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Human polymorphonuclear neutrophil activation with arachidonic acid

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1 The capacity of arachidonic acid (AA) to stimulate granule exocytosis from human polymorphonuclear neutrophils (PMNs) was investigated.

2 AA induced the selected extracellular release of azurophil (myeloperoxidase, lysozyme) and specific (lysozyme, vitamin B_{12} binding protein) granule constituents from human PMNs in a time- and concentration-dependent manner.

3 Cytochalasin B (CB) enhanced but was not required for PMN activation with AA.

4 Although extracellular calcium had no effect on granule exocytosis, AA did stimulate the mobilization of intracellular sequestered Ca^{2+} which resulted in an increase in cytosolic-free Ca^{2+} ($[Ca^{2+}]_i$) as reflected by increased fluorescence of Fura-2-treated cells.

5 AA stimulated Ca^{2+}/phospholipid-dependent protein kinase C (PK-C) activity in PMNs.

6 4,4'-Diisothiocyano-2,2'-disulphonic acid stilbene (DIDS), an anion channel blocker, caused a concentration-dependent inhibition of granule enzyme release.

7 Activation of PMNs with AA was unaffected by the lipoxygenase/cyclo-oxygenase inhibitors, 5,8,11, 14-eicosatetraynoic acid (ETYA) and benoxaprofen, a lipoxygenase inhibitor, 6, 9, deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin 1_1 (piriprost potassium) or a pure cyclo-oxygenase inhibitor, flurbiprofen.

8 These data define the properties of AA as a secretory stimulus for human PMNs.

Introduction

A hallmark of inflammatory reactions is the presence of polymorphonuclear neutrophils (PMNs) releasing their granule constituents into the surrounding tissues in response to various stimuli. The mechanism of stimulus-response coupling described for PMN activation involves the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Berridge, 1986; Smith *et al.*, 1985; Smith *et al.*, 1986) which results in the generation of two second messengers, inositol-1,4,5-trisphosphate and 1,2-diacylglycerol (Berridge, 1984; Berridge & Irvine, 1984) which mediate the mobilization of intracellular Ca^{2+} (Dougherty *et al.*, 1984; Berridge & Irvine, 1984; Prentki *et al.*, 1984) and phospholipid-dependent protein kinase (PK-C) activation (Nishizuka, 1984), respectively. PMNs are capable of generating metabolites of arachidonic acid (AA) lipoxygenation such as 5(**S**),12(**R**)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid (LTB_4) (Payan *et al.*, 1984), which stimulates these cells to express various functions (Payan *et al.*, 1984; Smith *et al.*, 1984a). To the extent that lipoxygenase inhibitors suppress neutrophil activation (Naccache *et al.*, 1979; Smolen & Weissmann, 1980; Duque *et al.*, 1986; Smith *et al.*, 1982a; 1986a,b), products of AA lipoxygenation have also been implicated in the PMN activation pathway. However, AA itself has been demonstrated to induce PMN aggregation (O'Flaherty *et al.*, 1979) and oxygen radical production (Badwey *et al.*, 1981; 1984). Therefore, we have investigated the capacity of AA to stimulate human PMN degranulation.

Methods

Purification of human PMNs

Blood from normal human donors was drawn by venipuncture into 0.1 volume of 3.8% citrate in conical plastic tubes. PMNs were purified by standard techniques of dextran sedimentation, centrifugation

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on Hypaque-Ficoll, and hypotonic lysis of erythrocytes. Final cell suspensions contained a minimum of 98% PMNs and cell viability was always greater than 98% as determined by trypan blue exclusion.

Incubation conditions

PMNs suspended in physiological solution (PBS) pH 7.4, containing (mM): NaCl 138, Na_2HPO_4 8.1, KH_2PO_4 1.5, KCl 2.7, $CaCl_2$ 0.6, $MgCl_2$ 1.0 and 0.1% glucose were incubated at 37°C in a Dubnoff shaker according to the various procedures described in the Results section. After incubation, the samples were centrifuged at 750 *g* (4°C), and the clear supernatants were assayed for enzyme (myeloperoxidase, lysozyme) and vitamin B_{12}-binding protein activities. For the kinetic studies, the incubations were terminated by addition of 100 μM N-ethylmaleimide to the reaction vessels. The net percentage release of enzyme and vitamin B_{12}-binding protein activities was calculated by subtracting the percentage release in buffer or vehicle from that attributable to a given test agent. The release of granule constituents from PMNs is expressed as the percentage of total activity released by 0.2% Triton X-100 in simultaneously run duplicate reaction mixtures.

