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CALCIUM STIMULATION OF A NOVEL LIPOXYGENASE

Barbara A. Jakschik, Frank F. Sun*, Lieh-hauh Lee and Maggie M. Steinhoff

Department of Pharmacology, Washington University School of Medicine, St. Louis, MO 63110, and the *Upjohn Company, Kalamazoo, MI 49001

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SUMMARY: Homogenates of rat basophilic leukemia (RBL-1) cells have a novel lipoxygenase which was stimulated by calcium in a concentration dependent fashion and inhibited by epinephrine. The major compounds formed from [^{14}C]-arachidonic acid were identified by gas chromatography - mass spectrometry to be 5-hydroxyeicosatetraenoic acid and 5,12-dihydroxyeicosatetraenoic acid. Other compounds present in small amounts were 12-hydroxy- and 5,6-dihydroxyeicosatetraenoic acid. The stimulation by calcium of this pathway in basophils links it closely to the release reaction which is calcium dependent.

INTRODUCTION

During anaphylactic or allergic reactions mast cells and basophils are stimulated to release various mediators including histamine and slow reacting substance of anaphylaxis (SRS-A)[1]. It is well established that this reaction is calcium dependent (1). It has been shown recently that SRS as well as 5,12-dihydroxyeicosatetraenoic acid (5,12-DiHETE) are formed via a novel lipoxygenase pathway and the compounds have been named leukotriene C and B respectively (2,3). Leukotriene A (5,6-oxido-7,9,11,14-eicosatetraenoic acid) is an unstable intermediate in the formation of leukotriene B and leukotriene C. We have reported earlier that rat basophilic leukemia (RBL-1) cells make SRS when incubated with the ionophore A23187 or arachidonic acid and that SRS synthesis is markedly potentiated when RBL-1 cells are exposed to arachidonic acid and A23187 simultaneously (4-6). Recently it has been shown by Parker et al. (7) that the structure of SRS obtained from RBL-1 cells is very similar or even identical to the structure of SRS reported by Samuelsson et al. (3,8) for SRS obtained from a mouse mastocytoma. We also observed a potentiation by A23187 of the formation of certain arachidonate metabolites which migrated by thin layer chromatography

[1] Abbreviations used: SRS, slow reacting substance; SRS-A, slow reacting substance of anaphylaxis; RBL-1 cells, rat basophilic leukemia cells; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography.

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just above and below prostaglandin A_2 (6). We now identified these compounds as 5-hydroxy- and 5,12-dihydroxyeicosatetraenoates (HETE). A potentiation of 5-HETE and 5,12-DiHETE synthesis by A23187 has also been reported by Borgeat and Samuelsson (9). These observations led us to investigate the effect of calcium on the formation of mono- and dihydroxyeicosatatraenoic acids.

MATERIALS AND METHODS

RBL-1 cells were grown and harvested as described previously (10). The cells were washed with 50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 0.1% gelatin and 14 μM indomethacin, resuspended at 5 x 10^7 cells/ml and homogenized as described (10). Preliminary experiments indicated no difference in products formed between pH 6.5 to 9. The homogenate was centrifuged at 10,000 x g for 20 min to remove debris, nuclei and granules.

Samples for structure analysis were prepared as follows: 10,000 x g supernatant (1 mM calcium) of 2-5 x 10^9 cells was incubated with 33 μM arachidonic acid ([^{14}C]arachidonic acid 50,000 cpm/ml) for 20 min at 37°C. The mixture was extracted twice with equal volumes of ethyl acetate (pH 3.2-3.5). The concentrated extract was chromatographed on a silicic acid column (9). Aliquots of 5 ml fractions containing radioactivity were chromatographed in solvent system A9 to determine the presence of hydroxy-arachidonic acids. Fractions containing these compounds were used for high performance liquid chromatography and gas chromatography - mass spectrometry. In one experiment bands I and II (Fig. 2) were eluted and subjected to gas chromatography mass spectrometry. The conditions for gas chromatography with radioactivity monitoring and mass spectrometry were described earlier (13). The compounds were hydrolyzed with aqueous sodium carbonate to open any δ-lactone present. Then conversion to the methyl ester and trimethylsilyl (TMS) derivatives was performed. Catalytic hydrogenation was performed in a Sulpelco microhydrogenator with 10% palladium in charcoal as catalyst. The saturated hydroxyicosanoates were analyzed as TMS derivatives.

