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Activation of Leukotriene Synthesis in Human Neutrophils by Exogenous Arachidonic Acid: Inhibition by Adenosine A_{2a} Receptor Agonists and Crucial Role of Autocrine Activation by Leukotriene B_4

MARC E. SURETTE, ERIC KRUMP, SERGE PICARD, and PIERRE BORGEAT

Centre de Recherche en Rhumatologie et Immunologie, Centre de Recherche du Centre Hospitalier Universitaire de Québec, Pavillon CHUL and Faculté de Médecine, Université Laval, Quebec, Canada

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ABSTRACT

We report here that the apparent inability of isolated human polymorphonuclear leukocytes (PMNs) to efficiently transform arachidonic acid (AA) is the consequence of A_{2a} receptor engagement by endogenous adenosine accumulating in incubation media. Indeed, when adenosine is eliminated from PMN suspensions by the addition of adenosine deaminase, or when cells are incubated with adenosine A_{2a} receptor antagonists, important quantities (40–80 pmol/10^6 cells) of 5-lipoxygenase products are synthesized by PMN incubated with 1 to 5 μM exogenous AA. The selective A_{2a} receptor agonist CGS21680 was a very potent inhibitor of the AA-induced leukotriene (LT) synthesis, showing an IC_{50} of ~1 nM. The mechanism of AA-induced stimulation of LT synthesis observed in the absence of extracellular adenosine was investigated. In adenosine deaminase-treated PMN, exogenous AA induced Ca^{2+} mobilization and the translocation of 5-lipoxygenase to nuclear structures. A time lag of 20 to 60 s (variable between PMN preparations) was observed consistently between the addition of AA and the elevation of intracellular Ca^{2+} concentration (and LT synthesis), indicating that AA itself did not trigger the Ca^{2+} mobilization in PMN. This AA-induced Ca^{2+} mobilization, as well as the corresponding 5-lipoxygenase translocation and stimulation of LT synthesis, was blocked efficiently by the LT synthesis inhibitor MK0591, the LTB_4 receptor antagonists CP105696 and LY223982, and the LTA_4 hydrolase inhibitor SC57461A. These data demonstrate that AA is a highly potent and effective activator of LT synthesis and acts through a mechanism that requires an autocrine stimulatory loop by LTB_4.

The stimulation of human leukocytes by various agents such as calcium ionophores, soluble agonists, or phagocytic stimuli results in the biosynthesis of the bioactive arachidonic acid (AA)-derived leukotrienes (LTs). In human polymorphonuclear leukocytes (PMN), the major AA-derived metabolites synthesized are LTs A_4 and B_4 (LTA_4, LTB_4); LTB_4 is one of the most potent naturally occurring leukocyte chemoattractants (Borgeat and Naccache, 1990). Its synthesis after cell stimulation is the result of the calcium-dependent release of AA from cellular phospholipids after the activation of phospholipase(s) (PL) A_2 and the stereospecific transformation of AA to 5-hydroperoxyeicosatetraenoic acid (5-HpETE) by the 5-lipoxygenase (5-LO) (Borgeat and Naccache, 1990). 5-HpETE then can be reduced to 5-hydroxyeicosatetraenoic acid (5-HETE) or converted further by 5-LO to LTA_4, which then is rapidly converted to LTB_4 by LTA_4 hydrolase. LTB_4 and 5-HETE are both biologically active compounds that stimulate leukocytes by interacting with distinct cell surface receptors (Yokomizo et al., 1997).

In addition to being transformed to oxygenated metabolites, free AA generated by PL A_2 activity also is released from activated cells in vitro and also has been measured in inflammatory foci (Barr et al., 1984; Lundy et al., 1990). This extracellular AA can be utilized for LT biosynthesis by agonist-stimulated neutrophils; such transcellular metabolism of AA is believed to play an important role in the overall production of the lipid mediators of inflammation (Maclouf et al., 1989; Serhan and Sheppard, 1990). AA also can act as a multifunctional agonist because the addition of AA to PMN

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ABBREVIATIONS: AA, arachidonic acid; PMN, polymorphonuclear leukocytes; ADA, adenosine deaminase; fura-2-acetoxymethly ester (fura-2/AM); 5-LO, 5-lipoxygenase; LT, leukotriene; 5-HpETE, 5-hydroperoxyeicosatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; PG, prostaglandin; PMSF, phenylmethanesulphonyl fluoride; EPA, 5,8,11,14,17-eicosapentaenoic acid; 20-OH-LTB_4, 20-hydroxy-LTB_4; 20-COOH-LTB_4, 20-carboxy-LTB_4; DMSO, dimethyl sulfoxide; RP, reversed phase; HBSS, Hanks' balanced salt solution; PL, phospholipase; fMLP, formyl-methionyl-leucyl-phenylalanine; PAF, platelet-activating factor; PGB_2, prostaglandin B_2.

stimulates the mobilization of calcium, degranulation, and superoxide anion production (Smith et al., 1987).

It is now well documented that adenosine acting via its A_{2a} receptor is a potent suppressor of PMN functions (Cronstein et al., 1983; Newby et al., 1983; Cronstein, 1994; Krump et al., 1997). Recently, it has been shown that PMN suspensions release adenosine in quantities that can severely inhibit functional responses to various agonists (Cronstein et al., 1983; Newby et al., 1983; Krump et al., 1997). In particular, the accumulation of adenosine in the incubation medium after a 30-min preincubation period at 37°C nearly completely inhibits LTB_4 production by formyl-methionyl-leucyl-phenylalanine (fMLP)- or platelet-activating factor (PAF)-stimulated PMN (Krump et al., 1997).

In the present study, we have investigated the impact of adenosine A_{2a} receptor occupancy on the ability of exogenous AA to stimulate PMN for 5-LO product synthesis. These studies reveal that in the absence of the suppressive effect of adenosine, AA is a highly potent and efficient activator of LT synthesis involving an autocrine stimulatory loop that includes LTB_4 itself. These studies indicate further that adenosine exerts its potent inhibitory effect on AA-induced LTB_4 synthesis by inhibiting the ability of exogenous AA to activate cellular 5-LO. Finally, these results suggest that the pharmacological manipulation of adenosine levels at inflammatory sites may have an important impact on the transcellular biosynthesis of LT.