Measurement of Fura-2 fluorescence

PMNs were suspended in HEPES buffer, pH 7.4 containing (mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.29, $MgCl_2$ 1.0 and HEPES 10. Fura-2 loading and fluorescence measurements were performed according to a modification of the method of Grynkiewicz *et al.* (1985). PMNs (10^8 ml^{-1}) in HEPES buffer were loaded with 10 μM Fura-2/acetoxymethyl ester (A/M) and incubated for 5 min at 37°C. The cells were then diluted 10 fold and incubated at 37°C for an additional 20 min. After loading, the cells were centrifuged at 150 *g* for 8 min and resuspended in HEPES buffer at a final concentration of 10^7 ml^{-1}. For fluorescence measurements, 2.9 ml of PMNs (containing 7.5×10^6 cells) were placed in a thermostated 1 cm^2 quartz curvette. AA was added to the cell suspensions in 100 μl aliquots via a direct injection system using a Hamilton syringe to bring the final volume to 3 ml. Fluorescent changes were monitored without stirring at 37°C with an excitation wavelength of 360 nm and emission wavelength of 510 nm utilizing a SLM 4800S fluorescence spectrophotometer.

Enzyme and vitamin B_{12}-binding protein assays

Myeloperoxidase (MPO:EC1.11.1.7), lysozyme (EC3.2.1.17) and lactate dehydrogenase (LDH:EC1.1.1.27) activities were determined as previously described (Henson *et al.*, 1978; Smith & Iden, 1979; Bergmeyer *et al.*, 1965). Vitamin B_{12}-binding protein (B_{12}-BP) activity was assessed according to a modification of the method of Gottlieb *et al.* (1965).

Preparation of PMN-associated protein kinase C

PMNs (~9×10^8) were resuspended in 10 ml 25 mM Tris, pH 7.5 containing 5 mM dithiothreitol (DTT), 2 mM EGTA, 0.1% Triton X-100, 1 mM phenylmethylsulphonyl fluoride and 100 μg ml^{-1} leupeptin. The suspension was sonicated for 3–15 s bursts (on ice) with a Vibra Cell sonicator (Sonics and Materials, Danbury, CT) followed by incubation for 60 min at 4°C. The homogenate was centrifuged at 100,000 *g* for 60 min. The resulting supernatant was purified by DEAE cellulose chromatography on 0.6 ml Econo columns (Bio-Rad) equilibrated with column buffer (25 mM Tris, pH 7.5, 5 mM DTT, 2 mM EDTA and 0.5 mM EGTA), washed with 10 ml column buffer, and eluted with 1 ml column buffer containing 100 mM NaCl. The eluate (referred to as PMN-DEAE) was used as the source of PK-C activity, and was stored at 4°C.

Protein kinase C assay

The PK-C assay contained, in a total volume of 50 μl: 0.75 mM $CaCl_2$, 2 mM DTT, 7.5 mM Mg acetate, 750 μg ml^{-1} histone IIIS (Sigma), 11 μg PMN-DEAE, 25 μM ^{32}P-ATP (80–120 c.p.m. pmol^{-1}) and 1 μg ml^{-1} phosphatidylserine (PS). In the experiments containing PS, aliquots of the lipids (PS in chloroform) were dried under a stream of nitrogen, resuspended in 25 mM Tris, pH 7.5, and sonicated for two 20 s bursts at 50% output with a Vibra Cell sonicator. AA was dissolved in methanol, and PMA was dissolved in dimethylsulphoxide (DMSO) and both solutions were diluted with 25 mM Tris before addition to the assay. The kinase assay tubes were incubated for 5 min at 30°C, placed on ice, and a 25 μl aliquot from each tube was spotted on phosphocellulose paper (Whatman). The paper was washed three times in water, once in acetone, dried, and the filters were counted in 4 ml ACS (Amersham) in a Packard TriCarb Model 4660 for 1 min. PK-C activity is expressed as pmol ^{32}P incorporated min^{-1} mg^{-1} protein.

Materials

AA (Nu-Chek-Prep., Inc., Elysian, MN) and cytochalasin B (Aldrich Chemical Company, Milwaukee, WI) were dissolved in methanol and ethanol, respectively; 8-eicosenoic acid, 11-eicosenoic acid, 11,14-eicosenoic acid, 8,11,14-eicosenoic acid, γ-linolenic acid, linoleic acid, oleic acid, myristic acid, and

palmitic acid (Nu-Check-Prep) were dissolved in methanol. Stearic acid (Nu-Check-Prep) and phorbol 12-myristate 13-acetate (LC Services Corporation, Woburn, MA) were prepared in ethanol and DMSO, respectively. 4,4'-Diisothyocyano-2,2'-disulphonic acid stilbene (DIDS, Sigma Chemical Company, St Louis, MO) and Fura-2/A/M (Molecular Probes, Inc., Junction City, OR) were dissolved in PBS and DMSO, respectively. ETYA, piriprost potassium, flurbiprofen (The Upjohn Company) and benoxaprofen (Eli Lilly and Company, Indianapolis, IN) were prepared in DMSO. The small amounts of ethanol, methanol and DMSO (final concentration of 0.05–0.1%) employed as vehicles did not alter cell viability or granule exocytosis.

