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INHIBITION OF THE A-23187-STIMULATED LEUKOTRIENE AND PROSTAGLANDIN BIOSYNTHESIS OF RAT BASOPHIL LEUKEMIA (RBL-1) CELLS BY NON-STEROIDAL ANTI-INFLAMMATORY DRUGS, ANTI-OXIDANTS, AND CALCIUM CHANNEL BLOCKERS

LAWRENCE LEVINE
Department of Biochemistry, Brandeis University, Waltham, MA 02254, U.S.A.

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Abstract—Rat basophil leukemia cells (RBL-1), when grown in monolayer, synthesize from endogenous substrates the prostaglandins (PG) E_2, $F_{2\alpha}$, and I_2 (measured as 6-keto-$PGF_{1\alpha}$) and 6-sulfidopeptide-containing leukotrienes (SRS), as well as materials that react serologically with anti-12-hydroxyeicosatetraenoic acid (HETE). The non-steroidal anti-inflammatory drugs indomethacin and aspirin inhibited PGE_2 synthesis by RBL-1 cells, which had been stimulated with the calcium ionophore A-23187, in a dose-dependent manner with an IC_{50} of 0.7 and 7.8 μM respectively. Indomethacin, when used at higher concentrations, also inhibited iSRS synthesis with an IC_{50} of 230 μM. Benoxaprofen, also a non-steroidal anti-inflammatory drug, inhibited both PGE_2 and iSRS production in a dose-dependent manner, but inhibition of the iSRS biosynthesis was three times more effective than inhibition of PGE_2 production. The anti-oxidants gossypol, butylated hydroxyanisole (BHA), nordihydroguariatic acid (NDGA), and 3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline (BW755c) also inhibited iSRS synthesis more effectively than PGE_2 biosynthesis. The IC_{50} values for inhibition of iSRS production were 0.2 μM (gossypol), 0.5 μM (BW755c), 0.6 μM (BHA), and 0.6 μM (NDGA) compared to 2.8 μM (gossypol), 2.0 μM (BW755c), 4.8 μM (BHA) and 2.6 μM (NDGA) for inhibition of PGE_2 synthesis. Gossypol, BW755C, BHA, and NDGA, as well as benoxaprofen, inhibited i12-HETE biosynthesis (IC_{50} for gossypol, 0.32 μM; and for benoxaprofen, 0.5 μM). Two calcium channel blockers, verapamil and nifedipine, inhibited PGE_2, iSRS and i12-HETE synthesis in a dose-dependent manner. The calcium channel blockers inhibited iSRS synthesis ten times more effectively than PGE_2 production.

Rat basophil leukemia cells (RBL-1), when grown in monolayers, synthesize from endogenous arachidonic acid the prostaglandins E_2, $F_{2\alpha}$ and I_2 (measured as 6-keto-$PGF_{1\alpha}$), hydroxyfatty acids that react serologically with anti-12-hydroxyeicosatetraenoic acid (HETE), and 6-sulfidopeptide-containing leukotrienes (SRS) that react with anti-SRS [1, 2]. When incubated with the calcium ionophore A-23187, RBL-1 cells are stimulated to produce increased levels of PGH_2 synthetase and lipoxygenase pathway products [2]. With the use of this model, the specificity of inhibition, with respect to PGH_2 synthetase and lipoxygenase pathways, can be monitored. Such specificities of inhibition by three non-steroidal anti-inflammatory drugs, four anti-oxidants, and two calcium channel blockers have been investigated and are described in this report.

