Protein Kinase Cε Dependence of the Recovery from Down-regulation of S1P1 G Protein-coupled Receptors of T Lymphocytes*

Markus H. Graeler, Yvonne Kong, Joel S. Karliner‡, and Edward J. Goetzl§

From the Departments of Medicine and Microbiology-Immunology, University of California, San Francisco, California 94143-0711 and ‡The Cardiology Section of the Veterans Affairs Medical Center, San Francisco, California 94121

Received for publication, April 7, 2003, and in revised form, May 13, 2003
Published, JBC Papers in Press, June 2, 2003, DOI 10.1074/jbc.C300147200

Sphingosine 1-phosphate (S1P) from mononuclear phagocytes and platelets signals T cells predominantly through S1P1 G protein-coupled receptors (Rs) to enhance other immunologically relevant responses. Cellular S1P1 Rs and their signaling functions are rapidly down-regulated by S1P, through a protein kinase C (PKC)-independent mechanism, but characteristics of cell-surface re-expression of down-regulated S1P1 Rs have not been elucidated. T cell chemotactic responses (CT) to 10 and 100 nM S1P and inhibition of T cell chemotaxis to chemokines (CI) by 1 and 3 μM S1P were suppressed after 1 h of preincubation with 100 nM S1P, but recovered fully after 12–24 h of exposure to S1P. Late recovery of down-regulated CT and CI, but not early down-regulation, was suppressed by PKC and PKCε-selective inhibitors and was absent in T cells from PKCε-null mice. The same PKCe inhibitors blocked S1P-evoked increases in T cell nuclear levels of c-Fos and phosphorylated c-Jun and JunD after 24 h, but not 1 h. A mixture of c-Fos plus c-Jun antisense oligonucleotides prevented late recovery of down-regulated CT and CI, without affecting S1P induction of down-regulation. Similarly, S1P-elicited threonine phosphorylation of S1P1 Rs was suppressed by a selective inhibitor of PKCe after 24 h, but not 1 h. Biochemical requisites for recovery of down-regulated S1P1 Rs thus differ from those for S1P induction of down-regulation.

The lysophospholipids sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are generated and secreted by many types of stimulated cells (1, 2). At concentrations of 0.1–1 micromolar found normally in plasma and other extracellular fluids, S1P and LPA evoke cellular proliferation and diverse other functional responses through at least eight members of a family of homologous G protein-coupled receptors (GPCRs) (3, 4). Originally termed endothelial differentiation gene-encoded or Edg receptors (Rs), those specific for S1P now are officially re-named S1P1 (Edg-1), S1P2 (Edg-5), S1P3 (Edg-3), S1P4 (Edg-6), and S1P5 (Edg-8), and those for LPA are LPA1 (Edg-2), LPA2 (Edg-4), and LPA3 (Edg-7). Stimulated mononuclear phagocytes and platelets are the predominant sources of S1P and LPA in the immune system. Blood and lymphoid tissue CD4+ and CD8+ T cells, B cells, and mononuclear phagocytes all express S1P and LPA GPCRs, in cell type-specific patterns, which are regulated distinctively by their respective ligands and by cellular immune activation (5–8).

T cells express predominantly S1P1 and S1P4, of which S1P1 transduces two distinct effects of S1P on T cell migration and also regulates other T cell functional responses (7, 8). S1P is chemotactic for T cells at 0.001–0.1 μM, enhances chemotactic responses to chemokines at 0.01–0.1 μM, and suppresses T cell chemotaxis to numerous stimuli at 0.3–3 μM. As T cell antigen receptor-dependent activation of T cells suppresses expression of S1P GPCRs and functional responses to S1P in parallel, the S1P-S1P1 R axis is considered most important in controlling recruitment and stimulation of naïve and memory T cells by setting their response threshold to other stimuli. One unanswered critical question about S1P1 Rs of T cells and other types of cells is how they are maintained at a level of full functional expression in tissues and fluids where there are completely saturating micromolar concentrations of S1P. Epitope-tagged or fluorescent protein-containing recombinant S1P1 Rs introduced by transfection into several different cell lines were down-regulated by S1P through phosphorylation, internalization, and translocation to a caveolar compartment by a G protein-coupled receptor kinase 2-dependent process (9, 10). Rapid down-regulation and internalization, but not S1P1 R binding and signaling functions, were facilitated by N-linked glycans of S1P1 Rs (11). However, recovery and stabilization of cell-surface expression of S1P1 Rs after down-regulation have not been examined previously. We now report that T cell S1P1 R recovery from S1P-induced down-regulation requires protein kinase Cε (PKCe) activity and AP-1 transcriptional complex, and involves PKCe-dependent late phosphorylation of the S1P1 Rs.