The sources of reagents were as follows: arachidonic acid was obtained from Nu Check Prep. (Elyson, Minn.), [^{14}C]arachidonic acid from Amersham (Arlington Heights, Ill.), L-epinephrine from Sigma (St. Louis, Mo.) and the solvents for extraction and chromatography from Fisher Scientific (St. Louis, Mo.). Prostaglandins were kindly supplied by Dr. J. Pike, 12-HETE by Dr. R.R. Gorman and 5-HETE by Dr. R.C. Kelly of the Upjohn Co. (Kalamazoo, Mich.) and indomethacin by Merck, Sharpe and Dohme (West Point, Pa.).

RESULTS AND DISCUSSION

When the 10,000 x g supernatant (1 mM EDTA) of RBL-1 cell homogenates was incubated with [^{14}C]arachidonic acid, only small amounts of lipoxygenase products were synthesized. No prostaglandins were observed since indomethacin was present. Addition of calcium stimulated the formation of hydroxy acids (Fig. 1A and 2A). This stimulation by calcium was concentration dependent (Fig. 1A). The same results were obtained when EGTA was present in the buffer instead of EDTA (data not shown). No such enhancement of hydroxy-arachidonate formation

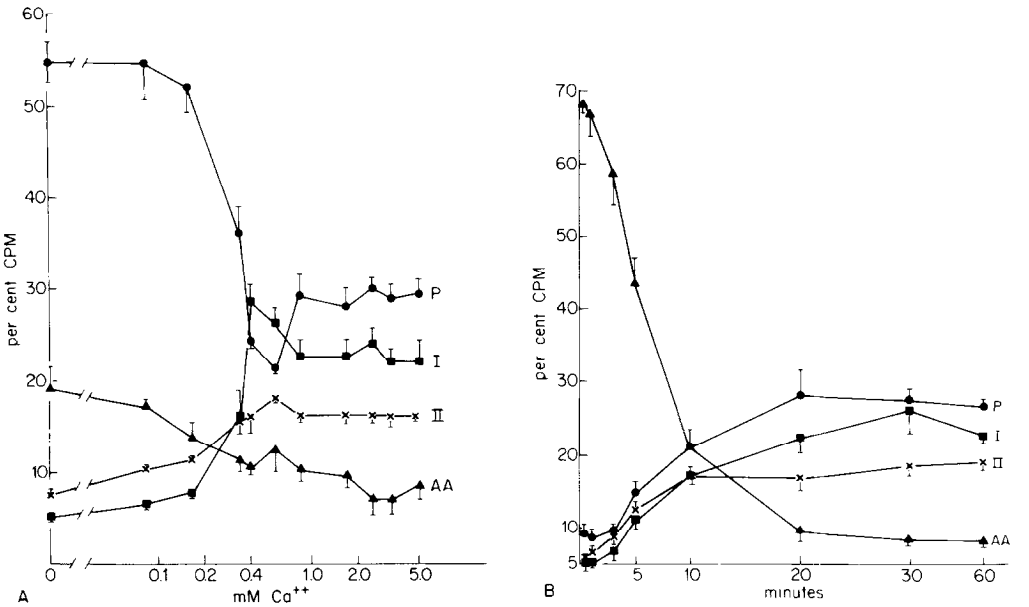

Fig. 1: Calcium Dependence (A) and Time Course (B) of the Formation of Hydroxyeicosatetraenoates. 10,000 x g supernatant (0.5 ml) of RBL-1 cell homogenates was incubated with [^{14}C]arachidonic acid (200,000 cpm). The reaction was stopped by the addition of 1 ml of acetone, 0.5 ml of cold saline and 2 N formic acid to adjust the pH to 3.2 to 3.5. This mixture was extracted twice with 2 ml of chloroform. Thin layer chromatography was performed in solvent system A9 the organic phase of ethyl acetate:2,2,4-trimethylpentane:acidic acid:water (110:50:20:100) (11). Standards were visualized by iodine staining. Radioactive peaks were located by fluorography (12). Quantitation was achieved by scraping and liquid scintillation counting. P, designates the polar material that remained at the origin; I, consists mainly of 5,12-DiHETE; II consists of two bands which were scraped together since they were too close to achieve accurate separation. The upper band comigrated with authentic 5-HETE. Gas chromatography - mass spectrometry of the compounds eluted from this area only showed evidence for 5-HETE. Therefore, the two bands may represent two isomers of 5-HETE. The bars designate SEM. n=5-11. A. Incubations were performed at the calcium concentrations indicated for 20 min at 37°C. B. Incubations were performed with 2 mM calcium for the times indicated at room temperature.

was observed when magnesium (same range of concentrations as calcium) was added to the incubation mixture (data not shown). No products were formed when boiled supernatant was incubated with calcium and [^{14}C]arachidonic acid. These data, therefore, indicate that RBL-1 cells have a calcium dependent lipoxygenase. Since it is very likely that some calcium is chelated by EDTA or EGTA during homogenization, it is difficult to calculate the actual amount of calcium needed to activate the enzyme. When platelet 8,000 x g supernatant (1 mM EDTA, 14 μM indomethacin) was incubated with [^{14}C]arachidonate without and with calcium no such calcium dependence of the lipoxygenase was observed (Fig. 2B).

