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DICLOFENAC BINDING TO HUMAN POLYMORPHONUCLEAR NEUTROPHILS: EFFECT ON RESPIRATORY BURST AND *N*-FORMYLATED PEPTIDE BINDING

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Abstract—The respiratory burst of human polymorphonuclear neutrophils (PMN) induced by particle or soluble stimuli was measured in the presence of the nonsteroidal anti-inflammatory drug, diclofenac sodium (Voltaren). Diclofenac (25–100 μg/ml) inhibited the oxygen consumption of PMN stimulated by 5×10^{-7} M of *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). The inhibition was linearly correlated to diclofenac concentration. By contrast, diclofenac did not affect the rate of heat-killed *Klebsiella pneumoniae* ingestion of PMN, or the PMN O_2-uptake induced by (0.67 μg/ml) serum-opsonized zymosan or (1 μg/ml) phorbol myristate acetate (PMA). The PMN production of superoxide anion induced by various FMLP concentrations (10^{-7}, 10^{-6} and 10^{-5} M) was also decreased by diclofenac. However, this inhibition declined when the formylated peptide concentration was raised suggesting that diclofenac could alter FMLP binding to the PMN membrane. Binding experiments of tritiated FMLP to intact PMN performed at 22° and 4° showed high- and low-affinity FMLP sites with dissociation constant (K_d) values of approximately 2×10^{-8} M and 10^{-5} M respectively. Diclofenac did not significantly alter the low-affinity component but induced modifications of the high-affinity component which were different at 22° and 4°. At 22° only the dissociation constant value was enhanced by diclofenac (competitive inhibition) whereas at 4° both binding parameters (i.e. dissociation constant and number of available binding sites) were modified (mixed inhibition). Diclofenac was also shown to bind to PMN with a low affinity. This binding was not diminished at 4° by various concentrations of FMLP which even increased the number of diclofenac binding sites on PMN at 22°. These data suggest that diclofenac binding to PMN may decrease FMLP-induced PMN respiratory burst by interfering with the peptide recognition by specific FMLP receptors.

Polymorphonuclear neutrophils (PMN) are involved in host defence against microorganisms, and in all types of inflammation [1, 2]. Under a variety of stimuli, they release oxygen by-products such as superoxide anion, hydrogen peroxide and hydroxyl radical as part of oxidative burst [3]. The purpose of these oxygen by-products is to kill invading micro-organisms [4]. In cases of misdirected or excessive inflammatory responses, the oxygen by-products released by PMN may be detrimental to the host [5, 6]. Anti-inflammatory agents (AIA) reduce inflammatory reaction development through various mechanisms including interference with PMN functions. Many AIA alter PMN responses induced by chemotactic formylated peptides [7–12], and most of them act by interfering with the specific binding of these peptides to PMN [13–20]. Moreover some AIA behave as competitive antagonists of the peptide binding to PMN [13, 14, 18–20]. If these agents do really compete with these peptides for their specific binding sites on PMN, then AIA binding to PMN should also be inhibited by formylated peptides, but this, to our knowledge, has not been shown. To gain further insight into the mechanisms by which AIA alter formylated peptide binding to PMN, and particularly to determine whether or not AIA binding to PMN is altered by formylated peptides, we studied the interactions of the nonsteroidal anti-inflammatory drug (NSAID) diclofenac sodium and peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) with PMN. Diclofenac inhibited both the PMN respiratory burst induced by FMLP and the FMLP binding to PMN. However, FMLP failed to reduce diclofenac binding to PMN.

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MATERIALS AND METHODS

Reagents. Diclofenac sodium (Ciba-Geigy, Basel, Switzerland) was diluted immediately prior to use in dimethylsulfoxide (DMSO) and then in 0.15 M Krebs-Ringer phosphate buffer (KRP), pH 7.4 to the final desired concentrations. The final concentration of DMSO in the assays was less than 0.5% and had no effect on PMN functions. FMLP, PMA, zymosan and superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (St Louis, MO). FMLP and PMA were dissolved in DMSO and stored

in aliquots at −80°. Appropriate dilutions were made in KRP and used extemporaneously. Zymosan was boiled in saline, washed twice, and stored in 40 mg/ml aliquots at −80°. Zymosan was opsonized by incubating 250 μl of zymosan suspension (10 mg) with 750 μl of human serum for 30 min at 37°. After high-speed centrifugation of the suspension, the pellet was resuspended in 1 ml of KRP containing 0.72 mM glucose. Dextran T 500 was from Pharmacia (Uppsala, Sweden), and radio-selectan, from Shering (Lyz-Les-Lannoy, France). Sera obtained from 10 healthy volunteers were pooled and stored in aliquots at −80°. ^{3}H-FMLP (specific activity 60 Ci/mmole) was obtained from New England Nuclear Corp. (Boston, MA) and ^{14}C-labeled diclofenac (specific activity: 3.44 mCi/mmole) was a generous gift from Ciba-Geigy (Basel, Switzerland). Soluene-dimilume R 30 was from Packard, BV (Breda, The Netherlands).