EXPERIMENTAL PROCEDURES

Isolation, Transfection, Culture, and S1P Treatment of Cells—Mouse CD4+ T cells were isolated from splenocytes of 6 to 8 week-old C57BL/6 female mice at a minimum purity of 97% using metallic beads bearing anti-CD4 monoclonal antibodies (MoAbs) and two cycles of magnetic retention chromatography (Miltenyi-Biotec, Auburn, CA), as described (8). HTC4 rat hepatoma cells, which lack any endogenous S1P or LPA Rs, were LipofectAMINE-transfected with a pcDNA3.1 (+) construct (Invitrogen) encoding influenza hemagglutinin peptide (HA)-NH2-terminally tagged human S1P1 Rs and selected by culture with 400 μg/ml of geneticin. The level of expression of S1P1 Rs was established by TaqMan real-time PCR (8). Suspensions of 0.5 × 10⁶ purified CD4+ T cells per ml of RPMI 1640 with 5% charcoal-adsorbed FBS were transfected with 50 μg/ml each of the c-Jun plus c-Fos antisense or corresponding sense phosphorothioated oligonucleotides (Biomol, Plymouth, PA, USA).
15 to 22% of the total number of CD4 T cells initially added to the top compartment of the chemotactic chambers with 100 nM S1P was re-introduced at 48, 36, 24, and 12 h (48 on the abscissa), 12 and 24 h (24), 12 h (12), or 1 h (1). Each column and bar depicts the mean ± S.D. of the results of four separate studies conducted in duplicate. Control values (100%) in the left frame are migration of CD4 T cells preincubated for the same times without S1P and ranged from 15 to 35% of the total number of CD4 T cells initially added to the top compartment of the chemotactic chambers with 100 nM S1P, 19 to 30% with 10−7 M S1P, and 21 to 32% with 200 nM CCL21. The range of control values for inhibition of chemotaxis to 200 nM CCL21 when preincubation was for 1, 12, 24, and 48 h without S1P was 43–58% for 10−6 M S1P and 47–62% for 3 × 10−6 M S1P in the T cell compartment (right frame). The statistical significance of each difference between the values for an S1P-preincubated sample and the respective control was calculated by a paired Student’s t test and shown by * = p < 0.05 and ** = p < 0.01.

BACKGROUND

During residence in lymphoid tissue, CD4 T cells are continuously exposed to ambient S1P levels that serve as autocrine regulators of their homeostasis. In order to stably sequester CD4 T cells in lymphoid tissue, S1P must be removed from the extracellular environment. Several studies have suggested that the recovery of S1P-deprived T cells is mediated by the expression of S1P1 GPCRs and subsequent PKC activation. Herein, we report that the PKCe-null mouse model (PKCe−/−) of mice with a selective absence of PKCε (results in down-regulation and internalization of S1P1 Rs, but T cells freshly isolated from fully saturating ambient S1P levels express a full complement of S1P1 Rs (9, 10). To determine the course of recovery and persistence of T cell functional S1P1 Rs, mouse CD4 T cells were isolated, preincubated in medium without S1P for 16 h, and then incubated for 48 h with 100 nM S1P present for the entire period or for shorter times prior to assessment of chemotaxis. In the absence of re-exposure to S1P, mean control chemotactic responses ± S.D. to 10−6 M and 10−7 M S1P, respectively, were 17 ± 4% and 24 ± 6% for all intervals of preincubation as a group and mean and phosphorothioated antisense and sense c-Fos and c-Jun oligonucleotides (Active Motif, Carlsbad, CA) and anti-HA rat MoAb. Lysophospholipids and Biochemical Inhibitors—LPA and S1P (Sigma); LipofectAMINE (Invitrogen); the broadly specific PKC inhibitor calphostin C, myristoylated peptide subtype-selective PKC inhibitors PKCα V1–2 (N-myristoyl-D-A-V-S-L-K-P-T) and PKCβII C2–4 (N-myristoyl-S-L-N-P-O-W-N-o-T), the protein phosphatase inhibitor okadaic acid, and phosphorothioated antisense and sense c-Fos and c-Jun oligonucleotides for the first 18 bases following the AUG sequence (Biomol); and the PKA-selective inhibitor KT5720 and broadly specific PKC inhibitor Ro318220 (Calbiochem) were obtained from the suppliers noted.

