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STIMULATION OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES POTENTIATES THE UPTAKE OF DICLOFENAC AND THE INHIBITION OF CHEMOTAXIS

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Abstract—Diclofenac sodium, a non-steroidal anti-inflammatory drug, has been shown to impair the stimulation of human polymorphonuclear leukocytes (PMNs) by chemoattractants. To gain insight into the mechanism of action of this agent, we investigated the uptake of diclofenac by resting and activated PMNs and the effect of the drug on PMN locomotion. During incubation of resting PMNs at 37° in the presence of 78 μM (25 μg/mL) diclofenac, drug uptake reached a plateau in less than 2 min. The resulting cellular to extracellular diclofenac concentration ratio (C/E) was 1.01 ± 0.13 (mean ± SD). Stimulation of PMNs at 37° but not at 4° with the chemoattractant formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol myristate acetate (PMA), induced a rise in diclofenac uptake, which was dependent on incubation time and diclofenac and stimulus concentrations. Maximal C/E was 1.83 ± 0.18 and 4.40 ± 0.60 (mean ± SD) for PMNs stimulated with 10 μM fMLP and 0.16 μM PMA, respectively. The diclofenac associated with PMNs was predominantly present in the soluble fraction of disrupted cells. Interestingly, PMNs which were pretreated with diclofenac and stimulated with fMLP, exhibited impaired random and directional locomotion induced by activated serum, as compared to controls, i.e. PMNs treated with diclofenac alone or fMLP alone. Thus, stimulation of PMNs enhances diclofenac uptake and potentiates the drug impairment of chemotactic activity. These findings could explain, in part, the observed anti-inflammatory properties of this compound.

Stimulation of polymorphonuclear leukocytes (PMNs‡) by chemoattractants such as N-formylated peptide formyl-methionyl-leucyl-phenylalanine (fMLP), complement-derived C5a or leukotriene B4, leads to the activation of various cellular responses including directed migration (chemotaxis), production of reactive oxygen species (respiratory burst) and release of lysosomal enzymes (exocytosis). These biological cellular functions account for important microbicidal, tumoricidal and inflammatory properties of phagocytes [1, 2]. Non-steroidal anti-inflammatory drugs (NSAID) have been shown to induce *in vivo* and *in vitro* alterations in PMN functions [3–12]. Certain agents such as diclofenac sodium have been found to inhibit the PMN stimulation induced by fMLP and C5a [6, 7], whereas other drugs such as indomethacin or phenylbutazone [6–10] affected PMN responses induced by fMLP more selectively, through a mechanism resembling a competitive inhibition of fMLP binding to its specific receptors on PMNs [8, 9, 11]. Diclofenac binds to PMNs non-specifically [12], suggesting that this drug may alter PMN functions through various mechanisms. This study was undertaken to determine the influence of PMN stimulation on diclofenac uptake and the potential effect of cellular diclofenac on subsequent chemotactic stimulation of PMNs.

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‡ Abbreviations used: PMN, polymorphonuclear leukocyte; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; NSAID, nonsteroidal antiinflammatory drugs; KRPB, Krebs–Ringer phosphate buffer; C/E, cell-to-medium ratio.

MATERIALS AND METHODS

Reagents. Cold and [^{14}C]diclofenac sodium (sp. act. 3.44 mCi/mmol) were gifts from Ciba–Geigy (Basel, Switzerland). Stock solutions of labeled diclofenac at 50 mg/mL in dimethylsulfoxide (DMSO) were stored at a temperature of −80° until use. Cold diclofenac was diluted immediately prior to use in DMSO and then in 0.15 M Krebs–Ringer phosphate buffer (KRPB) pH 7.4 to the final desired concentration. fMLP and phorbol myristate acetate (PMA) from Sigma Chemical Co. (St Louis, MO, U.S.A.) were dissolved in DMSO and stored at −80°. Appropriate dilutions were made in KRPB. Dimilume R30 was from Packard Becker B.V. (Breda, The Netherlands).

PMN preparation. Venous blood was obtained from healthy adults, in preservative-free lithium heparin (10 units/mL of blood). Leukocytes were isolated by one step centrifugation of blood on a mixture of Ficoll-Hypaque (Monopoly resolving medium from Flow Laboratories, France) as previously described [13]. The remaining erythrocytes were removed by one hypotonic lysis (30 sec). Leukocyte suspension was finally suspended in KRPB and contained approximately 98% PMNs.

Assay of diclofenac association with PMNs. Assays were performed in duplicate in 1.5 mL propylene Eppendorf tubes in a final volume of 200 μL of KRPB containing 10^7 PMNs/mL. PMNs were preincubated

with labeled diclofenac either at 37° or 4° and then treated with fMLP, PMA or the drug solvent (control). After incubation at 37°, tubes were placed in an ice bath for 5 min and spun down at 11,000 *g* for 5 sec. Supernatants were removed and the cell pellets were washed twice with cold saline. To remove free labeled diclofenac from cell pellets, PMNs were finally harvested on glass fiber filters with a multiple cell culture processor (Skatron, A.S. Norway) as previously described [12]. Radioactivity measurements were performed in 4 mL of dimilume R30 using a β-counter SL 3050 (Kontron, Plaisir, France). Blank values, i.e. the amount of radioactivity retained by filters in the absence of PMNs in the medium did not exceed 50 cpm. Results are expressed as percentages of the total measured binding.

