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EFFECTS OF INDOMETHACIN, 5,8,11,14-EICOSATETRAYNOIC ACID, AND *p*-BROMOPHENACYL BROMIDE ON LYSOSOMAL ENZYME RELEASE AND SUPEROXIDE ANION GENERATION BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES*

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Abstract—Preincubation of cytochalasin B-treated, human polymorphonuclear leukocytes (PMN) with indomethacin (a cyclo-oxygenase inhibitor), 5,8,11,14-eicosatetraynoic acid (ETYA) (a lipoxygenase/cyclo-oxygenase inhibitor), or *p*-bromophenacyl bromide (BPB) (a phospholipase A_2 inhibitor) resulted in dose-dependent inhibition of lysosomal enzyme release elicited by the chemotactic peptide *N*-formylmethionylleucylphenylalanine (FMLP); 50 per cent inhibition was seen at approximately 50, 12, 8 μM respectively. BPB also inhibited superoxide anion generation. The effects of indomethacin and ETYA were dependent upon the type of stimulus presented to the cells. Lysosomal enzyme release stimulated by zymosan-treated serum and serum-treated zymosan was relatively unaffected by these two inhibitors. Indomethacin and ETYA did not appear to exert their effects by specific inhibition of prostaglandin and thromboxane synthesis; the inhibition offered by both agents was reversible, and aspirin had no similar inhibitory capacity. Our results indicate not only that indomethacin may exert effects independent of its inhibition of the cyclo-oxygenase pathway but also that products formed via phospholipase and lipoxygenase may be mediators of lysosomal enzyme release and superoxide anion generation.

Human polymorphonuclear leukocytes (PMN)‡, exposed to surface stimuli, respond by generation of superoxide anion radicals and by secretion of lysosomal enzymes, stable prostaglandins [1] and thromboxanes [2]. Stable prostaglandins, in turn, inhibit lysosomal enzyme release [3]. Since non-steroidal anti-inflammatory agents, such as indomethacin, inhibit cyclo-oxygenase activity [4] and hence prostaglandin [1] and thromboxane [2] synthesis, it is conceivable that these drugs could modulate the degranulation process. Indeed, Northover [5] has reported that, at concentrations greater than 50 μM, indomethacin inhibits lysosomal enzyme release from rabbit PMN stimulated by calcium ions. Indomethacin (100–200 μM) also inhibited the release of myeloperoxidase from human PMN in response to *N*-formylmethionylleucylphenylalanine (FMLP) and C5a [6]. Other non-steroidal anti-inflammatory agents modulated degranulation of guinea pig PMN in response to serum-treated zymosan [7]. In contrast, mouse macrophages, exposed to zymosan, degranulated normally in the presence of 30 μM indomethacin [8]. Similarly, human PMN, stimulated with serum-treated zymosan to produce superoxide anions and to secrete lysosomal enzymes. were not affected by 50 μM indomethacin [2].

To examine the regulatory role of prostaglandins and thromboxanes in human PMN we studied the effects of indomethacin and the lipoxygenase/cyclo-oxygenase inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA) [4] on lysosomal enzyme release and superoxide anion generation elicited by several stimuli. Moreover, since indomethacin has been reported to inhibit phospholipase A_2 in rabbit PMN [9], *p*-bromophenacyl bromide, another known phospholipase A_2 inhibitor [10], was also studied. Data reported below indicate that each of these agents can inhibit lysosomal enzyme release (depending upon the stimulus) and may share the property of inhibiting phospholipase A_2 of granulocytes.

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‡ Abbreviations: PMN, polymorphonuclear leukocytes; ETYA, 5,8,11,14-eicosatetraynoic acid; FMLP, *N*-formylmethionylleucylphenylalanine; STZ, serum-treated zymosan; BSA/anti-BSA, bovine serum albumin/anti-bovine serum albumin immune complex; ZTS, zymosan-treated serum; Con A, concanavalin A; $O_2^{\cdot-}$, superoxide anion radical and BPB, *p*-bromophenacyl bromide.

