Journal of Cellular Biochemistry 88:732–743 (2003)

Biochemical Regulation of Breast Cancer Cell Expression of $S1P_2$ (Edg-5) and $S1P_3$ (Edg-3) G Protein-Coupled Receptors for Sphingosine 1-Phosphate

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Abstract G protein-coupled receptors (GPCRs) for lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) transduce signals to many functions of normal cells. Most human cancer cells upregulate S1P and LPA GPCRs, in patterns distinctive for each type of tumor. The findings that 1-alpha, 25-dihydroxy-vitamin D_3 (VD3) and all-trans retinoic acid (RA) differentially alter expression of the predominant $S1P_3$ (Edg-3) R and $S1P_2$ (Edg-5) R in human breast cancer cells (BCCs) permitted analyses of their individual activities, despite a lack of selective pharmacological probes. S1P-evoked increases in $[Ca^{2+}]_i$ in $S1P_3$ R-predominant BCCs were suppressed by concentrations of VD3 and RA which decreased expression of $S1P_3$ Rs, despite RA-induced increases in $S1P_2$ Rs. S1P-elicited chemokinetic migration of $S1P_3$ R-predominant BCCs across a type IV collagen-coated micropore filter also was inhibited by concentrations of VD3 and RA which decreased expression of $S1P_3$ Rs. The RA-induced increase in expression of $S1P_2$ Rs did not prevent suppression by RA of S1P-elicited chemokinesis, which appears to be mediated by $S1P_3$ Rs, but instead exposed $S1P_2$ R-mediated inhibition of epidermal growth factor-stimulated chemotaxis of BCCs. In contrast, expression of the predominant LPA_2 Rs, LPA-evoked increase in $[Ca^{2+}]_i$ and LPA-stimulated chemokinetic migration were suppressed concomitantly by RA but not VD3. Thus two structurally-homologous S1P Rs of BCCs differ in coupling to $[Ca^{2+}]_i$ signaling and have opposite effects on protein growth factor-stimulated chemotaxis. J. Cell. Biochem. 88: 732–743, 2003.

Key words: lysophospholipids; growth factors; 1-alpha, 25-dihydroxy-vitamin D_3; all-trans retinoic acid; transcription; chemotaxis

G protein-coupled receptors (GPCRs) for lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) transduce signals for cellular proliferation and diverse cytoskeleton-based functions [Chun et al., 1999; Goetzl and Lynch, 2000; Spiegel and Milstien, 2000; Tigyi and Goetzl, 2002]. Cells of several human tumors express higher levels of one or more LPA or S1P GPCRs than untransformed cells of the same lineage. For example, most ovarian cancer cells have abundant LPA_2 GPCRs, originally designated Edg-4 Rs, which are detected at very low levels or not at all in normal ovarian surface epithelial cells [Goetzl et al., 1999a; Hu et al., 2001]. Similarly, human breast cancer cells express predominantly $S1P_3$ GPCRs, formerly Edg-3 Rs, and only low levels of $S1P_2$ GPCRs, formerly Edg-5 Rs, and LPA_1 and LPA_2 GPCRs [Goetzl et al., 1999b]. LPA and S1P effects on

Abbreviations used: GPCRs, G protein-coupled receptors; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; FAF-BSA, fatty acid free-bovine serum albumin; FBS, fetal bovine serum; VD3, 1-alpha, 25-dihydroxy-vitamin D_3; RA, all-trans retinoic acid; Edg, endothelial differentiation gene; C_T, threshold cycle; R, receptor; Gus, β-glucuronidase; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

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Grant sponsor: Department of the Army.

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Received 13 August 2002; Accepted 26 September 2002
DOI 10.1002/jcb.10394

tumor cells encompass increases and decreases in proliferation, migration, and generation of numerous secreted and cell-surface proteins [Tigyi and Goetzl, 2002]. The nature of coupling of S1P and LPA GPCRs to individual G proteins, subsequent transductional pathways, and functional responses have been elucidated largely in transfectants overexpressing a single such GPCR. Far less is known about the distinctive properties of each of these GPCRs in the usual setting of co-expression of multiple S1P and LPA GPCRs by tumor cells, in part because of a lack of selective agonists and antagonists. We show here that 1, 25-dihydroxy-vitamin D_3 (VD3) suppresses the levels of both $S1P_2$ Rs and $S1P_3$ Rs, whereas retinoic acid (RA) suppresses the level of $S1P_3$ but strikingly enhances that of $S1P_2$ in human breast cancer cells (BCCs). These findings suggested the possibility of delineating separate functional roles for the S1P GPCRs in untreated BCCs ($S1P_3$ Rs predominate), BCCs pretreated with RA ($S1P_2$ Rs predominate), and BCCs pretreated with VD3 (low levels of both S1P Rs). That neither the level nor signals of related LPA_2 Rs in BCCs were affected by VD3 suggested a mechanistic relationship between altered expression and functions of S1P Rs.

EXPERIMENTAL PROCEDURES

Reagents and Cell Culture

1-alpha, 25-dihydroxy-vitamin D_3 (VD3) and D-erythro sphingosine 1A phosphate (S1P) (Biomol Research Laboratories, Inc., Plymouth Meeting, PA), all-trans retinoic acid (RA), fatty acid-free bovine serum albumin (FAF-BSA), purified type IV human collagen and L-alpha-lysophosphatidic acid (LPA) (Sigma Chemical Co., St. Louis, MO) were obtained from the designated suppliers. The human breast cancer cell line MDA-MB-453 (estrogen receptor negative, American Type Culture Collection, HTB-131, Manassas, VI) was cultured in DMEM H21 with 4.5 g of glucose/100 ml (UCSF Cell Culture Facility) supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Inc., Grand Island, NY), 2 mM glutamine, 100 U/ml of penicillin G, and 100 μg/ml of streptomycin. LPA/S1P R-null rat HTC4 hepatoma cells [An et al., 1998] were stably transfected with human $S1P_2$ and $S1P_3$ Rs separately to respective levels characteristic of RA-stimulated and unstimulated MDA-MB-453 cells using a retroviral kit (BD Biosciences-Clontech, Palo Alto, CA) and then cultured in DMEM with 10% FBS, penicillin G, and streptomycin. Proteins extracted from HTC4-$S1P_2$ and HTC4-$S1P_3$ cells were used as S1P R standards for Western blots [Zheng et al., 2001].

Quantification of S1P and LPA (Edg) R Expression by Real-Time PCR

MDA-MB-453 cells were serum-starved overnight in the presence of 50 μg of (FAF-BSA) per ml of DMEM, washed with serum-free DMEM, and incubated for 8, 24, 48, and 72 h with 1, 10, and 100 nM VD3 or 10 μM RA. Total RNA was isolated by the TRIzol method and treated with DNase I according to the manufacturer's protocol (Life Technologies). Specific primers and probes for the S1P and LPA Rs and the control genes used for standardization were designed using the Primer Express software package (PE Applied Biosystems, Foster City, CA). TaqMan probes were labeled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5′-end and the quencher dye TAMRA (6-carboxytetra-methylrhodamine) at the 3′-end. The primers and probes were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). The probes are:

$S1P_1$: 5′-CCT AGA ACT GGA AGC TGT CCA CCC ACC-3′ (Edg-1)
$S1P_2$: 5′-ACC TTC TGG TGC TCA TTG CGG TGG-3′ (Edg-5)
$S1P_3$: 5′-TGG TGA AGT CCA GCA GCC GTA AGG TG-3′ (Edg-3)
LPA_1: 5′-TTG GTC ATG GTG GCA ATC TAT GTC AAC C-3′ (Edg-2)
LPA_2: 5′-CGT TCG TGG TCT GCT GGA CAC CA-3′ (Edg-4)
LPA_3: 5′-CATTTTGCTTGTCTGGGCCATC-GC-3′ (Edg-7)

