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DISSOCIATION BY PIROXICAM OF DEGRANULATION AND SUPEROXIDE ANION GENERATION FROM DECREMENTS IN CHLORTETRACYCLINE FLUORESCENCE OF ACTIVATED HUMAN NEUTROPHILS

Henry S. Edelson, Howard B. Kaplan, Helen M. Korchak, James E. Smolen, and Gerald Weissmann

Division of Rheumatology of the Department of Medicine, New York University School of Medicine, New York, NY 10016.

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SUMMARY: Human neutrophils (PMN), activated by appropriate stimuli, aggregate, generate $O_2^{\cdot-}$, secrete lysosomal enzymes, and exhibit a characteristic decrease in chlortetracycline (CTC) fluorescence, which has been attributed to mobilization of membrane-bound Ca, a possible requirement for stimulus-secretion coupling in PMN. Preincubation of PMN with piroxicam (50 μM) before stimulation with the chemotactic peptide f-met-leu-phe (FMLP) inhibited each of these responses. In addition, binding of ^{3}H-FMLP was significantly reduced in the presence of piroxicam. In contrast, when either concanavalin A (Con A) or phorbol myristate acetate (PMA) were used as stimuli in the presence of piroxicam, aggregation and secretion of lysozyme proceeded normally, whereas $O_2^{\cdot-}$ generation and CTC fluorescence decrements were significantly inhibited. The binding of ^{3}H-Con A was unaffected by piroxicam. Since secretion and aggregation could be dissociated from the CTC-fluorescence response, it is unlikely that an event monitored by decrements in CTC fluorescence is necessary for stimulus-secretion coupling.

INTRODUCTION

Neutrophils undergo stimulus-secretion coupling when exposed to surface stimuli and it has been suggested that mobilization of intracellular calcium is an immediate, and necessary, response to stimulation (1,2). Chlortetracycline has been used as a probe of the mobilization of calcium from intracellular sites since CTC preferentially partitions into hydrophobic regions of lipid bilayers and generates characteristic fluorescence emission spectra when complexed with calcium (3). Changes in the fluorescence of CTC-preloaded cells have been recorded in a variety of stimulated cells, including pancreatic islet (4) and acinar (5) cells, platelets (6,7), endosperm (8), neurons (9) and neutrophils of various species

Abbreviations: PMN, polymorphonuclear leukocytes or neutrophils; FMLP, N-formy-methionyl-leucyl-phenylalanine; Con A, concanavalin A; PMA, phorbol myristate acetate; DMSO, dimethyl sulfoxide; $O_2^{\cdot-}$, superoxide anion; CTC, chlortetracycline; TMB-8, 8-(N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate hydrochloride.

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(10,11). In each instance, secretion was anteceded, or accompanied, by rapid decrements of CTC fluorescence, changes which have been attributed to mobilization of a "trigger" pool of calcium from intracellular loci.

However, Gains (12) has recently indicated that CTC fluorescence may not accurately monitor the fate of membrane-associated calcium. He suggested that it was not possible to distinguish whether changes in CTC fluorescence were due to loss of membrane-associated calcium or due to electrostatic interactions between CTC-Ca^{2+} complexes and hydrophilic (neutral or negative) groupings at the plasmalemma of stimulated cells.

Whatever the basis of the CTC-fluorescence response, a necessary role for this aspect of stimulus-secretion coupling would be excluded were it possible to dissociate secretion from changes in CTC-fluorescence under any circumstance. In this report, we document that the new, non-steroidal anti-inflammatory agent, piroxicam, inhibits changes in CTC-fluorescence provoked by three secretagogues (FMLP, Con A, PMA) but has no effect on secretion of lysozyme from the cells induced by two of these secretagogues (Con A, PMA).

MATERIALS AND METHODS

Reagents: N-formyl-methionyl-leucyl-phenylalanine (Peninsula Laboratories, CA) was prepared in DMSO (0.01%) (Fisher Scientific, Pittsburgh, PA). Concanavalin A, phorbol myristate acetate, chlortetracycline, cytochrome c and xanthine oxidase were obtained from Sigma Chemical Co., St. Louis, MO; acetaldehyde was obtained from Eastman Organic Chemicals, Inc., Rochester, NY; piroxicam from Pfizer, Inc., New York, NY.

Preparation of Cell Suspension. Heparinized (10 µg/ml) venous blood was obtained from healthy donors. Purified preparations of PMN were isolated from this blood by means of Hypaque/Ficoll gradients (13), followed by dextran sedimentation and hypotonic lysis of erythrocytes (14). These cells were then 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, 1mM $MgCl_2$ and 0.6 mM $CaCl_2$, pH 7.4 (PiCM).