MATERIALS AND METHODS

Cytochalasin B and *p*-bromophenacyl bromide (BPB; α,*p*-dibromoacetophenone) were purchased from the Aldrich Chemical Co., Milwaukee, WI. Indomethacin, concanavalin A (Con A) and human albumin (Fraction V) were obtained from the Sigma Chemical Co., St. Louis, MO. FMLP was purchased from the Peninsula Laboratories, San Carlos, CA, and calcium ionophore A23187 was a gift from Eli

Lilly & Co., Indianapolis, IN. ETYA was a gift of Dr. Aaron Marcus. All other materials were reagent grade.

Preparation of cell suspensions. Heparinized (10 units/ml) venous blood was obtained from healthy adult donors. Purified preparations of PMN were isolated from this blood by means of Hypaque/Ficoll gradients [11] followed by standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes [12]. This allowed studies of cell suspensions containing 98 ± 2% PMN with few contaminating erythrocytes or platelets. The cells were suspended in a buffered salt solution consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.0 mM $MgCl_2$ and 0.6 mM $CaCl_2$, pH 7.4 (hereafter referred to as PiCM).

Purified platelet suspensions were isolated according to the method of Hamberg *et al.* [13], except that the platelet pellet was washed and resuspended in pre-warmed (37°) phosphate-buffered saline, pH 7.4.

Lysosomal enzyme release and enzyme assays. Washed PMN (5×10^6 in 0.9 ml PiCM) were preincubated for 5 min at 37° with cytochalasin B (5 μg/ml, plus 0.05% dimethylsulfoxide contributed by the stock solution) and with or without ETYA, indomethacin or BPB. Since the inhibitors were added from stock solutions in ethanol, appropriate amounts of ethanol (usually 0.2%) were added to control samples. Lysosomal enzyme secretion was initiated by addition of 0.1 ml PiCM containing the concentrated stimulus; final stimulus concentrations were 10^{-7} M FMLP, 2 mg/ml serum-treated zymosan (STZ; zymosan from ICN Pharmaceuticals, Irvine, CA, opsonized with fresh serum for 30 min at 37°, then washed), 10^{-5} M calcium ionophore A23187, 150 μg/ml bovine serum albumin/anti-bovine serum albumin (BSA/anti-BSA; prepared according to Ref. 14, using anti-BSA IgG from Cappel, Cochranville, PA), 30 μg/ml concanavalin A and 10% zymosan-treated serum (ZTS; fresh serum was opsonized with 1 mg/ml zymosan particles for 15 min at 37°; the zymosan particles were removed from the serum by centrifugation and the resulting ZTS was used immediately). After incubation at 37° for 5 min, the cell suspensions were centrifuged, and aliquots of the medium were taken for standard determinations of β-glucuronidase [15], lysozyme [16] and lactate dehydrogenase [17] activities. Results were initially determined as the percentage of total cellular enzyme released into the medium.

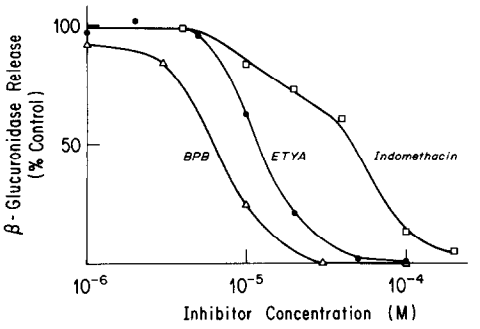

Fig. 1. Effects of ETYA, indomethacin and BPB on β-glucuronidase secretion in response to FMLP. Purified PMN (6×10^6) were preincubated for 5 min at 37° with cytochalasin B (5 μg/ml) and the indicated concentrations of inhibitors. FMLP was added to a final concentration of 10^{-7}M and the suspension was incubated for an additional 5 min. Cell-free supernatant fractions were then obtained and assayed for β-glucuronidase activity. The data represent averages of duplicate samples and are expressed as percentages of that quantity released by untreated control samples (48.6 per cent of total cellular enzyme for the indomethacin and ETYA series and 62.7 per cent for the BPB series).