MATERIALS AND METHODS

Rat basophil leukemia (RBL-1) cells were cultured as previously described [1, 2]. Twenty-four hours prior to the start of an experiment, exponentially growing cells were treated with 1 ml of trypsin (0.5 mg)/EDTA (0.2 mg) solution and plated at a density of approximately 5×10^5 cells per 60 mm culture dish in 4 ml of Eagle's Minimal Essential Medium containing 10% fetal bovine serum and 2 mM L-glutamine. To begin an experiment, the cells in each dish were washed three times with 2 ml of Minimal Essential Medium containing penicillin (250 units/ml) and streptomycin (250 μg/ml) and then incubated in 2 ml of Eagle's Minimal Essential Medium plus 2 mM L-glutamine and antibiotics and containing the vehicle, A-23187 and inhibitors. The cells were incubated at 37° in an atmosphere of 5% CO_2/95% air for 60 min, usually in three replicates for each inhibitor of A-23187 mixture and three replicates for A-23187 alone. The effect of the inhibitor only, at the concentrations used in this study, on iSRS, i12-HETE and PGE_2 production was measured, but no effect was observed. Gossypol, however, at a higher concentration than that used in this study (10 μM), did stimulate arachidonic acid metabolism in RBL-1 cells and in a dog kidney cell line (MDCK). After 60 min, the medium was removed for determination of PGE_2, iSRS and i12-HETE serologic activity. If cells were detached from the dishes because of the treatment with A-23187, they were removed by centrifugation.

In experiments in which the media were to be analyzed by radioimmunoassay (RIA) after resolution of the arachidonic acid metabolites by high performance liquid chromatography (HPLC), the media from the appropriate replicate dishes were pooled and extracted with ethanol. The culture media were extracted by addition of 3 vol. of ethanol. Each ethanolic suspension was mixed every 20 min

during incubation at room temperature for 1 hr; insoluble material was removed by centrifugation at 800 *g* for 15 min at 4°. The supernatant fluid was evaporated to dryness under negative pressure, and the residue was dissolved in ethanol–water (1:1, v/v). The remaining insoluble material was removed by centrifugation at 800 *g* for 15 min at 4°. The supernatant fluid was dried under nitrogen gas and then redissolved either in the appropriate solvent for chromatographic separation or in Tris buffer (0.01 M Tris·HCl, pH 7.4, containing 0.14 M NaCl and 0.1% gelatin) for assay by RIA. The methodology for high performance liquid chromatography (HPLC) has been reported previously [2–5]. Briefly, we used the Waters model 600A pumps, model 660 solvent programmer, model U6K injector, and a 300 × 3.9 mm fatty acid analysis column in the reverse phase model. The cyclooxygenase product, PGE_2, was eluted isocratically with a solvent system containing acetonitrile, benzene, acetic acid and water (230:2:1:767, by vol.). The flow rate was 2.0 ml/min, and sixty 2-ml fractions were collected. SRS-leukotrienes were separated using a linear gradient program from 100% solvent A to 100% solvent B over 100 min at a flow rate of 1 ml/min, and 1-ml fractions were collected. Solvent A consisted of 93.4% 0.01 M phosphate buffer (pH 7.4), 6% methanol (HPLC grade, Fisher), and 0.6% *t*-amyl alcohol (Aldrich); solvent B was 99.4% methanol with 0.6% *t*-amyl alcohol (% by volume). HETEs were separated using the same column, solvent systems and conditions as for the SRS-leukotrienes, the difference being that the gradient was interrupted at 45 min at which time the elution became isocratic; 80 1-ml tubes were collected during the isocratic elution. The HPLC fractions were dried under nitrogen gas and resuspended in Tris buffer for RIA.

The serologic specificities of antisera to the various arachidonic acid metabolic products assayed in the present study have been described [2, 6]. The LTB_4 immune system, whose serologic specificities have been described [7], was a gift from Dr. Robert A. Lewis. The RIAs were performed in 3.5 ml polypropylene test tubes (No. 535; Sarsdedt, Princeton, NJ). The diluent for all reagents was Tris buffer. ^3H-labeled standards for each compound assayed were purchased from the New England Nuclear Corp., Boston, MA.