PMN Aggregation, Enzyme Release and $O_2^{\cdot-}$ Generation. PMN aggregation was studied as described by Craddock et al (15). The generation of superoxide anion ($O_2^{\cdot-}$) from PMN and generation of $O_2^{\cdot-}$ by acetaldehyde-xanthine oxidase was performed as previously described (16,17). Extracellular release of PMN granule-associated enzymes (lysozyme, beta-glucuronidase) was described in ref. 16. Release of cytoplasmic lactate dehydrogenase was determined as in ref. 18 as an indicator of cell viability. In no experiment described below was total LDH release > 3%. Piroxicam was preincubated with PMN for 5 minutes at 37°C. Enzyme release is expressed as percent of total enzyme activity released from PMN exposed to 0.2% Triton X-100 (Rohm and Haas Co., Philadelphia, PA).

^{3}H-FMLP and ^{3}H-Con A Binding to PMN. ^{3}H-Con A (New England Nuclear Corp.) binding was performed as previously described (19). ^{3}H-FMLP (New England Nuclear Corp.) binding was performed by incubating 4 X 10^6 PMN with 10^{-7} M ^{3}H-FMLP,

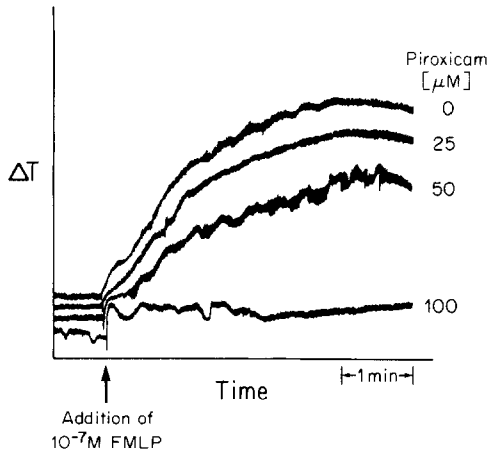

Figure 1. Inhibition of PMN aggregation by piroxicam with respect to concentration of PMN by piroxicam. Piroxicam was incubated with PMN for 5 minutes at 37°C before addition of stimulus (FMLP, 10^{-7} M). Piroxicam, 50 μM, inhibited FMLP induced aggregation by 50%.

in the presence of either 10^{-4} M FMLP or buffer for 2 minutes, and then rapid filtration through a celotate filter (Millipore Co.). Addition of 10^{-4} M FMLP permitted corrections to be made for non-specific binding by subtracting the total radioactivity of ^{3}H-FMLP with 10^{-4} M FMLP from the total activity of ^{3}H-FMLP alone.

CTC Fluorescence Measurements of PMN. CTC fluorescence was measured by a modification of the method of Naccache et al (10). Briefly, PMN (20 X 10^{6} PMN/ml) were incubated with 50 μM CTC in phosphate buffered saline pH 7.4 (PBS) for 20 minutes at 37°C. PMN were then washed 3 times with PBS and resuspended to their final concentration in PiCM. Fluorescence measurements were made in a Perkin Elmer model 650-10S spectrofluorimeter (emission wavelength: 560 nm, excitation wavelength: 370 nm). These wavelengths were chosen to distinguish Ca^{2+}·CTC from Mg^{2+}·CTC complexes. Slit widths were adjusted for maximum efficiency. PMN were place in cuvettes, positioned in a jacketed holder to maintain a temperature of 37°C throughout monitoring. Secretagogues were injected by a Hamilton syringe through an injection post on the spectrofluorimeter, and their volumes never exceeded 10% (v/v).

RESULTS

Activation of PMN by FMLP and Inhibition by Piroxicam. Human PMN exposed to the chemotactic peptide N-formyl-methionyl-leucy-phenylalanine (FMLP, 10^{-7} M) aggregate, generate $O_2^{\cdot-}$, and release lysozyme with no concomitant release of beta-glucuronidase (20). The concentration of piroxicam which inhibited FMLP-induced aggregation by 50% (ID_{50}) was 50 μM (Figure 1). The effects of this concentration of piroxicam next were studied on other neutrophil responses to FMLP. Preincubation of PMN with piroxicam (50 μM) for 5 minutes not only inhibited FMLP-induced PMN aggregation but also $O_2^{\cdot-}$ generation and lysozyme release (Table 1). Rapid decreases of CTC fluorescence were observed in PMN stimulated with 10^{-7} M FMLP (Figure 2A);