Experimental Procedures

Materials. Fura-2-acetoxymethly ester (fura-2/AM), prostaglandin B_2 (PGB_2), 19-hydroxy-PGB_2, leupeptin, aprotinin, phenylmethanesulfonyl fluoride (PMSF), adenosine deaminase (ADA), and horseradish peroxidase-linked donkey anti-rabbit antibodies were obtained from Sigma Chemical Co. (St. Louis, MO). Methyl arachidonyl fluorophosphonate, AA, and 5,8,11,14,17-eicosapentaenoic acid (EPA) were obtained from Cayman Chemical Co. (Ann Arbor, MI). CGS21680 HCl and CGS15943 were obtained from Research Biochemicals International (Natick, MA). CP105696 was a gift from Dr. Henry Showell (Pfizer, Groton, CT), and MK0591 was a gift from Dr. Robert Young (Merck Frosst, Dorval, Canada). BN50730, LY223982, and L659989 were obtained from the Institut Henri Beaufour (Paris, France), Eli Lilly Laboratories (Indianapolis, IN), and Merck Sharp and Dohme (Rahway, NJ), respectively. SC57461A was a gift from Dr. Walter G. Smith (Searle & Co., Skokie, IL). Drug stock solutions were in dimethyl sulfoxide (DMSO) and were added directly to cell suspensions; the maximal final concentration of DMSO in the cell suspensions was 0.2%.

Rabbit polyclonal anti-5-LO (5-LO 32) was kindly supplied by Dr. Jillian F. Evans of Merck Frosst. The enhanced chemiluminescence detection kit was obtained from Amersham Canada (Oakville, Ontario, Canada). Immobilon-P polyvinylidene difluoride blotting membrane was obtained from Millipore (Mississauga, Ontario, Canada). Ficoll-Paque was obtained from Pharmacia (Montréal, Canada).

Isolated Cell Preparations. Venous blood was obtained from healthy donors and collected into 10-ml glass tubes (100 × 16 mm; Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing 143 USP units of heparin. PMN were isolated from peripheral blood after dextran sedimentation and centrifugation on Ficoll-Paque cushions (Pharmacia, Dorval, Canada), as described previously (Boyum, 1968). Final preparations contained 95% PMN, and viability was >95% as assessed by trypan blue exclusion.

Stimulation of Cells and Analysis of 5-LO. The cells were suspended in Hanks' balanced salt solution (HBSS) (1×10^7 cells/ml) at 37°C and stimulated with the indicated concentrations of fatty acid for the indicated time periods. For experiments in which cells were pretreated with ADA, 0.4 U ADA/ml was added to the cell suspension 2 min before stimulation. In some experiments, cells were preincubated at 37°C with the indicated concentrations of receptor agonists or antagonists before stimulation. For the determination of 5-LO products, reactions were stopped at the indicated times by the addition of 1 volume of ice-cold methanol/acetonitrile (1:1, v/v) containing 12.5 ng each of PGB_2 and 19-hydroxy-PGB_2 as internal standards, and the samples were processed and analyzed by reversed-phase (RP) HPLC with the use of an on-line extraction procedure, as described previously (Borgeat et al., 1990).

Preparation of Nuclei. For the preparation of nuclei, neutrophils (2×10^7 cells/2 ml) incubated under the conditions described were pelleted and resuspended in 600 μl of ice-cold Nonidet P-40 lysis buffer containing 0.1% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF (Pouliot et al., 1996). The cells were vortexed for 15 s, kept on ice for 5 min, and centrifuged at 300*g* (10 min, 4°C). The resulting supernatants (i.e., the non-nuclear fractions) and pellets (the nuclei-containing fractions) then were solubilized immediately in electrophoresis sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol, 0.01% bromophenol blue, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF) and boiled for 5 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis, as described by Laemmli (1970) on 9% acrylamide gels. Proteins then were transferred at 0.5 A for 4 to 15 h at 4°C onto an Immobilon-P polyvinylidene difluoride blotting membrane. Transfer efficiency was visualized by Ponceau Red staining. For the determination of 5-LO, the membranes were soaked for 30 min at 25°C in Tris-buffered saline (25 mM Tris-HCl, pH 7.6, 0.2 M NaCl, 0.15% Tween 20) containing 5% dried milk (w/v), blotted with anti-5-LO, and revealed using a horseradish peroxidase-coupled monoclonal antibody and the enhanced chemiluminescence detection kit.

Measurement of Intracellular Calcium Concentration $[Ca^{2+}]_i$. Fura-2 fluorescence was monitored, as described previously (Faucher and Naccache, 1987). Briefly, cells (1×10^7/ml) were incubated for 30 min with 1 μM fura-2 AM at 37°C. The cells then were washed, resuspended at 5×10^6/ml, and transferred into the thermally controlled (37°C) and magnetically stirred cuvette compartment of the spectrofluorometer (Aminco-Bowman series 2, SLM-Aminco, Urbana, IL). The excitation and emission wavelengths for Ca^{2+} measurements were 340 and 510, respectively.

Results

The Transformation of AA by 5-LO in Human PMN Is Inhibited by Endogenous Adenosine. A first series of experiments was designed to determine whether endogenous adenosine in isolated PMN preparations interferes with the transformation of exogenous AA into 5-LO products. It was shown previously that isolated PMN release endogenous adenosine into the incubation medium in a time- and cell concentration-dependent manner and that this release affects cell functions (Cronstein et al., 1983; Newby et al., 1983; Krump et al., 1997). As can be seen on the HPLC chromatogram in Fig. 1A, the addition of 3 μM AA to isolated PMN resulted in the synthesis of small amounts of LTB_4 and its ω-oxidation products 20-hydroxy-LTB_4 (20-OH-LTB_4) and 20-carboxy-LTB_4 (20-COOH-LTB_4). This is in accord with results of many previous reports that AA is a weak stimulus for LT synthesis and that the threshold concentration of AA required to induce basal LTB_4 synthesis is in the range of 3 to 10 μM (Clancy et al., 1983; McColl et al., 1989). However, when cells were pretreated with the adenosine A_{2a} receptor antagonist CGS15943 (Ghai et al., 1987), the capacity of the