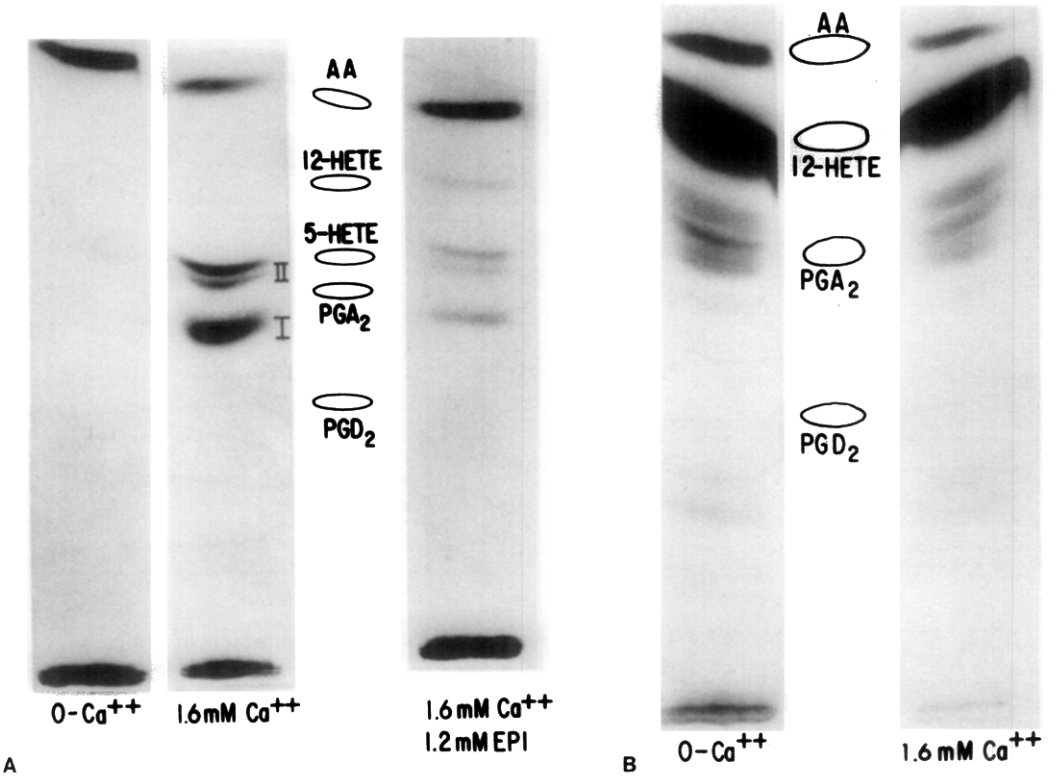

Fig. 2: Radioautogram of Thin Layer Chromatograms of Lipoxygenase Products. A. 10,000 x g supernatant (0.5 ml) of RBL-1 homogenates was incubated with [^{14}C]-arachidonic acid (200,000 cpm) for 20 min, 37°C with the agents indicated and processed as described in legend of Fig. 1. B. 8,000 x g supernatant (1 ml) of platelet lysates was incubated with [^{14}C]AA (500,000 cpm) and processed as described for RBL-1 supernatant. EPI: L-epinephrine; PG: prostaglandin; I, II and P: see legend of Fig. 1.

As can be seen in Fig. 1A, calcium had two major effects on the metabolism of [^{14}C]arachidonic acid by RBL-1 10,000 x g supernatant. As discussed above, calcium increased hydroxy acid formation. Secondly, it decreased the formation of polar [^{14}C]AA-derived material (P), again in a concentration dependent fashion. This polar material remained close to the origin in the thin layer chromatographic system where phospholipids are usually found. Preliminary experiments indicate that phospholipids are present in this band. It, therefore, appears that calcium might inhibit the incorporation of arachidonic acid into or cause its release from phospholipids. However, this decrease in the polar material might also be due to the greater affinity of arachidonic acid for the lipoxygenase as compared to the enzyme(s) forming the polar material. Since

some arachidonate was left unreacted, the latter possibility is unlikely. Experiments are in progress characterizing this polar material and investigating its formation. The observed effect of calcium is of great interest since calcium is needed for the release reaction by basophils or mast cells to occur. The increase in intracellular calcium would therefore enhance the conversion of arachidonic acid to its hydroxy derivatives and make larger amounts of arachidonic acid available to the lipoxygenase.

