Vol. 118, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1984 Pages 629-635

CALMODULIN ANTAGONISTS INHIBIT FORMATION OF PLATELET-ACTIVATING FACTOR IN STIMULATED HUMAN NEUTROPHILS

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Received December 9, 1983

SUMMARY: Human polymorphonuclear leukocytes in the presence of the Ca^{2+} ionophore A23187 plus Ca^{2+} incorporated exogenously-added [^{3}H]acetate or 1-O-[^{3}H]-alkyl-sn-glycero-3-phosphocholine (lysoplatelet-activating factor) into platelet-activating factor (PAF,1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine). The incorporation of these radiolabels into PAF by stimulated neutrophils was inhibited by the calmodulin antagonists, trifluoperazine and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide. These two drugs were active at concentrations similar to their respective binding constants to purified calmodulin, suggesting a possible involvement of calcium-activated calmodulin in PAF biosynthesis.

Platelet-activating factor (PAF)[1](1), a phospholipid mediator of acute allergic responses (2) is produced and released by a variety of proinflammatory cells in response to immunologic and nonimmunologic challenges (3-5).The structure of PAF has been elucidated as 1-O-alkyl-2-acetyl-GPC (6-8). In neutrophils (9) and macrophages (10), the biosynthesis of PAF appears to involve two sequential enzymatic steps: 1) activation of phospholipase A_2 which acts on pre-existing 1-O-alkyl-2-acyl-GPC to increase the levels of precursor 1-O-alkyl-GPC (lysoPAF) and 2) stimulation of an acetyltransferase activity which transfers acetate from acetylCoA to lysoPAF to produce PAF. Acetyltransferase appears to be the rate-limiting enzyme (10) and requires Ca^{2+} for activity (10,11).

It is now apparent that most of the Ca^{2+} regulation in eukaryotes is mediated by the intracellular calcium-receptor calmodulin (12). Specific calmodulin antagonists have been used to assess the possible involvement of calmodulin in the responses of intact cells to stimuli (12). This report

[1]The abbreviations are: PAF, platelet-activating factor (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine); GPC, sn-glycero-3-phosphocholine; PMN, polymorphonuclear leukocyte; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid; TFP, trifluoperazine; W7, N-(6-aminohexyl)-5-chloro-1-naphthaline sulfonamide; EGTA, ethylene glycol-bis (β-aminoethylether) N,N,N',N'-tetraacetic.

0006-291X/84 $1.50

documents that ionophore A23187-induced formation of PAF in human PMN's can be conveniently followed by using radiolabeled acetate or lysoPAF. In addition, these studies reveal that ionophore A23187-induced incorporation of these radiolabels into PAF is potently inhibited by low concentrations of two specific calmodulin antagonists, trifluoperazine (13) and W7 (14), indicating a possible role of calmodulin in PAF formation by activated neutrophils.

MATERIALS AND METHODS

Materials: [^{3}H]acetic$_3$acid, Na-salt$_3$(2 Ci/mmol) was from New England Nuclear, Boston, MA. 1-O-[^{3}H]alkyl-GPC([^{3}H]lysoPAF, 90 Ci/mmol) was from Amersham, Arlington Heights, IL. Ionophore A23187 and PAF were purchased from Calbiochem, La Jolla, CA. N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7) was kindly provided by Dr. Mike Green, Schering Corp., Bloomfield, N.J. Silica gel G plates were from Brinkman Instruments, Westbury, N.Y. All the other reagents were from Sigma, St. Louis, MO.

Preparation of human PMN's (15): Freshly drawn human blood containing citrate/dextrose anticoaugulant was mixed with 0.1 vol of 6% solution of dextran T500 in saline and the erythrocytes were allowed to settle for 30 min. at 37°C. Ten ml aliquots of supernatant plasma containing leukocytes and platelets were layered over 9 ml of Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) in 50 ml polycarbonate centrifuge tubes (Nalge, Rochester, N.Y.) and centrifuged at 200 x g for 45 min. at 4°. The pellets were suspended in ice-cold HEPES-saline buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM dextrose and 0.5 mM EGTA) and then diluted 6-fold with water to lyse contaminating erythrocytes. Granulocytes were pelleted at 200 x g for 5 min., the erythrocyte stroma was aspirated and discarded, and the neutrophil pellet was washed once and resuspended in HEPES-saline buffer, pH 7.4. Cell viability was determined by trypan blue exclusion and was consistently found to be over 95%. Cell counts were obtained using a Neubauer chamber. Appropriate dilutions were made to obtain a final concentration of 10^7 cells/ml.