PMN preparation. Blood was obtained from peripheral veins of healthy adults, in preservative-free lithium heparin (10 IU/ml of blood). Whole blood was first centrifuged on Ficoll-Hypaque gradient. The resulting pellet which contained erythrocytes and polymorphonuclear cells was suspended in diluted isologous plasma. Red cells were removed by sedimentation of the cellular suspension using a mixture comprising 6.35% dextran and 10.5% radio-selectan as previously described [10]. The remaining erythrocytes were removed by one hypotonic lysis (30 sec). The final leukocyte suspension was suspended in KRP and contained more than 98% PMN.

Bacterial ingestion rate. The rate at which PMN ingested heat-killed ^{14}C-labeled *Klebsiella pneumoniae* was measured as previously described [21] in the presence of 100 microorganisms per PMN, except that PMN were incubated for 10 min at 37° with diclofenac at various concentrations before the microorganisms were added. Diclofenac was not removed during the assays.

Respiratory burst. Cyanide-insensitive oxygen uptake by PMN stimulated with opsonized zymosan (i.e. serum-treated zymosan: STZ), PMA or FMLP, was measured polarographically as previously described [22] in the presence of varying amounts of diclofenac. PMN were preincubated with the diclofenac which was not removed during the assays.

Superoxide anion production by PMN stimulated by FMLP or PMA was measured according to the method of Cohen and Chovaniec [23] by continuously recording the reduction of cytochrome *c* with an Uvikon 810/820 spectrophotometer. The incubation medium in a thermostatted cuvette (37°) contained 80 μM cytochrome *c*, 10% decomplemented fetal calf serum (FCS) and 5×10^5 PMN/ml KRP. As for other functional tests, PMN were preincubated for 10 min at 37° with varying amounts of diclofenac which was not removed during the assays. No reduction of cytochrome *c* occurred when superoxide dismutase (20 μg/ml) was added to the incubation medium. The given values were assessed under the conditions of maximal rate of cytochrome *c* reduction, i.e. in the linear part of the curve.

Results used for statistical analyses were the means of assays performed in duplicate, after subtraction of appropriate blanks. Results were discarded when a variation of more than 10% was observed between the duplicate measurements. Control experiments were performed by incubating the PMN for 10 min at 37° with the drug solvent (DMSO) which was not removed during the measurements. Drug solvent (less than 0.5%) did not affect PMN functions. More than 95% of the PMN incubated for 30 min at 37° with the largest amount of diclofenac used in the assays (i.e. 100 μg/ml) excluded Trypan blue. This suggested that diclofenac was not cytotoxic to the PMN under our experimental conditions. Moreover, as will be shown below, several of the fine functions of PMN were not altered by diclofenac.

FMLP and diclofenac binding assays. ^{3}H-FMLP binding assays were performed as previously described [24] with slight modifications. Two × 10^6 PMN in 180 μl KRP containing various amounts of diclofenac or diclofenac solvent solution (control) in propylene tubes were preincubated for 10 min at either 22° or at 4° in an ice-bath. Each determination was performed in duplicate. Diclofenac was not removed during the FMLP binding assays. For measurement of total binding, 20 μl of ^{3}H-FMLP was added to the incubation medium, leading to final FMLP concentrations ranging from 0.05 to 2×10^{-7}M. For each concentration of tritiated FMLP, nonspecific binding was measured in the presence of cold FMLP at a concentration 500 times that of the labeled FLMP. Cell suspensions were incubated with gentle shaking at 22° (binding isotherms) for 60 min [24] or at 4° for 120 min [25]. It has been verified that under these conditions binding reactions were at a steady state. The cells were then rapidly harvested on glass filters and washed with cold saline with a multiple cell culture processor (Skatron A.S., Norway). Filters were dried and placed in 4 ml of soluene-dimilume for radioactivity measurement (Kontron, Intertechnique, Plaisir, France). Nonspecific binding did not exceed 15% of the total binding counts.

^{14}C-diclofenac binding to the PMN was measured in the same way as ^{3}H-FMLP binding, except that unlabeled diclofenac was added at only 100 times the concentration of the labeled drug, because higher doses of cold diclofenac were found to be toxic to the PMN.

