change using an aggregometer (Chrono-log Corp., Havertown, PA) equipped with a computer analyzer.

RESULTS

Effect of SPN, TMS, DMS, H-7, and Calphostin C on GMP-140 Expression in Platelets. Agonist-induced GMP-140 redistribution to the cell surface from secretory granules of platelets was determined by flow cytometry as described under Materials and Methods. Figure 1 shows representative cytofluorographs of resting and PMA-activated platelets with or without pretreatment with SPN derivatives. SPN, either free form or in salt form, showed the same effect (data not shown). Effects of various compounds at various concentrations, expressed in terms of percent activity of thrombin-activated platelets without preincubation with reagent, are summarized in Figure 2A. TMS and DMS, at 10–20 μM, strongly inhibited thrombin-induced GMP-140 expression, whereas SPN and H-7 produced no significant inhibition. Calphostin C produced weak inhibition at 10–20 μM; however, this compound has been reported to inhibit PKC at a concentration of 0.05 μM (IC_{50}) in vitro (Hidaka et al., 1984). In our previous study on thrombin- and ADP-induced platelet aggregation, TMS showed strong inhibitory activity at 10–20 μM, while DMS in this concentration range showed only weak activity, regardless of salt form (Okoshi et al., 1991).

Similar results were obtained for PMA-induced platelet activation; i.e., both TMS and DMS, at 10–20 μM, strongly

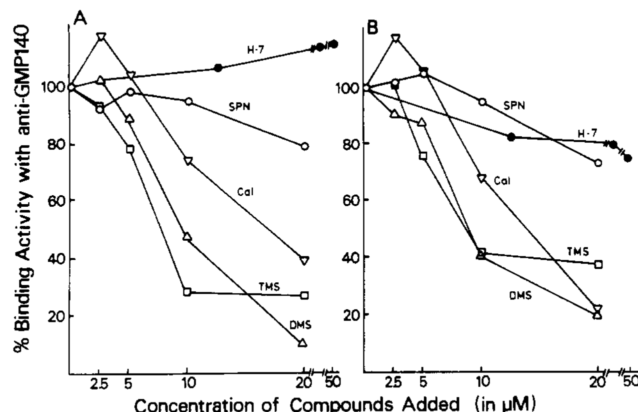

FIGURE 2: Effect of various compounds on thrombin- (panel A) and PMA- (panel B) induced GMP-140 expression, detected by flow cytometry. A total of 25 μL of the platelet suspension (see Figure 1 legend) was mixed with 225 μL of Tyrode's buffer, pH 7.2, containing SPN derivatives or PKC inhibitors H-7 or calphostin C, as indicated on the abscissa. The cell suspensions were incubated at 37 °C for 5 min and then stimulated by addition of 10 μL of thrombin (final concentration 1 unit/mL) or PMA (final concentration 10^{-7} M), followed by incubation at 37 °C for 10 min. After fixation, the platelets were stained by MAb AC1.2 and analyzed by flow cytometry as in Figure 1. The mean fluorescence intensity of the resting platelets (incubated in the absence of inhibitor and activator) was subtracted from the value of each activated platelet sample. Addition of ethanol (0.5% and 1.0%) alone, which was used for preparing 10 or 20 μM DMS or TMS in Tyrode's, had no effect. Results represent an average of three similar experiments using different platelet sources.

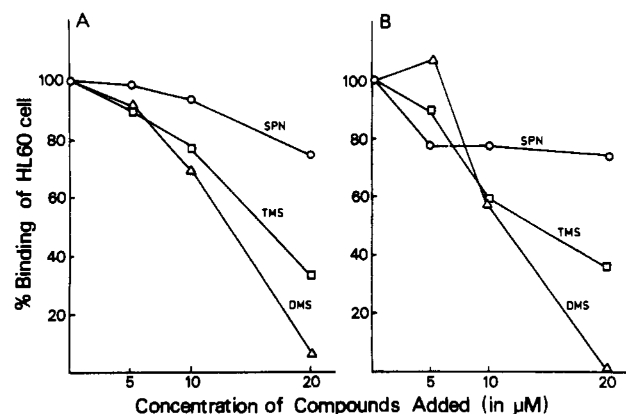

FIGURE 3: Effect of various compounds on thrombin- (panel A) and PMA- (panel B) induced GMP-140 expression, detected by HL60 cell adhesion. Platelets were preincubated and activated as described in Figures 1 and 2, except that this experiment was done on a large scale (3 mL). Platelets were fixed on 48-well plates precoated with poly(L-lysine) as described under Materials and Methods. [^{3}H]-Thymidine-labeled HL60 cells (1×10^6 cells) were added to each well, and the mixture was incubated at room temperature for 45 min. After washing, bound cells were detached and counted in a scintillation counter. Experiments were performed in quadruplicate, and the mean value for HL60 cell binding on resting platelets (incubated in the absence of inhibitors and stimulators) was subtracted from that on experimental platelets. Results represent an average of three similar experiments using different platelet sources.

inhibited GMP-140 redistribution to the cell surface (Figure 2B). Neither SPN nor H-7 showed significant inhibition, even though H-7 was reported to inhibit PKC at a concentration of 15 μM.