Table 1

Effects of piroxicam (50 μM) on PMN functions. All data are expressed as % control ± S.E.M. (n = number of experiments). N.S. = not significant, N.A. = not available. Binding was determined as described in Materials and Methods. Control counts per minute (cpm) of ^{3}H-Con A binding was 4961 ± 470 cpm. Aggregation was quantified by measuring area under curves over 5 minutes using a planimeter: each cm^2 is designated an "aggregation unit". Control FMLP induced aggregation units 15.0 ± 1.6. CTC fluorescence was quantified by measuring the slope of the decrease in fluorescence over time. Lysozyme release was measured as % lysozyme release of total lysozyme (released by Triton X-100 (0.2%, v/v)). FMLP induced lysozyme release was 9.9 ± 0.6%, Con A induced lysozyme release was 10.3 ± 0.3% and PMA induced lysozyme release was 17.6 ± 2.3%. $O_2^{\cdot-}$ generation expressed as nmoles cytochrome c reduced/minute/10^6 PMN. Control FMLP induced $O_2^{\cdot-}$ generation 12.7 ± 6.7 nmoles/min/10^6 PMN, Con A induced $O_2^{\cdot-}$ generation was 9.7 ± 0.9 nmoles, and PMN induced $O_2^{\cdot-}$ generation 9.9 ± 1.0 nmoles.

FUNCTION	STIMULUS		
	FMLP + Piroxicam 50 μM	Con A + Piroxicam 50 μM	PMA + Piroxicam 50 μM
Binding (%)	42.5 ± 8.5 (3) p < 0.01	97.2 ± 3.7 (3) N.S.	N.A.
Aggregation (%)	56.0 ± 12.0 (5) p < 0.01	100 (4)	100 (3)
CTC Fluorescence (%)	42.3 ± 8.6 (4) p < 0.004	72.3 ± 5.1 (3) p < 0.01	62.2 ± 4.2 (3) p < 0.01
Lysozyme Release (%)	52.3 ± 6.0 (5) p < 0.001	147.7 ± 6.9 (3) N.S.	119.4 ± 8.0 (3) N.S.
$O_2^{\cdot-}$ Generation (%)	55.2 ± 9.9 (5) p < 0.005	22.4 ± 6.9 (3) p < 0.008	43.3 ± 3.7 (3) p < 0.006

exposure to piroxicam (50 μM) for 5 minutes before FMLP was introduced significantly inhibited decreases in CTC fluorescence.

Binding of ^{3}H-FMLP to PMN and Inhibition by Piroxicam. To determine whether piroxicam exerted its effect on stimulus-secretion coupling or simply prevented receptor-ligand interaction, we examined binding of ^{3}H-FMLP to PMN; binding was inhibited by 57.7% (Table 1). Since inhibition by piroxicam of FMLP-induced aggregation, $O_2^{\cdot-}$ generation, lysozyme release and CTC fluorescence decrements might simply reflect decreases in receptor availability, we examined effects of two other secretagogues, concanavalin A and phorbol myristate acetate.

Activation of PMN by Concanavalin A and PMA and Inhibition by Piroxicam. Table 1 illustrates the effects of concanavalin A (Con A, 30 μg/ml) and phorbol myristate acetate (PMA, 50 ng/ml) on PMN activation and inhibition of these effects

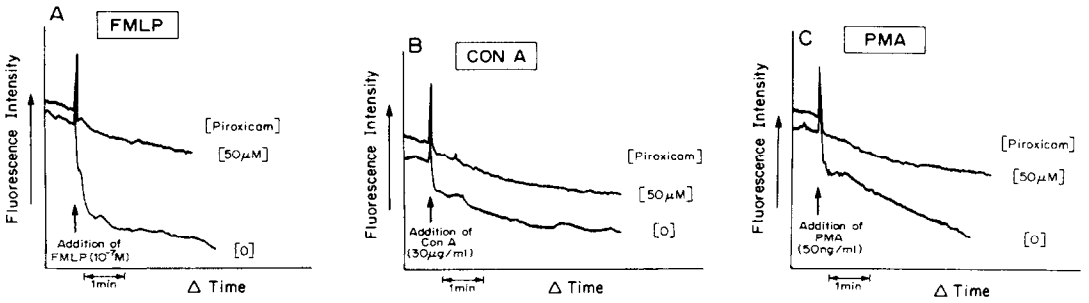

Figure 2. Effect of piroxicam on the CTC fluorescence response of PMN to stimulation with FMLP, Con A and PMA. The concentration of piroxicam employed was that which inhibited FMLP induced aggregation by 50% (50 μM). Arrow marks injection of indicated stimulus.