RESULTS AND DISCUSSION

The addition of S1P to S1P-deprived cells of many types results in down-regulation and internalization of S1P1 Rs, but T cells freshly isolated from fully saturating ambient S1P levels express a full complement of S1P1 Rs (9, 10). To determine the course of recovery and persistence of T cell functional S1P1 Rs, mouse CD4 T cells were isolated, preincubated in medium without S1P for 16 h, and then incubated for 48 h with 100 nM S1P present for the entire period or for shorter times prior to assessment of chemotaxis. In the absence of re-exposure to S1P, mean control chemotactic responses ± S.D. to 10−6 M and 10−7 M S1P, respectively, were 17 ± 4% and 24 ± 6% for all intervals of preincubation as a group and mean control inhibition of chemotaxis to CCL21 by 10−6 M and 3 ×...
10⁻⁶ M S1P as a group were 47 ± 14% and 53 ± 6%. A 1-h exposure, which down-regulates S1P₁ Rs, significantly decreased the chemotactic response of CD4 T cells to 10⁻⁶ M and 10⁻⁷ M S1P and concurrently reduced suppression of CD4 T cell chemotactic responses to CCL21 by 10⁻⁶ M and 3 × 10⁻⁷ M S1P (Fig. 1). Similar decreases were observed for suppression of chemotactic responses to CCL5 by S1P. At 12 h and for up to 48 h of exposure to S1P, the S1P₁R-mediated direct chemotactic effects and chemotactic inhibitory effects of S1P both returned to control levels similar to those for CD4 T cells not exposed to down-regulating levels of S1P.

The capacity of inhibitors of potentially relevant signal transducers to prevent recovery of function of down-regulated S1P₁ Rs also was assessed in chemotactic assays. After 24 h of preincubation with S1P, the S1P₁-mediated direct chemotactic effects and chemotactic inhibitory effects of S1P both returned to control levels similar to those for CD4 T cells not exposed to down-regulating levels of S1P.
exposure of S1P-deprived CD4 T cells to S1P, the direct chemotactic effects and inhibitory actions on chemokine-evoked chemotaxis of $10^{-8}$ and $10^{-6} \text{M S1P}$, respectively, had returned to control levels (Fig. 2A, right frame). The PKC type-specific inhibitors calphostin C and Ro318220, and the PKCβ subtype-selective inhibitor m-PKCβ-V1–2 significantly suppressed the recovery of both functions of the S1P1-Rs. In contrast, the PKA inhibitor KT5720 and the PKCα/β/γ inhibitory peptide m-PKCαβC2–4 had no effect on recovery of down-regulated S1P1-Rs (Fig. 2A). In addition, neither calphostin C nor m-PKCβ-V1–2 altered the S1P-elicited down-regulation of functional S1P1-Rs after 1 h of exposure of CD4 T cells to S1P (Fig. 2A, left frame). To confirm involvement of PKCε in the recovery of down-regulated S1P1-Rs, similar studies were conducted with CD4 T cells from selective PKCε-null mice. The patterns of down-regulation of functional S1P1-Rs by a 1-h exposure to 100 nM S1P, reflected in reduced direct chemotactic responses and suppressed inhibition of chemotaxis to CCL21 relative to controls not preincubated with S1P1, were identical in wild-type and PKCε-null mice (Fig. 2B, left two sets). In contrast, only the wild-type CD4 T cells had recovered S1P1-R-mediated chemotactic and inhibitory responses after 24 h of exposure to S1P (Fig. 2B, first of right two sets). PKCε-null mouse-derived CD4 T cells continued to show impaired direct chemotactic responses to S1P without suppression by S1P of CCL21-evoked chemotaxis after 24 h (Fig. 2B, second of right two sets), which was a pattern indistinguishable from that observed after 1 h (Fig. 2B, second of left two sets).

