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Research Article

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Regulation of Arachidonic Acid, Eicosanoid, and Phospholipase A_2 Levels in Murine Mast Cells by Recombinant Stem Cell Factor

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Abstract

The current study evaluates the capacity of recombinant rat stem cell factor (rrSCF) to regulate enzymes that control AA release and eicosanoid generation in mouse bone marrow–derived mast cells (BMMCs). Initial studies indicated that rrSCF provided for 24 h inhibited the release of AA into supernatant fluids of antigen- and ionophore A23187-stimulated BMMCs. Agonist-induced increases in cellular levels of AA were also inhibited, albeit to a lesser degree by rrSCF. To determine the inhibitory mechanism, several steps (e.g., mobilization of cytosolic calcium, release of BMMC granules, and regulation of phospholipase A_2 [PLA_2] activity) that could influence AA release were measured in rrSCF-treated cells. rrSCF did not alter the capacity of BMMCs to mobilize cytosolic calcium or release histamine in response to antigen or ionophore. BMMCs released large amounts of PLA_2 with characteristics of the group II family in response to antigen and ionophore A23187. rrSCF treatment of BMMCs reduced the secretion of this PLA_2 activity by BMMCs. Partial purification of acid-extractable PLA_2 from rrSCF-treated and untreated BMMCs suggested that rrSCF decreased the quantity of acid-stable PLA_2 within the cell. In contrast to group II PLA_2, the quantity of $cPLA_2$ (as determined by Western blot analysis) increased in response to rrSCF.

To assess the ramifications of rrSCF-induced reductions in AA and group II PLA_2, eicosanoid formation was measured in antigen- and ionophore-stimulated BMMCs. rrSCF-inhibited (100 ng/ml, 24 h) prostaglandin D_2 (PGD_2), thromboxane B_2, and leukotriene B_4 by 48.4±7.7%, 61.1±10.0% and 38.1±3.6%, respectively, in antigen-stimulated cells. Similar patterns of inhibition were observed in ionophore-stimulated BMMCs. The addition of a group I PLA_2 or exogenous AA to BMMCs reversed the inhibition of eicosanoid generation induced by rrSCF. Together, these data indicate that rrSCF differentially regulates group II and cytosolic PLA_2 activities in BMMCs. The resultant reductions in eicosanoid generation suggest that group II PLA_2 provides a portion of AA that is used for eicosanoid biosynthesis by BMMCs. (*J. Clin. Invest.* 1985. 96:1432–1439.) **Key words: arachidonic acid • eicosanoids • phospholiphase A_2 • stem cell factor • mast cells**

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Introduction

The generation of AA and its subsequent conversion to eicosanoids are pivotal events associated with inflammation and allergy (1). The initial step in eicosanoid generation is the mobilization of AA from the *sn*-2 position of the glycerol backbone of membrane phospholipids by the activities of phospholipase A_2 (PLA_2)[1] enzymes (2–5). In the last 5 yr, several PLA_2 activities that may participate in this critical step have been purified and cloned (6–20). For example, a relatively high molecular weight PLA_2 (70–110 kD) has been isolated from the cytosolic fractions of several cell types. This enzyme has a preference for phospholipids that contain AA and translocates to cellular membranes in response to nanomolar calcium concentrations (21–24). There also exists a family of low molecular weight PLA_2 (~ 14 kD in size, called group II PLA_2) that is released from cells during activation (25–28). Group II PLA_2 are present in high quantities in inflammatory sites such as peritoneal exudates and synovial fluids from patients with rheumatoid arthritis and are implicated in the pathogenesis of many diseases (29–34).

Since the discovery of these two families of PLA_2, there has been considerable research using several approaches to establish whether one or both of these $PLAs_2$ are necessary for AA release leading to eicosanoid generation. Studies showing the importance of group II PLA_2 include those of Pfeilschifter et al., who showed that 85–90% of group II PLA_2 is secreted in parallel with PGE_2 biosynthesis from mesangial cells in response to IL-1β and $TNF_{1\alpha}$ (26). Furthermore, several groups demonstrated that overexpression, or adding of group II PLA_2 to different cell types, leads to a marked increase in the release of AA and the synthesis of eicosanoids (35–42). Our studies indicated that group II PLA_2 is rapidly released from mast cells in response to antigen (27). This PLA_2 then specifically releases AA, a proportion of which can be used for leukotriene B_4 and prostanoid formation. In addition, specific inhibitors of group II PLA_2 or antibodies directed against group II PLA_2 markedly reduce eicosanoid generation in mast cells, mesangial cells, endothelial cells, and monocytes. Barbour and Dennis used an antisense oligonucleotide to reduce the synthesis of group II PLA_2 and PGE_2 in macrophages (43). Taken together, these studies provide strong evidence for a role of group II PLA_2 in AA mobilization and the generation of eicosanoids.

1. *Abbreviations used in this paper:* BMMC, bone marrow–derived mast cell; C, cytosolic; GC/MS, gas chromotography/mass spectroscopy; LTB_4, leukotriene B_4; m/z, mass/charge; PLA_2, phospholipase A_2; rrSCF, recombinant rat stem cell factor; TXB_2, thromboxane B_2.