Incubation Conditions: To a 0.4 ml aliquot of the neutrophil suspension was added 0.1 ml of HEPES-saline buffer containing $CaCl_2$ (7.5 mM) and [^{3}H]acetate (20 μCi) or [^{3}H]lysoPAF (0.5-1 μCi). After 2 min at 37°C, ionophore A23187 (0.5 μM) was added for different periods of time or different concentrations of A23187 were added for a fixed period. Preincubations with inhibitors were performed for 1-5 min. Ionophore A23187 was dissolved in ethanol. The final concentration of ethanol did not exceed 0.6% which had no effect on the incorporation studies.

Lipid extraction and thin-layer chromatography: Reactions were stopped by adding 1.9 ml of $CHCl_3$:CH_3OH:CH_3COOH (100:200:4, by vol.). Standard samples of sphingomyelin (10 μg) and lysophosphatidylcholine (10 μg) were added and the phases separated by adding 0.6 ml of $CHCl_3$ and 0.6 ml of H_2O(16). The lower $CHCl_3$ phase was transferred, washed once with 2 ml of CH_3OH:H_2O (10:9, v/v) and dried under a flow of nitrogen. Dried samples were dissolved in $CHCl_3$ and spotted on silica gel G plates. The plates were dried over anhydrous $CaSO_4$ for 90 min and then developed with $CHCl_3$:CH_3OH:CH_3COOH:H_2O (50:30:8:6, by vol.) (17). In this system, PAF chromatographs slower than sphingomyelin and separates well from lysophosphatidylcholine and all the other phospholipids (18). The lipids were localized by exposure to iodine vapors, and the appropriate areas containing PAF were scraped and counted in a liquid scintillation counter. All experiments presented are one of at least three giving similar results.

RESULTS

Incubation of human PMN's with the Ca^{2+}-ionophore A23187 plus Ca^{2+} induced incorporation of exogenously-added [^{3}H]acetate or [^{3}H]lysoPAF into PAF (Fig. 1). With [^{3}H]acetate as the radiolabel, a peak of radioactivity was found to comigrate with standard PAF by thin-layer chromatography. However, with [^{3}H]lysoPAF as the radiolabel, an additional radioactive spot was found to be associated with phosphatidylcholine. While [^{3}H]lysoPAF incorporation into PAF area was ionophore-dependent, that into phosphatidylcholine area was observed in both the control and the ionophore-treated cells. These results are indicative of a rapid uptake of [^{3}H]lysoPAF by human PMN's and its subsequent metabolism by both acetylation and acylation to produce [^{3}H]PAF and 1-O-[^{3}H]alkyl-2-acyl-GPC respectively (see also ref. 9).

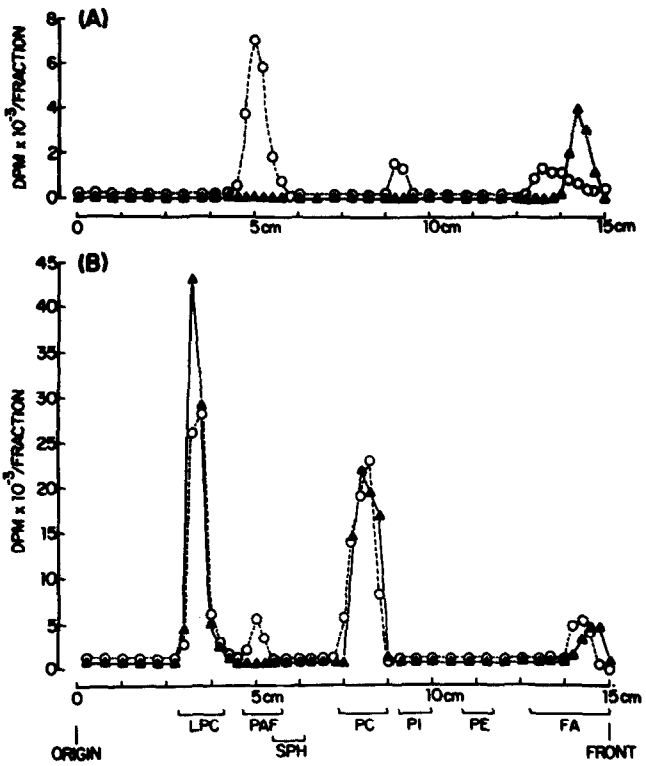

Fig. 1 Incorporation of [^{3}H]acetate or [^{3}H]lysoPAF into phospholipids by human neutrophils. Samples (0.5 ml) containing $4x10^6$ cells and 1 mM $CaCl_2$ were preincubated with 20 μCi of [^{3}H]acetate (Panel A) or 0.5 μCi of [^{3}H]lysoPAF (Panel B) for 2 min before adding either buffer (▲) or 1 μM A23187 (○) for 10 min. Extracted lipids were separated by thin-layer chromatography using $CHCl_3$:CH_3OH:CH_3COOH:H_2O(50:30:8:6, by vol.). Phospholipids were visualized by iodine vapors and 0.5 cm regions of the chromatograms were scraped and counted in a liquid scintillation. LPC, lysophosphatidylcholine; SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; FA, fatty acids.