Results

Arachidonic acid-induced human PMN degranulation

The data in Figure 1a and b show AA to stimulate a time-and concentration-dependent release of MPO and B_{12}-BP from CB-treated PMNs. The kinetics of granule exocytosis indicate B_{12}-BP release to be more rapid than that of MPO with the maximum rates of release occurring approximately 5 (B_{12}-BP) and 15 (MPO) min after contact with AA (Figure 1a). Higher concentrations of AA did not induce the release of quantities of B_{12}-BP and MPO which exceeded those demonstrated for 67 μM AA. Therefore, the EC_{50}s (concentrations of AA required to elicit 50% of the maximal secretory response) are approximately 20 and 40 μM for B_{12}-BP and MPO, respectively (Figure 1b).

The release of less than 6% of total cytoplasmic LDH activity under the defined experimental conditions demonstrates AA-induced PMN degranulation to be a selective, noncytotoxic process. Cohen *et al.* (1986) reported human PMNs to discharge significant amounts of LDH (> 20% of total cell activity) after exposure for 10 min to AA (~42 μM). We suggest that our PMN preparations are less sensitive to AA in that we find comparable LDH release (as measured with a continuous spectrophotometric method) to occur following 10 to 15 min of cell contact with 80 to 100 μM AA. In further contrast to the observations of Cohen *et al.* (1986), we have found that LDH release from AA-stimulated PMNs is not enhanced in the absence of extracellular Ca^{2+} or in the presence of EGTA.

Granule exocytosis from human PMNs stimulated with congeners of arachidonic acid

8-Eicosenoic acid, 11-eicosenoic acid, 11,14-eicosenoic acid and 8,11,14-eicosenoic acid initiated a concentration-dependent release of B_{12}-BP but not MPO from PMNs (Figure 2). All four unsaturated fatty acids were less effective than AA in stimulating degranulation.

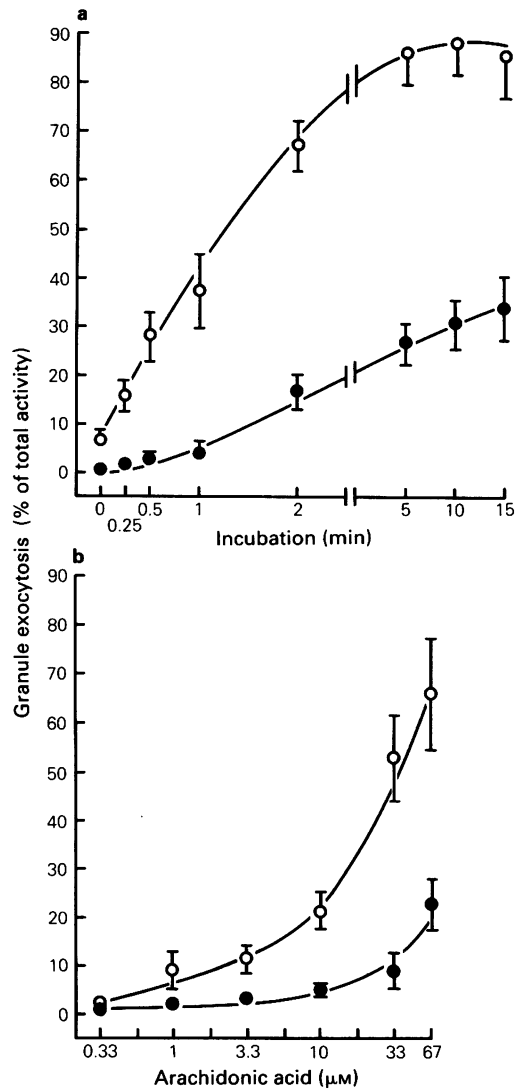

Figure 1 Granule exocytosis from human PMNs stimulated with arachidonic acid (AA) versus (a) time of incubation and (b) concentration of AA. PMNs (5×10^6) were preincubated with cytochalasin B ($5\,\mu g\,ml^{-1}$) for 10 min and either incubated with AA (67 μM) for the time periods indicated or exposed to various concentrations of AA for 15 min. Total cell activity for the respective granule constituents was: 0.690 ± 0.003 ΔABS 460 nm for myeloperoxidase (●) and 1004.4 ± 90.5 ng vitamin B_{12}-binding protein per 5×10^6 cells for B_{12}-BP (○). Data represent the mean of three to four separate experiments run in duplicate; vertical lines show s.e.mean.