However, there are also studies that suggest that the release of AA used for eicosanoids requires cytosolic (c) PLA_2 (44–52). Several studies indicate that cytokines such as IL-1α, IL-1β, $TNF_{1\alpha}$, and $TGF_{\beta 2}$ induce the synthesis of PLA_2 and eicosanoid generation. For example, Lin et al. demonstrated that IL-1α induces the accumulation of cytosolic PLA_2 and PGE_2 in lung fibroblasts (46). Moreover, the glucocorticoid, dexamethasone, blocks IL-1α–mediated increase in both $cPLA_2$ and PGE_2 biosynthesis. Similarly, glucocorticoids block the TNF1α-induced increase of $cPLA_2$ in an epithelial carcinoma cell line (52). Antisense oligonucleotides have also recently been used to reduce $cPLA_2$ and LPS–induced PGE_2 formation in monocytes, suggesting that $cPLA_2$ is responsible for eicosanoid generation in these cells (53).

Stem cell factor is a cytokine that influences the development and function of mast cells in rodents and primates (54–56). When provided in vitro for short periods of time (< 24 h), recombinant rat stem cell factor (rrSCF) induces mediator release from human mast cells and activates phospholipase D in rat mast cells (57–60). In the current study, we utilized rrSCF as a tool to alter the activities and levels of cytosolic PLA_2 and group II PLA_2 in mouse bone marrow–derived mast cells (BMMCs). Incubation with rrSCF attenuated the release of free AA from BMMCs. In addition, rrSCF reduced antigen- and A23187-induced generation of eicosanoids. This inhibition of AA release and eicosanoid formation was accompanied by a reduction of group II PLA_2 activity within BMMCs and the inhibition of group II PLA_2 release upon stimulation of these cells. Cellular levels of cytosolic PLA_2 were increased under these same conditions. These studies suggest that rrSCF can differentially influence the cellular levels of PLA_2 isozymes, thereby regulating AA metabolism in BMMCs.

Methods

Materials. Deuterated eicosanoid standards ($^2H_4PGD_2$, $^2H_4TXB_2$, and $^2H_4LTB_4$ and octadeuterated (5,6,8,9,11,12,14,15-^{2}H) AA were purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Leukotrene B_4LTB_4 was purchased from Cayman Chemical Co. (Ann Arbor, MI). Essentially fatty-acid–free HSA, snake venom PLA_2 from *Naja naja,* essential and nonessential amino acids, mouse IgE anti-dinitrophenol (IgE anti-DNP), heat-inactivated FBS, and ionophore A23187 were purchased from Sigma Chemical Co. (St. Louis, MO). Methoxylamine HCl (MOX in pyridine), acetonitrile, pentafluorobenzyl bromide (20% in acetonitrile), diisoprophylethylamine (20% in acetonitrile), and *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Pierce (Rockford, IL). RPMI 1640 cell culture medium and HBSS were from Gibco Laboratories (Grand Island, NY). HPLC-grade organic solvents were purchased from Fisher Scientific (Norcross, GA). A rabbit antipeptide antibody for $cPLA_2$ was kindly provided by Hoffman-LaRoche (Nutley, NJ). rrSCF expressed in *Escherichia coli* was a generous gift from Amgen Biologicals (Thousand Oaks, CA).

Mast cell culture and activation. BMMCs were obtained from CBA/J mice (Jackson Laboratories, Bar Harbor, ME) and grown in RPMI 1640 culture medium (Gibco Laboratories) supplemented with 10% (vol/vol) FCS, 50 μM 2-mercaptoethanol, 1% essential amino acids, 1% nonessential amino acids, 2 mM L-glutamine, 5 μg/ml gentamycin, and 1% (vol/vol) penicillin/streptomycin. The culture medium was periodically enriched with a 40% WEHI supernatant fluid as a source of cytokines.

BMMCs were harvested after 3 wk in culture. In all experiments, BMMCs were placed in newly prepared culture media containing 40% WEHI 24 h before they were used for the described experiments. This was found to be necessary to obtain consistent responses from BMMCs. Cell viability (> 95%) was determined by trypan blue exclusion. After resuspending the BMMCs in fresh WEHI-conditioned growth media, they were incubated with different concentrations of rrSCF (0–100 ng/ml) for 24 h or with 100 ng/ml rrSCF for different periods of time as indicated in the figure legends. When BMMCs were stimulated with antigen, they were also sensitized overnight with IgE anti-DNP (0.5 μg/ml). Subsequently, cells were removed from culture and placed in HBSS containing calcium, 0.1 mg/ml gelatin, and 0.01 mg/ml HSA. BMMCs were then stimulated with antigen (2 μg/ml) or with ionophore A23187 (1 μM) for 5 min at 37°C. At the end of the incubations, cells were quickly removed from supernatant fluids by centrifugation (400 *g* for 5 min). Four volumes of ethanol were then added to supernant fluids, and the mole quantities of fatty acids and eicosanoids were determined by gas chromotography/mass spectroscopy (GC/MS) as described later.

In experiments designed to restore AA release and eicosanoid biosynthesis, BMMCs were stimulated with antigen in combination with a group I PLA_2 (1 μg/ml) or free AA (1 μM) for 5 min at 37°C. The amount of PLA_2 and AA added to these cells did not compromise cell viability.

Quantitation of free fatty acids in supernatant fluids. After the addition of 100 ng ^2H_8AA as an internal standard to supernatant fluids, solvents were removed from extracts of supernatant fluids under a stream of nitrogen. Fatty acids were then converted to pentafluorobenzyl esters, and the mole quantities of free fatty acids determined by combined negative-ion chemical ionization GC/MS using a Hewlett Packard instrument (model 5989; Pal. Alto, CA) (61). Carboxylate anions mass/charge (m/z) were monitored at 303 and 311 for AA and ^2H_8AA, respectively, in the single ion monitoring mode. In experiments where cellular AA was determined, lipids were extracted from cellular pellets (62). A fatty-acid–enriched fraction was obtained using Bakerbond silica gel disposable columns (J. T. Baker Inc., Phillipsburg, NJ) (63). After solvent removal using a stream of nitrogen, mole quantities of AA were determined as described above.