PKCε in T cells has been linked to recruitment and functional activation of the AP-1 and N-FAT-1 transcription factors (13). Two approaches were used to examine independently the course and PKCε dependence of activation of AP-1 by S1P and the involvement of components of AP-1 in recovery of down-regulated functional S1P1-Rs of CD4 T cells. First, the characteristics of S1P activation of c-Fos and c-Jun/JunD in CD4 T cells were investigated, as some growth effects of S1P involve engagement of the c-Fos promoter. S1P increased the nuclear contents of c-Fos and phosphorylated c-Jun/JunD by 1 h, and the levels were sustained for up to 24 h in the continued presence of a plasma concentration of 100 nM S1P (Fig. 3A). These increases were blocked significantly by the type-specific PKCε inhibitor calphostin C and the PKCε-selective inhibitor m-PKCε-V1–2 at 24 h, but not at 1 h, and not at either time point by the PKCαβγ inhibitory peptide m-PKCαβC2–4 (Fig. 3A) (14). Second, the involvement of AP-1 components in recovery of chemotactic signaling by down-regulated functional S1P1-Rs of CD4 T cells was demonstrated by introducing c-Fos plus c-Jun antisense oligonucleotides into CD4 T cells, in amounts proven to

**Fig. 3. PKCε dependence of the role of AP-1 in S1P effects on CD4 T cell migration.** A, quantification by ELISAs of the involvement of PKCε in S1P effects on expression of c-Fos and c-Jun/JunD in nuclear extracts of CD4 T cells. Each column and bar depicts the mean ± S.D. of the results of two separate studies conducted in duplicate. All samples were incubated for 24 h with inhibitors and S1P added at 24, 12, and 1 h for the four “24-h” sets and at 1 h prior to termination for the four “1-h” sets. The c-Jun values include phosphorylated c-Jun and JunD. Mean net base-line values without S1P were 0.11 and 0.14 for c-Fos and c-Jun/JunD, respectively, at 1 h and 0.16 and 0.12 at 24 h. B, prevention of recovery of S1P1-GPCR effects on migration of CD4 T cells by c-Fos + c-Jun antisense oligonucleotides. Each column and bar depicts the mean ± S.D. of the results of two separate studies conducted in triplicate. Control values (100% on the left ordinate) without S1P pretreatment ranged from 23 to 28% and 31 to 35% of the total number of CD4 T cells initially added to the top compartment of the chemotactic chambers for sense (S)- and antisense (AS)-treated sets, respectively, with $10^{-8}$ (left-hand bar) and $10^{-6}$ M S1P (left-hand bar) and 23 to 29% and 24 to 27% for 200 nM CCL21 (middle bar). The range of control values without S1P pretreatment to 200 nM CCL21 was 37–45% and 39–44% for sense- and antisense-treated sets, respectively, with $10^{-6}$ M S1P in the T cell compartment (right-hand bar and right ordinate). Statistical significance was calculated for differences in corresponding values at the same time between sense- and antisense-treated sets, and symbols are the same as described in the legend to Fig. 1.
decrease AP-1 sufficiently for functional alterations (15), prior to assessing S1P effects on chemotaxis. Suppression of both direct chemotactic effects of $10^{-7} \text{M} \ S1P$ and of the inhibition by $10^{-6} \text{M} \ S1P$ of CCL21-induced chemotaxis by 1 h of prior exposure to S1P was not affected by the mixture of AP-1 antisense oligonucleotides nor the corresponding sense oligonucleotides (Fig. 3B, left-hand set). In contrast, recovery of both chemotactic stimulation by S1P and S1P inhibition of chemokine-elicted chemotaxis after 24 h was prevented by c-Fos plus c-Jun antisense oligonucleotides (Fig. 3B, set furthest to the right), whereas timely recovery was complete for CD4 T cells pretreated only with corresponding sense oligonucleotides. The migration response patterns were no different after 24 h than 1 h for the antisense oligonucleotide-pretreated CD4 T cells. S1P1 (Edg-1) Rs of S1P-deprived HTC4-S1P1 transfectants re-exposed to 100 nM S1P were threonine-phosphorylated after 1 and 24 h (Fig. 4). Selective inhibition of PKCe suppressed S1P-evoked threonine phosphorylation after 24 h, but not after 1 h. Protein phosphatase inhibition enhanced threonine phosphorylation of S1P1 Rs slightly after 24 h but not after 1 h. Thus only late threonine phosphorylation of S1P1(Rs) appears to be PKCe-dependent and occurs in the same time period as reappearance of functional S1P1 Rs. The PKCe dependence of recovery and persistence of functional S1P1 Rs in the presence of micromolar concentrations of S1P, which fully saturate the S1P1 Rs, thus also may require S1P R specific phosphorylation.