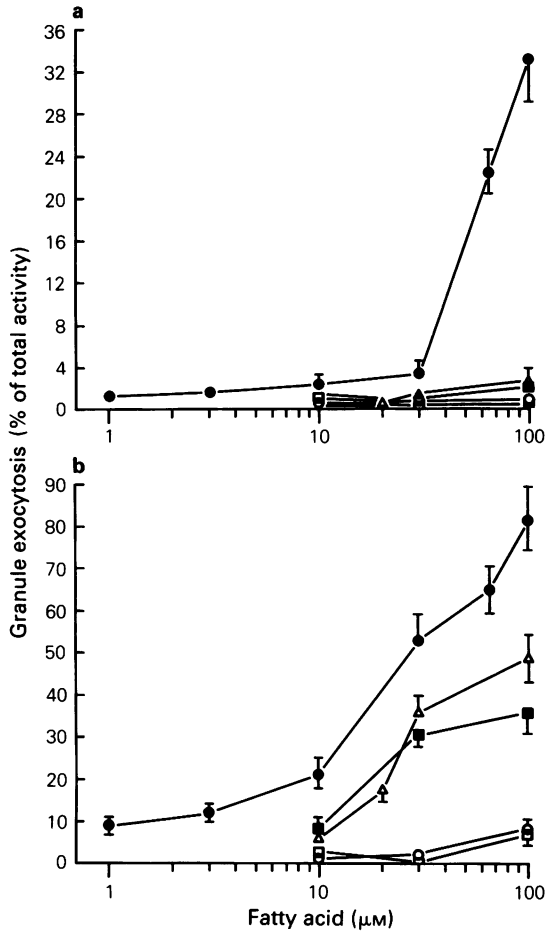

Figure 2 Granule exocytosis from human PMNs stimulated with congeners of arachidonic acid (AA). PMNs (5×10^6) were preincubated with cytochalasin B ($5\,\mu g\,ml^{-1}$) for 10 min followed by a 10 min incubation with the indicated concentrations of arachidonic acid (●), 8-eicosenoic acid (○), 11-eicosenoic acid (□). 11,14-eicosenoic acid (■) or 8,11,14-eicosenoic acid (△). Total cell myeloperoxidase lysozyme (MPO) (a) and vitamin B_{12}-binding protein (B_{12}-BP) (b) activities were: 0.964 ± 0.04 ΔABS 460 nm for MPO and 1026.4 ± 43.1 ng B_{12}-BP per 5×10^6 cells for B_{12}-BP. Data represent the mean of two to three separate experiments run in duplicate; vertical lines show s.e.mean.

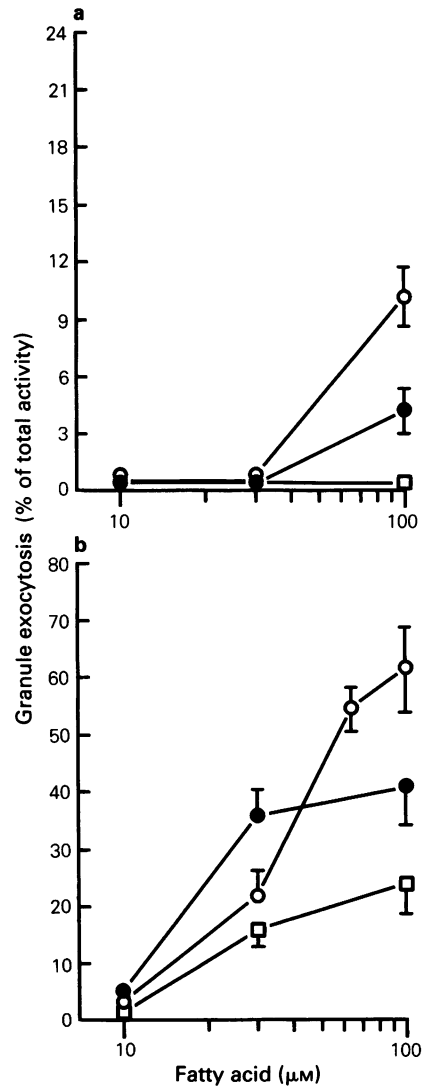

Figure 3 Granule exocytosis from human PMNs stimulated with *cis*-polyunsaturated fatty acids. PMNs (5×10^6) were preincubated with cytochalasin B ($5\,\mu g\,ml^{-1}$) for 10 min followed by a 10 min incubation with the stipulated concentrations of γ-linolenic acid (○), linoleic acid (●) or oleic acid (□): In (a) meyeloperoxidase and in (b) vitamin B_{12}-binding protein. Data represent the mean of three separate experiments run in duplicate; vertical lines show s.e.mean.