For superoxide anion (O_2^-) generation, the same procedure was followed, with the following exceptions: (1) PMN concentrations were 0.75 to 2.5×10^6/ml; and (2) ferricytochrome *c* (horse heart, Sigma Type III) and superoxide dismutase (Miles Biochemicals, Elkhart, IN) were added just prior to stimulation. Superoxide generation was determined by measuring superoxide dismutase-sensitive ferricytochrome *c* reduction, as specified in Ref. 18.

RESULTS

The cyclo-oxygenase inhibitor, indomethacin, and the cyclo-oxygenase/lipoxygenase inhibitor, eicosatetraynoic acid (ETYA), both proved to be potent

Table 1. Effects of FMLP, ethanol, ETYA, indomethacin and BPB on the release of β-glucuronidase, lysozyme and lactate dehydrogenase

Additions	Enzyme release (% of control levels)		
	β-Glucuronidase	Lysozyme	Lactate dehydrogenase
None	0	0	5.9 ± 4.8
FMLP	49.2 ± 10.1	54.9 ± 6.9	3.3 ± 4.0
Ethanol; FMLP	46.4 ± 6.0	45.2 ± 15.0	3.4 ± 2.7
Indomethacin (+ ethanol); FMLP	12.1 ± 4.2	12.2 ± 7.7	4.4 ± 4.1
ETYA (+ ethanol); FMLP	12.2 ± 1.9	10.6 ± 5.7	4.5 ± 3.8
BPB (+ ethanol); FMLP	2.7 ± 4.5	2.9 ± 1.7	2.9 ± 2.5

* PMN (5×10^6) were preincubated with cytochalasin B (5 μg/ml) and ethanol (0.2%), indomethacin (100 μM), ETYA (20 μM) or BPB (20 μM) for 5 min at 37°. After challenge with FMLP (10^{-7} M, where indicated) for 5 min at 37°, the extracellular medium was obtained by centrifugation. The amounts of β-glucuronidase, lysozyme and lactate dehydrogenase secreted into the medium were determined and are expressed as percentages of the total cellular content. Each value represents the mean ± S.D. of three or more experiments.

inhibitors of lysosomal enzyme release from cytochalasin B-treated human PMN. Pretreatment with cytochalasin B was used to maximize the amount of granule enzymes and superoxide anion released into the extracellular medium. As shown in Fig. 1, high micromolar concentrations of indomethacin and ETYA virtually blocked enzyme release effected by the chemotactic peptide FMLP. ETYA was a more potent inhibitor than indomethacin, and the concentrations required to obtain 50 per cent inhibition of degranulation were 12 and 50 μM, respectively. Neither drug affected the enzyme assay within the concentration range shown. FMLP, indomethacin and ETYA apparently did not damage cell integrity, as extracellular lactate dehydrogenase levels were constant (Table 1). Inhibition of lysozyme release was similar to that seen for β-glucuronidase release.

PMN, which were preincubated with indomethacin and ETYA and then washed before challenge with FMLP, released normal amounts of β-glucuronidase (Table 2); thus, the effects of these agents were completely reversible. Furthermore, aspirin at concentrations up to 5 mM (titrated with NaOH to maintain pH 7.4) was without effect on β-glucuronidase and lysozyme release (Table 3). These two observations suggested that inhibition of lysosomal enzyme release by ETYA and indomethacin was not mediated by specific inhibition of the cyclo-oxygenase or lipoxygenase systems.

Because indomethacin is known to inhibit phospholipase A_2 [9] and since it is conceivable that ETYA could also interact with this enzyme, the effect of BPB, an inhibitor of phospholipase A_2 [10], was tested on the enzyme release system. BPB inhibited β-glucuronidase release, with 50 per cent inhibition observed at 6–10 μM (Fig. 1). BPB similarly inhibited lysozyme release (not shown) and did not affect either enzyme assay at the concentrations tested (Fig. 1). Unlike ETYA and indomethacin, BPB was irreversible in its effects: cells pretreated with 20 μM BPB released 0.4 per cent of control amounts of β-glucuronidase before washing and only 0.5 per cent after washing (Table 2). Similar irreversibility was seen with respect to lysozyme secretion.