The primers are: $S1P_1$ forward 5′-GCA GCA GCA AGA TGC GAA G-3′ and reverse 5′-CGA TGA GTG ATC CAG GCT TTT-3′; $S1P_2$ forward 5′-GCG CCA TTG TGG TGG AA-3′ and reverse 5′-GAG CCA GAG AGC AAG GTA TTG G-3′; $S1P_3$ forward 5′-CTG GTG ACC ATC GTG ATC CTC-3′ and reverse 5′-ACG CTC ACC ACA ATC ACC AC-3′; LPA_1 forward 5′-GCT GGT GAT GGG ACT TGG AAT-3′ and reverse 5′-CAA CCC AGC AAA GAA GTC TGC-3′; LPA_2 forward 5′-ACG CTC AGC CTG GTC AAG AC-3′ and

reverse 5′-AAC CAT CCA GGA GCA GTA CCA C-3′; and LPA_3 forward 5′-GGTCCATAG-CAACCTGACCAA-3′ and reverse 5′-GGGAA-GAGCAGGCAGAGATGT-3′.

PCR conditions were optimized according to guidelines suggested in the PRISM 7700 sequence detection system manual. PCR reactions were carried out with 20–100 ng of DNase I-pretreated RNA, 0.3 μM each primer, 0.1 μM probe, 2.5 mM MgCl, 0.2 mM dNTPs, 5 U of RNase inhibitor (Roche Molecular Biochemicals, Mannheim, Germany), 12.5 U of M-MuLV reverse transcriptase, and 1.25 U of AmpliTaq Gold DNA polymerase (PE Applied Biosystems) in a total volume of 25 μl. The first reaction began with 30 min at 48°C (first-strand cDNA synthesis), 5 min at 99°C (inactivate reverse transcriptase and activate AmpliTaq Gold DNA polymerase), and 2 min at 95°C, followed by 40 cycles of denaturation for 15 s at 94°C and annealing/extension for 1 min at 60°C. Real-time PCR was performed with an ABI PRISM 7700 Sequence Detector (UCSF Genome Analysis Core Facility) using TaqMan PCR reagents. Standard curves for S1P/LPA Rs and reference sequences showed interanalysis coefficients of variation lower than 4%. The comparative C_T (threshold cycle) method for quantification of gene expression was applied, as described [Dorsam and Goetzl, 2002]. Analyses of data generated by this method are dependent upon the inverse exponential relationship between starting quantities (copies) of target sequence in the reactions and the corresponding levels of C_T. The mean C_T values for a reference sequence (for example, β-glucuronidase, Gus or hypoxanthine-guanine phospho-ribosyltransferase, HPRT) were subtracted from the mean C_T values for each target S1P/LPA R sequence for both the VD3-treated and RA-treated samples and the untreated control samples, so that $\Delta C_T = C_T$ (Edg) $- C_T$(Gus). The ΔC_T values of known quantity-calibrated samples then were subtracted from ΔC_T values of the test samples to obtain $\Delta\Delta C_T$ values: $\Delta\Delta C_T = \Delta C_T$ (S1P/LPA R, VD3 or RA) $- \Delta C_T$ (S1P/LPA R, calibrator). An exponential formula ($2^{-\Delta\Delta CT}$) was used to determine the levels of S1P/LPA R target sequences in the unknown experimental samples from $\Delta\Delta C_T$ values. The standard calibration curve for each gene was generated by plotting the C_T values against the logarithm of the known concentrations of serially diluted RNA and the concentration of each unknown was determined from the calibration curve. Statistical significance of differences between group mean values were calculated by a paired Students *t*-test.

Quantification of S1P and LPA (Edg) R Expression by Western Blots

Replicate suspensions of 10^7 MDA-MB-453 cells, which had been preincubated with VD3, RA or medium alone were washed three times and resuspended in 0.3 ml of cold 10 mM Tris-HCl (pH 7.4) containing 0.12 M sucrose, 5% glycerol (v:v), and a mixture of protease inhibitors (Sigma Chemical Co.). Each sample was homogenized with a Teflon pestle for 2 min at 250 *g*, centrifuged at $400 \times g$ for 5 min at 4°C, and the $400 \times g$ supernate centrifuged at $300{,}000 \times g$ for 30 min at 4°C. Each $300{,}000 \times g$ pellet was resuspended in 0.1 ml of 10 mM Tris-HCl (pH 7.4) with 5% glycerol (v:v), the mixture of protease inhibitors, and 1% (v:v) NP-40 to be rehomogenized, incubated for 2 h at 4°C and recentrifuged at $300{,}000 \times g$ for 30 min at 4°C. Aliquots of MDA-MB-453 cell extracts and of similar extracts of HTC4-$S1P_2$ and HTC4-$S1P_3$ stable transfectants, containing 10–20 μg of protein, were electrophoresed, transferred to a nitrocellulose membrane, and labeled with mouse monoclonal anti-$S1P_2$ and anti-$S1P_3$ directed to carboxy- and amino-terminal substituent peptides, respectively [Goetzl et al., 2000], and a peroxidase-conjugated second antibody for detection with a standard ECL kit (Amersham, Inc., Arlington Heights, IL).

Quantification of Intracellular Concentration of Calcium ($[Ca^{2+}]_i$)

MDA-MB-453 cells were cultured in 384 well plates (Greiner Bio-One, Inc., Lake Mary, FL) at a density of 6×10^4 cells/well in DMEM containing 10% FBS, 4 mM glutamine, and 1 mM sodium pyruvate. After 8 h, medium was replaced with serum-free DMEM-50 μg/ml of FAF-BSA for an additional 15 h of incubation, and then some replicate wells received 1, 10, or 100 nM VD3 or 10 μM RA and were incubated for an additional 8, 24, 48, or 72 h. The plates were washed and refilled with Hanks' balanced salt solution-10 mM HEPES-0.1 g % FAF-BSA (pH 7.4) and the cells were incubated with a calcium dye loading kit (Molecular Devices, Sunnyvale, CA) for 1 h at room temperature. The dye-labeled cells then were

placed in the FLIPR^{384} (Molecular Devices, Inc.) for stimulation with 10 nM, 100 nM, and 1 μM S1P and LPA. An integrated robotic pipettor allowed for simultaneous addition of a stimulus into each well of a plate. Calcium fluorescence signals were detected (Molecular Devices, Inc.) using a cooled CCD camera.