Human PMN activation with cis*-polyunsaturated fatty acids*

Gamma-linolenic acid, linoleic acid and oleic acid induced a concentration-dependent release of MPO and B_{12}-BP from PMNs (Figure 3). All three *cis*-unsaturated fatty acids were more effective in stimulating the release of B_{12}-BP than MPO.

Stimulation of human PMN degranulation with saturated fatty acids

Myristic acid induced the release of a marginal amount of B_{12}-BP from PMNs (Table 1). Palmitic acid

Table 1 Stimulation of human PMN degranulation with saturated fatty acids

Experimental Conditions[a]	*Conc* (μM)	*Granule exocytosis (% of total activity)*[b]	
		MPO	*B_{12}-BP*
Myristic acid	10	0[c]	0
	30	0	3.0 ± 2.10
	100	0	5.9 ± 3.47
Palmitic acid	10	0	0
	30	0	0
	100	0	1.6 ± 1.3
Stearic acid	10	0	0
	30	0	0
	100	0	0

[a] PMNs (5×10^6) were preincubated with cytochalasin B ($5\,\mu g\,ml^{-1}$) for 10 min followed by a 10 min incubation with the respective fatty acids.
[b] Total cell activity for the respective granule constituents was: 0.964 ± 0.04 ΔABS 460 nm for myeloperoxide (MPO) and 1026 ± 43.14 ng vitamin B_{12}-binding protein (B_{12}-BP) per 5×10^6 cells for B_{12}-BP.
[c] Data represent the mean ± s.e.mean of two to three separate experiments performed in duplicate.

and stearic acid had no effect on B_{12}-BP release and all three saturated fatty acids had no capacity to induce MPO release.

Influence of cytochalasin B on arachidonic acid-stimulated granule exocytosis from human PMNs

Preincubation of PMNs with CB before exposure to AA caused an enhancement of lysozyme and MPO release stimulated with the respective concentrations of AA (Figure 4).

Degranulation of arachidonic acid-activated human PMNs in the presence and absence of extracellular calcium

AA stimulated the release of 21.2 ± 3.5 and 20.6 ± 2.5% of total lysozyme from PMNs in the presence and absence of extracellular calcium (0.6 mM), respectively.

Incubation of PMNs with EGTA (2 mM) in calcium-free PBS during the entire incubation period had no effect on AA-stimulated lysozyme release (control = 18.9 ± 0.1 and EGTA = 20.7 ± 1.8% of total lysozyme activity released).

Arachidonic acid-induced changes in fluorescence of Fura-2-loaded human PMNs

AA stimulated a concentration-dependent increase in fluorescence of Fura-2-loaded PMNs (Figure 5) with a maximum fluorescent response occurring with 3 μM