cells to synthesize LTB_4, its ω-oxidation products, and the nonenzymatic hydrolysis products of LTA_4, 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4, in response to 3 μM AA was greatly enhanced (greater than 10-fold), as shown in Fig. 1B. The cells' capacity to respond to exogenous AA was similarly enhanced when adenosine was removed enzymatically from the incubation medium by the addition of ADA (Fig. 1C). However, when the adenosine A_{2a} receptor agonist CGS21680 was included in these incubations containing ADA, the enhanced response to AA was reversed (Fig. 1D). In fact, the adenosine A_{2a} receptor agonist CGS21680 was shown to be a very potent inhibitor (IC_{50} of ~1 nM) of the AA-induced synthesis of 5-LO products in PMN (treated with ADA) (Fig. 2).

In a parallel set of experiments, washing cells by centrifugation followed by immediate resuspension in prewarmed HBSS (37°C) and stimulation with 3 μM AA also resulted in an enhanced capacity to synthesize 5-LO products. When the washed cells (1×10^7/ml) were allowed to incubate at 37°C for as little as 3 min before stimulation, their response to AA was reduced to 60 to 70% of that of freshly washed cells and, within 15 min, the enhanced capacity to respond had all but disappeared (data not shown). Therefore, the removal of endogenous adenosine by washing the cells, by the action of added ADA or the treatment of cells with the A_{2a} receptor antagonist, resulted in a greatly enhanced ability of the cells to synthesize 5-LO products in response to low micromolar concentrations of exogenous AA.

Kinetics of the Synthesis of 5-LO Products. The synthesis of 5-LO products normally requires both AA release from phospholipids and activation of the 5-LO enzyme. However, because substrate availability should not be limiting when exogenous AA is added to the incubation media (as in the present experimental conditions), the kinetics of the synthesis of 5-LO products under such conditions are likely only reliant on 5-LO activation. The kinetics of 5-LO product synthesis in PMN exposed to AA in the presence of ADA were evaluated; as can be seen in Fig. 3A, a lag period of approximately 40 s transpired before a measurable synthesis of 5-LO products could be detected, although the substrate AA should be available immediately for transformation. This observed lag period varied between 20 and 60 s, depending on the PMN preparation. The addition of 3 μM AA to PMN induced a synthesis of 5-LO products that was maximal within 2 min. The decrease observed in the quantities of detectable 5-HETE when incubations are extended to >3 min is because 5-HETE is efficiently acylated into cellular glycerolipids (Stenson and Parker, 1979).

Figure 3B shows that when cells were stimulated with various concentrations of AA for 5 min, the optimal concentration for 5-LO product synthesis was 3 μM. When the incubations contained the higher AA concentrations of 10 to 30 μM, the cells eventually synthesized larger amounts of 5-LO products, but only after a 15- to 20-min incubation period (data not shown). Another difference in the response to higher concentrations of AA is the synthesis of increasing

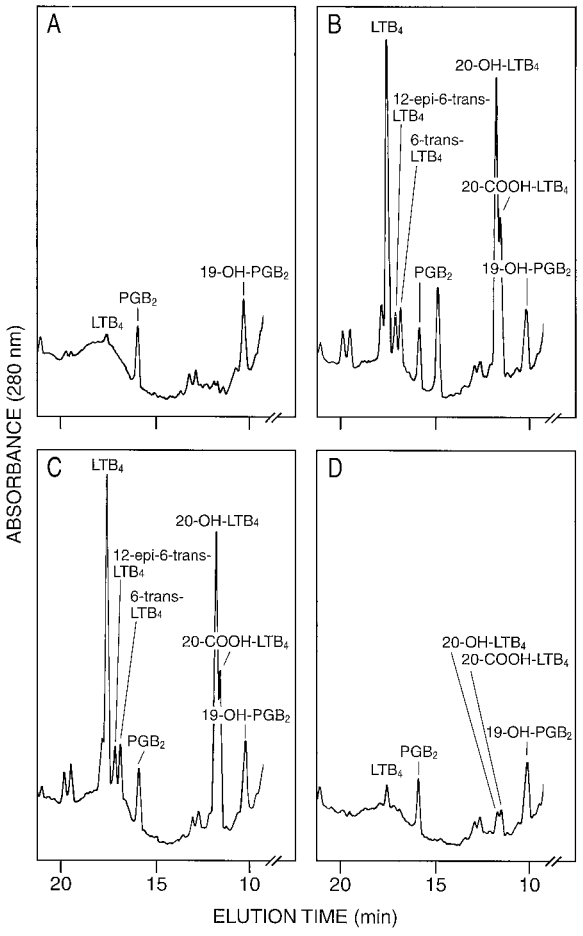

Fig. 1. RP-HPLC chromatograms of eicosanoids from human PMN treated with exogenous AA. PMN (1×10^7/ml) were preincubated for 5 min at 37°C in HBSS (A), in HBSS containing 1 μM CGS15943 (B), in HBSS containing 0.4 U/ml ADA (C), or in HBSS containing adenosine deaminase and 0.1 μM CGS21680 (D). The cells then were stimulated for 5 min with 3 μM AA and the reactions stopped by the addition of organic solvents containing the internal standards PGB_2 and 19-OH-PGB_2 (12.5 ng each), as described in *Experimental Procedures*. LTB_4, its ω-oxidation products 20-OH-LTB_4 and 20-COOH-LTB_4, and the nonenzymatic hydrolysis products of LTA_4 (6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4) were analyzed by RP-HPLC, as described in *Experimental Procedures*. Attenuation setting of the UV detector was 0.01 absorbance unit at full scale at 280 nm.