Analyses of S1P R-Dependent Migration of MDA-MB-453 Cells

MDA-MB-453 cells were washed twice and incubated at 30–60% confluence in DMEM-50 μg/ml of FAF-BSA for 48 h without and with 10 nM VD3 or 10 μM RA. After harvesting in protease-free dissociating buffer, replicate 0.3 ml aliquots of suspensions of 1.5×10^6 MDA-MB-453 cells in DMEM-50 μg/ml of FAF-BSA then were added to the 13 mm diameter upper inserts of Transwell chambers (Costar, Cambridge, MA) over 12 μm pore diameter polycarbonate filters (Nucleopore Corp., Pleasanton, CA) coated evenly on the bottom surface with 25 μl of 10 μg/ml of purified type IV human collagen (Sigma Chemical Co.) [Xia et al., 1996]. Each insert was placed into 800 μl of DMEM-50 μg/ml of FAF-BSA in the lower compartment so that the fluid levels inside and outside the insert were at the same height. Some duplicate sets of Transwell chambers had 10^{-8} or 10^{-6} M S1P in the lower compartment alone (chemotaxis) or both the upper and lower compartments (chemokinesis) and other duplicate sets had 10^{-10} M human purified recombinant EGF (Peprotech, Inc., Rocky Hill, NJ) in the lower compartment without or with 10^{-8} or 10^{-6} M S1P in both the upper and lower compartments. Chambers were incubated at 37°C in 5% CO_2 in humidified air for 12–48 h prior to shaking inserts into lower compartments for 3 min to detach cells adherent to the bottom surface of the filters, and counting the number of cells in each lower compartment. The migration responses were expressed as a percentage of the total number of MDA-MB-453 cells added to the upper insert or a percentage of a control response (100%). The mean (n = 3) magnitude of responses of untreated MDA-MB-453 cells to 10^{-8} and 10^{-6} M S1P and to 10^{-10} M EGF were 2.9, 5.1, and 8.2% for chemotaxis and 4.5, 7.8, and 1.1% for chemokinesis at 12 h; 7.3, 11, and 18% for chemotaxis and 9.1, 14, and 5% for chemokinesis at 24 h; and 9.2, 13, and 22% for chemotaxis and 12, 17, and 6% for chemokinesis at 48 h, respectively. As nearly maximal responses were attained by 24 h, all studies were carried out for this period.

RESULTS

Expression of S1P Rs and LPA Rs

Real-time polymerase chain reaction (PCR) analyses, using the TaqMan fluorogenic probe system and an Applied Biosystems Prism model 7700 sequence detection instrument, quantified the levels of mRNA encoding each S1P R and LPA R in unstimulated MDA-MB-453 BCCs and those exposed to VD3 and RA. Of several control genes examined in pilot studies, Gus was chosen as the primary standard for measurement of mRNA encoding S1P/LPA Rs by the comparative C_T method because VD3 and RA did not affect its expression. In contrast, VD3 and RA changed the expression of GAPDH, β-actin, and cyclophilin sufficiently to prevent their application as standards. Basal levels of mRNAs encoding S1P/LPA Rs in unstimulated MDA-MB-453 BCCs confirmed our previously observed rank-order of $S1P_3$ (Edg-3) > LPA_2 (Edg-4) ≫ $S1P_2$ (Edg-5) ≥ LPA_1 (Edg-2), without detectable $S1P_1$ (Edg-1) or LPA_3 (Edg-7) Rs (Table I). More recent analyses also have not detected $S1P_4$ or $S1P_5$.

To examine effects of 1-alpha, 25-dihydroxyvitamin D_3 (VD3) on expression of S1P/LPA Rs, MDA-MB-453 BCCs were serum-starved and incubated with 1, 10, and 100 nM VD3 for 8, 24, and 48 h. After 8 h, all three concentrations of VD3 suppressed the levels of mRNA encoding

TABLE I. Relative Levels of S1P Rs and LPA Rs in MDA-MB-453 BCCs

S1P Rs			LPA Rs		
$S1P_1$ (Edg-1)	$S1P_2$ (Edg-5)	$S1P_3$ (Edg-3)	LPA_1 (Edg-2)	LPA_2 (Edg-4)	LPA_3 (Edg-7)
ND	3.3 ± 0.4	75 ± 13*	1.0 ± 0.1	$26 \pm 3.1^+$	ND

Each value is the mean ± SEM of the results of four different determinations performed in triplicate and expressed as a percentage of the Gus endogenous reference standard. ND, not detected. The symbols denoting statistical significance are: +, $P < 0.01$ relative to $S1P_2$; *, $P < 0.01$ relative to LPA_2.

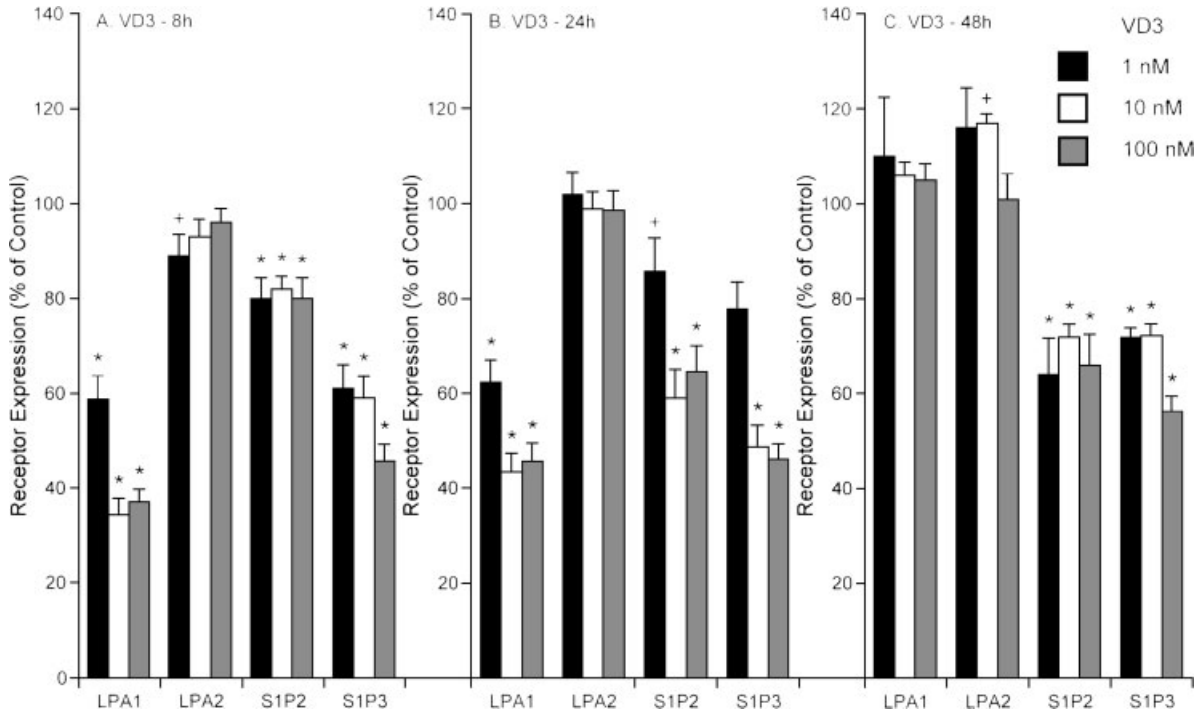

Fig. 1. Real-time PCR quantification of VD3 effects on S1P/LPA R mRNAs. Each bar value is the mean ± SEM of the results of three determinations, normalized with the Gus endogenous standard, and expressed as a percentage of the control incubated without VD3 (100%). The symbols denoting statistical significance of differences from control are: +, $P < 0.05$; *, $P < 0.01$. **A**: Incubation with VD3 for 8 h. **B**: Incubation with VD3 for 24 h. **C**: Incubation with VD3 for 48 h.