Elucidation of the different requisites for S1P and phorbol ester induction of rapid phosphorylation and internalization of epitope-tagged recombinant S1P1 receptors in a line of hamster fibroblast transfectants revealed independent mechanisms (9). Immediate down-regulation evoked by S1P depended on 12 amino acids of the carboxyl terminus, was resistant to suppression by PKC inhibitors, and was mediated in part by G protein-coupled receptor kinase-2. In contrast, elicitation of immediate down-regulation by a phorbol ester was not dependent on the carboxyl terminus and was completely suppressed by PKC inhibitors. Dissociation of early S1P-evoked down-regulation of S1P1 Rs from PKC activity was confirmed for T cells by the lack of effect of PKC inhibitors or of PKCe genetic deletion on down-regulation of functional S1P1 Rs (Fig. 2, A and B). Late recovery and persistence of S1P1 Rs down-regulated by S1P were shown to depend on PKCe by the suppressive effects of selective and broadly specific PKC inhibitors, PKCe gene deletion, and antisense inhibition of portions of the S1P1-coupled signaling pathways (Figs. 2–4).

Coupling of expression of S1P1 Rs to the activation of PKCe was considered because of the known capacity of PKCe to recruit components of the AP-1 transcription complex, which are implicated in S1P signaling (13). These observations have been extended to the demonstration of involvement of PKCe-evoked AP-1 in late-phase recovery of down-regulated S1P1 Rs (Fig. 3). A sustained rise in expression of the c-Fos and phosphorylated c-Jun/JunD components of AP-1 resulted in peak levels for all at 24 h after S1P stimulation (Fig. 3). Antisense suppression of these same components of AP-1 blocked PKCe-dependent late recovery of S1P1 Rs from down-regulation by S1P, without altering PKCe-independent S1P induction of early down-regulation. Although the site(s) of S1P-evoked and PKCe-dependent phosphorylation of S1P1 Rs have not been established, threonine (Fig. 4), and presumably serine, are preferred phosphorylation targets consistent with the specificity of PKC. That PKCe-dependent phosphorylation of Edg-1, recruitment of AP-1 components, and recovery of down-regulated Edg-1 receptors follow a similar late time course suggests mechanistic relationships. However, the precise roles and interrelationships of late phosphorylation of S1P1 Rs and AP-1 regulation of specific transcriptional events remain to be elucidated. Ongoing studies are examining effects of AP-1 antisense oligonucleotides on late phosphorylation of S1P1 Rs receptors, distinctive characteristics of S1P1 Rs phosphorylation in PKCe-null mice, and a range of mutant Edg-1 Rs to determine which will not be PKCe-phosphorylated.

That distinct mechanisms govern S1P1 R down-regulation and recovery has numerous implications for cell biology and immunology. S1P effects on S1P1 Rs of T cells differ substantially from those of T cell antigen receptor activation, which persistently suppresses expression of all S1P GPCRs with a distinctive rank order of efficacy. It explains how S1P1 Rs may be internalized by acute exposure to S1P, but avoid persistent down-regulation in the presence of plasma and lymph levels of 100–300 nM S1P. It also suggests the possibility that agents may be developed which will distinguish and separately regulate down-regulation and recovery of S1P1 Rs pharmacologically.