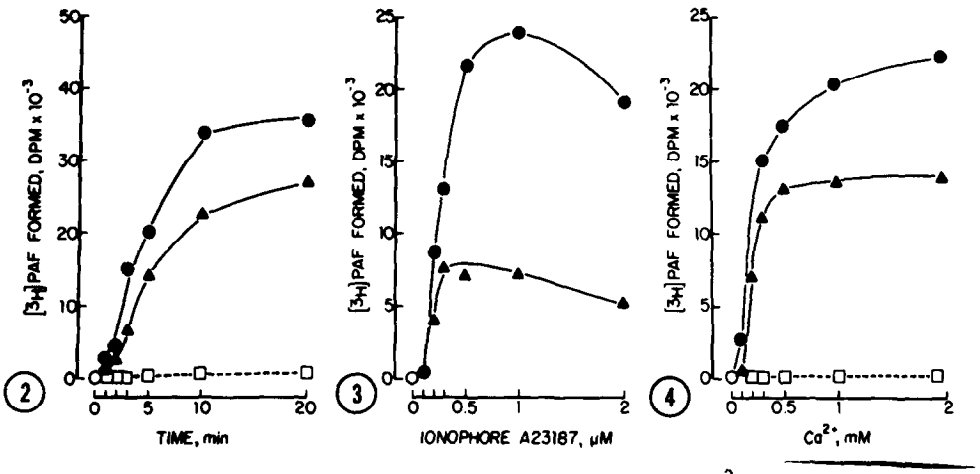

Fig. 2 (left) Time course of A23187-induced incorporation of [^{3}H]acetate or [^{3}H]lysoPAF into PAF by human neutrophils. Samples (0.5 ml) containing $4x10^6$ cells and 1 mM Ca^{2+} were preincubated with either 20 μCi of [^{3}H]acetate (●) or 0.5-1.0 μCi of [^{3}H]-lysoPAF (▲) for 2 min before adding either buffer (□) or 0.5 μM A23187 (●,▲) for different periods of time. Other conditions are as in 'Materials and Methods'.

Fig. 3 (center) Effect of A23187 concentrations on the incorporation of [^{3}H]-acetate or [^{3}H]lysoPAF into PAF by human neutrophils. Different concentrations of A23187 were added for 5 min in the presence of 1mM Ca^{2+}. For other conditions and symbols, see Fig. 2.

Fig. 4 (right) Effect of Ca^{2+} on the incorporation of [^{3}H]acetate or [^{3}H]lysoPAF into PAF by human neutrophils. A23187 (0.5 μM) was added for 5 min in the presence of 0.5 mM EGTA (and no added Ca^{2+}) or different concentrations of Ca^{2+}. Other conditions and symbols are as in Fig 2.

In human PMN's, ionophore A23187 induced the incorporation of [^{3}H]acetate or [^{3}H]lysoPAF into PAF in a time and dose-dependent manner (Fig. 2 and 3). In the presence of the Ca^{2+}-chelator, EGTA (and no added Ca^{2+}) the ionophore was ineffective. The formation of [^{3}H]PAF by ionophore-treated PMN's increased with increasing concentrations of Ca^{2+} reaching a maximum at 1 mM (Fig. 4).

Trifluoperazine (TFP) and other phenothiazines are known to bind to Ca^{2+}-calmodulin complexes and thereby block their action on target enzymes (13). W7, a naphthalene sulfonamide derivative represents another distinct class of compounds which also inhibit calmodulin-mediated reactions by interacting with Ca^{2+}-calmodulin complexes (14). As illustrated in Fig. 5, TFP and W7 inhibited [^{3}H]acetate incorporation into PAF in a dose-dependent manner. The IC_{50} values for these drugs were dependent on several factors including concentration of the ionophore, time of incubation with the ionophore and concentration of extracellular Ca^{2+}. The IC_{50} values increased when ionophore concentrations were increased, incubation time was prolonged (Table 1) or extracellular Ca^{2+} concentrations were increased

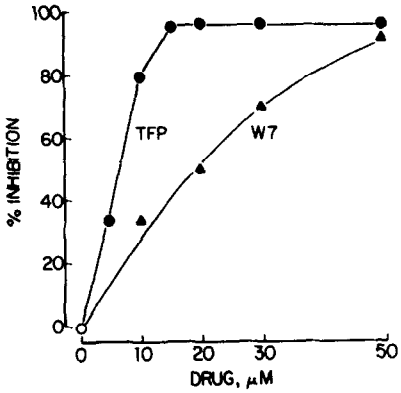

Fig. 5 Effects of TFP and W7 on the incorporation of [^{3}H]acetate into PAF by stimulated human neutrophils. Samples (0.5 ml) containing 4×10^6 cells and 1 mM Ca^{2+} were preincubated with [^{3}H]acetate (20 μCi) and different concentrations of TFP (●) or W7 (▲) for 1 min before adding either buffer or A23187 (0.5 μM) for 3 min. Other experimental conditions are as in "Materials and Methods".