Determination of mole quantities of eicosanoids in supernatant fluids. GC/MS was utilized to determine the mole quantities of eicosanoids in supernatant fluids of mast cells. Initially, $^2H_4PGD_2$, $^2H_4TXB_2$, and $^2H_4LTB_4$ (10 ng of each) were added to supernatant fluids as internal standards. Eicosanoids were converted to methoxime-pentafluorobenzyl ester trimethylsilyl ether derivatives. Derivatized eicosanoids were then extracted with hexane and analyzed using combined negative-ion chemical ionization GC/MS (64). Carboxylate anions for LTB_4 (m/z 479), $^2H_4LTB_4$ (m/z 483), PGD_2 (m/z 524), $^2H_4PGD_2$ (m/z 528), TXB_2 (m/z 614), and $^2H_4TXB_2$ (m/z 618) were analyzed in the single ion monitoring mode.

Histamine release assays. Supernatant fluids were obtained from unstimulated or stimulated BMMC that had been incubated without or with 100 ng/ml rrSCF for 24 h. Total histamine was determined using perchloric acid extracts of BMMCs. Histamine released into supernatant fluids and total histamine content of mast cells were determined using an RIA kit following the recommendations of the manufacturer (Amac Inc., Westbrook ME).

Intracellular calcium determination. BMMC (5 × 10^6/ml) in HBSS containing 0.1 mM calcium were incubated with 5 μM fura-2 for 30 min at 37°C. The fura-2 loaded cells were then washed twice and resuspended in HBSS, and cytosolic calcium concentrations were monitored using a fluorometer (Deltascan; Photon Technology International Inc., Princeton, NJ). Suspensions of BMMMCs (10^6/ml) were incubated for 5 min at 37°C to establish the baseline fluorescence. Fluorescence signals obtained at excitation wavelengths of 340 and 380 nm were measured at an emission wavelength of 508 nm for 2 min before and 5 min after the addition of antigen (0.5 μm/ml) or ionomycin (50 nM). The ratio of the fluorescence signals obtained at the two excitation wavelengths was recorded (65). Total calcium was determined by lysis of mast cells using 10 μl 5% Triton X-100. Results are expressed as the fluorescence intensity ratio (340 nm/380 nm) per million BMMCs.

Determination of PLA_2 activity in supernatant fluid. BMMC (5 million/ml) in HBSS containing 0.1% gelatin (wt/vol) and 0.01% HSA were maintained at 37°C for 5 min and then challenged with antigen or ionophore A23187 for 5 min. Reactions were terminated by the addition

of 0.2 ml ice cold HBSS/gelatin buffer containing 1 mM EDTA, and supernatant fluids were obtained by pelleting the cells at 4°C (400 *g*, 5 min). A portion of the supernatant fluid was utilized to determine PLA_2 activity. The PLA_2 reaction was initiated by the addition of 0.02 μCi (9.7 nmol) [^{3}H]-AA-labeled *E. coli* membranes (New England Nuclear, Boston, MA). After incubation (90 min at 37°C) in a water bath, the reaction was stopped by extracting lipids by the method of Bligh and Dyer (62). Free fatty acids were isolated from phospholipids by TLC on silica gel G developed in hexane/ethyl ether/formic acid (90:60:6, vol/vol). The radioactivity in lipids was located using a radiochromatogram imaging system (Bioscan Inc., Washington, DC). Free AA and phospholipids were isolated using TLC zonal scraping, and the quantity of radioactivity was determined using liquid scintillation counting. PLA_2 activity was calculated and expressed as picomoles of AA released per milligram of protein per hour.

Isolation of PLA_2 activity. Secretory group II PLA_2 was extracted from BMMCs using 0.18 M H_2SO_4 overnight at 4°C (11). The acid extract was neutralized using 10 M NaOH before the addition of trifluoroacetic acid (TFA) (final concentration 0.1%). Chromatography was performed using solvents of the following composition; solvent A, 0.1% TFA; solvent B, acetonitrile/0.1% TFA (55:45 vol/vol); solvent C, acetonitrile containing 0.1% TFA. Samples were loaded onto a Supelcosil DP column (Supelco Inc., Bellefonte, PA) that had been conditioned with solvent A at a flow rate of 0.8 ml/min. After 5 min, proteins were eluted from the column by increasing the acetonitrile content of the solvent to 55% within a 25-min period (100% solvent B) followed by an increase of the acetonitrile content to 100% over 5 min (100% solvent C). This final solvent composition was maintained for 15 min for total elution of proteins. Fractions (1 min) were collected, and PLA_2 activity was determined in each fraction after solvent removal.

SDS-PAGE and Western blot analysis of $cPLA_2$. Amounts of $cPLA_2$ were determined in 1–2 million total lysates of BMMCs. Briefly, BMMCs were incubated in lysis buffer (100 mM Tris-/HCl, pH 7.5, containing 0.1 M NaCl, 2 mM EDTA, 1% NP-40, 1 mM Na_3VO_4, 50 mM NaF, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, 0.1 mM quercetin, 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) for 10 min on ice. After removal of nuclei by centrifugation, SDS-PAGE and Western blot analysis were performed. Briefly, extracts were mixed with an equal volume of 2× loading buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% mercaptoethanol, and 0.05% bromophenol blue) and boiled for 5 min. Proteins were separated by SDS-PAGE on a 4–20% polyacrylamide gel. Separated proteins were transferred onto nitrocellulose membranes, and the blots were incubated overnight with an anti-$cPLA_2$ antibody. Detection of $cPLA_2$ was accomplished using horseradish peroxidase conjugate anti-rabbit IgG and enhanced chemiluminescence reagents (New England Nuclear).