Computer modeling. The data from FMLP binding assays were subjected to a non-linear least-squares curve fitting using a LIGAND program developed by Munson and Rodbard [26] and provided by the Biomedical computing technology information center (BCTIC, Nashville) for an Apple II computer. This method is based on the law of mass action and allows the analysis of the binding of a ligand to multiple classes of binding sites. This procedure consists of a series of iterations in which the parameters are systematically adjusted until a least-squares solution is reached. Two statistical tests are performed: a runs test to examine the randomness of the distribution of the data points around the fitted curve and an analysis of variance together with an F-test of the significance of the regression. The data were fitted to one- and two-site models successively and the two-site model was accepted only when the fit of the data was significantly improved ($P < 0.05$).

Statistics were performed on a Compucorp 445

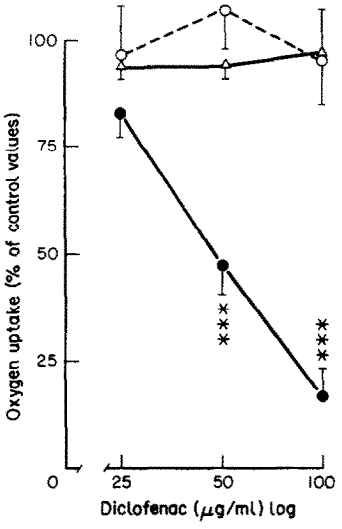

Fig. 1. Effect of diclofenac on soluble and particle stimulation of O_2-uptake by PMN. Diclofenac was present in the incubation medium throughout the experiments. PMN stimulation by 5×10^{-7} M FMLP (●), 1 μg/ml PMA (△) or 0.67 μg/ml STZ (○) started 10 min after PMN preincubation with diclofenac. Control and assay experiments were performed simultaneously using two oxygraph apparatuses. Results are expressed in percentages of control values. Mean control values (100%) for PMN O_2-uptake induced STZ, PMA and FMLP in nmole O_2 consumed per 10^6 PMN per min were respectively 8.77 ± 0.83 (mean ± 1 SD of 5 experiments); 6.48 ± 0.94 (6 experiments) and 7.46 ± 0.51 (5 experiments). Significant differences between control and assay values are indicated by *** ($P < 0.001$).

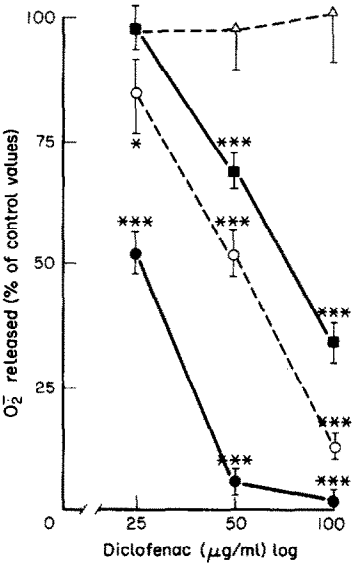

Fig. 2. Effect of diclofenac on FMLP-induced production of superoxide anion by PMN. PMN (5×10^5) were preincubated for 10 min at 37° in the absence or presence of diclofenac in plastic microcuvettes containing 10% fetal calf serum and 80 μM cytochrome *c*. The reduction of cytochrome *c* by control and diclofenac-treated PMN, was simultaneously and continuously monitored after addition of PMA (△) at the final concentration of 0.1 μg/ml or FMLP at 10^{-7} M (●), 10^{-6} M (○) and 10^{-5} M (■) into the cuvettes. Results are expressed in percentages of control values and represent the initial rate of PMN production of superoxide anion. Each value is the mean ± 1 SD of 5 experiments. The corresponding control values (100%) represent a production of 2.9 ± 0.2, 3.2 ± 0.4, 2.9 ± 0.5 and 2.8 ± 0.2 nmoles of superoxide anion per 5×10^5 PMN per min in the presence of FMLP concentration of 10^{-7}, 10^{-6}, 10^{-5} M and PMA respectively. Significant differences between control assay experiments are designated by * ($P < 0.05$) and *** ($P < 0.001$).

Statistician. The significance of differences between control and assay values was assessed by the Student *t*-test (paired or unpaired). Linear regression parameters were calculated by the least-square method.