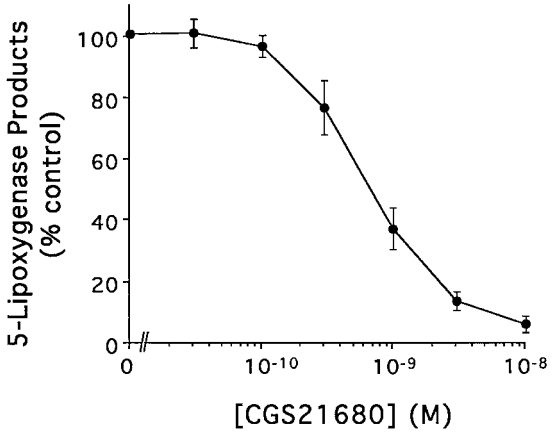

Fig. 2. The effect of CGS21680 on the biosynthesis of 5-LO products by human PMN stimulated with exogenous AA. PMN (1×10^7/ml) were preincubated at 37°C for 5 min in HBSS containing ADA (0.4 U/ml) and the indicated concentrations of CGS21680. The cells then were stimulated for 5 min with 3 μM AA and the reactions stopped by the addition of organic solvents containing the internal standards PGB_2 and 19-OH-PGB_2 (12.5 ng each), and 5-LO products were determined by RP-HPLC, as described in *Experimental Procedures*. The data show the relative amounts of LTB_4 and its metabolites generated. Values are the means ± S.E. of three separate experiments, each performed in duplicate.

quantities of the 15-LO product 15-HETE.15-HETE is known to exhibit inhibitory effects on PMN functions including LT synthesis (Borgeat et al., 1983). Therefore, its synthesis may be partially responsible for the slower accumulation of 5-LO products at high AA concentrations.

LTB_4 Receptor Antagonists and an LTA_4 Hydrolase Inhibitor Block the AA-Induced Synthesis of 5-LO Products. Although no specific cell surface receptor capable of transmitting a signal has been described for fatty acids including AA, previous studies have shown that AA-induced signals can be blocked with pertussis toxin, suggesting that AA may act either directly or indirectly via G protein-coupled receptors (McColl et al., 1989). It also has been suggested that these may be LTB_4 receptors (Naccache et al., 1989). To determine whether the induction of 5-LO product synthesis by AA may involve the LTB_4 receptor, PMN were preincubated for 2 min with two different LTB_4 receptor antagonists, CP105696 and LY223982, before cell stimulation in the presence of ADA. Figure 4 shows that both LTB_4 receptor antagonists blocked the synthesis of 5-LO products induced by AA at concentrations that are consistent with their previously reported IC_{50} for inhibiting PMN responses to LTB_4 (74 nM for LY223982 and 5 nM for CP105696) (Jackson et al., 1992; Showell et al., 1995). When AA was added along with the agonists fMLP and PAF, which stimulate PMN via their own receptors, LTB_4 receptor antagonists were without effect on 5-LO product synthesis, indicating that LTB_4 receptor antagonists were acting in a receptor-specific manner (data not shown). These results suggest that either AA itself can act as an agonist of the LTB_4 receptor or that small amounts of LTB_4 generated in response to AA can then act in an autocrine manner on its receptor and stimulate the cell for a more important biosynthesis of 5-LO products. This hypothesis of an autocrine stimulation of the PMN by endogenous LTB_4 was verified further by using the LTA_4 hydrolase inhibitor SC57461A. As can be seen in Fig. 5A, pretreatment of PMN with SC57461A inhibited in a dose-dependent manner the ability of exogenous AA to induce the synthesis of all 5-LO products (including 5-HETE and 6-*trans* isomers of LTB_4, whose synthesis is not dependent on LTA_4-hydrolase activity), indicating that the transformation of LTA_4 to LTB_4 is absolutely required for the AA-induced synthesis of 5-LO products in the experimental conditions used. In contrast, when SC57461A-treated PMN were stimulated with the calcium ionophore A23187, only LTB_4 synthesis was blocked, not that of LTA_4 (as evidenced by the detection of its nonenzymatic hydrolysis products 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4) or 5-HETE (Fig. 5B). This is in agreement with our previous report that endogenous LTB_4 does not exert a significant autocrine stimulatory effect on LT synthesis in human PMN stimulated with 1 μM A23187 (McDonald et al., 1994).

AA Induces the Translocation of 5-LO to the Nucleus. One of the features of 5-LO activation after stimulation of human PMN is the translocation of the enzyme from the cytosol to the nuclear envelope where the 5-LO activating

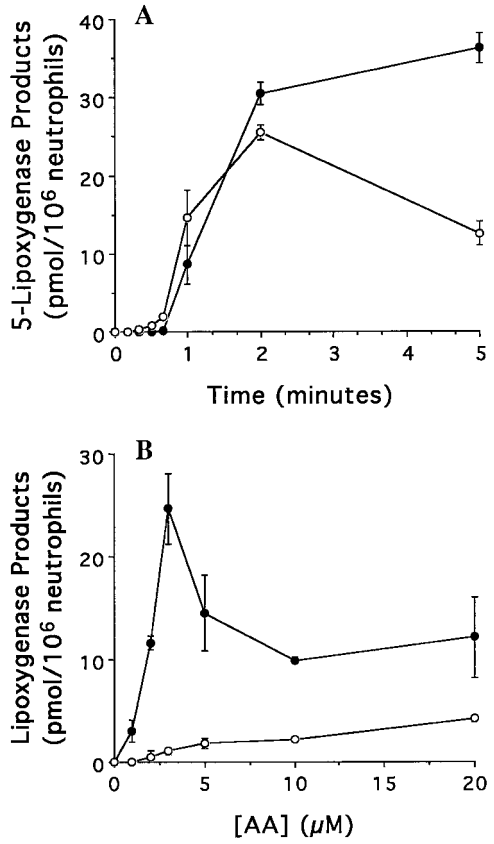

Fig. 3. The kinetics and dose-response to exogenous AA for the biosynthesis of 5-LO products in human PMN. PMN (1×10^7/ml) were preincubated at 37°C for 2 min in HBSS containing ADA (0.4 U/ml). A, cells were stimulated with 3 μM AA and, at the indicated times reactions, were stopped by the addition of organic solvents. ●, LTB_4; ○, 5-HETE. B, cells were stimulated with the indicated concentrations of AA for 7 min and reactions stopped by the addition of organic solvents. LO products were determined by RP-HPLC, as described in *Experimental Procedures*. ●, 5-LO products; ○, 15-HETE. Values for 5-LO products represent the sum of LTB_4, its ω-oxidation products 20-OH-LTB_4 and 20-COOH-LTB_4, and the nonenzymatic hydrolysis products of LTA_4 (6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4). Values are the means ± range of duplicate incubations from one experiment, which is representative of four separate experiments.