Statistical analysis. All data are expressed as the means±SEM of separate experiments. Statistics (*P* values) are obtained from Student's *t* test for paired samples. Notations used on figures and legends are $*P < 0.05$; $**P < 0.01$; $***P < 0.005$.

Results

Influence of rrSCF on AA release from BMMCs. Initial studies were designed to examine the influence of rrSCF on AA release by BMMCs. BMMCs were incubated with various concentrations of rrSCF for different periods of time and the mobilization of AA examined after immunologic and nonimmunologic challenge. As illustrated in Fig. 1 *A*, rrSCF attenuated antigen- and ionophore-induced AA release into supernatant fluids in a dose-dependent manner with IC_{50} of ~ 9 and 5 ng/ml, respectively. Kinetics studies demonstrated that rrSCF induced a time-dependent inhibition of AA release from BMMCs with significant inhibition occurring after 12 h (Table I). Prolonged stimulation (30 min) of BMMCs that had been incubated with 100 ng/ml rrSCF for 24 h did not restore their capacity to release AA (unpublished observation). The effects of rrSCF on the accu-

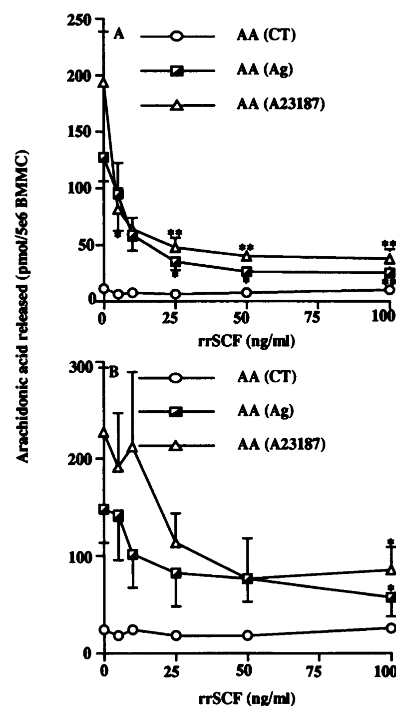

Figure 1. AA release from phospholipids of BMMCs. (*A*) Effect of various concentrations of rrSCF on extracellular AA. BMMCs were incubated with different concentrations of rrSCF (0–100 ng/ml) for 24 h. BMMCs were then placed in HBSS and stimulated with antigen or A23187 for 5 min. Control cells were not exposed to any stimuli. Supernatant fluids were obtained, and mole quantities of free AA released by BMMCs were determined by GC/MS as described in Methods. These data are the means ±SEM of eight different experiments. $*P < 0.05$; $**P < 0.01$; $n = 8$, compared with stimulated BMMCs (−rrSCF). (*B* Effect of various concentrations of rrSCF on cellular AA. BMMCs were incubated with different concentrations of rrSCF (0–100 ng/ml) for 24 h. BMMCs were incubated without (controls) or with antigen or A23187 for 5 min. After removal of supernatant fluids, free AA was extracted from BMMC pellets, and mole quantities (cellular AA) were determined by GC/MS as described in Methods. These data are the means±SEM of three separate experiments that were performed in duplicate. $*P < 0.05$; $n = 3$, compared with stimulated BMMCs (−rrSCF).

mulation of AA in BMMC pellets (cellular AA) were also examined (Fig. 1 *B*). rrSCF inhibited cellular AA when BMMCs were stimulated with antigen or ionophore. However, only the highest concentration of rrSCF (100 ng/ml) significantly inhibited the mobilization of AA that remained cell associated. It is important to point out that inhibition of AA release occurred only when BMMCs were maintained in 40% WEHI before and while they were treated with rrSCF. If BMMCs were removed from WEHI overnight and then provided rrSCF in the absence of WEHI, BMMCs released much less AA, and the inhibitory effect of rrSCF was abolished.

Mechanism of rrSCF inhibition. The aforementioned data suggested that rrSCF interacts with immunologic and nonimmunologic processes involved in AA release from BMMCs. In an attempt to uncover this mechanism, several key steps involved in AA mobilization from cellular phospholipids were examined. An early step in the transmembrane signaling of antigen binding to IgE receptors and phospholipase activation is the mobilization of cytosolic calcium. Therefore, experiments were designed to determine if rrSCF altered the capacity of BMMCs to mobilize cytosolic calcium from intra- and extracellular calcium pools. Both antigen and ionomycin induced a rise in cytosolic calcium concentration within the BMMCs as determined by fura-2 fluorescence (0.26±0.07 and 0.56±0.03 fluorescence intensity ratio per million BMMCs, respectively; $n = 6$). High concentrations (100 ng/ml) of rrSCF for 24 h had no measurable effect on the magnitude or kinetics of antigen- or ionomycin-induced increase in free calcium concentrations (0.21±0.05 and 0.51±0.08 fluorescence intensity ratio per mil-

Table I. Time-dependent Inhibition of AA Release by rrSCF

Time (h) of incubation with 100 ng/ml rrSCF	AA (pmol/5 × 10^6 mast cells)	
	Control	Antigen
0	18.6±3.4	259.3±31.7
6	20.1±6.8	183.1±40.8
12	35.6±1.3	76.1±8.8
24	15.7±4.0	31.4±5.6*

BMMCs were incubated with 100 ng/ml rrSCF for different periods of time. The cells were then removed from culture media, placed in HBSS, and stimulated with antigen. Mole quantitites of free AA released by BMMCs into supernatant fluids were determined by GC/MS as described in Methods. These data are the means±SEM of five different experiments. * $P < 0.05$; $n = 5$, compared with antigen-stimulated BMMCs (−rrSCF).