Indomethacin, aspirin, gossypol, butylated hydroxyanisole (BHA) and nordihydroguariatic acid (NDGA) were purchased from the Sigma Chemical Co., St. Louis, MO. A-23187 was purchased from Calbiochem, La Jolla, CA. Benoxaprofen and 3-amino-1-[*m*-trifluromethyl)phenyl]-2-pyrazoline (BW755c) were obtained from Dr. Robert A. Lewis. Nifedipine was provided by Pfizer, Inc., New York, NY; and verapamil was a gift from Dr. Michael A. Moskowitz. Stocks of the reagents (1–10 mg/ml) were made in dimethyl sulfoxide (DMSO); the final concentrations of the DMSO never exceeded 0.1%, amounts which had no effect on arachidonic metabolism by RBL-1 cells.

RESULTS

The non-steroidal anti-inflammatory drug indomethacin inhibited PGE_2 production in a dose-dependent manner, and only at relatively high concentrations (20–200 μM) did indomethacin inhibit iSRS production (Fig. 1). The 50% inhibiting concentrations (IC_{50}) for PGE_2 by indomethacin and aspirin were 0.7 and 7.8 μM respectively (Table 1). Indomethacin, at 200 μM, inhibited il2-HETE production 35%. Benoxaprofen [8] also inhibited PGE_2, iSRS and i12-HETE production after stimulation by A-23187 but, unlike indomethacin, it inhibited the lipoxygenase pathway more effectively than it did cyclooxygenase metabolism. The IC_{50} values for benoxaprofen were 0.4 μM for iSRS, 0.45 μM for i12-HETE and 1.3 μM for PGE_2. The anti-oxidants BHA, NDGA, BW755c, and gossypol (1,1',6,6',-7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl-

Table 1. Inhibition of A-23187-stimulated arachidonic acid metabolism in RBL-1 cells by non-steroidal anti-inflammatory drugs, anti-oxidants, and calcium channel blockers

Inhibitor	IC_{50} (μM)		iSRS IC_{50} / PGE_2 IC_{50}
	iSRS	PGE_2	
Non-steroidal anti-inflammatory drugs			
Indomethacin	230	0.7	329
Aspirin	*	7.8	
Benoxaprofen	0.4	1.3	0.31
Anti-oxidants			
Gossypol	0.2	2.8	0.07
3-Amino-1-[*m*-trifluromethyl)phenyl]-2-pyrazoline (BW755c)	0.5	2.0	0.25
Butylated hydroxyanisole (BHA)	0.6	4.8	0.13
Nordihydroguariatic acid (NDGA)	0.6	2.6	0.23
Calcium channel blockers			
Verapamil	1.8	15.0	0.12
Nifedipine	7.5	75	0.10

* No inhibition with 500 μM.