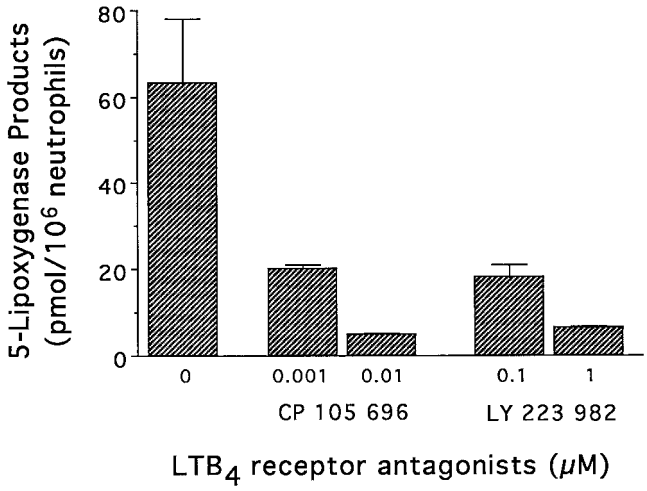

Fig. 4. Effect of LTB_4 receptor antagonists on the synthesis of 5-LO products in PMN stimulated with exogenous AA. PMN (1×10^7/ml) were preincubated at 37°C for 5 min in HBSS containing ADA (0.4 U/ml) and the indicated concentrations of LTB_4 receptor antagonists. Cells then were stimulated with 3 μM AA for 7 min and the reactions stopped by the addition of organic solvents, and 5-LO products were determined by RP-HPLC, as described in *Experimental Procedures*. Values represent the sum of LTB_4, its ω-oxidation products, and the nonenzymatic hydrolysis products of LTA_4. Values are the means ± S.D. of triplicate incubations from one experiment, which is representative of four separate experiments.

protein is present (Woods et al., 1993; Pouliot et al., 1996). Therefore, the translocation of 5-LO to the nucleus after activation of PMN by AA was assessed in cells treated with ADA alone and in combination with the adenosine A_{2a} receptor agonist CGS21680. Figure 6 shows that the stimulation of human PMN with 5 μM AA efficiently induces the translocation of 5-LO to the nucleus. Importantly, when cells were preincubated with the 5-LO activating protein antagonist MK0591, both 5-LO product biosynthesis and 5-LO translocation were inhibited. Stimulation of the adenosine A_{2a} receptor with the agonist CGS21680 also completely blocked AA-induced translocation of 5-LO and 5-LO product synthesis. Similarly, the LTB_4 receptor antagonist CP105696 also blocked the translocation of 5-LO, supporting the idea that a signal transmitted through this receptor is required for the AA-induced translocation of 5-LO. This is consistent with the effects of this antagonist on LTB_4 synthesis (Fig. 4). As with the biosynthesis of 5-LO products, the PAF receptor antagonist BN50730 did not block 5-LO translocation induced by AA.

AA Induces Calcium Mobilization: Effect of CGS21680, MK0591, CP105696, and SC57461A. The mechanism of the stimulation of 5-LO product biosynthesis by exogenous AA in human PMN and the inhibitory effects of adenosine on this process was investigated further in Ca^{2+} mobilization studies. As shown in Fig. 7, stimulation of PMN with 3 μM AA induced a transient elevation of $[Ca^{2+}]i$; furthermore, as observed in the kinetics of AA-induced 5-LO product biosynthesis, a 20- to 60-s lag was observed between the time of addition of AA to the PMN suspensions and the elevation of intracellular Ca^{2+} concentrations, clearly indicating that AA does not directly trigger the Ca^{2+} mobilization, and supporting a causal relationship between these events. The treatment of cells with CGS21680 resulted in a profound inhibition of the biosynthesis of 5-LO products and 5-LO translocation induced by AA; similarly, the adenosine A_{2a} receptor agonist CGS21680 showed a concentration-dependent inhibitory effect on the Ca^{2+} transient induced by AA (Fig. 7A).

The LTB_4 receptor antagonist CP105696, which effectively blocked both AA-induced 5-LO product biosynthesis and 5-LO translocation, was also investigated for its effect on Ca^{2+} mobilization. As shown in Fig. 7B, blocking the LTB_4 receptor also completely prevented the AA-induced mobilization of Ca^{2+}. When cells were treated with the 5-LO biosynthesis inhibitor MK0591 or with the LTA4 hydrolase inhibitor SC57461A, calcium mobilization also was completely blocked (Fig. 7, C and D). These results demonstrate that the synthesis of a 5-LO product is involved in AA-induced calcium mobilization and that LTB_4 itself ultimately stimulates PMN in an autocrine manner. Thus, the data also suggest that engagement of the A_{2a} receptor blocks AA-induced LT biosynthesis by preventing the autocrine activation of the PMN by LTB_4.