All three agents inhibited O_2^- generation by PMN in response to FMLP (Fig. 2). BPB was again the most potent; no O_2^- was produced at inhibitor concentrations of 5 μM and above. At concentrations of 40 μM and above, BPB inhibited the O_2^- assay system itself. Both ETYA and indomethacin were relatively poor inhibitors of O_2^- generation, being generally more effective against lysosomal enzyme release (compare Fig. 1 with Fig. 2). This suggested that inhibition of enzyme release by ETYA and indomethacin was not due to general effects of these agents on the plasma membrane, where the O_2^- generating system is located. The inhibitors were similarly effective in the absence of cytochalasin B, which was normally included to maximize O_2^- release (data not shown).

In view of the pronounced effect of ETYA, indomethacin and BPB on enzyme release elicited by FMLP, it was of interest to see if these agents had similar effects when other stimuli were employed. Table 4 shows that the three agents were most effective in inhibiting β-glucuronidase release when FMLP was used as the stimulus. BPB was a potent inhibitor when any of the five stimuli were presented. ETYA failed to produce inhibition when the cells were exposed to ZTS and was only partially effective when A23187 was employed; indomethacin failed

Table 2. Reversibility of ETYA, indomethacin and BPB inhibition of β-glucuronidase secretion*

Pretreatment	Enzyme release (% of control levels)
ETYA (20 μM)	33 ± 16
Indomethacin (100 μM)	53 ± 28
BPB (20 μM)	0.4 ± 0.6
ETYA (20 μM), washed	81 ± 26
Indomethacin (100 μM), washed	102 ± 12
BPB (20 μM), washed	0.5 ± 0.9

* PMN (5×10^6) were preincubated with cytochalasin B (5 μg/ml) and ETYA, indomethacin or BPB for 5 min at 37°. They were then challenged with 10^{-7} M FMLP (upper three samples) or else washed once in PiCM before resuspension in PiCM (with cytochalasin B) and subsequent FMLP challenge (lower three samples). The amounts of β-glucuronidase released into the extracellular medium were determined and are expressed as percentages of that released by stimulated control cells exposed to appropriate amounts of ethanol. Each value represents the mean ± S.D. of four experiments.

Table 3. Effect of aspirin on lysosomal enzyme release*

	Enzyme release (% control)	
	β-Glucuronidase	Lysozyme
Aspirin (5 mM)	80 ± 42	62 ± 21
Aspirin (5 mM) + NaOH (5 mM)	109 ± 19	100 ± 11

* PMN (5×10^6) were preincubated with cytochalasin B (5 μg/ml) and aspirin, or aspirin plus NaOH (to restore pH 7.4) for 5 min at 37°. In addition, control cells were preincubated with 0.5% ethanol, the concentration contributed by the aspirin stock solution. The PMN were challenged with FMLP (10^{-7}) for 5 min at 37° and the cell-free medium was then assayed for β-glucuronidase and lysozyme. Release of granule enzymes is expressed as the percentage of the amount of enzyme released from control cells to which only ethanol was added. Each value represents the mean ± S.D. of three experiments.

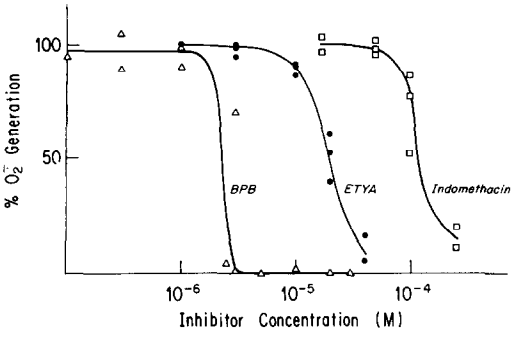

Fig. 2. Effects of ETYA, indomethacin and BPB on superoxide generation in response to FMLP. Purified PMN (0.75×10^6) were preincubated for 5 min at 37° with cytochalasin B (5 μg/ml) and the indicated concentrations of ETYA, indomethacin and BPB. Cytochrome *c* (0.9 mg) and superoxide dismutase (10 μg, to appropriate control samples) were then added, followed by stimulation with FMLP (10^{-7} M). The samples were incubated for an additional 5 min after which cell-free supernatant fractions were isolated and cytochrome *c* reduction was determined. The results were compiled from three experiments and data are expressed as percentages of that activity found for untreated control cells (63 ± 1/nmoles cytochrome *c* reduced/10^6 cells/5 min, N = 3).