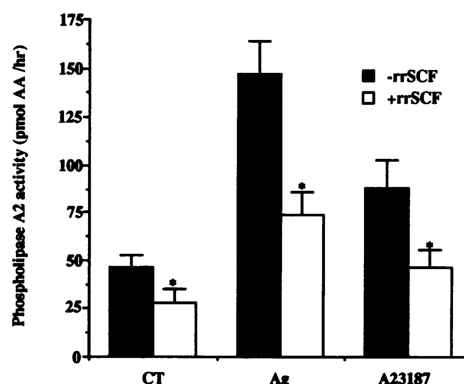

Figure 2. Effect of rrSCF on the secretion of PLA_2 activity by stimulated BMMCs. BMMCs that had been incubated without (−rrSCF) or with 100 ng/ml rrSCF for 24 h were stimulated with antigen or A23187 for 5 min or incubated in buffer alone (*CT*). Supernatant fluids were obtained by centrifugation, and PLA_2 activity was determined as described in Methods. PLA_2 activity (fatty acid release from labeled *E. coli* membranes) is expressed in pmol AA release/h, and these data are the means±SEM of four separate experiments performed in duplicate. *$P < 0.05$; $n = 4$, ±rrSCF.

lion BMMCs, respectively; $P > 0.2$, $n = 6$). These data suggested that a reduction in cytosolic calcium was not associated with the inhibition of AA release by stimulated mast cells that have been exposed to rrSCF.

The degranulation of histamine-containing granules is also an important event associated with the antigen activation of mast cells. Therefore, the histamine content of BMMCs and the capacity of BMMCs to release histamine in response to antigen were measured in cells treated with rrSCF and in nontreated cells. Histamine content was similar in both untreated and rrSCF-treated BMMCs (data not shown). Moreover, the incubation of BMMCs with 100 ng/ml rrSCF for 24 h did not influence their ability to release histamine in response to antigen or ionophore A23187 (antigen − rrSCF, 38.7±4.0%; antigen + rrSCF, 30.2±4.9%; ionophore − rrSCF, 36.8±3.3%; ionophore + rrSCF, 40.4±4.4%; $n = 6$).

Earlier studies in our laboratory indicated that BMMCs rapidly secret a PLA_2 activity that has biochemical characteristics of group II PLA_2 (27). In addition, the incubation of BMMCs with secretory PLA_2 resulted in a profile of fatty acid release that resembled that recovered after BMMCs are stimulated with antigen. The next group of experiments examined the consequences of rrSCF treatment on the release of group II PLA_2 from BMMCs. Antigen induced a 3–4-fold increase in PLA_2 activity in supernatant fluids of BMMCs (Fig. 2). By contrast, there was no significant increase in PLA_2 activity (compared with unstimulated BMMCs) in supernatant fluids from BMMCs that had been incubated with 100 ng/ml rrSCF for 24 h before antigen activation. Similarly, the incubation of BMMCs with rrSCF resulted in significant inhibition of PLA_2 activity in supernatant fluids of ionophore-stimulated cells ($P < 0.05$, $n = 4$). These data suggest that the failure of rrSCF-treated cells to release AA was due in part to a decrease in the capacity of these cells to secrete PLA_2 activity.

It was unclear from these experiments whether the absence of PLA_2 in supernatant fluids was a result of inhibition of some step(s) involved in PLA_2 secretion or a reduction in the total amount of PLA_2 within the cell. To examine the latter question, the quantities of total PLA_2 activity as well as two individual PLA_2 isotypes were measured in nontreated and rrSCF-treated cells. There was no significant change in the total PLA_2 activity measured in BMMC sonicates from control and rrSCF-treated BMMCs (untreated, 354±48 pmol AA release/h; treated, 476±82 pmol AA release/h; $n = 6$). Although total PLA_2 activity was not altered, it was possible that quantities or activities of individual PLA_2 isotypes found within BMMCs changed upon treatment with rrSCF. Therefore, the activity of group II PLA_2 in BMMCs was measured after acid extraction of cell pellets. During acid extraction, $cPLA_2$ was completely eliminated, allowing for determination of group II–like PLA_2 activity. In these experiments, the acid stable activity was then partially purified utilizing a diphenyl reverse phase column and PLA_2 activity measured in HPLC fractions. As illustrated in Fig. 3, the bulk of acid extractable PLA_2 activity eluted in two distinct peaks (A and B) in untreated cells. Examination of acid extracts from BMMCs that had been treated with rrSCF