Acknowledgment—We are grateful to Robert Chan for expert graphics and electronic submission of this manuscript.

REFERENCES
1. Moolenaar, W. H., Kranenburg, O., Postma, F. R., and Zondag, G. C. (1997) Curr. Opin. Cell Biol. 9, 168–173
2. Speegel, S., Cuvillier, O., Edsall, L. C., Kohama, T., Menezleev, R., Olah, Z., Olivera, A., Pirianov, G., Thomas, D. M., Tu, Z., Van Brooklyn, J. R., and Wang, F. (1998) Ann. N. Y. Acad. Sci. 845, 11–18
3. Goetzl, E. J., and Lynch, K. R. (2000) Ann. N. Y. Acad. Sci. 905, 1–357
4. Chen, J., Goetzl, E. J., Hla, T., Igarashi, Y., Lynch, K. R., Moolenaar, W., Pyne, S., and Tigi, G. (2002) Pharmacol. Res. 45, 265–269
5. Goetzl, E. J., Kong, Y., and Voice, J. K. (2000) J. Immunol. 164, 4964–4999
6. Zheng, Y., Voice, J. K., Kong, Y., and Goetzl, E. J. (2000) FASEB J. 14, 2387–2394
7. Graeber, M., Shankar, G., and Goetzl, E. J. (2002) J. Immunol. 169, 4084–4087
8. Graeber, M., and Goetzl, E. J. (2002) FASEB J. 16, 1874–1878
9. Waterson, K. R., Johnston, E., Chalmers, C., Pronin, A., Cook, J. S., Benovic, J. L., and Palmer, T. M. (2002) J. Biol. Chem. 277, 5767–5777
10. Igarashi, J., and Michel, T. (2000) J. Biol. Chem. 275, 32363–32370
11. Kohn, T., Wada, A., and Igarashi, Y. (2002) FASEB J. 16, 983–992
12. Khasar, S. G., Lin, Y. H., Martin, A., Dadgar, J., McMahon, T., Wang, D., Hundle, B., Aley, K. O., Isenberg, W., McCarter, G., Green, P. G., Hodge, C. W., Levine, J. D., and Messing, R. O. (1999) Neuron 24, 253–260
13. Genot, E. M., Parker, P. J., and Cantrell, D. A. (1995) J. Biol. Chem. 270, 9833–9839
14. Ward, N. E., and O’Brian, C. A. (1995) Biochemistry 32, 11903–11909
15. Kondo, T., Matsuda, T., Kitano, T., Takahashi, A., Tashima, M., Ishikura, H., Umehara, H., Domae, N., Uchiyama, T., and Okazaki, T. (2000) J. Biol. Chem. 275, 7668–7676

Fig. 4. Western blot analyses of threonine phosphorylation of S1P1 (Edg-1) GPCRs in HTC4-S1P1(HA) transfectants. The upper row shows immunoblotting of phosphothreonine-(p-Thr) in the ~55-kDa immunoprecipitated HA epitope-tagged S1P1 GPCRs, and the lower row shows the total amount of S1P1(HA) receptor. All samples were incubated for 24 h, but with inhibitors and S1P added at 24, 12, and 1 h for the three 24-h sets and at 1 h prior to termination for the three 1-h sets. N-Myristoyl-PKCeV1–2 was used at 100 μM and okadaic acid at 100 nM. The left-hand values are for molecular mass standards.
Protein Kinase C \(\text{\textcircled{1}}\) Dependence of the Recovery from Down-regulation of S1P\(\text{\textcircled{1}}\) G Protein-coupled Receptors of T Lymphocytes
Markus H. Graeler, Yvonne Kong, Joel S. Karliner and Edward J. Goetzl

J. Biol. Chem. 2003, 278:27737-27741.
doi: 10.1074/jbc.C300147200 originally published online June 2, 2003

Access the most updated version of this article at doi: 10.1074/jbc.C300147200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 14 references, 7 of which can be accessed free at http://www.jbc.org/content/278/30/27737.full.html#ref-list-1