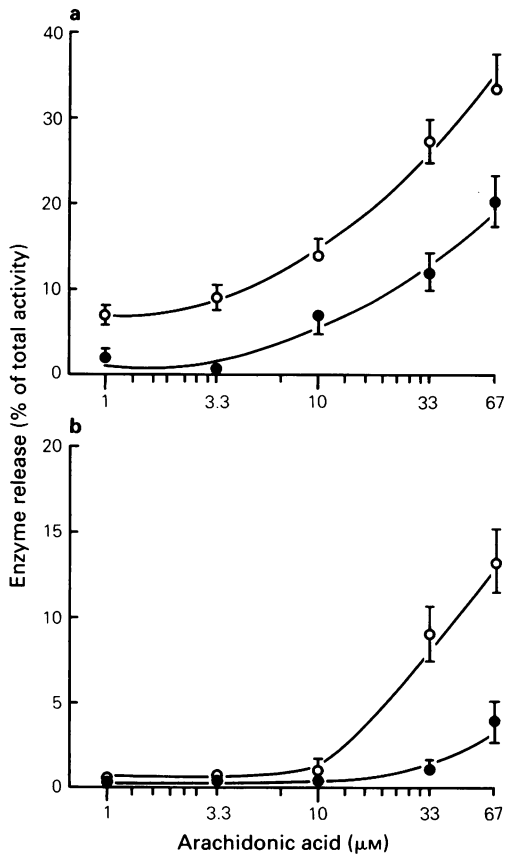

Figure 4 Arachidonic acid (AA)-induced granule enzyme release from human PMNs in the presence and absence of cytochalasin B (CB). Neutrophils (5×10^6) were preincubated with (○) or without (●) CB ($5\,\mu g\,ml^{-1}$) for 10 min followed by a 15 min incubation with the indicated concentrations of AA. Total cell enzyme activity was: 0.862 ± 0.01 ΔABS 460 nm for myeloperoxidase (b) and 28.1 ± 0.1 μg lysozyme std. $3\,min^{-1}$ per 5×10^6 cells for lysozyme (a). Data represent the mean of three separate experiments run in duplicate; vertical lines show s.e.mean.

AA. The fluorescent response of PMNs to AA, which reflects an increase in $[Ca^{2+}]_i$ is quite rapid with no measurable lag phase between the time of cell contact with AA and the production of a quantifiable increase in fluorescence.

Concentration-dependence of the effect of DIDS on arachidonic acid-stimulated human PMN degranulation

DIDS caused a concentration-related inhibition of AA-induced lysozyme release from PMNs (Figure 6).

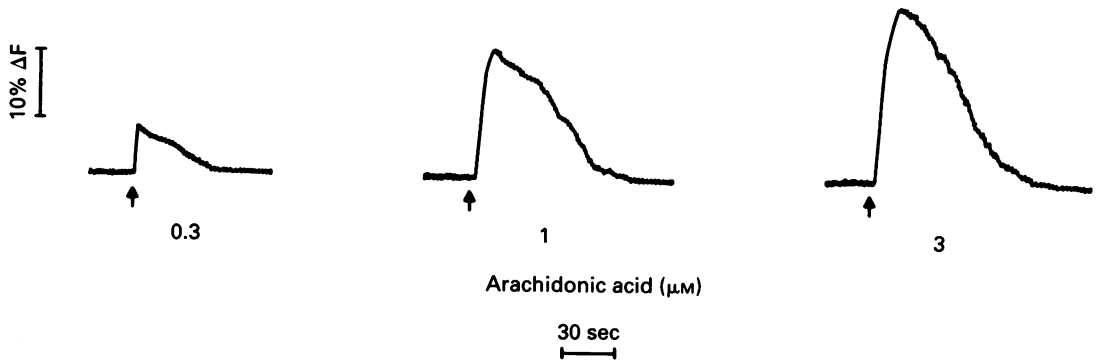

Figure 5 Concentration-dependence of the effect of arachidonic acid (AA) on the fluorescent response of Fura-2-loaded human PMNs. Fura-2-loaded PMNs (7.5×10^6) were preincubated with cytochalasin B (5 μg ml^{-1}) for 5 min at 37°C. The respective concentrations of AA were added at the arrows. The $[Ca^{2+}]_i$ in resting PMNs was 63 nM. The data constitute the results of a single experiment representative of a minimum of six separate experiments.

The IC_{50} (concentration exerting a 50% inhibitory effect on granule exocytosis) for DIDS was approximately 150 μM. DIDS had no effect on lysozyme itself.

Effect of arachidonic acid on soluble protein kinase C activity in human PMNs

AA caused a concentration-dependent stimulation of soluble PK-C activity in PMNs (Table 2). The concentrations of AA that activate PK-C are comparable to those that stimulate PMN degranulation. Phorbol myristate acetate, a well defined activator of PK-C, also stimulated PMN PK-C activity (PMA, 18.5 ng ml^{-1}: 1418 ± 71 vs control: 950 ± 69 pmol-min^{-1} mg^{-1} protein).

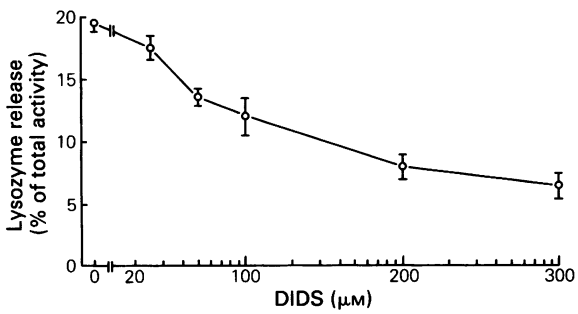

Figure 6 Concentration-dependence of the effect of 4,4'-diisothiocyano-2,2'-disulphonic stilbene (DIDS) on arachidonic acid (AA)-stimulated human PMN degranulation. PMNs (5×10^6) were preincubated with the stipulated cocentrations of DIDS and cytochalasin B (5 μg ml^{-1}) for 10 min followed by a 5 min incubation with AA (67 μM). Total cell lysozyme activity was: 23.8 ± 1.0 μg lysozyme std. 3 min^{-1} per 5×10^6 cells. Data represent the mean of three separate experiments run in duplicate; s.e.mean shown by vertical lines.