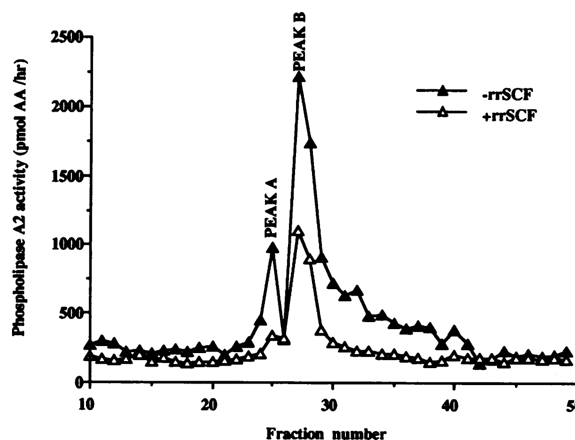

Figure 3. Partial purification of $sPLA_2$ from BMMCs. BMMCs that had been incubated without (−rrSCF) or with 100 ng/ml rrSCF (+rrSCF) were incubated with 0.18 M H_2SO_4 overnight at 4°C. Extracts were neutralized, suspended in solvent A (0.1% TFA), and then loaded onto a Supercosil DP column (0.46-cm internal diameter, 24 cm long). PLA_2 activity (fatty acid release from labeled *E. coli* membrane) was determined in each 1-min fraction as described in Methods. PLA_2 activity is expressed as the amount of AA hydrolyzed from *E. coli* phospholipids (pmol/h). These data are representative of four separate experiments.

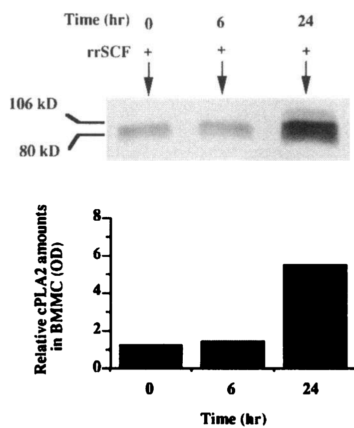

Figure 4. SDS-PAGE of $cPLA_2$ from BMMCs. BMMCs were incubated with or without 100 ng/ml rrSCF for different periods of time. BMMCs were then treated with lysis buffer for 10 min on ice. Solubilized proteins obtained after centrifugation were suspended in SDS-PAGE loading buffer. SDS-PAGE and detection of $cPLA_2$ amounts were performed as described in Methods. These data are representative of three separate experiments.

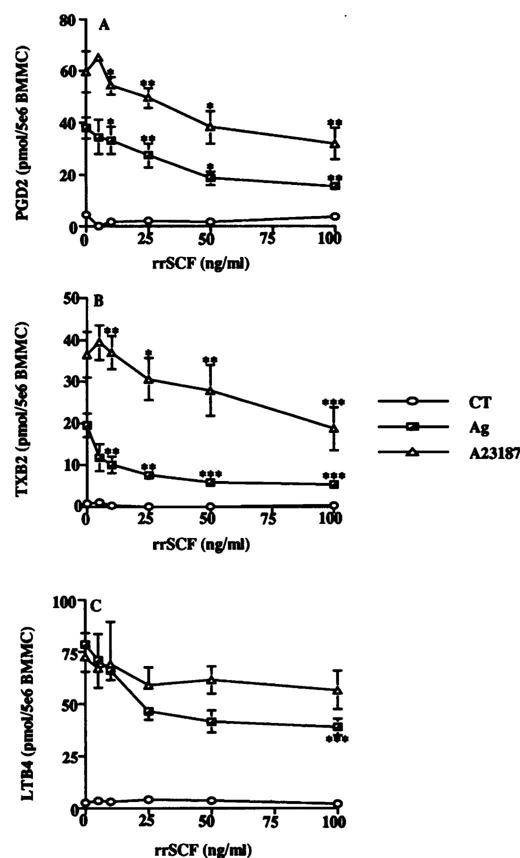

Figure 5. Influence of rrSCF on eicosanoid biosynthesis by BMMCs. BMMCs were incubated without or with 100 ng/ml rrSCF for 24 h. The cells were then stimulated with antigen or A23187 for 5 min. Mole quantities of eicosanoids released into supernatant fluids were obtained after ethanol extraction, derivatization, and analysis by GC/MS as described in Methods. These data are the means±SEM of five separate experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$; $n = 5$, compared with stimulated BMMCs (−rrSCF).

indicated that there was a reproducible decrease in PLA_2 activity found in peaks A and peak B (55.3±3.2% decrease, $n = 4$).

Levels of $cPLA_2$ were measured in rrSCF-treated and untreated BMMC lysates utilizing SDS-PAGE and Western blot analysis. Fig. 4 illustrates that rrSCF induces a marked increase in the quantity of $cPLA_2$ when compared with untreated controls. Densitometric analysis of untreated and rrSCF-treated BMMCs confirmed the increase in $cPLA_2$ (optical density of 1.84±0.32 and 6.02±0.29 [$n = 3$] for control and rrSCF-treated BMMCs, respectively). These data suggest that the initial findings, indicating little or no change in total PLA_2 activity of BMMC sonicates, were probably a result of an increase in $cPLA_2$ concomitant with a decrease in group II-like PLA_2 activity. Further, these data insinuate that rrSCF may inhibit PLA_2 secretion by BMMCs by decreasing the amount of enzyme available (likely group II PLA_2) for release upon stimulation. Finally, these results reveal that the quantities of AA produced by BMMCs are reduced under circumstances where there is an increase in $cPLA_2$ and a reduction in group II–like PLA_2 activity.