LPA_1 and $S1P_3$ significantly and by means of 39% or more (Fig. 1A). Levels of mRNA encoding S1P2 also were reduced significantly, but by means of only 20% or less, whereas those for LPA_2 were lowered only marginally or were unaffected. After 24 h, all concentrations of VD3 significantly and similarly suppressed expression of LPA_1, $S1P_2$ and $S1P_3$, except for the lack of effect of 1 nM VD3 on $S1P_2$, but again did not alter significantly expression of LPA_2 (Fig. 1B). At 48 h after introduction of VD3, significant suppression of levels of mRNA encoding $S1P_2$ and $S1P_3$ was maintained for all concentrations of VD3, those for LPA_1 had returned to control levels, and those for LPA_2 remained unaffected (Fig. 1C). Thus VD3 modified expression of mRNAs encoding each LPA/S1P R with a unique time-course encompassing early and sustained suppression of $S1P_3$, early and subsiding inhibition of LPA_1, later and sustained reductions in $S1P_2$, and no substantial effect at any time on LPA_2. In contrast, the pattern of effects of 10 μM all-trans RA on each receptor was established after 24 h (Fig. 2A) and maintained for 72 h (Fig. 2B,C). At each time, incubation of MDA-MB-453 BCCs with 10 μM RA suppressed the levels of mRNAs encoding LPA_1, LPA_2, and $S1P_3$ by more than 50%, but increased expression of $S1P_2$ by at least threefold (Fig. 2A,B,C). At 72 h, levels of mRNA were assessed by real-time PCR using both Gus and HPRT as primary endogenous standards unaffected by RA and the results were the same (Fig. 2C). At 1 μM, RA had no effect on the levels of mRNA encoding LPA_1, LPA_2, and $S1P_3$, but increased that for $S1P_2$ by a mean of 176%.

Western blots of proteins extracted from MDA-MB-453 cells after pretreatment with 10 nM VD3, 10 μM RA or medium alone for 24 h showed changes qualitatively similar to the results of real-time PCR analyses. Expression of $S1P_3$ Rs (Edg-3 Rs) was suppressed by both VD3 and RA (Fig. 3, left frame). In contrast, the level of $S1P_2$ Rs (Edg-5 Rs) was decreased by VD3 and increased by RA (Fig. 3, right frame). Thus three sets of MDA-MB-453 BCCs, each expressing different levels of S1P and LPA Rs, were available for biochemical and functional studies. Native untreated MDA-MB-453 BCCs have predominantly $S1P_3$ and LPA_2 Rs, VD3-treated MDA-MB-453 BCCs have

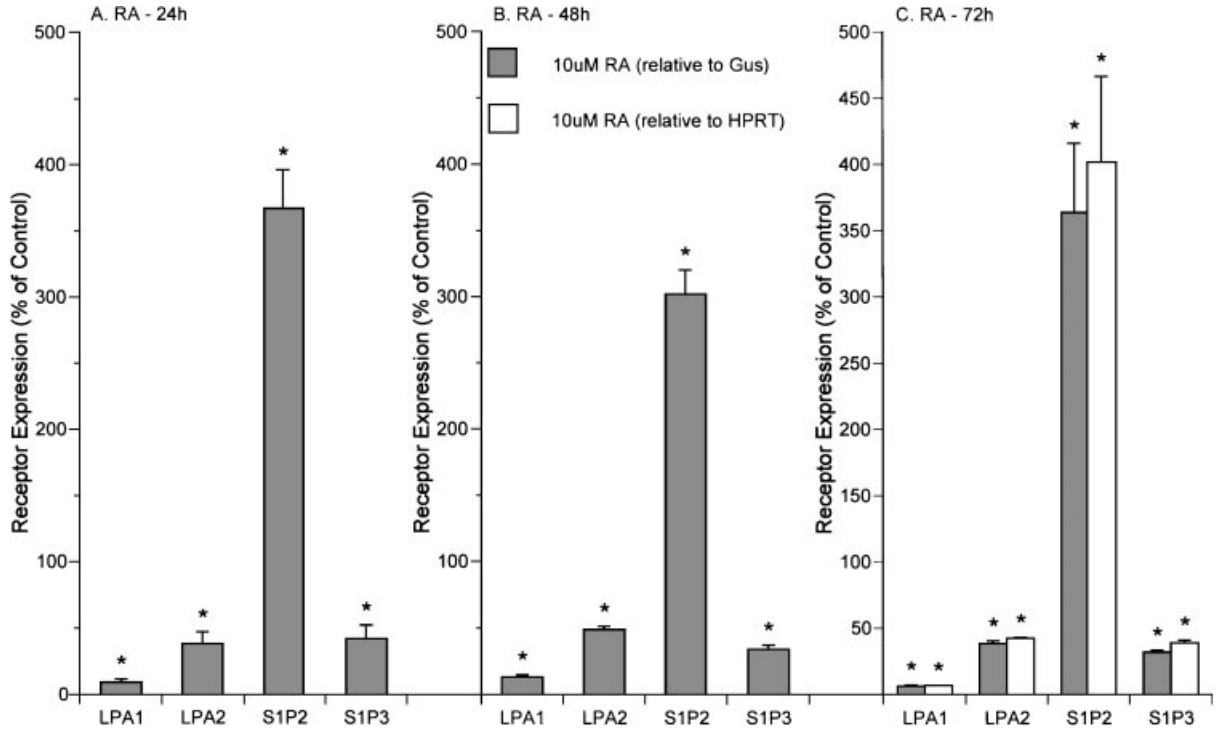

Fig. 2. Real-time PCR quantification of RA effects on S1P/LPA R mRNAs. Each bar value is the mean ± SEM of the results of six measurements, normalized to the endogenous standards Gus and for Figure 2C only to HPRT, and expressed as a percentage of the control incubated without RA (100%). The symbols denoting statistical significance of differences from control are: +, $P < 0.05$; *, $P < 0.01$. **A**: Incubation with 10 μM RA for 24 h. **B**: Incubation with 10 μM RA for 48 h. **C**: Incubation with 10 μM RA for 72 h.

decreased levels of $S1P_3$ and other receptors in the subfamily, but normal expression of LPA_2 Rs, and RA-treated MDA-MB-453 BCCs have increased expression of $S1P_2$ Rs and reduced levels of all other receptors in the subfamily.

Altered Responses of the Intracellular Concentration of Calcium ($[Ca^{2+}]_i$)

S1P and LPA both evoke concentration-dependent increases in $[Ca^{2+}]_i$ in native MDA-MB-453 cells (Fig. 4). The response to LPA is

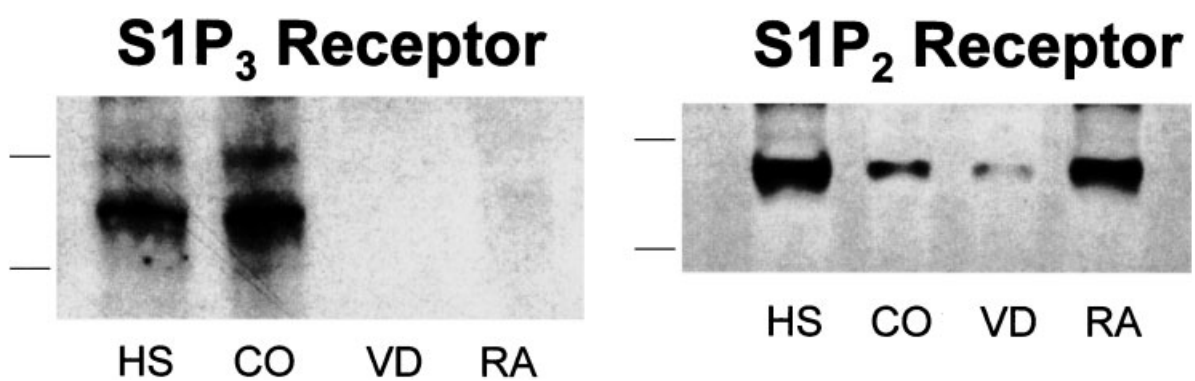

Fig. 3. Western blot analyses of the effects of VD3 and RA on S1P/LPA Rs. $S1P_3$ Rs (**left frame**) and $S1P_2$ Rs (**right frame**) were immunoquantified in extracts of MDA-MB-453 cells by standard Western blotting techniques. The lane designations indicate extracts of HTC4 cell transfectant positive control (HS), untreated control MDA-MB-453 cells (CO), MDA-MB-453 cells treated with 10 nM VD3 for 24 h (VD), and MDA-MB-453 cells treated with 10 μM RA for 48 h (RA). The HS lanes received 5 μg of protein and each MDA-MB-453 lane received 10 μg of protein. Marginal horizontal lines denote positions of protein mw standards of 51 and 38 kDa (Life Technologies).