Effects of inhibitors of arachidonic acid metabolism on AA-elicited granule exocytosis from human PMNs

The cyclo-oxygenase/lipoxygenase inhibitors, ETYA and benoxaprofen, a lipoxygenase inhibitor, piriprost potassium and a cyclo-oxygenase inhibitor, flurbiprofen, had no effect on AA-stimulated degranulation by PMNs (Table 3).

Discussion

We show here that AA elicits granule exocytosis from human PMNs. Cytochalasin B enhances but is not required for the expression of a secretory response. In contrast to phorbol myristate acetate which induces the discharge of only specific granule constituents and is thus viewed as an incomplete secretagogue (Smith & Iden, 1979), AA stimulates the release of both azurophil and specific granules constituents and is, therefore, a complete secretagogue. The data indicate that B_{12}-BP release precedes the discharge of MPO (0–30 s following PMN exposure to AA) and that the rate of B_{12}-BP secretion clearly exceeds that of MPO during the first 5 min of cell contact with AA. This pattern of granule exocytosis wherein the discharge of specific granule constituents precedes the release of azurophil granule constituents is defined as sequential degranulation and has been described in PMNs activated with various secretagogues (Bentwood & Henson, 1980; Smith *et al.*, 1982b).

An investigation of the PMN stimulatory capacity of a series of structural analogues of AA revealed

Table 2 Effect of arachidonic acid (AA) on soluble protein kinase C activity in human PMNs[a]

Experimental conditions[a]	*PK-C activity* (pmol min^{-1} mg^{-1} protein)[b]
Arachidonic acid (μM)	
3	974 ± 69
10	984 ± 29
30	1476 ± 62
100	1818 ± 488

[a] Phospholipid-dependent protein kinase (PK-C) activity was assayed as described in Methods in the presence of the indicated concentrations of AA. Control PKC activity was 980 ± 170 pmol min^{-1} mg^{-1} protein.
[b] Data represent the average ± range of two experiments.

8-,11-,11,14-, and 8,11,14-eicosenoic acid to have no effect on MPO release whereas all four analogues did elicit B_{12}-BP release and the increased capacity to do so was correlated with the degree of unsaturation of these 20-carbon *cis*-unsaturated fatty acids (i.e., 8,11, 14 > 11,14 > 8 = 11-eicosenoic acid). Additional *cis*-polyunsaturated fatty acids were found to stimulate PMN degranulation and the respective secretory capacities of these fatty acids to elicit MPO and B_{12}-BP release were also correlated with the degree of unsaturation (i.e., γ-linolenic > linoleic > oleic acid). The requirement for unsaturation relative to secretory capacity is emphasized by our finding that myristic, palmitic and stearic acid which are 14-, 16-, and 18-carbon saturated fatty acids, respectively, did not stimulate granule exocytosis from PMNs. The ability of fatty acids to activate PMNs as a function of the degree of unsaturation has also been reported for superoxide anion production (Badwey *et al.*, 1981).

Table 3 Effects of inhibitors of arachidonic acid metabolism on arachidonic acid-elicited granule exocytosis from human PMNs

Experimental conditions[a]	*Conc* (μM)	*B_{12}-BP release (% of control)*[b]
ETYA	30	97.5 ± 1.8[c]
	100	87.6 ± 5.9
Piriprost potassium	30	97.4 ± 4.0
	100	89.4 ± 1.9
Benoxaprofen	30	95.4 ± 0.6
	100	83.4 ± 7.2
Flurbiprofen	30	96.1 ± 1.5
	100	93.4 ± 4.7

[a] PMNs (5×10^6) were preincubated with cytochalasin B (5 μg ml^{-1}) for 9 min followed by a one min incubation with or without the respective inhibitors. Cells were then incubated with arachidonic acid (67 μM) for 5 min at 37°C.
[b] Total cell vitamin B_{12}-protein binding (B_{12}-BP) activity was: 1136 ± 120.9 ng B_{12}-BP per 5×10^6 cells.
[c] Data represent the mean ± s.e.mean of three separate experiments performed in duplicate.