Effect of SPN, DMS, and TMS on HL60 Cell Adhesion. Inhibition of thrombin- and PMA-induced cell surface expression of GMP-140 by DMS and TMS was in accord with prevention of thrombin- and PMA-induced stimulation of platelet adhesion to sialosyl-Le^x-expressing HL60 cells (Figure 3). SPN had minimal effect (Figure 3), in accord with its minor effect on GMP-140 expression as estimated by MAb

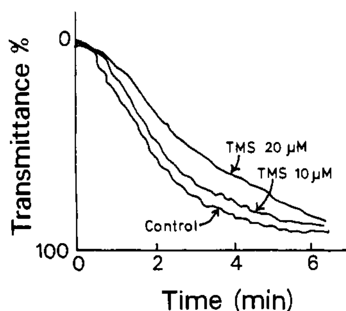

FIGURE 4: Effect of TMS on ristocetin-induced platelet aggregation. Platelets (3.5×10^8/mL) were preincubated in 450 µL of Tyrode's buffer (with or without TMS) and stimulated by addition of 50 µL of ristocetin (final concentration 0.75 mg/mL) solution. Platelet aggregation was measured as the change of light transmittance (410-nm wavelength) recorded by an aggregometer as described under Materials and Methods.

(Figure 2). The inhibitory effect of DMS and TMS on GMP-140 expression is not likely due to cytotoxicity, since platelets preincubated with these compounds showed a similar degree of aggregation to control in the presence of ristocetin (Figure 4). Binding of HL60 cells to activated platelets is considered to depend solely on recognition by GMP-140 of sialosyl-Le^x expressed on HL60 cells, since the binding was specifically inhibited by liposome containing sialosyl-Le^x but not by liposome containing other GSLs (Polley et al., 1991).

Discussion

Platelet activation is of central importance for initiation of numerous biological processes related to hemostasis, inflammation, wound healing, and tumor cell metastasis and invasion. There are many factors and mechanisms which influence platelet activation and many consequences of this activation [for reviews see Hawiger (1989) and Jamieson (1988)]. The present study is focused on expression of GMP-140 (CD62) by redistribution from α-granules to the cell surface as a "secretory response" of platelets induced by agonist, thrombin, or PMA. GMP-140 is a member of the selectin family and binds to neutrophils and monocytes or tumor cells which express the sialosyl-Le^x or Le^x epitope (Polley et al., 1991). Mechanisms of expression of GMP-140 on the cell surface from secretory granules, and its subsequent binding to sialosyl-Le^x or Le^x, are of central importance for initiation of inflammatory processes as well as tumor cell metastasis. Our results clearly indicate that GMP-140 expression due to redistribution from α-granules to platelet cell surface can be blocked by preincubation of platelets with DMS or TMS, or to a lesser extent by calphostin C, based on flow cytometric analysis with anti-GMP-140 MAb AC1.2 or on HL60 cell adhesion to activated platelets. HL60 cell adhesion on activated platelets was previously shown to be based on recognition of sialosyl-Le^x by GMP-140 (Polley et al., 1991).

A remarkable feature of adhesion molecules expressed on endothelial cells and platelets is their inducibility by lymphokine, IL-1β, TNFα, TGFβ (for ELAM-1), ADP, PMA (for GMP-140), and many other compounds which have physiological relevance in initiation of inflammatory processes. Expression of these adhesion molecules is also downregulated by endogenous factors, particularly those involved in transmembrane signaling. Intercellular adhesion molecule 1 (ICAM-1), a member of the Ig receptor family, was previously shown to be downregulated by H-7, a PKC inhibitor (Lane et al., 1990). A similar mechanism appears to operate in downregulation of the selectin family. As demonstrated by the present study, H-7 had no effect on GMP-140 expression, despite its potent inhibition of ICAM-1 expression.