RESULTS

Effect of diclofenac on PMN ingestion and oxidative burst

The rate at which PMN ingested heat-killed opsonized *Klebsiella pneumoniae* at a microorganism-to-PMN ratio of 100 was 9.2 ± 2.6 bacteria per PMN for a 10 min incubation (mean ± 1 SD from 5 experiments). In the presence of diclofenac concentrations below 100 μg/ml, PMN bacterial uptake was not modified (results not shown). Cyanide-insensitive oxygen uptake by the PMN, stimulated by PMA, STZ or FMLP was altered only with the latter stimulus (Fig. 1). The decrease in oxygen uptake by FMLP-stimulated PMN was linearly correlated to the logarithm of the diclofenac concentration (Fig. 1). The concentration of diclofenac required for half-inhibition (IC_{50}) of PMN O_2-uptake was approximately 50 μg/ml. Diclofenac also reduced the rate of superoxide anion production when PMN were stimulated by various FMLP concentrations (Fig. 2), but not by PMA. Half-inhibition of PMN superoxide production induced by 10^{-7}, 10^{-6} and 10^{-5} M FMLP occurred in the presence of a diclofenac concentration of 26, 52 and 72 μg/ml respectively. The presence of diclofenac in the incubation medium seemed essential for inhibiting FMLP-induced PMN responses since PMN preincubated with diclofenac for 10 min at 37° and then washed displayed normal responses (results not shown). This data, as well as the observation that diclofenac inhibited the PMN oxidative burst induced by FMLP but not that induced by PMA or STZ, seems to indicate that diclofenac could interfere with FMLP binding to PMN. The fact that increasing FMLP concentrations reversed diclofenac-induced inhibition of PMN response suggests that FMLP and diclofenac could compete for the same binding site on PMN. In other respects, more than 95% of PMN incubated with the largest amount of diclofenac tested (i.e. 100 μg/ml) excluded Trypan blue. This result as well as the absence of an inhibitory effect of diclofenac on PMN bacterial ingestion or PMA and STZ-induced PMN respiratory burst indicates that diclofenac is not toxic to PMN at the concentrations used, and that it does not modify the effector system of the oxidative burst.

Effect of diclofenac on FMLP binding

The results of Fig. 3 indicate that diclofenac reduced the specific binding of FMLP (10^{-7} M) to PMN at temperatures of 4° and 22° (room temperature). This inhibition was linearly correlated to the logarithm of diclofenac concentration. However, low concentrations of diclofenac (10, 20 μg/ml) induced greater inhibition ($P < 0.01$) of FMLP binding at 4° than at 22°. The concentration required for half-inhibition of FMLP binding was 34 μg and 48 μg/ml of diclofenac at 4° and 22° respectively. It is worthy to note that these values and those inhibiting PMN oxidative burst induced by FMLP are of the same order of magnitude (Figs 1 and 2). To further characterize this inhibition, we measured FMLP binding parameters for various peptide concentrations in the presence of diclofenac (10 and 20 μg/ml) at both temperatures. Accurate binding parameters were analysed by the use of the LIGAND program [26]. Each series of experiments (i.e. in the presence or absence of diclofenac) was first fitted by a one-site model and then by a two-site model. All experiments provided a best fit by the two-site model. The average binding parameters (4 separate experiments) corresponding to the two classes of FMLP sites are summarized in Table 1. Diclofenac enhanced the dissociation constant (K_d) of the high-affinity component (Site 1) of FMLP binding isotherms (22°) but failed to alter the corresponding total PMN binding capacity. Binding experiments performed at 4° indicated that the dissociation constant of the high-affinity component (Site 1) was similar to that measured at 22° whereas the total number of binding sites was reduced ($P < 0.001$). At 4°, diclofenac decreased both the affinity and number of Site 1. The low-affinity component (Site 2) of FMLP binding was characterized by a high, and apparently similar K_d value (close to 10^{-5}M), at 22° and 4°. The corresponding number of available binding sites was much less at 4° than at 22°. Both binding parameters of Site 2 were unaltered by diclofenac. Data from a representative experiment with a Scatchard plot [27] showing the alteration of the high-affinity component of FMLP binding by diclofenac, are shown in Fig. 4 (22°) and Fig. 5 (4°). From these data, the inhibition constant (K_i) for diclofenac reduction of FMLP binding to Site 1 was calculated using the equation $K_i = I/(K'_a/K_a - 1)$ [28] where K_a and K'_a are the dissociation constants of FMLP binding in the absence and presence of diclofenac respectively and I the diclofenac concentration. In the presence of 10 and 20 μg/ml diclofenac, the K_i values were found to be 6.25 and 5.32 μg/ml respectively at 22°, and 7.06 and 5.32 μg/ml respectively at 4°.

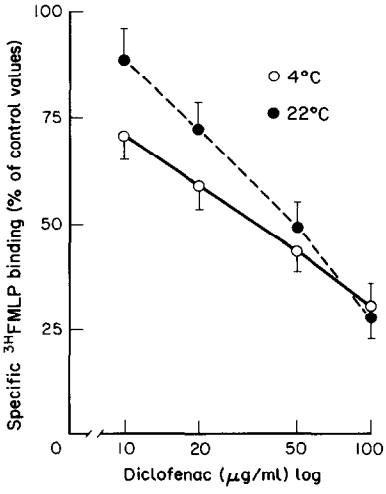

Fig. 3. Dose dependent inhibitory effect of diclofenac on ^{3}H-FMLP binding to PMN. PMN were preincubated for 10 min at 22° or 4° with various doses of diclofenac, and then with 10^{-7} M labeled FMLP still in the presence of diclofenac. The total incubation time lasted 60 min at 22° and 120 min at 4°. Nonspecific binding of labeled FMLP to PMN was determined in the presence of a 500-fold excess of unlabeled peptide and was less than 15% of total binding. Specific binding is defined as total minus nonspecific binding. Results are expressed in percentages of control values and are means ± 1 SD of 5 experiments. Corresponding control values (100%) in fmole of FMLP per 2×10^6 PMN were 169 ± 15.5 and 45.4 ± 2.5 at 22° and 4° respectively.