Influence of rrSCF on eicosanoid generation by BMMCs. Previous studies from our laboratory suggest that AA released by the application of extracellular PLA_2 to BMMCs is utilized for eicosanoid generation (PGD_2, TXB_2, and LTB_4). We next examined the effect of reduced secretion of PLA_2 (by rrSCF treatment) on the capacity of antigen- or ionophore-treated BMMCs to produce eicosanoids. Fig. 5 illustrates that BMMCs which had been incubated with rrSCF generated significantly smaller quantities of eicosanoids. Quantities of PGD_2, TXB_2, and LTB_4 decreased as a function of the amount of rrSCF provided to BMMCs. Maximum inhibition of eicosanoid formation (PGD_2, 48.4±7.7%; TXB_2, 61.1±10.0%, and LTB_4, 38.1±3.6%) was observed in antigen-stimulated BMMCs at 100 ng/ml rrSCF. A similar pattern of inhibition in PGD_2 (36.2±12.1%), TXB_2 (40.1±7.6%), and LTB_4 (17.2±4.4%) was observed in A23187-stimulated BMMCs.

To determine whether the decrease in eicosanoid generation was a result of the reduction of AA observed after rrSCF treatment, extracellular AA was provided to rrSCF-treated BMMCs during activation. Fig. 6 shows that the addition of exogenous AA reversed the inhibition of eicosanoid levels observed in rrSCF-treated BMMCs. To ensure that rrSCF was not directly inhibiting group II PLA_2 activity and that phospholipid substrate was still available to group II PLA_2, exogenous PLA_2 was added to BMMCs and the quantities of AA measured. There was no significant difference in the quantities of AA mobilized by exogenous PLA_2 in rrSCF treated cells and in nontreated cells (Table II). In addition, eicosanoid formation was restored when rrSCF-treated BMMCs were stimulated with Ag in combination with exogenous PLA_2. Data from these studies indicated that the reduction of AA release by rrSCF treatment of BMMCs resulted in significant inhibition of eicosanoid generation.

Discussion

These results support the hypothesis that rrSCF inhibits the lipase-catalyzed cleavage of AA from membrane phospholipids that would otherwise go on to form eicosanoids. This hypothesis is supported by the following five lines of evidence. First, rrSCF inhibited the capacity of both antigen and ionophore to mobilize AA, which is ultimately released from mast cells. Second, rrSCF inhibits the secretion of a PLA_2 activity with characteristics of the group II family. The inhibition of PLA_2 secretion by rrSCF is likely due to reduction of group II PLA_2 amounts within mast cells. Third, both antigen and ionophore formation of cyclooxygenase and 5-lipoxygenase products are reduced by rrSCF. Fourth, addition of exogenous PLA_2 or AA restores the capacity of rrSCF-treated mast cells to release AA and produce eicosa-

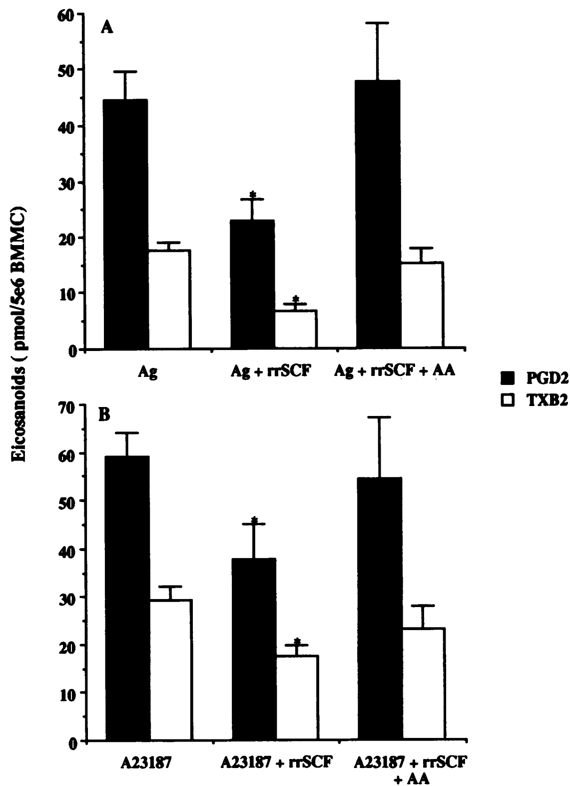

Figure 6. Incorporation of extracellular AA into eicosanoids. BMMCs were incubated without or with 100 ng/ml rrSCF for 24 h. The cells were then stimulated (*A*) with antigen or (*B*) with A23187 for 5 min in the presence of AA (1 μM). Mole quantities of eicosanoids released into supernatant fluids were obtained after ethanol extraction, derivatization, and analysis by GC/MS as described in Methods. These data are the means±SEM of three separate experiment. $*P < 0.05$; $n = 8$, compared with stimulated BMMCs (−rrSCF).

noids. Fifth, it is unlikely that the inhibition in AA and eicosanoid generation is due to attenuation of cytosolic calcium mobilization or the release of histamine-containing granules, because we found rrSCF to be without effect on these processes.

Over the last 5 yr, there has been considerable research using a variety of approaches to establish the importance of different PLA_2 isotypes. As pointed out in the introduction, there is ample evidence that both group II PLA_2 and the higher molecular weight $cPLA_2$ have a role in AA release and eicosanoid generation. The current study utilizes a cytokine/growth factor, rrSCF, to reduce group II PLA_2 while increasing $cPLA_2$ in mast cells. To our knowledge, this is the first study to show differential regulation of these two PLA_2 isotypes by a cytokine. The mechanism responsible for reduced group II PLA_2 activity in rrSCF-treated cells has not yet been uncovered. In contrast to the current study, studies by Van den Bosch et al. indicate that IL-1β increases message levels for group II PLA_2 in rat mesangial cells (35). Perhaps rrSCF attenuates the transcription of the group II PLA_2 gene. Alternatively, rrSCF may prevent the secretion of group II PLA_2 or induce the synthesis of endogenous inhibitors of group II PLA_2 activity. rrSCF may also block the synthesis and secretion of activators of group II PLA_2. Two such activators (transglutaminases and phospholipase-activating protein) have been isolated from many cell types (5, 66, 67). Finally, it is also possible that rrSCF is altering phospholipid substrates or is blocking the capacity of group II PLA_2 to interact with phospholipids at the surface of the cell. Recently, putative receptors of secretory PLA_2 that may facilitate the interaction of $sPLA_2$ with phospholipids have been identified on a number of cells (39, 68, 69). However, the fact that similar quantities of AA are mobilized by exogenously added PLA_2 in rrSCF-treated and nontreated cells (Table II) suggests that rrSCF does not influence substrate availability. Future studies will be directed at understanding the mechanism by which rrSCF inhibits group II PLA_2 activity.