Stimulatory or inhibitory effects on the secretory response of platelets may take place through at least three transmembrane signal pathways: (i) through the channel of PKC/47-kDa phosphoprotein, (ii) through inositol phosphate turnover associated with Ca^{2+} mobility change, and (iii) through the prostaglandin/thromboxane A_2 channel [for review see Hawiger (1989)]. The mechanisms operating in up- and downregulation of GMP-140 expression must be highly complex but could be closely associated with these pathways, particularly pathway i, since phosphorylation of the 47-kDa phosphoprotein, the substrate of PKC, was greatly enhanced on platelet activation by agonist and inhibited in the presence of 10–20 µM TMS and 30 µM DMS (Okoshi et al., 1991). A brief preincubation of platelets in 10–20 µM DMS or TMS strongly inhibited surface expression of GMP-140 as described in this paper, and similar treatment of platelets with a similar concentration of DMS or TMS inhibited agonist-induced phosphorylation of the 47-kDa protein, as well as platelet aggregation (Okoshi et al., 1991). Thus, involvement of the sphingoid base in modulation of transmembrane pathway i above, as a part of the secretory response of platelets, is strongly suggested. TMS-treated platelets, which are not susceptible to aggregation by thrombin or ADP, can be aggregated in the presence of ristocetin, which induces platelet aggregation through a channel independent from pathway i above.

Involvement of PKC in GMP-140 expression was further suggested by the observation that calphostin C, which binds specifically to the regulatory domain of PKC (Hidaka et al., 1984), also inhibited GMP-140 expression in the 10–20 µM range, whereas this reagent inhibited PKC in vitro at very low concentration (10–50 nM). Calphostin C may not penetrate readily into platelets, therefore requiring a thousandfold concentration for inhibition of pathway i above. This reagent is incapable of inhibiting platelet aggregation by thrombin or ADP (Okoshi et al., 1991), to which pathways ii and iii above may also contribute. GMP-140 expression is not inhibited when intact platelets are preincubated with the PKC inhibitor H-7. Thus H-7, like calphostin C, may not penetrate easily into platelets under the experimental conditions. Our results suggest that TMS and DMS, in contrast, penetrate easily into platelets and inhibit platelet secretory response through inhibition of PKC/47-kDa protein phosphorylation.

Both SPN and DMS are physiological modulators of transmembrane signaling, as is DAG [see, for review, Hannun and Bell (1989), Hakomori (1990), and Igarashi (1990)]. SPN and DMS have been detected in various animal cells as minor physiological components, with a concentration on the order of 20–50 nM, similar to that of DAG. However, concentrations of SPN, DMS, or DAG required to affect in vitro PKC activity are as high as 50–100 µM (Wilson et al., 1988; Igarashi et al., 1989). The rationale for this discrepancy is not known. DMS was metabolically labeled by [^{3}H]Ser as the major sphingoid base in human epidermoid carcinoma A431 cells (Igarashi et al., 1990) and mouse T-cell lymphoma CTLL cells (Felding-Habermann et al., 1990) and is physiologically derived from SPN (Igarashi & Hakomori, 1989; Goldkorn et al., 1991). While the chemical quantity of SPN has been determined to be in the range of 20–50 pmol per 10^7 cells by detection of the N-fluorescent derivative (Wilson et al., 1988; Kolesnick, 1989), that of DMS cannot be determined under these conditions. The quantity and identification of DMS and SPN in various cells and tissues, including platelets, are currently determined by gas chromatography–mass

spectrometry (S. B. Levery, E. Nudelman, Y. Igarashi, K. Handa, and S. Hakomori, unpublished results). Data on exact levels of SPN and DMS in platelets and their changes in response to agonist stimulation will be presented elsewhere.

ACKNOWLEDGMENTS

MAb AC1.2 was provided by Dr. Bruce Furie (Division of Hematology/Oncology, Tufts University, Boston, MA) through arrangement with Becton-Dickinson Co. We thank Erik Lium for technical assistance and Dr. Stephen Anderson for scientific editing and preparation of the manuscript.

Registry No. PMA, 16561-29-8; thrombin, 9002-04-4; protein kinase, 9026-43-1.

REFERENCES

Bevilacqua, M. P., Stengelin, S., Gimbrone, M. A., Jr., & Seed, B. (1989) *Science 243*, 1160–1172.

Brandley, B. K., Swiedler, S. J., & Robbins, P. W. (1990) *Cell 63*, 861–863.

Bremer, E., & Hakomori, S. (1984) in *Ganglioside structure, function, and biomedical potential* (Ledeen, R. W., Yu, R. K., Rapport, M. M., & Suzuki, K., Eds.) pp 355–367, Plenum Press, New York.

Bremer, E., Hakomori, S., Bowen-Pope, D. F., Raines, E., & Ross, R. (1984) *J. Biol. Chem. 259*, 6818–6825.

Carmody, M. W., Ault, K. A., Mitchell, J. G., Rote, N. S., & Ng, A.-K. (1990) *Hybridoma 9*, 631–641.

Cavanaugh, P. G., Sloane, B. F., & Honn, K. V. (1988) *Haemostasis 18*, 37–46.