Table 1. Effect of diclofenac on FMLP binding to PMN

Temperature (°C)	Diclofenac (μg/ml)	Binding parameters (in % of control values) Site 1 K_d (1) (M)	Site 1 R (1) (Sites/PMN)	Site 2 K_d (2) (M)	Site 2 R (2) (Sites/PMN)
22		100%	100%	100%	100%
	0	(2.58 ± 1.74 10^{-8})	(6.4 ± 1.08 10^4)	(10.65 ± 3.9 10^{-6})	(1.93 ± 0.34 10^6)
	10	260 ± 16% ***	94 ± 19%	98 ± 6%	91 ± 4%
	20	451 ± 123% *	99 ± 56%	88 ± 19%	91 ± 8.4%
4		100%	100%	100%	100%
	0	(2.05 ± 1.24 10^{-8})	(1.6 ± 0.34 10^4)	(7.29 ± 5.2 10^{-6})	(2.55 ± 1.95 10^5)
	10	256 ± 63% *	84 ± 11%	104 ± 25%	70 ± 24%
	20	476 ± 218% *	39 ± 9% ***	117 ± 67%	79 ± 30%

Data from each experiment were analysed by the use of the LIGAND program [26]. Each series of data was found to be better fitted by a two-site model. The parameters of the high- (Site 1) and low- (Site 2) affinity binding sites were designated by K_d (dissociation constant) and R (total number of available FMLP binding sites). For experiments performed in the presence of diclofenac, the two binding parameters are expressed as the percentages of control values. The corresponding control values (100%) are given in brackets. Results are the mean ± 1 SD of 4 separate experiments. Significant differences between control and assay values are designated by * ($P < 0.05$) and *** ($P < 0.001$).

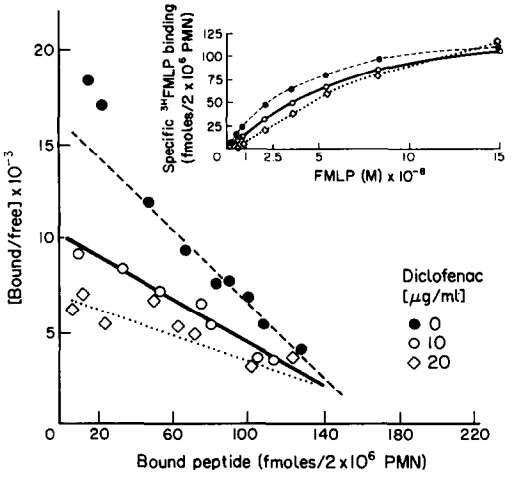

Fig. 4. Effect of diclofenac on the FMLP binding to PMN at 22°. PMN were preincubated in the absence (●) or presence of 10 (○) or 20 (◇) μg/ml diclofenac for 10 min at room temperature and then with various concentrations of labeled FMLP for 60 min at the same temperature. For each dose of labeled FMLP, nonspecific binding was determined in the presence of a 500-fold excess of cold FMLP, and did not exceed 15% of the total counts. Results shown are those of a representative experiment. The Scatchard plots represents the values corresponding to the high-affinity component of FMLP binding (Site 1, in Table 1).

Diclofenac binding to PMN and FMLP effect

Because diclofenac acted kinetically as a competitive inhibitor of ^{3}H-FMLP binding isotherms to PMN, it was important to find out whether diclofenac bound to the PMN, and if so, whether or not FMLP altered this binding. We measured the binding of labeled diclofenac in concentrations ranging from 1.25 to 50 μg/ml (i.e. at concentrations which altered FMLP binding) in the absence and presence of a 100-fold excess of cold diclofenac (i.e. from 0.125 to 5 mg/ml). Higher concentrations were not used because they were toxic to PMN as assessed by the Trypan blue exclusion test. Results shown in Fig. 6 indicate that diclofenac did bind to PMN at 4° or 22°. The dissociation constants (mean ± SD of three experiments) of this binding at 4° and 22° were 79 ± 23 μM (i.e. 25.1 ± 7.33 μg/ml) and 98 ± 31 μM (i.e. 31.4 ± 10 μg/ml) respectively with a corresponding total binding capacity of 11.3 ± 2.8 × 10^{6} (at 4°) and 54.4 ± 9.8 × 10^{6} (at 22°) sites per PMN respectively. In order to gain further insight into the mechanism by which diclofenac inhibits FMLP binding to PMN, we measured FMLP effects on diclofenac binding to PMN. Figure 7 shows that FMLP did not reduce the amount of diclofenac bound to PMN at a temperature of 4° and even enhanced it at 22°.