(not shown). TFP and W7 also inhibited the incorporation of [^{3}H]lysoPAF into PAF by ionophore-stimulated neutrophils (Fig. 6). With either radioactive precursor of PAF, the degree of inhibition by these drugs was the same. The antimalarial drug quinacrine has been shown to inhibit phospholipase A_2 activity in platelets (19) and the formation and release of PAF in macrophages (10). Quinacrine also inhibited the incorporation of both [^{3}H]acetate and [^{3}H]lysoPAF into PAF by human neutrophils although this compound was much less potent than TFP or W7 (Fig. 6).

TFP and W7, at concentrations at or above 20 μM and 50 μM respectively, have been shown to cause lysis of rabbit neutrophils (20). However, in the present experiments, incubation of human PMN's with 50 μM TFP for 10 min. had little or no effect on cell viability as judged by trypan blue exclusion.

DISCUSSION

The present results clearly indicate that in human PMN's, ionophore A23187 induces a rapid incorporation of [^{3}H]acetate or [^{3}H]lysoPAF into

TABLE I
Inhibition by calmodulin antagonists of PAF formation in human PMN.

A23187 conc. (μM)	Incubation with A23187 (min)	IC_{50} values (μM) TFP	W7
0.5	3	7	20
0.5	7	10	30
5.0	3	13	--
5.0	7	27	57

Experimental conditions are as in Fig. 5.

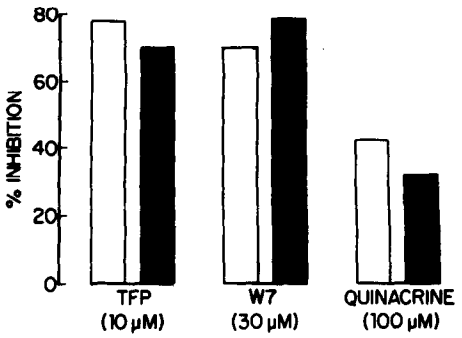

Fig. 6 Effects of various drugs on the incorporation of [^{3}H]acetate or [^{3}H]lysoPAF into PAF by stimulated human neutrophils. Experimental conditions are as in Fig. 5. Open bars, [^{3}H]acetate incorporation. Solid bars, [^{3}H]lysoPAF incorporation.

PAF. Similar observations have recently been reported for rabbit peritoneal neutrophils (9) and rabbit platelets (21). It may thus be concluded that the incorporation of these radiolabels into PAF is a valid measure of PAF formation in stimulated neutrophils.

Ca^{2+} is obligatory for PAF biosynthesis (9-11) and calmodulin has been detected in human neutrophils (22). The results of the present study indicate that specific calmodulin antagonists of two chemically distinct types block PAF formation in human PMN (Fig. 5 and 6). Importantly, both TFP and W7 are active at concentrations (Table I) similar to their respective binding constants to purified calmodulin (13,14). Furthermore, these drugs have been shown not to inhibit chemotactic factor-induced uptake of $^{45}Ca^{2+}$ into rabbit PMN (20). Taken together, these results strongly suggest that PAF formation in human neutrophils is mediated by calcium-activated calmodulin.

In most tissues, stimulus-sensitive phospholipase A_2 is Ca^{2+}-dependent and recent reports suggest an involvement of calmodulin in phospholipase A_2 activation (12,23). Whereas the Ca^{2+}-dependency of acetyltransferase has been established (10,11), such information on the phospholipase A_2 that acts on 1-O-alkyl-2-acyl-GPC has not yet been obtained. Nevertheless, it may be argued that calmodulin antagonists inhibit phospholipase A_2 activity, thereby limiting the availability of precursor lysoPAF and thus inhibiting [^{3}H]acetate incorporation into PAF. However, since these drugs also inhibit the incorporation of [^{3}H]lysoPAF into PAF to the same extent as they do [^{3}H]acetate incorporation (Fig. 6), these observations suggest that calmodulin antagonists can act at the level of acetyltransferase activity as well.

Notably, several cellular proteins, in addition to calmodulin, have been shown to bind TFP or W7 in a Ca^{2+}-dependent manner (24). Thus,

criteria other than drug inhibition are needed to confirm the involvement of calmodulin in the mechanisms of PAF formation in stimulated neutrophils.

ACKNOWLEDGEMENTS

We are grateful to Ms. Cora Atienza for technical assistance and Ms. Carol Shave for editorial assistance. We thank Dr. Robert Bryant for many helpful discussions.

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