by piroxicam (50 μM). $O_2^{\cdot-}$ generation was significantly reduced whereas aggregation and lysozyme release were totally unaffected. Since the possibility remained that piroxicam might interfere with the detection of the superoxide anion, $O_2^{\cdot-}$ was generated using the xanthine oxidase-acetaldehyde, cell-free, $O_2^{\cdot-}$ generating system, with cytochrome c as the indicator. The concentration of xanthine oxidase was adjusted so that 2 nanomoles of cytochrome c were reduced per minute. The presence of piroxicam (50 μM) did not alter this rate, nor did piroxicam reduce cytochrome c when added in the absence of xanthine oxidase.

Binding of ^{3}H-Con A to PMN. Binding of ^{3}H-Con A was unaffected by the presence of piroxicam (Table 1). Thus, the effect of piroxicam on $O_2^{\cdot-}$ generation by neutrophils in response to Con A cannot be due to inhibition of receptor-ligand binding (as was the case for FMLP) but must be due to the inhibition of a specific step in the activation of the $O_2^{\cdot-}$ generating system of the neutrophil.

CTC Fluorescence Response of PMN to Con A and PMA. Figures 2B and 2C illustrate the response of CTC labelled neutrophils to Con A (30 μg/ml) and PMA (50 ng/ml). Each stimulus provoked a rapid and characteristic decrease in CTC fluorescence (Figure 2A). Preincubation of PMN with piroxicam (50 μM) for 5 minutes inhibited the CTC-fluorescence response (Figures 2B, 2C).

DISCUSSION

Neutrophils stimulated by FMLP, Con A, or PMA aggregate, generate $O_2^{\cdot-}$, secrete lysozyme, and show a characteristic decrease in CTC-fluorescence. Piroxicam inhibited each neutrophil response to FMLP but also inhibited the binding of ^{3}H-

FMLP to PMN. Piroxicam also inhibited $O_2^{\cdot-}$ generation and the CTC fluorescence response when either Con A or PMA were used as stimuli, whereas lysozyme release, aggregation, and ligand binding were unaffected. The ability of piroxicam to inhibi various neutrophil responses aggres with earlier reports of the inhibitory effects of several inhibitors of arachidonic acid metabolism (21). However, inhibition of PMN responses by piroxicam is stimulus-dependent, indicating that multiple pathways to stimulus-secretion coupling must be operative in the neutrophil.

Our data do not permit conclusions as to the validity of CTC fluorescence as a probe of membrane-associated calcium. Naccache et al (10) reported that PMN treated with FMLP and C5a showed a rapid decrease in CTC fluorescence. Takeshige et al (11) reported similar results in guinea pig PMN stimulated by cytochalasin B or E. Coli, and found that TMB-8 inhibited this CTC-fluorescence response, suggestin that CTC monitored mobilization of intracellular calcium. Evidence obtained from platelets, and pancreatic acinar cells also indicates that changes in CTC fluorescen report an early event in activation of secretory cells (5,7). Feinstein (6) argued that decrements in CTC fluorescence of platelets are a necessary antecedent of aggregation and secretion, since secretion was never observed without decrease in CTC fluorescence, and inhibition of CTC fluorescence was invariably accompanied by inhibition of secretion. Our data show that secretion is possible in the absence of a CTC fluorescence response. One possible explanation is that CTC fluorescence may not monitor the mobilization of intracellular calcium. Another possibility is that piroxicam "uncouples" stimulus-secretion coupling. The latter is unlikely, since inhibition by piroxicam of the CTC-response to FMLP was accompanied by inhibition of secretion, whereas inhibition of CTC fluorescence responses to PMA and Con A was observed in the presence of secretion.

The data do not permit one to exclude a role for release of a pool of "trigger" calcium from intracellular sites during neutrophil activation. Thus, even if it is assumed that CTC fluorescence accurately reflects changes in that pool, sufficient calcium may be lost from CTC-reporting sites to initiate secretion even when fluorescence decrements were almost completely abolished by piroxicam. Finally in the presence of piroxicam, influxes of calcium from the extracellular medium may

substitute for intracellular sources (1). However, our observations clearly demonstrate that a normal CTC fluorescence response is not a necessary step in stimulus-secretion coupling.

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