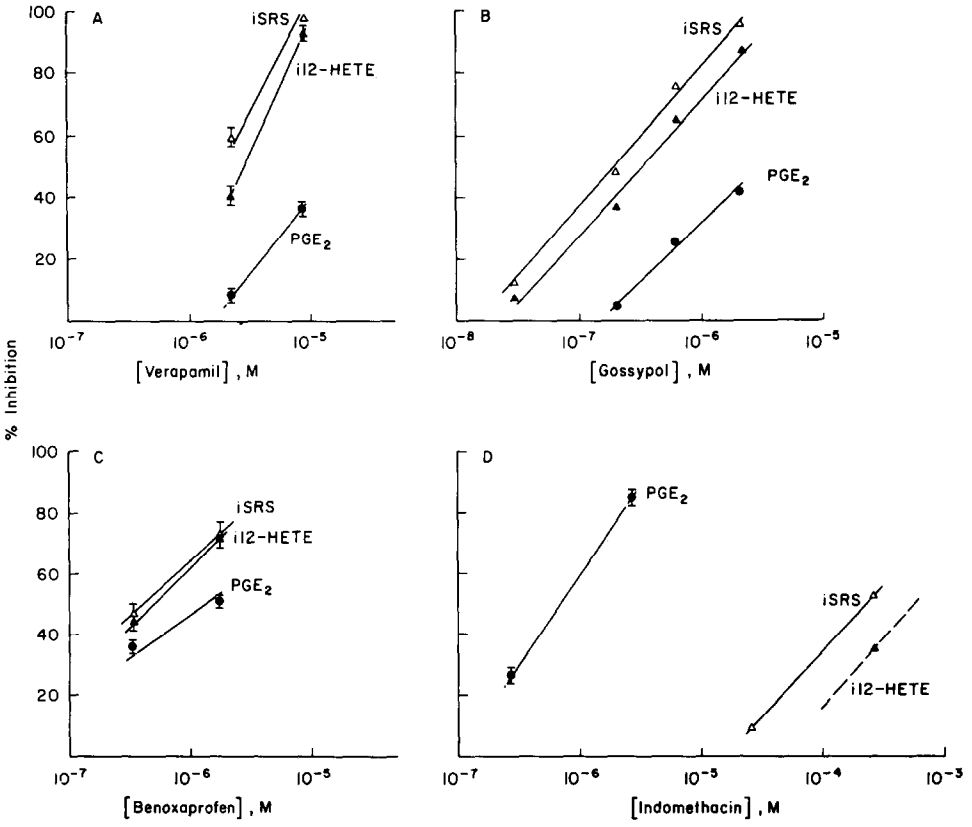

Fig. 1. Inhibition of arachidonic acid metabolism by increasing amounts of verapamil (A), gossypol (B), benoxaprofen (C), and indomethacin (D) of 0.5 μM A-23187-stimulated RBL-1 cells. The culture fluids were assayed for iSRS (△), i12-HETE (▲) and PGE_2 (●). The data are presented as the average found in the culture fluids of triplicate dishes and their standard deviations. In some analyses, the RIAs were done on culture fluids of duplicate dishes; the values agreed within 20%.

[2,2′-binapthalene]-8,8′-dicarboxaldehyde), a polyphenol found in certain species of cotton plant, inhibited iSRS, i12-HETE and $iPGE_2$ [9] production (Fig. 1 and Table 1). The IC_{50} values with gossypol were, for iSRS, i12-HETE and PGE_2, 0.2, 0.28 and 2.8 μM respectively. Both iSRS and i12-HETE production were inhibited, indicating that the activity of the lipoxygenase was being blocked. The anti-oxidants inhibited iSRS and i12-HETE biosynthesis mor effectively than PGE_2 production (14 times more effectively), whereas indomethacin inhibited PGE_2 production 330 times more effectively than iSRS biosynthesis.

A third class of inhibitors was tested for its effect on A-23187-stimulated arachidonic acid metabolism by RBL-1 cells. As can be seen in Fig. 1 and Table 1, both nifedipine and verapamil inhibited iSRS and i12-HETE production about ten times more effectively than PGE_2 production.

The serologic specificity of our SRS and 12-HETE immune systems is such that absolute identification can only be made from both chromatographic and serologic properties. Thus, inhibition of the A-23187-stimulated arachidonic acid metabolism of RBL-1 cells by gossypol and nifedipine also was measured after analyses of the culture fluids by immunochromatography [2, 4, 5, 10]. The data obtained after inhibition with nifedipine are shown in Fig. 2, but similar results were also found after treatment with gossypol. Treatment of RBL-1 cells (5×10^5/60 mm dish) for 60 min with 0.5 μM A-23187

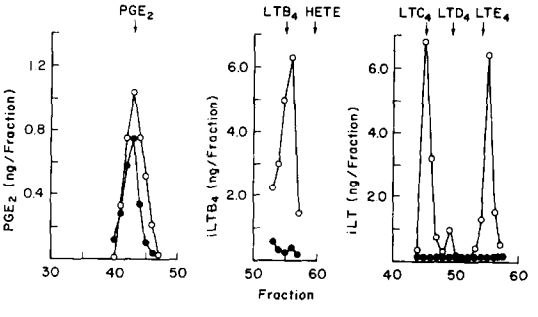

Fig. 2. Inhibition of arachidonic acid metabolism by 29 μM nifedipine of 0.5 μM A-23187-stimulated RBL-1 cells. Tissue culture fluids were pooled and extracted as described in Materials and Methods. The arachidonic acid metabolites in the extracts were resolved by HPLC. The data presented are RIA analysis of appropriate fractions with anti-PGE_2, anti-LTB_4, and anti-SRS. Key: A-23187-stimulated cells (○); and A-23187-stimulated cells in the presence of 29 μM nefedipine (●). The arrows represent the retention of authentic PGE_2, LTB_4, 12-HETE, LTC_4, LTD_4, and LTE_4.