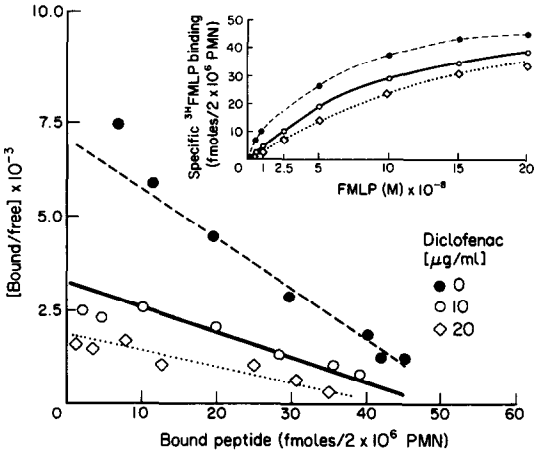

Fig. 5. Effect of diclofenac on the FMLP binding to PMN at 4°. PMN were preincubated in the absence (●) or presence of 10 (○) or 20 (◇) μg/ml diclofenac for 10 min at 4° and then in the presence of various amounts of labeled peptide for 120 min at 4°. Nonspecific binding of FMLP, determined in the presence of a 500-fold excess of cold FMLP did not exceed 20% of the total bound counts. Results shown are those of a typical experiment. The Scatchard plot reproduces the data corresponding to the high-affinity component of FMLP binding (Site 1, in Table 1).

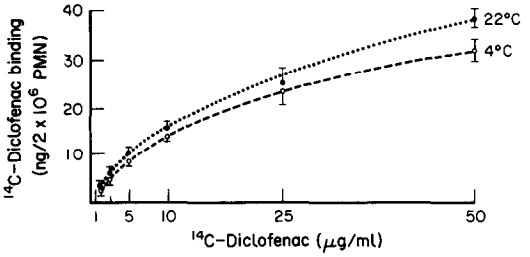

Fig. 6. ^{14}C-diclofenac binding to PMN. PMN were incubated at 22° (●), or at 4° (○) with various concentrations of ^{14}C-diclofenac. For each concentration of labeled diclofenac, a 100-fold excess of cold diclofenac was tested to determine the "nonspecific binding". Results are expressed as ng diclofenac bound to 2 × 10^{6} PMN (mean ± 1 SD of four experiments) and represent the difference between diclofenac amounts bound to PMN in the absence and presence of cold diclofenac.

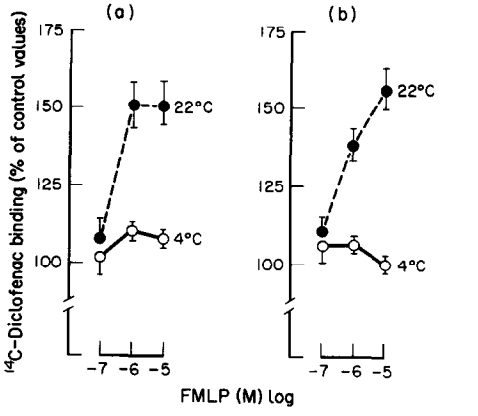

Fig. 7. Effect of FMLP on diclofenac binding to PMN. PMN were incubated with 2.5 (part a) or 25 μg/ml (part b) ^{14}C-diclofenac in the absence (control) or presence of various FMLP concentrations (10^{-7}–10^{-5} M) either at room temperature (22°) or 4° for 1 hr. Results are the mean ± 1 SD of three experiments and are expressed in percentages of control values.

DISCUSSION

The results reported here confirm that PMN bear high- and low-affinity binding sites for FMLP [25, 29–32]. They further indicate that diclofenac possesses *in vitro* antagonistic properties against both FMLP-induced PMN respiratory burst and the high-affinity component of FMLP binding to PMN. These data suggest that PMN receptors of high-affinity for FMLP are responsible for FMLP-induced oxidative burst. The inhibition by diclofenac of FMLP-induced PMN stimulation as well as of FMLP binding will be discussed, bearing in mind that the drug in concentrations of 10–100 μg/ml is not toxic to the PMN because it does not alter the ability of the cell to exclude Trypan blue, ingest heat-killed *Klebsiella pneumoniae,* or develop an oxidative burst when stimulated by opsonized zymosan or PMA.