The time course (Fig. 1B) indicates that all the compounds synthesized (I, II and P) seem to be formed in a parallel fashion. The time course was performed at room temperature in order to be able to follow the initial portion more readily. In preliminary experiments at 37°C the plateau was reached at approximately 5 min.

Epinephrine significantly inhibited (35.7±1.3%, n=5) this enzyme system (Fig. 2A). This is in contrast to its action on cyclooxygenase. It has been reported by a number of laboratories (14,15) including our own (10) that epinephrine markedly enhances cyclooxygenase activity. These different effects of epinephrine may indicate an opposing regulation by certain factors of the two enzyme pathways in the conversion of arachidonic acid within the cell.

Gas chromatographic - mass spectrometric analysis of the lipoxygenase compounds formed by the 10,000 x g supernatant of RBL-1 homogenates indicate that No. I in Fig. 1A consists mainly of 5,12-DiHETE and No. II of 5-HETE. A radioactive peak (gas chromatography) with the retention time of monoHETE exhibited a mass spectrum essentially identical to that of 5-HETE (Fig. 3). Three successive radioactive peaks between C-23 and C-25 with the same mass spectrum (Fig. 3) indicated the presence of isomers. The spectrum was identical to that described for three isomers of 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (16). Gas chromatography - mass spectrometry of the products of catalytic hydrogenation identified the two major components as methyl esters of 5-hydroxy- and 5,12-dihydroxyeicosanoate confirming the presence of 5-HETE and 5,12-DiHETE. Upon reverse phase HPLC the major 280 nm absorbing species resolved into a triplet (Fig. 4), resembling the chromatogram for 5,12-DiHETE (16). Gas chroma-