Although extracellular calcium is neither required for nor enhances AA-induced degranulation, the capacity of AA to stimulate a fluorescent response by PMNs loaded with fluorescent calcium indicator, Fura-2, reflects an increase in $[Ca^{2+}]_i$ which has been mobilized from a site of intracellular sequestration. The possibility that AA-induced Ca^{2+} mobilization was mediated by phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate with the release of inositol-1,4,5-trisphosphate would appear to be negated by the report that AA has no effect on phosphatidylinositol breakdown in PMNs (Volpi *et al.*, 1984). However, AA could facilitate Ca^{2+} mobilization via a direct effect on the intracellular Ca^{2+} storage site(s). In this regard, AA has been demonstrated to stimulate calcium release from the endoplasmic reticulum in isolated pancreatic islets (Wolf *et al.*, 1986), and the endoplasmic reticulum is believed to be one of perhaps several intracellular calcium storage sites in PMNs and other cell types (Burgess *et al.*, 1984; Suematsu *et al.*, 1985). Further, several of the aforementioned *cis*-unsaturated fatty acids have been found to stimulate Ca^{2+} release from the sacroplasmic reticulum, an intracellular Ca^{2+} storage site, in skeletal muscle (Cheah, 1981). Consistent with our data is the observation that AA increases the $[Ca^{2+}]_i$ in human (Lew *et al.*, 1984) and rabbit (Volpi *et al.*, 1984) PMNs and degranulation by rabbit PMNs (Naccache *et al.*, 1979). Thus $[Ca^{2+}]_i$ would appear to be a component of the activation pathway associated with AA stimulation of granule exocytosis. The observation that AA elicits Fura-2 fluorescence at subsecretory and marginal PMN activating concentrations implies that $[Ca^{2+}]_i$ represents but one component of the PMN signal transduction system. Protein kinase C (PK-C) is also believed to play a role in mediating the expression of functions by activated PMNs (Kajikawa *et al.*, 1983; Nishizuka, 1984) and our data show AA to stimulate PMN PK-C at concentrations that induce degranulation. In addition, γ-linolenic, linoleic and oleic acid have also been demonstrated to activate human PMN PK-C (McPhail *et al.*, 1984) and their activating potencies correlate with their capacities to induced granule exocytosis from PMNs. However, palmitic acid, which we have described as being inactive as a PMN secretory stimulus, does not activate PMN PK-C. These findings confirm and extend reports that AA stimulates PK-C from PMNs and other tissue (Leach & Blumberg, 1985; Murakami & Routtenberg, 1985) and further implicate PK-C in the mechanism of

stimulus-response coupling described for PMN activation with AA and other stimuli.

Reports from several laboratories have demonstrated anion channel blockers to inhibit PMN degranulation induced with various secretagogues (Korchak *et al.*, 1980; Smith *et al.*, 1984b). A role for anions in the mechanism of AA-induced PMN degranulation is demonstrated by the finding that DIDS, an anion channel blocker, inhibits the secretory process. We have preliminary data (Smith, unpublished observations) which suggest and others have reported (Korchak *et al.*, 1980) that anion channel blockers suppress degranulation by inhibiting the fusion of granule and cell membranes.

PMNs exposed to AA generate 5- and 15-monohydroxyeicosatetraenoic acids (Payan *et al.*, 1984), and 5-HETE has been reported to stimulate PMN degranulation (Stenson & Parker, 1980). It is conceivable, therefore, that AA-induced PMN activation is mediated by metabolites of lipoxygenase-catalysed reactions for which AA serves as substrate. However, we have found a lipoxygenase inhibitor, piriprost potassium, and the lipoxygenase/cyclo-oxygenase inhibitors, ETYA and benoxaprofen, to have no effect on granule exocytosis from AA-treated PMNs. Flurbiprofen, a cyclo-oxygenase inhibitor is also inactive. Oxygen-derived free radical production by AA stimulated PMNs is also unaffected by inhibitors of AA metabolism (Badwey *et al.*, 1981).

We have demonstrated that AA stimulates human PMN degranulation in which cytosolic-free Ca^{2+} and PK-C appear to be two components of the signal transduction system coupled to the activation pathway. These data together with the observations that AA can induce an inflammatory reaction *in vivo* associated with PMN infiltrates (Carlson *et al.*, 1985; Chang *et al.*, 1986) suggest that AA is an important inflammatory mediator. In addition, our data provide insight into the mechanism of PMN activation with AA. Furthermore, to the extent that the free AA is made available in activated PMNs via phospholipase A_2 and phospholipase C/diacylglycerol lipase-catalysed reactions, our data also suggest a possible role for AA as a second messenger of Ca^{2+}-mediated events associated with stimulus-response coupling in human PMNs.

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