FMLP is known to bind to specific receptors on the PMN plasma membrane [14, 20, 25, 29–34]. Recently, several groups have obtained evidence for the existence of a high- and low-affinity state of FMLP binding sites [25, 31, 32]. Other authors, using membrane preparations, found that the high-affinity state was convertible to the low-affinity state in the presence of guanine nucleotides [33, 34]. Our binding data, obtained at 22° as well as 4° were better fitted by a two-site model, and showed high- and low-affinity binding sites on PMN. The high-affinity component was characterized by an apparent dissociation constant close to 2.6×10^{-8} M and 2.0×10^{-8} M at 22° and 4° respectively (Table 1, under Site 1) which is compatible with results reported previously [14, 20, 25, 33]. The low-affinity component was characterized by high K_d value close to 10^{-5} M at 22° and 4° (Table 1, under Site 2) which is similar to that of low-affinity site recently reported on membrane preparations [34]. The binding of FMLP to the high-affinity component, in contrast to the low one, was decreased by diclofenac (Table 1). These data and the observed inhibition by diclofenac of FMLP-induced PMN respiratory burst suggest that the high-affinity component could be responsible for the activation of the PMN oxidative burst induced by FMLP. The concentration of diclofenac required for half-inhibition of PMN oxidative burst (Figs 1 and 2) was of the same order of magnitude as both the K_d value of diclofenac binding to PMN (Fig. 6) and the concentration of diclofenac required for half-inhibition of the specific FMLP binding to PMN (Fig. 3). Moreover, in the presence of diclofenac, increasing concentrations of FMLP restored in part the oxidative burst. However, it has not been possible to establish a quantitative relationship between diclofenac concentration and PMN responses induced by increasing doses of FMLP because the functional responses of control PMN (in the absence of diclofenac) were not proportional to FMLP concentrations (Fig. 2).

The mechanism by which diclofenac alters the high-affinity sites was dependent on the temperature, since the number of available binding sites was reduced at 4° but not at 22°. At 22°, diclofenac acts kinetically as an apparent competitive inhibitor of FMLP binding to its high-affinity sites, i.e. decreasing the apparent affinity constant of the peptide without altering the total number of available receptors (Table 1). Similar effects have been reported for other AIA such as phenylbutazone [13, 14], indomethacin [20] and auranofin [18]. By contrast, at 4°, the effects of diclofenac were of the mixed type (i.e. a decrease in the affinity of the peptide as well as in the total number of available receptors). These types of effects have been previously reported for cetiedil [16] and auranofin [7]. This suggests that at 4° diclofenac could block, in an apparently irreversible way, some of the high-affinity FMLP receptors and would not be a true competitive antagonist of FMLP at this temperature. It is thus apparent that diclofenac does not act similarly on FMLP high-affinity receptors at 22° and 4° even though its calculated apparent K_i at these 2 temperatures is similar (5.3 μg/ml of diclofenac for a concentration of 20 μg/ml). This differential effect of diclofenac on the number of FMLP binding sites at 22° and 4° cannot be explained by the reported data. However, an effect on the traffic of membrane components (which occurs at 22° and not at 4°) [35–37] as suggested here by the great number of FMLP sites measured at 22° as compared to 4°, cannot be ignored. Whatever the reasons behind the discrepancies, we have measured the binding of diclofenac to the PMN and the effects of FMLP on this binding. Our results showed that diclofenac does bind to the PMN. The binding was characterized by a low-affinity constant value (apparent K_m of 98 and 79 μM at 22° and 4° respectively) and by the large number of available sites (54 and 11×10^6 sites per PMN at 22° and 4° respectively). The very high number of diclofenac binding sites compared to those of FMLP may explain that FMLP did not induce any noticeable decrease of diclofenac binding. It has, however, observed that the amount of diclofenac bound to PMN at 22° in the presence of FMLP was increased in comparison to that bound at 4°. This result argues for the increase of a traffic of membrane comonents which occurs at 22° and not at 4° and is known to be increased by FMLP [35–37].

In conclusion, diclofenac binds with low affinity to PMN. At 22° this binding, which may involve more than 10^7 sites per PMN, inhibits in a competitive-like manner the binding of FMLP to its high-affinity receptors (apparent K_d of FMLP binding decreases while the number of binding sites is not altered). At 4° diclofenac binds to PMN and inhibits FMLP binding to its high-affinity receptors in a mixed manner, i.e. decreases both the affinity and the number of specific receptors for FMLP. The reason behind the differential effects of diclofenac on FMLP binding at 4° and 22° needs further study. The interference of diclofenac with the binding of FMLP may account for the effects of diclofenac on PMN respiratory burst induced by FMLP.