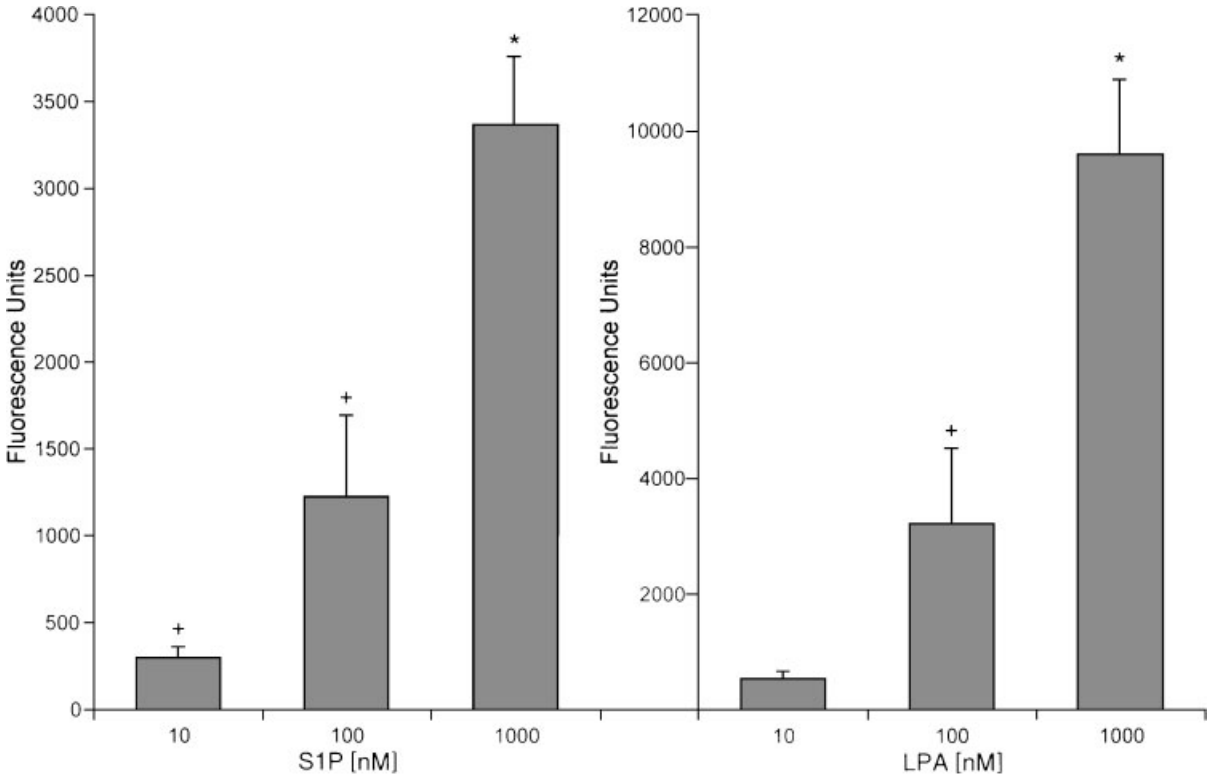

Fig. 4. S1P and LPA evoke increases in $[Ca^{2+}]_i$ in MDA-MB-453 BCCs. Each bar value depicts the mean ± SEM of results of six different assays. The symbols denoting statistical significance of differences from control are: +, $P < 0.05$; *, $P < 0.01$.

greater and a higher multiple of the background than that to S1P, despite the apparently higher level of mRNA expression for $S1P_3$ than LPA_2 Rs. In VD3-treated MDA-MB-453 cells, the $[Ca^{2+}]_i$ responses to 10 and 100 nM S1P were reduced very significantly, in parallel with decreases in expression of $S1P_3$ Rs, whereas the response to 1000 nM S1P was more modestly suppressed (Fig. 5). None of the $[Ca^{2+}]_i$ responses of VD3-treated MDA-MB-453 cells to LPA was inhibited significantly, which is consistent with the lack of decrease in expression of LPA_2 Rs. RA strikingly reduced the $[Ca^{2+}]_i$ responses of MDA-MB-453 cells to LPA, as expected from the prominent reduction in expression of both LPA_1 and LPA_2 Rs (Fig. 6). However, reduction of the response to the lowest concentration of 10 nM LPA was not apparent until after 48 h. Lesser decreases in $[Ca^{2+}]_i$ responses to S1P were induced by pretreatment with RA for 24–72 h, which is consistent with partial compensatory increases in $S1P_2$ Rs accompanying reductions in $S1P_3$ Rs (Fig. 6).

Differential Regulation by VD3 and RA of Migration Responses of MDA-MB-453 BCCs to S1P and EGF

The migration of control untreated MDA-MB-453 BCCs was stimulated significantly by 10^{-8} and 10^{-6} M S1P in a principally chemokinetic manner, as evidenced by the greater responses evoked when S1P was on both sides of the micropore filter than when it was only in the lower compartment at the same concentration (Fig. 7, left frame). The migration responses of control MDA-MB-453 BCCs to LPA reached a mean peak magnitude at 10^{-6} M of only 24% of that elicited by S1P and also was predominantly chemokinetic (data not shown). Pretreatment of MDA-MB-453 BCCs with 10 nM VD3 for 24 h, which significantly suppresses expression of $S1P_3$ and $S1P_2$ Rs (Figs. 1 and 3), decreased the migration responses to all concentrations of S1P significantly relative to the responses of control MDA-MB-453 BCCs (Fig. 7, left frame). Pretreatment of MDA-MB-453 BCCs with 10 μM RA for 24 h, which suppresses expression of $S1P_3$ Rs and enhances expression of $S1P_2$ Rs