with both ZTS and STZ. A similar pattern was seen for lysozyme release in response to these various stimuli (Table 4). In addition, lysozyme secretion in response to the lectin Con A (which does not provoke β-glucuronidase release) was reduced by all three inhibitors. Because the small number of platelets in our PMN preparation might be responsible for some of these results, we repeated the experiments using PMN suspensions which were purposely supplemented with ten times the number of platelets normally found as contaminants. Inhibition of β-glucuronidase and lysozyme release by BPB, ETYA and indomethacin was not significantly affected by these additional platelets when any of the stimuli were employed (data not shown).

In view of the fact that less inhibition of enzyme release was seen for those stimuli which contributed large amounts of protein to the incubation mixtures (i.e. STZ and ZTS), we tested the effect of added protein on the inhibition produced by ETYA and indomethacin. As shown in Table 5, both ETYA and indomethacin profoundly inhibited β-glucuronidase release in response to 10^{-7} M FMLP (A). This inhibition was partially blocked by the presence of 0.3 mg/ml human albumin (B); more blocking was observed when the albumin concentration was increased to 3 mg/ml (C). Similarly, the presence of 1% (D) and 10% (E) heat-inactivated serum blocked the effects of these two inhibitors. When samples of ETYA and indomethacin were treated with STZ (F) or BSA/anti-BSA (G) before addition of the drugs to PMN, partial blocking of inhibition was seen. It is apparent that addition of protein *per se* interferes with the effects of ETYA and indomethacin, probably by non-specific adsorption of the drugs. Consequently, inhibition of enzyme release in response to BSA/anti-BSA would probably be greater than that depicted in Table 4 were it not for this interference; the smaller amount of inhibition of enzyme release seen when BSA/anti-BSA (as opposed to FMLP) was used as the stimulus can be largely ascribed to protein contributed by this stimulus (Table 5G). Similarly, the fact that inhibition of enzyme release by ETYA and indomethacin can be almost completely blocked by 10% heat-inactivated serum (Table 5E) explains why no inhibition was seen when ZTS was used as the stimulus. However, preincubation with STZ did not completely block inhibition by indomethacin (Table 5F); thus, the failure of this drug to inhibit lysosomal enzyme release in response to STZ (Table 4) was most likely

Table 4. Effects of ETYA, indomethacin and BPB on lysosomal enzyme release in response to various stimuli*

Agent	FMLP	STZ	A23187	BSA/anti-BSA	ZTS	Con A
			β-Glucuronidase release (% control)			
ETYA	17 ± 9† (11)	56 ± 25‡ (7)	68 ± 35 (7)	32 ± 21† (8)	90 ± 9 (4)	
Indomethacin	26 ± 11† (11)	96 ± 13 (7)	55 ± 18† (7)	62 ± 27‡ (8)	93 ± 11 (4)	
BPB	6 ± 9† (3)	4 ± 3† (3)	3 ± 2† (3)	5 ± 2† (3)	24 ± 15† (3)	
			Lysozyme release (% control)			
ETYA	17 ± 10† (7)	65 ± 13‡ (5)	84 ± 38 (7)	45 ± 36‡ (7)	95 ± 22 (4)	45 ± 16† (6)
Indomethacin	16 ± 12† (7)	101 ± 34 (5)	66 ± 12† (7)	51 ± 20 (7)	90 ± 17 (4)	67 ± 17‡ (6)
BPB	9 ± 5†	5 ± 8† (3)	6 ± 6† (3)	6 ± 5† (3)	7 ± 12† (3)	1 ± 2† (3)