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REFERENCES

1. T. P. Stossel, *N. Engl. J. Med.* **290**, 717 (1974).
2. R. L. Baehner, *Clin. Haemat.* **4**, 609 (1975).
3. S. J. Klebanoff, *Ann. intern. Med.* **93**, 480 (1980).
4. S. J. Klebanoff and R. A. Clark, in *The Neutrophil; Function and Clinical Disorders*, p. 283. Elsevier: North-Holland Biomedical Press, Amsterdam (1978).
5. B. Halliwell, *Cell Biol. Int. Rep.* **6**, 529 (1982).
6. K. F. Austen, *J. Immun.* **121**, 793 (1978).
7. T. D. Coates, B. Wolach, D. Y. Tzeng, C. Higgins, R. L. Baehner and L. A. Boxer, *Blood.* **62**, 1070 (1983).
8. I. Rivkin, G. U. Foschi and C. H. Rosen, *Proc. Soc. exp. Biol. Med.* **153**, 236 (1976).
9. M. J. H. Smith and J. R. Walker, *Br. J. Pharmac.* **63**, 473 (1980).
10. A. Perianin, M. T. Labro and J. Hakim, *Biochem. Pharmac.* **31**, 3071 (1982).
11. D. Pham Huy, M. Roch-Arveiller, O. Muntaner and J. P. Giroud. *Eur. J. Pharmac.* **111**, 251 (1985).
12. K. Tanaka, K. Kanaoka, M. Egawa, N. Abe, I. Watanabe and S. Hirai, *Jap. J. Pharmac.* **35**, 181 (1984).
13. C. Dahinden and J. Fehr, *J. clin. Invest.* **66**, 884 (1980).
14. R. D. Nelson, J. M. Gracyck, V. D. Fiegel, J. M. Herron and D. E. Chenoweth, *Blood* **58**, 752 (1981).
15. K. Van Dyke, D. Peden, C. Van Dyke, G. Jones, V. Castranova and J. Ma, *Inflammation* **6**, 113 (1982).
16. J. B. Wolach, T. D. Coates, D. Y. Tzeng, R. L. Baehner and L. A. Boxer, *Blood* **62**, 274 (1983).
17. K. Lohr, J. B. Feix and C. Kurth, *J. infect. Dis.* **150**, 5 (1984).
18. I. Hafström, B. E. Seligman, M. M. Friedman and J. I. Gallin, *J. Immun.* **132**, 2007 (1984).
19. W. F. Stenson, J. Mehta and I. Spielberg, *Biochem. Pharmac.* **33**, 407 (1977).
20. H. Cost, C. Gespach and J. P. Abita, *FEBS Lett.* **132**, 85 (1981).
21. M. Torres, D. de Prost, J. Hakim and M.-A. Gougerot, *Eur. J. clin. Invest.* **9**, 209 (1979).
22. A. Perianin, M. Torres, M. T. Labro and J. Hakim, *Biochem. Pharmac.* **32**, 2819 (1983).
23. H. J. Cohen and M. E. Chovaniec, *J. clin. Invest.* **61**, 1081 (1978).
24. C. Koo, R. J. Lefkovitz and R. Snyderman, *Biochem. biophys. Res. Commun.* **196**, 442 (1982).
25. J. Mehta and I. Spielberg, *Inflammation* **7**, 301 (1983).
26. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
27. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
28. Y. C. Cheng and W.H. Prusoff, *Biochem. Pharmac.* **22**, 3099 (1983).
29. L. T. Williams, R. Snyderman, M. C. Ike and R. J. Lefkowitz, *Proc. natn. Acad. Sci. U.S.A.* **74**, 1204 (1977).
30. S. Aswanikumar, B. Corcoran, E. Schiffmann, A. R. Day, R. J. Feer, H. J. Showell, E. L. Becker and C. Pert, *Biochem. biophys. Res. Commun.* **74**, 810 (1977).
31. W. M. Mackin, C. K. Huang and E. Becker, *J. Immun.* **129**, 1608 (1982).
32. E. J. Goetz, D. W. Foster and D. W. Goldman, *Biochemistry* **19**, 5722 (1981).
33. C. Koo, R. J. Lefkowitz and R. Snyderman, *J. clin. Invest.* **72**, 748 (1983).
34. F. Okajima, T. Katada and M. Ui, *J. biol. Chem.* **260**, 6761 (1985).
35. L. A. Sklar, D. A. Finney, Z. G. Oades, A.J. Jesaitis, R. G. Painter and C. G. Cochrane, *J. biol. Chem.* **259**, 5661 (1984).
36. M. P. Fletcher and J. I. Gallin, *J. Immun.* **124**, 1585 (1980).
37. M. P. Fletcher, B. Seligman and J. I. Gallin, *J. Immun.* **128**, 941 (1982).