EPA Is a Weak Stimulus of Ca^{2+} Mobilization and 5-LO Product Biosynthesis. Another polyunsaturated fatty acid, EPA, which is also a substrate for 5-LO, was evaluated for its ability to induce the biosynthesis of 5-LO products in human PMN. Despite the fact that EPA is as good a substrate for 5-LO as is AA (Lee et al., 1984), the addition of 1 to 5 μM EPA to ADA-treated PMN resulted in little or no detectable synthesis of its 5-LO products (data not shown). Accordingly, EPA also was a much weaker stimulus than AA for the induction of Ca^{2+} mobilization in PMN (data not shown). Such data are entirely compatible with the hy-

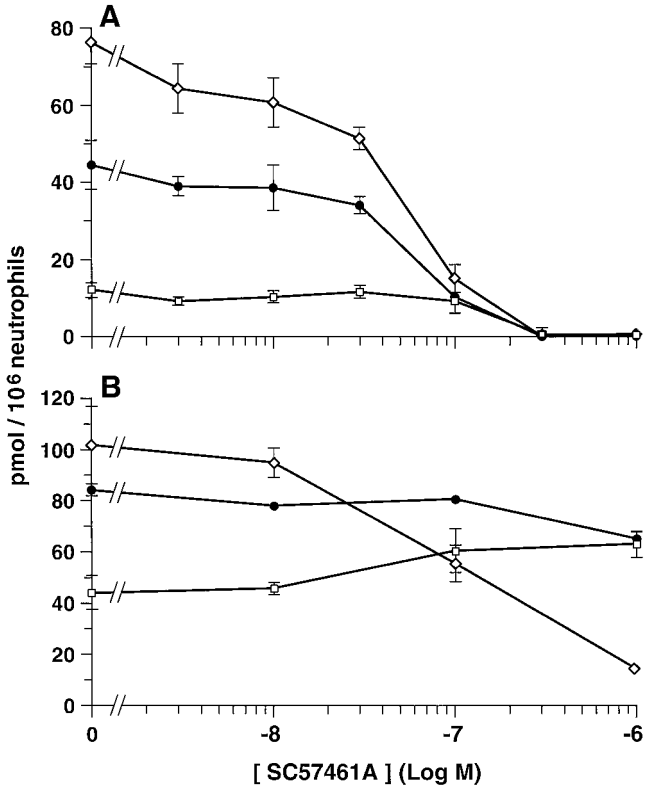

Fig. 5. Effect of SC57461A on the biosynthesis of 5-LO products in PMN stimulated with exogenous AA or ionophore A23187. PMN (1×10^7/ml) were preincubated for 5 min at 37°C in HBSS containing 0.4 U/ml ADA and SC57461A at the indicated concentrations. The cells then were stimulated for 5 min with 3 μM AA (A) or 1 μM A23187 (B), and the reactions were stopped by the addition of organic solvents containing the internal standards PGB_2 and 19-OH-PGB_2 (12.5 ng each), as described in *Experimental Procedures*. LTB_4 and its ω-oxidation products 20-OH-LTB_4 and 20-COOH-LTB_4 (◇, the nonenzymatic hydrolysis products of LTA_4 (6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4) (●), and 5-HETE (□)were analyzed by RP-HPLC, as described in *Experimental Procedures*. Values are the means ± S.D. of triplicate incubations from one experiment, which is representative of three separate experiments.

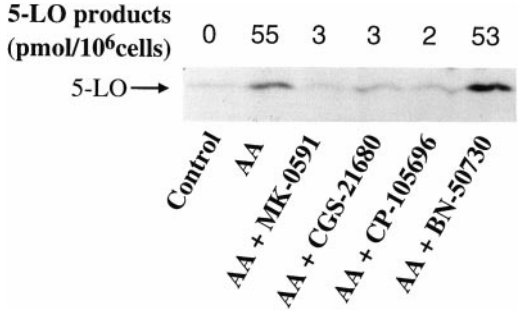

Fig. 6. 5-LO product synthesis and immunoblot analysis of 5-LO translocation to nuclei in AA-stimulated human PMN. Human PMN were pretreated for 5 min with ADA (0.4 U/ml) and the indicated compounds (0.1 μM), except for BN50730, which was at 1.0 μM. Cells then were stimulated with 3 μM AA for 5 min and centrifuged, and the supernatants were evaluated for 5-LO products. Nuclei were isolated from the cell pellets and prepared for gel electrophoresis and immunoblot analysis, as described in *Experimental Procedures*. Each lane was loaded with nuclei obtained from 15×10^6-cell equivalents of PMN. Results are from one experiment, which is representative of four separate experiments

pothesis proposed above, inasmuch as LTB_5, the product of EPA transformation by 5-LO, is a much weaker (25-fold) agonist of the LTB_4 receptor (as assessed by Ca^{2+} mobilization in human PMN) (Powell et al., 1996), which likely explains the inability of EPA to trigger the formation of 5-LO products. Interestingly, the addition of small concentrations (1 nM) of exogenous LTB_4 to ADA-treated PMN exposed to 5 μM EPA resulted in an enhanced biosynthesis of 5-LO products from EPA (26 $\pm$ 2 pmol/10^6 cells, $n = 3$).

Discussion

The incubation of isolated human PMN in vitro results in the rapid accumulation of endogenous adenosine, which has profound inhibitory effects on many cell functions, including LT synthesis. In the present study, we show that when endogenous adenosine is eliminated enzymatically from PMN suspensions, or its actions blocked with receptor antagonists, human PMN respond very strongly to low micromolar concentrations of AA for the synthesis of 5-LO products. This observation contrasts with the widely held perception that resting human PMN respond poorly to exogenous AA for LTB_4 synthesis and that the measurable synthesis of 5-LO products in the presence of AA requires the simultaneous stimulation with another agonist. Thus, our studies establish clearly that AA can trigger, in the absence of other added PMN stimuli, an important generation of 5-LO products and that the effect of exogenous AA is highly sensitive to the inhibitory effect of adenosine A_{2a} receptor engagement on PMN. Therefore, these findings are in line with our previous observations that A_{2a} receptor agonists as well as endogenous adenosine accumulating in PMN suspensions down-regulate agonist-induced LT biosynthesis in PMN (Krump et al., 1996, 1997) and add to the potential importance of adenosine as a natural regulator of LT synthesis.