Having a model system in which one PLA_2 isotype is upregulated while another is downregulated provides a unique opportunity to examine the importance of individual PLA_2 isotypes in AA mobilization. Addition of rrSCF with concomitant changes in PLA_2 isotypes leads to a reduction in the release of AA to the outside of mast cells and little influence on AA levels within the cell. These data suggested that group II PLA_2 was responsible for the release of extracellular AA. This hypothesis is consistent with our previous studies in this cell, which showed that group II PLA_2 is released during antigen activation and that adding exogenous group II PLA_2 initiates the release of extracellular AA (27).

This model also provides an opportunity to address the question of whether specific pools of AA, which are mobilized by individual PLA_2 isotypes, provide AA for further metabolism to eicosanoids. A previous study from our laboratory suggested that extracellular AA and leukotrienes are derived from different AA pools of mast cells (70). For example, it was demonstrated that the specific activity of supernatant fluid AA mimics that of phosphatidylethanolamine as a class, whereas the specific

Table II. Influence of Extracellular PLA_2 on AA Release by BMMCs

	AA and eicosanoid release from BMMCs (pmol/5 × 10^6 mast cells)					
	AA		PGD_2		TXB_2	
Conditions	−rrSCF	+rrSCF	−rrSCF	+rrSCF	−rrSCF	+rrSCF
Control	14.30±2.78	8.11±2.32	7.70±1.57	3.15±0.70	1.90±0.49	1.13±0.37
Ag	207.09±25.52	36.96±9.12*	44.55±5.04	22.97±3.66*	17.60±1.38	6.83±1.12*
Ag + PLA_2	644.66±129.72	558.59±178.42	70.20±8.75	47.56±10.52	21.61±2.26	15.16±2.75

BMMCs were maintained without (−rrSCF) or with 100 ng/ml rrSCF (+rrSCF) for 24 h. The cells were then stimulated with antigen alone or in combination with 1 μg/ml *Naja naja* PLA_2 for 5 min at 37°C. Control cells did not receive any stimuli. Mole quantities of free AA and eicosanoids released by BMMCs into supernatant fluids were determined by GC/MS as described in Methods. These data are the means±SEM of eight separate experiments. $*P < 0.05$; $n = 8$, compared with BMMCs that were not treated with rrSCF.

activity of leukotrienes resembles the average specific activity of a mixture of phospholipids. A subsequent study demonstrated that AA generated on the outside of the cell by group II PLA_2 hydrolysis (of primarily phosphatidylethanolamine) could be readily utilized for cyclooxygenase products, whereas a smaller proportion was utilized for the synthesis of 5-lipoxygenase products. Again, these studies suggested that much of AA utilized for LTB_4 is mobilized from within the cell. These data are consistent with recent findings which indicate that 5-lipoxygenase translocates to a nuclear membrane fraction during cell activation (71, 72). In the current study, cyclooxygenase products are more readily inhibited than are 5-lipoxygenase products when mast cells are incubated with rrSCF. An explanation consistent with previous studies is that group II PLA_2 is responsible for most of the AA released to the outside of mast cells and much of this AA can be utilized by cyclooxygenase. However, the highest concentrations of rrSCF, which maximally block extracellular AA release, cause only a modest decrease in LTB_4. Future studies are necessary to determine whether a cellular enzyme such as $cPLA_2$ is responsible for the mobilization of cellular AA, which supplies AA for 5-lipoxygenase.

It is important to emphasize that inhibition of AA metabolism by rrSCF is only seen in mast cells that are grown in a media enriched with cytokines found in WEHI supernatant fluids. If mast cells are deprived of this enriched environment and grown for 24 h in culture media only, subsequent supplementation of the growth media with rrSCF appears not only to rescue the cells but actually to increase the quantities of eicosanoids that they produce. However, it is likely that mast cells in vivo are bathed in a variety of cytokines, including rrSCF. Therefore, we have attempted to utilize a protocol in these experiments that better represents the complex in vivo situation.

This study centers on the observation that rrSCF provided in vitro to mast cells inhibits group II PLA_2 activity as well as AA and eicosanoid generation. The ramifications of this rrSCF inhibition in vivo remains to be assessed. However, it is worth noting that studies by Ando et al. have examined the chronic treatment of normal mice with rrSCF (73). This chronic treatment results in marked mast cell hyperplasia. Nevertheless, high doses of antigen in these rrSCF-treated mice produced significantly fewer fatalities compared with mice that had not received rrSCF. This suggested that rrSCF-treated mice, even though they contained more mast cells, had mast cells that were less reactive to antigen. The current study may begin to explain this paradoxical effect of rrSCF in vivo; rrSCF may diminish the severity of IgE-dependent anaphylaxis, in part, by its capacity to block PLA_2 activity and reduce eicosanoid generation in mast cells.

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