* Washed PMN (5×10^6) were preincubated for 5 min at 37° with cytochalasin B (5 μg/ml) and with ETYA (20 μM), indomethacin (100 μM) or BPB (20 μM). The cells were then stimulated by the addition of FMLP (10^{-7}), serum-treated zymosan (STZ, 2 mg), calcium ionophore A23187 (10^{-5} M), bovine serum albumin/anti-bovine serum albumin immune complex (BSA/anti-BSA, 150 μg), zymosan-treated serum (ZTS, 10%) or concanavalin A (Con A, 30 μg). These mixtures were incubated for an additional 5 min at 37°, after which the amount of β-glucuronidase and lysozyme released by the cells were determined. The results are given as mean percentages (±S.D.) of that amount of granule enzyme released by control samples to which no inhibitor was added and the number of experiments is given in parentheses. The control β-glucuronidase release values (percent of total cell enzyme released) were 49 ± 15 for FMLP, 8.6 ± 2.5 for STZ, 50 ± 14 for A23187, 26 ± 14 for BSA/anti-BSA and 33 ± 5 for ZTS. The control lysozyme release values were 33 ± 11 for FMLP, 11 ± 5 for STZ, 43 ± 17 for A23187, 25 ± 6 for BSA/anti-BSA, 22 ± 10 for Con A and 24 ± 2 for ZTS.

† $P < 0.001$.

‡ $P < 0.01$.

Table 5. Effect of protein on inhibition of β-glucuronidase release elicited by FMLP*

Additions	Enzyme release (% of control) after preincubation with: ETYA	Indomethacin
(A) None	27 ± 24	30 ± 10
(B) Human albumin (0.3 mg/ml)	41 ± 36	42 ± 19
(C) Human albumin (3 mg/ml)	104 ± 16	82 ± 9
(D) Heat-inactivated serum (1%)	62 ± 25	40 ± 10
(E) Heat-inactivated serum (10%)	107 ± 7	85 ± 5
(F) Drugs pretreated with STZ	82 ± 16	63 ± 18
(G) Drugs pretreated with BSA/anti-BSA	75 ± 4	42 ± 9

* Washed PMN (5×10^6) were preincubated with cytochalasin B (5 μg/ml) and with ETYA (20 μM) or indomethacin (100 μM) for 5 min at 37° after which the cells were stimulated to release β-glucuronidase with FMLP (10^{-7} M) as described previously. Sample A was preincubated without added protein. The preincubation media in samples B and C contained, respectively, 0.3 and 3.0 mg/ml human albumin. Samples D and E contained, respectively, 1 and 10% heat-inactivated serum. The ETYA and indomethacin presented to sample F were pretreated with STZ; solutions of ETYA (100 μM in 1.0 ml) and indomethacin (500 μM in 1.0 ml) were incubated with 10 mg STZ for 5 min at 37°. The STZ particles were centrifuged down and appropriate aliquots of the media were added to PMN during the preincubation phase. Sample G represents ETYA and indomethacin pretreated with BSA/anti-BSA as described above. Results are expressed as percentages of that amount of β-glucuronidase released by control samples to which no inhibitor was added. Each value represents the mean ± S.D. of three experiments.

specific to this stimulus (since it cannot be totally attributed to interference by protein). The patterns of inhibition seen for the soluble stimuli A23187 and Con A are unlikely to be attributable to similar interference since they contribute either little (e.g. Con A) or no (e.g. A23187) protein to the preincubation medium.

Because 20 μM ETYA and 100 μM indomethacin did not profoundly inhibit O_2^- generation (Fig 2), the effect of BPB alone was tested on O_2^- generation elicited by various stimuli (Table 6). As can be seen, BPB (5 μM) significantly inhibited O_2^- generation in response to all stimuli.

Table 6. Effect of BPB on superoxide anion generation in response to various stimuli*

Stimulus	O^- generation (% of control)
FMLP	2 ± 2
STZ	18 ± 29
A23187	3 ± 2
BSA/anti-BSA	45 ± 17
ZTS	29 ± 20
Con A	1 ± 1

* Washed PMN (0.75 to 2.5×10^6) were preincubated for 5 min at 37° with cytochalasin B (5 μg/ml) and BPB (5 μM), after which cytochrome *c* was added. Superoxide anion generation was initiated by addition of the indicated stimuli (see legend to Table 4). After incubation for 5 min at 37°, the cell-free media were obtained and O_2^- generation was determined by measuring superoxide dismutase-sensitive cytochrome *c* reduction. The data represent the means and standard deviations of three experiments and are presented as the percentages of activities of control samples to which no BPB was added. The control values for O_2^- generation (nmoles cytochrome *c* reduced/10^6 cells/5 min, N = 3) were 69 ± 11 for FMLP, 36 ± 11 for STZ, 19 ± 5 for A23187, 13 ± 4 for BSA/anti-BSA, 39 ± 12 for Con A and 1.5 ± 0.7 for ZTS.