The present observation that PMN do efficiently utilize exogenous AA (when released from the inhibitory constraint of adenosine) strongly supports the concept that the transcellular metabolism of exogenous AA by PMN could be an important source of biologically active eicosanoids such as lipoxins and LTs at inflammatory sites. Indeed, PMN can be exposed to elevated concentrations of free AA on close contact with activated cells releasing AA into the extracellular milieu. Such transcellular metabolism of AA has been shown to be an important pathway for the synthesis of a number of AA metabolites. In particular, AA released by stimulated platelets can be utilized by stimulated PMN for LTA_4 and LTB_4 synthesis (Palmantier and Borgeat, 1991), whereas LTA_4 released by stimulated PMN can be converted to lipoxins by the platelet 12-LO (Serhan and Sheppard, 1990). Thus, the present data indicate that PMN likely utilize exogenous AA in a much more efficient manner than was thought previously. In fact, the quantities of LTB_4 produced by PMN in the presence of 3 μM AA (without the addition of other stimulatory agents) exceed the quantities reported previously under most conditions of PMN stimulation involving priming agents and receptor-mediated agonists that activate PL A_2 and 5-LO for LT synthesis (Dahinden et al., 1988; Surette et al., 1998). Moreover, such efficient utilization of exogenous AA may take place in pathological situations in which secreted PL(s) A_2 accumulation and associated AA release occur, such as in adult respiratory distress syndrome, in the synovial fluid of arthritic patients, and in the asthmatic lung (Nevalainen, 1993). The two potent anti-inflammatory drugs methotrexate and sulfalazine have been shown to promote adenosine accumulation at inflammatory sites (Cronstein, 1995), an effect believed to be involved in their anti-inflammatory activity. Therefore, our data support the concept that the pharmacologically mediated accumulation of adenosine in inflammatory sites constitutes a promising approach for

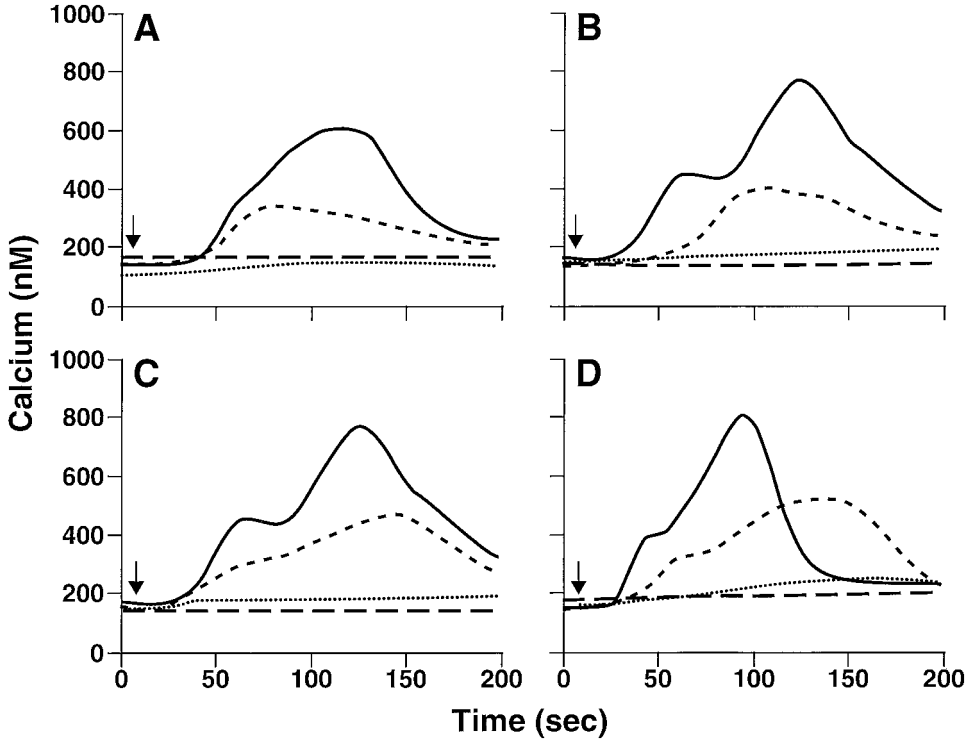

Fig. 7. Effect of CGS21680, CP105696, MK0591, and SC57461A on AA-induced calcium mobilization in human PMN. Fura-2-loaded PMN (5×10^6/ml) in HBSS were preincubated for 5 min at 37°C with ADA (0.4 U/ml) in the presence of CGS21680 at 0, 10, and 100 nM (A); CP105696 at 0, 1, and 10 nM (B); MK0591 at 0, 10, and 100 nM (C); and SC57461 at 0, 10, and 100 nM (D) before stimulation with 3 μM AA. Unstimulated cells (dotted lines) were incubated with diluent only (0.1% DMSO). $\downarrow$ indicate the time of AA additions; the fluorescence was monitored at the excitation and emission wavelengths of 340 nm and 510 nm, respectively, as described in *Experimental Procedures*. The results are representative of two to five separate experiments. Full traces indicate no drug added; dashed lines, low drug concentrations; interrupted lines, high drug concentrations.

the treatment of inflammatory diseases, inasmuch as LTB_4 might play important roles in some disease states.

AA-induced 5-LO product biosynthesis showed intriguing features that suggested a role of de novo synthesized LTB_4 in the activation of PMN. Indeed, our data demonstrate that the observed biosynthesis of 5-LO products in response to exogenous AA involves an absolute requirement for an autocrine/paracrine activation of the PMN by endogenous LTB_4, resulting in Ca^{2+} release from intracellular stores, the translocation and activation of 5-LO, and the important transformation of exogenous AA into 5-LO products (Fig. 8). Several lines of evidence support this mechanism. First, the addition of AA to PMN did not result in an immediate mobilization of Ca^{2+} or synthesis of 5-LO products, because a lag of 20 to 60 s (variable between PMN preparations) was observed consistently. This indicates that AA itself is not acting directly in a receptor-dependent manner as is observed when cells are stimulated with agonists such as fMLP, PAF, or LTB_4. Second, blocking the LTB_4 receptor with specific receptor antagonists strikingly inhibited the AA-induced delayed mobilization of Ca^{2+}, 5-LO translocation, and the delayed biosynthesis of 5-LO products. This suggests that LTB_4 receptor occupation is required for AA-induced PMN stimulation leading to 5-LO translocation and activation. Third, the specific LT synthesis inhibitor MK0591 also inhibited the mobilization of calcium, clearly indicating that 5-LO activity is required for this response to exogenous AA. That LTB_4 itself is responsible for this autocrine effect is supported further by studies with the LTA_4 hydrolase inhibitor SC57461A, which prevents the transformation of LTA_4 to LTB_4, but not the synthesis of other 5-LO product such as 5-HETE. In the present studies, SC57461A inhibited AA-induced synthesis of all 5-LO products and Ca^{2+} mobilization in PMN, indicating that LTB_4 formation is specifically required for these responses to exogenous AA. Additional evidence that an initial biosynthesis of LTB_4 is required for the further biosynthesis of large amounts of 5-LO products on the addition of AA to ADA-treated cells was obtained by using EPA instead of AA in similar experiments. Indeed EPA, which is as good a substrate for 5-LO as AA (Lee et al., 1984), did not induce an important synthesis of its 5-LO products (such as LTB_5) when added to PMN. This is likely because LTB_5, which is a much weaker agonist than LTB_4 for the LTB_4 receptor, did not induce cell activation as measured herein by Ca^{2+} mobilization. Altogether, these observations point to a requirement for an initial LTB_4 synthesis in response to exogenous AA, which triggers through an autocrine/paracrine mechanism the full activation of PMN for 5-LO product synthesis.