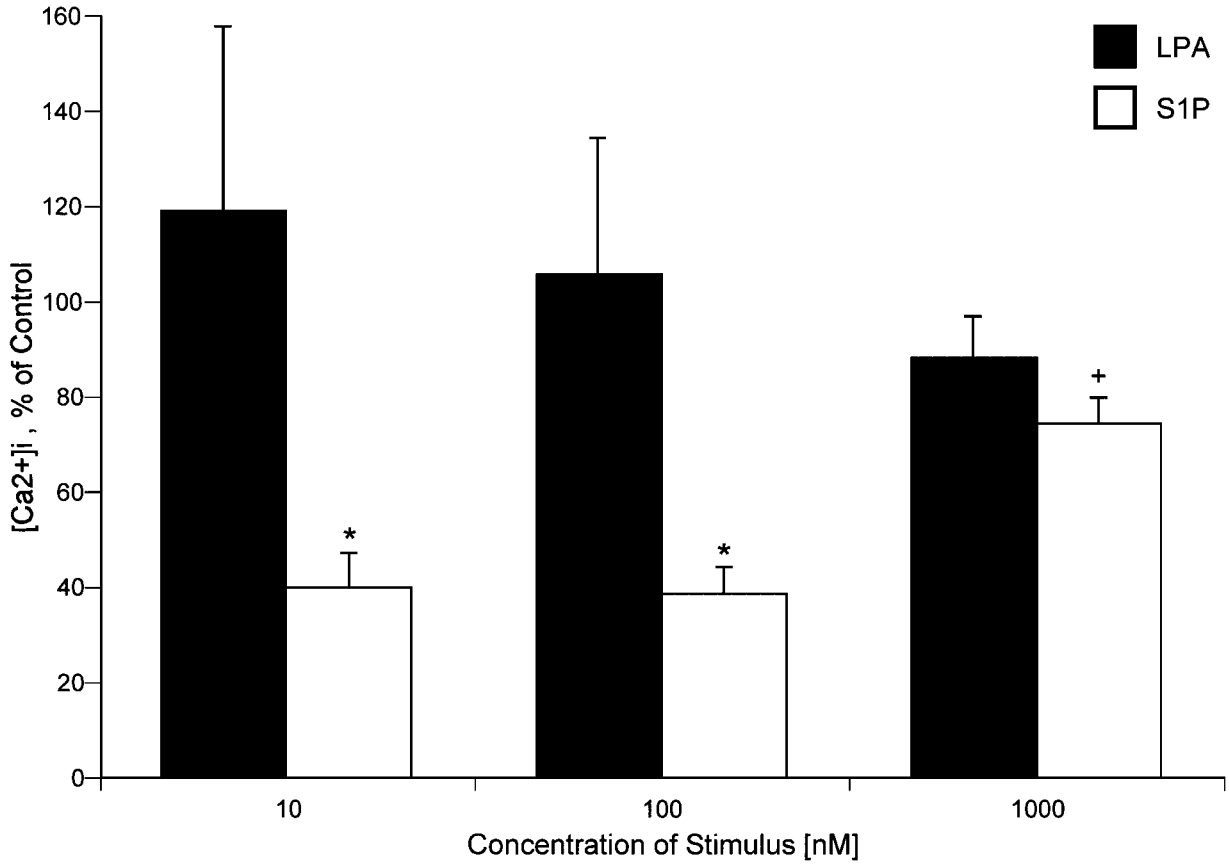

Fig. 5. Effect of VD3 on S1P- and LPA-elicited increases in $[Ca^{2+}]_i$ in MDA-MB-453 cells. MDA-MB-453 cells were pretreated with 10 nM VD3 for 24 h prior to stimulation with S1P (open bars) and LPA (filled bars). Each bar value depicts the mean ± SEM of the results of three triplicate assays as a percentage of the values obtained in vehicle-treated control cells. The symbols denoting statistical significance of differences from controls without VD3 are: +, $P < 0.05$; *, $P < 0.01$.

significantly (Figs. 2 and 3), also decreased migration responses to S1P significantly relative to the responses of control MDA-MB-453 BCCs (Fig. 7, left frame). Control MDA-MB-453 BCCs showed a prominent chemotactic response to human EGF, which was additive with S1P-elicited responses (Fig. 7, right frame, open bar in each set). VD3 pretreatment did not diminish the chemotactic response to EGF alone and the responses to EGF and S1P together were simply additive, as in control MDA-MB-453 BCCs (Fig. 7, right frame, diagonally cross-hatched bars). RA pre-treatment of MDA-MB-453 BCCs also did not diminish the response to EGF alone. However, S1P suppressed significantly and with concentration-dependence the chemotactic responses of RA-pretreated MDA-MB-453 BCCs to EGF (Fig. 7, right frame, vertically-striped bars). Thus, both VD3 suppression of $S1P_3$ Rs and $S1P_2$ Rs, and RA suppression of $S1P_3$ Rs with elevation of $S1P_2$ Rs decreased responses to S1P alone, suggesting that $S1P_3$ Rs are the principal transducer of chemokinesis. However, increases in expression of $S1P_2$ Rs failed to compensate for the loss of $S1P_3$ Rs in chemokinetic responses of RA-pretreated BCCs to S1P. $S1P_2$ Rs not only failed to transduce migration responses to S1P, but at the same level of expression mediated inhibition of the chemotactic response of RA-pretreated BCCs to EGF.

DISCUSSION

The micromolar concentrations of LPA, S1P, and related phospholipid mediators in blood and tissue fluids combined with the widespread organ-system distribution of their GPCRs have focused attention on the importance of organ-specific and response-coupled regulation of expression of these GPCRs and related signaling

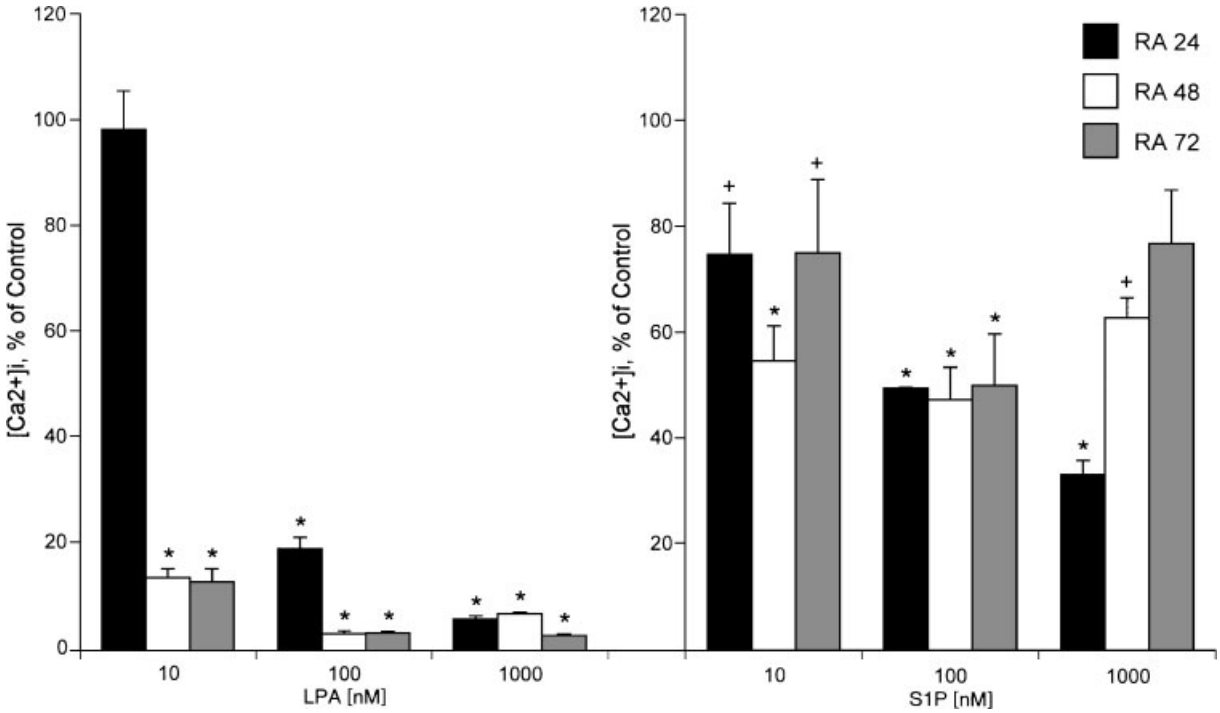

Fig. 6. Effects of RA on LPA- and S1P-evoked increases in $[Ca^{2+}]_i$ in MDA-MB-453 cells. MDA-MB-453 cells were pretreated with 10 μM RA for 24, 48, and 72 h prior to stimulation with LPA (left frame) or S1P (right frame). Each bar value depicts the mean ± SEM of the results of three triplicate assays as a percentage of the values obtained in vehicle-treated control cells (100%). The symbols denoting statistical significance of differences from controls without RA are: +, $P < 0.05$; *, $P < 0.01$.