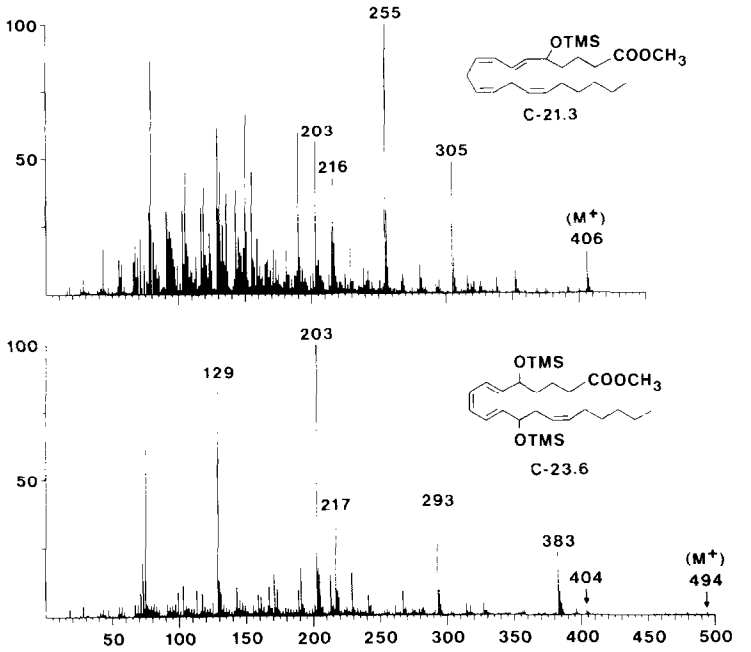

Fig. 3: Upper: Mass spectrum of the methyl ester trimethylsilyl (TMS) ether derivative of 5-hydroxyeicosatetraenoic acid (band II in Fig. 2). Lower: Mass spectrum of the methyl ester trimethylsilyl ether derivative of 5,12-dihydroxy-eicosatetraenoic acid (band I in Fig. 2).

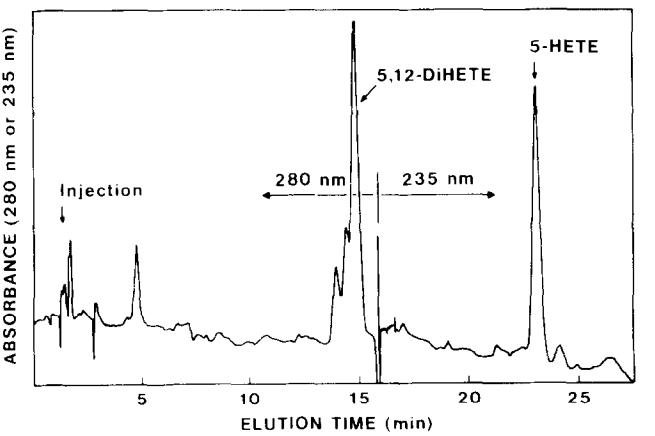

Fig. 4: High Performance Liquid Chromatogram of 5-HETE and 5,12-DiHETE. The methyl esters of the products were injected into a Varian Micropak MCH-10 column (Varian 5000). Actonitrile in water was delivered at a rate of 2.0 ml/min over a linear gradient of 40:60 to 75:25 in 60 min. The optical density of the effluent was monitored at 280 nm initially and changed to 235 nm after 16 min.

tography - mass spectrometry of these HPLC fractions confirmed the presence of three isomers of 5,12-DiHETE. A less polar peak which eluted at 23 minutes on HPLC was identified as 5-HETE. The indomethacin added proved to be a major interference in the analysis of 5,12-DiHETE. Indomethacin cochromatographed with 5,12-DiHETE in thin layer chromatography and HPLC and with one of the isomers in gas chromatography. A minor radioactive peak which appeared in the DiHETE region gave several characteristic mass ions (m/e 171, 203 and 291) of 5,6-DiHETE (16). A fraction which eluted from the silicic acid column prior to 5,12-DiHETE and 5-HETE contained 12-hydroxyicosatetraenoic acid (m/e 295).

Earlier we have shown that RBL-1 cells produce SRS from arachidonic acid via a lipoxygenase pathway (5). The work reported in this paper indicates that besides SRS, RBL-1 cells synthesize 5,12-DiHETE and the unstable precursor, 5,6-oxido-7,9,11,14-eicosatetraenoic acid as indicated by the presence of its spontaneous breakdown product 5,6-DiHETE. Therefore, RBL-1 cells have the complete enzyme system to form leukotrienes (3). The calcium dependence of this pathway in basophils suggests a close relationship between the release reaction of these cells and leukotriene formation.

ACKNOWLEDGEMENT

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