DISCUSSION

Pretreatment of human PMN with the cyclo-oxygenase inhibitor indomethacin resulted in a dose-dependent diminution of lysosomal enzyme release; inhibitory concentrations of this drug affected O_2^- generation far less. Because indomethacin would be expected to inhibit prostaglandin and thromboxane synthesis, the lipoxygenase/cyclo-oxygenase inhibitor ETYA was also tested; this latter agent gave results similar to those seen for indomethacin. Indeed, the data indicate that these two agents did not inhibit lysosomal enzyme release through specific effects (i.e. by inhibition of prostaglandin synthesis), since their inhibition was reversible and aspirin was inactive. The phospholipase A_2 inhibitor BPB irreversibly blocked enzyme release and was a potent inhibitor of O_2^- generation

These results are in agreement with other reports indicating that non-steriodal anti-inflammatory agents inhibit lysosomal enzyme release from rabbit PMN in response to calcium ions [4], guinea pig PMN in response to serum-treated zymosan [7], and human PMN in response to FMLP and C5a [6]. Our results also show that the effects of indomethacin and ETYA on lysosomal enzyme release can be strongly dependent upon the nature of the stimulus. In fact, the reported failure of indomethacin to affect secretion from human PMN [2] can be attributed to the use of STZ as the stimulus.

Lysosomal enzyme release in response to the soluble chemotactic peptide FMLP was greatly inhibited by both indomethacin and ETYA. When the soluble stimuli Con A and A23187 were used, far less inhibition was seen; thus, the effects of ETYA and indomethacin were not dependent upon the soluble or particulate nature of the stimulus presented. BPB was a potent inhibitor of lysosomal enzyme release and O_2^- generation when any of the stimuli were

employed. Inhibition of lysosomal enzyme release by indomethacin and ETYA was most noticeable when FMLP was the stimulus. The ineffectiveness of ETYA and indomethacin observed when cells were stimulated by ZTS or STZ could be partially attributed to inactivation or adsorption of the drugs by protein (Table 5); however, the complete lack of inhibition by indomethacin when STZ was employed was probably stimulus-specific. A similar stimulus-specific effect was the relative impotence of ETYA compared to indomethacin when A23187 was presented to the cells. Unlike BPB, which is a powerful inhibitor when any stimulus is used, ETYA and indomethacin are not general inhibitors of degranulation. These results emphasize that the mechanisms of specific receptor-secretion coupling are not identical for all stimuli.

Although indomethacin and ETYA are generally considered to be specific inhibitors of prostaglandin and thromboxane synthesis, the effects reported here are unlikely to be due to such specific interactions, since (1) both drugs are reversible, (2) aspirin is without effect, and (3) inhibition of enzyme release by the drugs takes place at relatively high concentrations (see review by Flower [4]). Non-specific effects of indomethacin have been reported. This drug inhibits cyclic AMP-dependent protein kinase in rabbit ileal mucosa [19] and phospholipase A_2 in rabbit PMN [9]. Since phospholipase A activity has been reported in human PMN [20] and since ETYA, a reactive fatty acid-like molecule, could conceivably interact with the active site of the enzyme, the 'specific' phospholipase A_2 inhibitor BPB was examined in our system. Because all three agents inhibited lysosomal enzyme release, our results are consistent with the hypothesis that inhibition resulted from diminished phospholipase activity. It is possible that phospholipase activity is required for local production of lysophosphatides which could promote the fusion of lysosomes and plasma membranes necessary for degranulation. It is also possible that arachidonic acid released by phospholipase is required for PMN responses. For rabbit PMN, this fatty acid is sufficient to induce both degranulation [21] and aggregation (which has several properties and requirements similar to those for lysosomal enzyme release) [22]. Both responses were inhibited by ETYA, but indomethacin was not tested. Stimulated PMN also produce hydroxy [23] and dihydroxy [24] derivatives of arachidonic acid, which may be of significance for stimulation of PMN. The effects of inhibitors on production of the dihydroxy compounds was not tested [24]; however, neither ETYA nor indomethacin affected production of the hydroxy derivatives [23].