pathways [Tigyi and Goetzl, 2002]. Few natural mechanisms have been identified which contribute to the selective control of cellular expression, turnover, or desensitization of individual LPA Rs and S1P Rs. The great redundancy of both tissue distribution and signaling pathways of the GPCRs for LPA and S1P have even complicated many genetically-based approaches to defining distinctive roles for each LPA R and S1P R. Overexpression of a single GPCR in the subfamily has allowed assignment of some functions to that GPCR in limited in vitro settings, but attempts at concurrent genetic suppression of the other GPCRs for LPLs in the same cell have generally been unsuccessful. Similar problems have confounded many in vivo genetic manipulations. For example, despite abundant evidence supporting developmental regulation of expression and critical functions of LPA_1 Rs (Edg-2 Rs) in the central nervous system, deletion of the mouse gene encoding LPA_1 Rs alters suckling behavior through facial defects but has no relevant effects on intrinsic neural functions [Contos et al., 2000]. Reactive upregulation of other LPA Rs in critical areas of the CNS in the LPA_1 R knock-out mouse is presumed to compensate functionally for the loss of LPA_1 Rs. Further difficulties arise from the lack of selective and bioavailable pharmacological agonists or antagonists for any of the S1P Rs or LPA Rs.

Our discovery that VD3 and RA differentially alter expression of $S1P_3$ Rs (Edg-3 Rs) and $S1P_2$ Rs (Edg-5 Rs) in several lines of BCCs suggested that elucidation of specific functional consequences of these effects in one well-studied line might increase our understanding of cellular mechanisms regulating LPL GPCRs. Interpretation of the relationships between alterations in expression and functions of S1P Rs was facilitated by finding that neither levels nor signals of the related and prominent LPA_2 Rs were affected by VD3, which suggested minimal or no impact of VD3 on transductional pathways common to both LPA and S1P Rs.

A line of BCCs expressing predominantly $S1P_3$ Rs and LPA_2 Rs was selected to examine the effects of VD3 and RA. The prominent depression of $S1P_3$ Rs by VD3 and RA, and concurrent elevation by RA of $S1P_2$ Rs, which are suppressed by VD3, created three patterns of expression of S1P Rs in the same BCC (Table I,

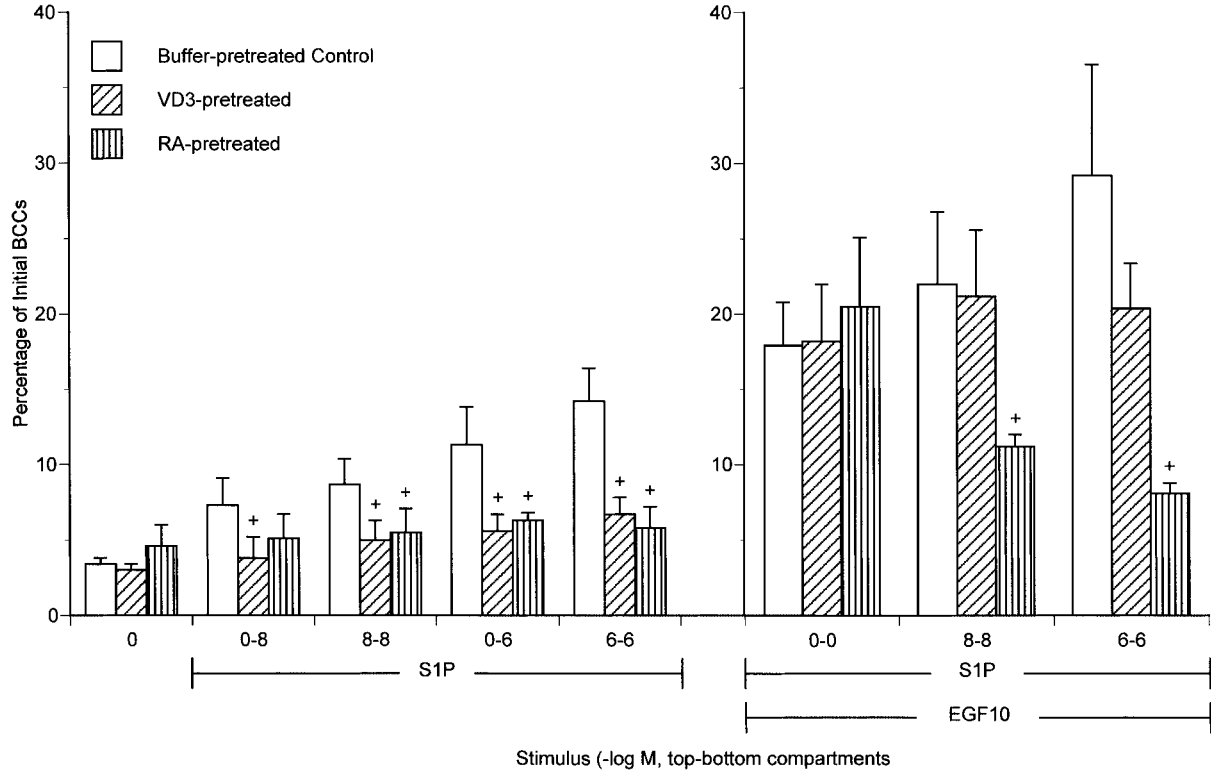

Fig. 7. Differential regulation of S1P-elicited migration of BCCs by VD3 and RA. After 24 h of pretreatment with VD3, RA or medium alone, migration responses to S1P and human EGF were quantified in chambers with type IV human collagen-coated filters. Each bar value is the mean ± SD of the results of three studies conducted in duplicate. Statistical significance of differences in responses to S1P from those of medium-pretreated controls (left frame) and from those of RA-pretreated BCCs to EGF alone (right frame) were calculated by a paired *t*-test and the symbols denoting results are: +, $P < 0.05$; *, $P < 0.01$.

Figs. 1 and 3). First, native MDA-MB-453 BCCs had only a high level of $S1P_3$ Rs. Second, VD3-treated BCCs had depressed levels of both S1P Rs. Third, RA-treated BCCs had reduced levels of $S1P_3$ Rs, but strikingly elevated levels of $S1P_2$ Rs. The increases in $[Ca^{2+}]_i$ in each of the three variants of this BCC line evoked by S1P were consistent with the timing and magnitude of their level of expression of S1P GPCRs (Figs. 4–6). Introduction of these pretreated BCCs into a migration assay system permitted definition of the separate roles of $S1P_3$ Rs and $S1P_2$ Rs in mediating a complex tumor cell functional response. The suppression of migration responses to S1P by concentrations of VD3 and RA shown to decrease expression of $S1P_3$ Rs was independent of the level of concurrent expression of $S1P_2$ Rs (Fig. 7). However, a high level of expression of $S1P_2$ Rs in RA-pretreated BCCs also reduced migration responses to EGF. Thus $S1P_3$ Rs signal stimulation of migration, whereas $S1P_2$ Rs transduce signals inhibitory of migration. Presumably the ratio of $S1P_2/S1P_3$ Rs is a crucial determinant of migration responses of BCCs to mixtures of S1P and other chemotactic factors.