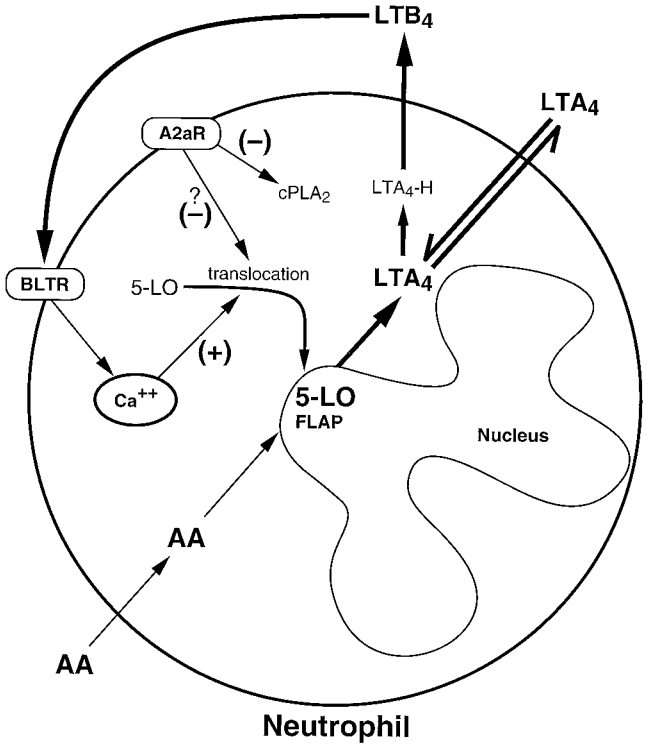

Fig. 8. Hypothetical mechanism of 5-LO product biosynthesis involving autocrine activation by LTB_4 in PMN exposed to exogenous AA. Unstimulated PMN exposed to AA generate LTA_4 and LTB_4; this initial synthesis might involve a small quantity of 5-LO present on the nuclear envelope and is likely Ca^{2+}-independent (Skorey and Gresser, 1998). The LTB_4 generated initially then plays a pivotal role in activating further the PMN through engagement of the LTB_4 receptors, which results in Ca^{2+} release from intracellular stores, stimulation of the translocation of cytosolic 5-LO, and enhanced LTA_4 and LTB_4 biosynthesis from exogenous AA. In these experimental conditions, this autocrine/paracrine stimulatory loop by endogenous LTB_4 is required for the full activation of LT biosynthesis by exogenous AA, as demonstrated by the almost complete inhibition of 5-LO product formation by LTB_4 receptor antagonists and an LTA_4-hydrolase inhibitor. Engagement of the A_{2a} receptor appears to interfere with the autocrine/paracrine activation loop by inhibiting 5-LO translocation (through a yet-unidentified mechanism; see *Discussion*).

Finally, it is noteworthy that the present studies with this particular model (AA-induced LT biosynthesis) unraveled an additional inhibitory mechanism of A_{2a} receptor engagement on LT synthesis in PMN. In previous studies, it was clearly established that the A_{2a} receptor agonist CGS21680 is a potent inhibitor of AA release in agonist-activated human PMN (Krump et al., 1999), strongly supporting that such a mechanism was involved in the inhibitory effects of the adenosine analog on LT synthesis. Because in these and other previous studies, the inhibition of AA release also was observed with a variety of other agents known to cause an elevation of intracellular cAMP in PMN (E-type prostaglandins, the β-adrenergic agent isoproterenol, and the type IV phosphodiesterase inhibitor Rolipram (Schering AG, Berlin,

Germany) (Ham et al., 1983; Fonteh et al., 1993; Krump et al., 1999), it was proposed that the effect of CGS21680 on AA release was cAMP-dependent. In the present studies, it is demonstrated that CGS21680 is a potent inhibitor of LT synthesis, even under conditions of PMN stimulation by exogenous substrate (AA), clearly indicating another site of action of A_{2a} receptor agonists in the inhibition of LT synthesis. CGS21680 does not inhibit the release of Ca^{2+} from intracellular stores induced by LTB_4 in human PMN (data not shown). However, as shown herein, CGS21680 interferes with the activation/translocation process of the 5-LO. Hypothetically, A_{2a} receptor engagement might down-regulate phosphorylation events necessary for 5-LO translocation; indeed, recent studies by Lepley et al. (1996) described the inhibitory effects of various tyrosine kinase inhibitors on LT synthesis and 5-LO phosphorylation and translocation in human PMN. Studies are in progress to define further the molecular events involved in the inhibition of AA release and 5-LO activation by A_{2a} agonists in human neutrophils.

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Send reprint requests to: Dr. Pierre Borgeat, Centre de Recherche en Rhumatologie et Immunologie, Centre Hospitalier Universitaire de Québec, Pavillon CHUL, 2705 Boul. Laurier, Room T 1–49, Ste-Foy, Québec, G1V 4G2, Canada. Email: pierre.borgeat@crchul.ulaval.ca