An interpretation such as this, which implicates phospholipase activity, must be tempered by the fact that BPB also appears to have non-specific effects (e.g. it inhibits O_2^- generation) and that other interpretations are plausible. It should also be noted that the effects of the three inhibitors on phospholipase, cyclo-oxygenase and lipoxygenase activities were not tested in this work and that the conclusions we have reached regarding the mode of action of these agents are necessarily speculative.

Our results demonstrate the pitfalls of assuming the specificity of such agents as indomethacin, ETYA and BPB. In addition, we have provided further evidence that the paths of stimulus-secretion coupling are not identical. Finally, our data indicate that indomethacin may owe some of its anti-inflammatory effects *in vitro* to actions which are probably unrelated to its specific inhibition of prostaglandin and thromboxane syntheses.

REFERENCES

1. R. B. Zurier and D. M. Sayadoff, *Inflammation* **1**, 93 (1975).
2. I. M. Goldstein, C. L. Malmsten, H. Kandahl, H. B. Kaplan, O. Radmark, B. Samuelsson and G. Weissmann, *J. exp. Med.* **148**, 787 (1978).
3. R. B. Zurier, G. Weissmann, S. Hoffstein, S. Kammerman and H. H. Tai, *J. clin. Invest.* **53**, 297 (1974).
4. R. J. Flower, *Pharmac. Rev.* **26**, 33 (1974).
5. B. J. Northover, *Br. J. Pharmac.* **59**, 25 (1977).
6. R. O. Webster and P. M. Henson, *Inflammation* **3**, 129 (1978).
7. R. J. Smith, *J. Pharmac. exp. Ther.* **207**, 618 (1978).
8. R. J. Bonney, P. D. Wightman, P. Davies, S. J. Sadowski, F. A. Kuehl and J. L. Humes, *Biochem. J.* **176**, 433 (1978).
9. L. Kaplan, J. Weiss and P. Elsbach, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2955 (1978).
10. M. F. Roberts, R. A. Deemer, T. C. Mincey and E. A. Dennis, *J. biol. Chem.* **252**, 2405 (1977).
11. A. Boyum, *Scand. J. clin. Lab. Invest.* **21**, 77 (1968).
12. R. B. Zurier, S. Hoffstein and G. Weissmann, *J. Cell Biol.* **58**, 27 (1973).
13. M. Hamberg, J. Svensson, T. Wakabayashi and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **71**, 345 (1974).
14. P. A. Ward and N. Zvaifler, *J. Immun.* **11**, 1771 (1973).
15. G. R. Brittinger, R. Hirschhorn, S. D. Douglas and G. Weissmann, *J. Cell Biol.* **37**, 394 (1968).
16. *Worthington Enzyme Manual*, p. 100. Worthington Biochemical Corp., Freehold, NJ (1972).
17. W. E. C. Wacker, D. D. Ulmer and B. L. Valee, *New Engl. J. Med.* **255**, 449 (1956).
18. I. M. Goldstein, D. Roos, H. B. Kaplan and G. Weissmann, *J. clin. Invest.* **56**, 1155 (1975).
19. H. S. Kantor and M. Hampton, *Nature, Lond.* **276**, 841 (1978).
20. R. Franson, J. Weiss, L. Martin, J. K. Spitznagel and P. Elsbach, *Biochem. J.* **167**, 839 (1977).
21. P. J. Naccache, H. J. Showell, E. L. Becker and R. I. Sha'afi, *Biochem. biophys. Res. Commun.* **87**, 292 (1979).
22. J. T. O'Flaherty, H. J. Showell, E. L. Becker and P. A. Ward, *Inflammation*, in press.
23. P. M. Borgeat, M. Hamberg and B. Samuelsson, *J. biol. Chem.* **251**, 7816 (1976).
24. P. Borgeat and B. Samuelsson, *J. biol. Chem.* **254**, 2643 (1979).