Corral, L., Singer, M. S., Macher, B. A., & Rosen, S. D. (1990) *Biochem. Biophys. Res. Commun. 172*, 1349–1356.

Felding-Habermann, B., Igarashi, Y., Fenderson, B. A., Park, L. S., Radin, N. S., Inokuchi, J., Strassmann, G., Handa, K., & Hakomori, S. (1990) *Biochemistry 29*, 6314–6322.

Goelz, S. E., Hession, C., Goff, D., Griffiths, B., Tizard, R., Newman, B., Chi-Rosso, G., & Lobb, R. (1990) *Cell 63*, 1349–1356.

Goldkorn, T., Dressler, K. A., Muindi, J., Radin, N. S., Mendelsohn, J., Menaldino, D., Liotta, D. C., & Kolesnick, R. N (1991) *J. Biol. Chem. 266*, 16092–16097.

Grignani, G., & Jamieson, G. A. (1988) *Blood 71*, 844–849.

Hakomori, S. (1990) *J. Biol. Chem. 265*, 18713–18716.

Hannun, Y. A., & Bell, R. M. (1989) *Science 243*, 500–507.

Hannun, Y. A., Greenberg, C. S., & Bell, R. M. (1987) *J. Biol. Chem. 262*, 13620–13626.

Hawiger, J. (1989) *Methods Enzymol. 169*, 191–195.

Hidaka, H., Inagaki, M., Kawamoto, S., & Sasaki, Y. (1984) *Biochemistry 23*, 5036–5041.

Igarashi, Y. (1990) *Trends Glycosci. Glycotechnol. 2*, 319–332.

Igarashi, Y., & Hakomori, S. (1989) *Biochem. Biophys. Res. Commun. 164*, 1411–1416.

Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K., & Racker, E. (1989) *Biochemistry 28*, 6796–6800.

Igarashi, Y., Kitamura, K., Toyokuni, T., Dean, B., Fenderson, B. A., Ogawa, T., & Hakomori, S. (1990) *J. Biol. Chem. 265*, 5385–5389.

Jamieson, G. A., Ed. (1988) *Platelet membrane receptors: Molecular biology, immunology, biochemistry, and pathology*, A. R. Liss, New York.

Karpatkin, S., Ambrogio, C., & Pearlstein, E. (1988) in *Platelet membrane receptors: Molecular biology, immunology, biochemistry, and pathology* (Jamieson, G. A., Ed.) pp 585–606, A. R. Liss, New York.

Kolesnick, R. N. (1989) *J. Biol. Chem. 264*, 7617–7623.

Lane, T. A., Lamkin, G. E., & Wancewicz, E. V. (1990) *Biochem. Biophys. Res. Commun. 172*, 1273–1281.

Lapetina, E. G. (1990) *FEBS Lett. 268*, 400–404.

Larsen, E., Palabrica, T., Sajer, S., Gilbert, G. E., Wagner, D. D., Furie, B. C., & Furie, B. (1990) *Cell 63*, 467–474.

Lowe, J. B., Stoolman, L. M., Nair, R. P., Larsen, R. D., Berhend, T. L., & Marks, R. M. (1990) *Cell 63*, 475–484.

Okoshi, H., Igarashi, Y., Nisar, M., Zhou, Q., Kimura, S., Tashiro, K., & Hakomori, S. (1991) *Cancer Res.* (in press).

Osborn, L. (1990) *Cell 62*, 3–6.

Phillips, M. L., Nudelman, E., Gaeta, F. C. A., Perez, M., Singhal, A. K., Hakomori, S., & Paulson, J. C. (1990) *Science 250*, 1130–1132.

Polley, M. J., Phillips, M. L., Wayner, E. A., Nudelman, E., Singhal, A. K., Hakomori, S., & Paulson, J. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6224–6228.

Springer, T. A. (1990) *Nature 346*, 425–434.

Stoolman, L. M. (1989) *Cell 56*, 907–910.

Tohgo, A., Tanaka, N. G., & Osada, Y. (1990) *Invasion Metastasis 10*, 1–17.

Ugen, K. E., Mahalingam, M., Klein, P. A., & Kao, K.-J. (1988) *J. Natl. Cancer Inst. 80*, 1461–1466.

Walz, G., Aruffo, A., Kolanus, W., Bevilacqua, M. P., & Seed, B. (1990) *Science 250*, 1132–1135.

Watanabe, M., Okochi, E., Sugimoto, Y., & Tsuruo, T. (1988) *Cancer Res. 48*, 6411–6416.

Wilson, E., Wang, E., Mullins, R. E., Uhlinger, D. J., Liotta, D. C., Lambeth, J. D., & Merrill, A. H., Jr. (1988) *J. Biol. Chem. 263*, 9304–9309.