$S1P_2$ and $S1P_3$ Rs are capable of coupling similarly to many G proteins, but the magnitude of transductionally relevant G protein signals elicited by S1P may differ for each depending on the host cell, density of R expression, and concurrent changes in activity of other receptors [Ancellin and Hla, 1999; An et al., 2000]. Functional differences between these two S1P Rs have been observed previously, perhaps because only $S1P_3$ Rs also couple efficiently to activation of Rho GTPases. In two other types of cells, $S1P_3$ Rs transduced S1P-elicited cellular migration, whereas $S1P_2$ Rs inhibited cell migration evoked by growth factors [Okamoto et al., 2000; Ryu et al., 2002]. The possibility of opposite effects of $S1P_2$ and $S1P_3$ Rs on cell migration is supported by the present findings that $S1P_3$ Rs mediate BCC chemokinesis, whereas $S1P_2$ Rs are far less active directly but suppress BCC chemotaxis to EGF (Fig. 7).

Both VD3 and all-trans RA, the major physiologically active metabolite of vitamin A, affect crucial elements of cellular proliferation, survival, gene expression and transcription, and differentiation [Ahn et al., 1995; Jones et al., 1998; Green et al., 1999; Stoica et al., 1999; Koli and Keski-Oja, 2000; Dow et al., 2001]. These effects together suppress some forms of tumorigenesis, directly and through induction of anti-tumor cytokines, and reduce activities of several proteases necessary for tumor growth and spread. It is now suggested that VD3 and RA also reduce tumorigenesis by suppressing expression of some of the GPCRs specific for the lysophospholipid growth factors LPA and S1P (Figs. 1 and 3). The suppression is presumed to be through transcriptional mechanisms, as mRNAs encoding the LPA/S1P GPCRs as well as the GPCR proteins were altered drastically. The clinical implication is that antagonists of $S1P_3$ Rs and agonists of $S1P_2$ Rs could suppress spread of breast cancers, which express the appropriate GPCRs or where RA or a derivative of RA would elevate expression of $S1P_2$ Rs. It will now be necessary to confirm the basic findings in vivo and by mimicking consequences of amplification and attenuation of LPA/S1P Rs with potent bioavailable agonists and antagonists for the same receptors.

ACKNOWLEDGMENTS

These studies were supported by a grant (to E.J.G.) and a fellowship (to H.D.) from the Department of the Army. The authors are grateful to Yvonne Kong for expert technical assistance and to Robert Chan for careful graphics.

REFERENCES

Ahn MJ, Langenfeld J, Moasser MM, Rusch V, Dmitrovsky E. 1995. Growth suppression of transformed human bronchial epithelial cells by all-trans retinoic acid occurs through specific retinoid receptors. Oncogene 11:2357–2364.

An S, Bleu T, Hallmark OG, Goetzl EJ. 1998. Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. J Biol Chem 273:7906–7910.

An S, Zheng Y, Bleu T. 2000. Sphingosine 1-phosphate-induced cell proliferation, survival, and related signaling events mediated by G protein-coupled receptors Edg-3 and Edg-5. J Biol Chem 275:288–296.

Ancellin N, Hla T. 1999. Differential pharmacological properties and signal transduction of the sphingosine 1-phosphate receptors Edg-1, Edg-3, and Edg-5. J Biol Chem 274:18997–19002.

Chun J, Contos JJ, Munroe D. 1999. A growing family of receptor genes for lysophosphatidic acid (LPA) and other lysophospholipids (LPs). Cell Biochem Biophys 30:213–242.

Contos JJ, Fukushima N, Weiner JA, Kaushal D, Chun J. 2000. Requirement for the lpA1 lysophosphatidic acid receptor gene in normal suckling behavior. Proc Natl Acad Sci USA 97:13384–13389.

Dorsam G, Goetzl EJ. 2002. Vasoactive intestinal peptide receptor-1 (VPAC-1) is a novel gene target of the hemolymphopoietic transcription factor Ikaros. J Biol Chem 277:13488–13493.

Dow R, Hendley J, Pirkmaier A, Musgrove EA, Germain D. 2001. Retinoic acid-mediated growth arrest requires ubiquitylation and degradation of the F-box protein Skp2. J Biol Chem 276:45945–45951.

Goetzl EJ, Lynch KR. 2000. Lysophospholipids and eicosanoids in biology and pathophysiology. Ann NY Acad Sci 905:1–357.

Goetzl EJ, Dolezalova H, Kong Y, Hu YL, Jaffe RB, Kalli KR, Conover CA. 1999a. Distinctive expression and functions of the type 4 endothelial differentiation gene-encoded G protein-coupled receptor for lysophosphatidic acid in ovarian cancer. Cancer Res 59:5370–5375.

Goetzl EJ, Dolezalova H, Kong Y, Zeng L. 1999b. Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. Cancer Res 59: 4732–4737.

Goetzl EJ, Kong Y, Voice JK. 2000. Cutting edge: Differential constitutive expression of functional receptors for lysophosphatidic acid by human blood lymphocytes. J Immunol 164:4996–4999.

Green A, Shilkaitis A, Christov K. 1999. 4-(hydroxyphenyl)-retinamide selectively inhibits the development and progression of ductal hyperplastic lesions and carcinoma in situ in mammary gland. Carcinogenesis 20:1535–1540.

Hu YL, Tee MK, Goetzl EJ, Auersperg N, Mills GB, Ferrara N, Jaffe RB. 2001. Lysophosphatidic acid induction of vascular endothelial growth factor expression in human ovarian cancer cells. J Natl Cancer Inst 93:762–768.

Jones G, Strugnell SA, DeLuca HF. 1998. Current understanding of the molecular actions of vitamin D. Physiol Rev 78:1193–1231.

Koli K, Keski-Oja J. 2000. 1alpha,25-dihydroxyvitamin D_3 and its analogues down-regulate cell invasion-associated proteases in cultured malignant cells. Cell Growth Differ 11:221–229.

Okamoto H, Takuwa N, Yokomizo T, Sugimoto N, Sakurada S, Shigematsu H, Takuwa Y. 2000. Inhibitory regulation of Rac activation, membrane ruffling, and cell migration by the G protein-coupled sphingosine 1-phosphate receptor Edg-5 but not Edg-1 or Edg-3. Mol Cell Biol 20:9247–9261.

Ryu Y, Takuwa N, Sugimoto N, Sakurada S, Usui S, Okamoto H, Matsui O, Takuwa Y. 2002. Sphingosine 1-phosphate, a platelet-derived lysophospholipid mediator, negatively regulates cellular Rac activity and cell migration in vascular smooth muscle cells. Circ Res 90: 325–332.

Spiegel S, Milstien S. 2000. Sphingosine 1-phosphate: Signaling inside and out. FEBS Lett 476:55–57.

Stoica A, Saceda M, Fakhro A, Solomon HB, Fenster BD, Martin MB. 1999. Regulation of estrogen receptor-alpha gene expression by 1, 25-dihydroxyvitamin D in MCF-7 cells. J Cell Biochem 75:640–651.

Tigyi G, Goetzl EJ. 2002. Lysolipid mediators in cell signaling and disease. Biochim Biophys Acta 1582: VII.

Xia M, Leppert D, Hauser SL, Sreedharan SP, Nelson PJ, Krensky AM, Goetzl EJ. 1996. Stimulus specificity of matrix metalloproteinase dependence of human T cell migration through a model basement membrane. J Immunol 156:160–167.

Zheng Y, Kong Y, Goetzl EJ. 2001. Lysophosphatidic acid receptor-selective effects on Jurkat T cell migration through a Matrigel model basement membrane. J Immunol 166:2317–2322.