results in stimulated production of cyclooxygenase and lipoxygenase products [2], probably as a result of increased acylhydrolase activity. Most of the 6-sulfidopeptide-containing leukotrienes present in the culture fluids are LTC_4 and LTE_4; presumably, the γ-glutamyltranspeptidase activity is the rate-limiting reaction in our experimental conditions. No LTC_4, LTD_4 or LTE_4 was found in the culture fluids of the A-23187-stimulated cells when incubated in the presence of 29 μM nifedipine. The production of LTB_4, measured with a serologically specific RIA, was also inhibited, but inhibition of PGE_2 biosynthesis was less marked [inhibition of iSRS production by nifedipine was ten times more effective than inhibition of PGE_2 release (Table 1)]. RBL-1 cells also synthesize 5-HETE, but this serologic activity is not recognized by the anti-LTB_4 [7].

DISCUSSION

Inhibition of iSRS, i12-HETE and PGE_2 biosynthesis by the compounds used in this study can result from blockage of several enzymes that metabolize arachidonic acid. For example, inhibition of iSRS production could have resulted from decreased acylhydrolase, 5-lipoxygenase, leukotriene A_2 synthetase and, since our anti-SRS is non-specific for *S*-sulfidopeptides, any of the terminal enzymes that are involved in SRS synthesis—i.e. glutathione-*S*-transferase, γ-glutamyltranspeptidase and aminopeptidase [11]. Inhibition of i12-HETE production could have resulted from decreased acylhydrolase and lipoxygenase activities. On the other hand, inhibition of PGE_2 biosynthesis could have resulted from decreased acylhydrolase, PGH_2 synthetase, PG hydroperoxidase and endoperoxide E_2 isomerase activities. If we assume that enzymes with identical functions have identical conformations and that the kinetic properties of each enzyme are similar, then we can eliminate some of these enzymes as the site of action of these inhibitors.

Indomethacin and aspirin inhibited PGE_2 production, but only at high concentrations did they affect iSRS or i12-HETE production. Thus, indomethacin and aspirin do not inhibit acylhydrolase, lipoxygenase or glutathione *S*-transferase activities. The properties of indomethacin and aspirin are consistent with their well known inhibition of PGH_2 synthetase activity [12–14]. The effects of benoxaprofen can be explained by inhibition of PGH_2 synthetase, lipoxygenase, and acylhydrolase activities. In preliminary experiments, benoxaprofen did not inhibit release of radiolabeled compounds from [^{14}C]arachidonic acid labeled cells. Thus, it appears that benoxaprofen is inhibiting both 5-lipoxygenase and PGH_2 synthetase, the 5-lipoxygenase more effectively than the PGH_2 synthetase. Since we have found that acylhydrolase activities were not affected by the antioxidants (unpublished data), the same mechanism of action of that deduced for benoxaprofen would explain the inhibitory properties of BHA, NDGA, gossypol and BW755c. The mechanism of inhibition by the calcium channel blockers would be consistent with blockage of acylhydrolase, 5-lipoxygenase and PGH_2 synthetase activities. Most likely it is the movement of calcium into the cell stimulated by the calcium ionophore A-23187 that is being blocked by nifedipine and verapamil. Thus, the calcium channel blockers may be decreasing calcium-dependent acylhydrolase [15, 16] and 5-lipoxygenase activities [17]. This is consistent with the findings that lipoxygenase products were inhibited more effectively than PGH_2 synthetase products. Regardless of the mechanism of inhibition by verapamil and nifedipine, decreased iSRS, iHETE and PGE_2 production as well as the decreased phospholipase activity in the cell membrane must be taken into account in interpreting the striking properties of the calcium channel blockers in cardiovascular responses [18].

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