Assessment of diclofenac in the PMN soluble and particulate fractions of PMNs. Cells were incubated with labeled diclofenac in the absence or presence of fMLP or PMA under conditions leading to an optimal drug uptake at 37°. PMNs were then washed twice, resuspended in 200 μL of cold KRPB and disrupted in an ice bath for 20 sec using a 20/200 S.V. Sonicator (C.I.T. Alcatel-Pons, France) at a setting of 1.5 (approx. 20 W). Examination of PMN sonicates at the microscope showed that approximately 97% of PMNs were disrupted. Aliquots of 100 μL of PMN homogenates were spun down for 30 min at 160,000 *g* (Beckman Airfuge, Palo Alto, CA, U.S.A.). Supernatants and pellets were carefully separated and their amount of radioactivity evaluated. Results are expressed as the total amount of radioactivity per 2×10^6 cells.

Determination of cellular concentration of diclofenac. The mean volume of PMN was electronically determined by the use of a ZB-ZBI Coulter counter (Coultronics, Margency, France) as described [14]. Calibration was performed with Coulter latex particles. PMNs were preincubated with 78 μM diclofenac at 37° then pretreated in the absence or presence of 10 μM fMLP or 0.16 μM PMA. Aliquots of the PMN suspension incubated at 37° were collected and used directly for evaluating PMN volume. Cellular diclofenac concentrations were determined in resting and stimulated PMNs and the cellular to extracellular concentration ratio was calculated on the basis of 2×10^6 PMNs in 200 μL KRP.

PMN migration assay. Random and directional migration of PMN under agarose were measured by the leading front method using the technique previously described [10], except that PMNs were pretreated with 0, 78 and 156 μM cold diclofenac in the absence (resting) or presence of 10 μM fMLP or 0.16 μM PMA. Cells were then washed twice with cold KRPB and adjusted at the concentration of 10^5 PMN/μL. Directional migration of PMN was induced by fMLP and activated serum [10]. In some experiments, labeled and unlabeled diclofenac were compared and were found to induce similar inhibition of PMN migration, as described [5]. Results are expressed in arbitrary units.

Statistics. Means, standard deviations (SD) and standard errors of the means (SE) were calculated for each series of experiments. Student's *t*-test was used to assess differences between control and assay experiments.

RESULTS

Characterization of diclofenac association with resting and stimulated PMNs

The time course of diclofenac association with resting and stimulated PMNs was first measured in the presence of 78 μM (25 μg/mL) labeled diclofenac (Fig. 1), a drug concentration that was previously found not to alter the PMN locomotion and respiratory burst induced by 10 μM fMLP [5, 12]. Resting PMNs incubated at 37° for 40 min with diclofenac, incorporated approximately 14 ng of drug per 2×10^6 PMNs, which represents approximately 0.25% of the total drug amount in the medium. This diclofenac amount was reached within 2 min and did not change significantly during the next 40 min of incubation. When PMNs were stimulated with 10 μM fMLP, the amount of diclofenac associated with PMNs rose during 15 min and declined weakly thereafter suggesting a drug release from PMNs. PMA also enhanced diclofenac uptake, which was greater and more rapid than that observed with fMLP. The maximal PMA effect peaked after 30 min of PMN stimulation and was also followed by a weak decline. Approximately 95% of the PMNs treated with diclofenac in the presence of fMLP or PMA were viable, as assessed by the Trypan blue exclusion test (results not shown), indicating that the diclofenac association with activated PMNs was not due to any drug cytotoxic effects.

Diclofenac uptake by resting, fMLP and PMA-stimulated PMNs was proportional to the labeled drug concentration from 3 to 304 μM (Fig. 2), reaching a maximal cellular amount of 23.1, 52.4 and 180.4 ng of drug/2×10^6 PMNs, respectively, for 30 min incubation. fMLP induced a diclofenac uptake which was proportional to the peptide concentration from 0.1 to 10 μM (Fig. 3). The diclofenac uptake induced by PMA was proportional to the stimulant concentration up to 0.16 μM and plateaued with higher concentrations (Fig. 4). The maximal measured incorporation induced by 10 μM fMLP or 0.16 μM PMA was approximately 36.7 and 84.6 ng diclofenac per 2×10^6 cells, respectively, after 30 min of incubation of cells at 37° with 78 μM diclofenac. At 4°, neither PMA nor fMLP enhanced diclofenac uptake (Figs 3 and 4).

To determine whether stimulation of PMNs induced diclofenac accumulation, we measured the drug concentration in cells which were treated with 78 μM diclofenac at 37° in the presence or absence of 10 μM fMLP or 0.16 μM PMA. The average volume of resting PMNs was approximately 260 μm^3, which represents a volume of 1.2 μL for 5×10^6 PMNs. This value is close to that found by others using the same technique or isotopic markers to assess intra- and extracellular spaces [15–18]. The volume of fMLP-stimulated PMNs was not different from that of resting cells, whereas that of PMA-stimulated PMNs was approximately 30% larger, as reported [18, 19]. The calculated maximal cellular to extracellular diclofenac concentration ratio (mean ± SD) for PMNs treated with 78 μM diclofenac in the absence or presence of 10 μM fMLP or 0.16 μM PMA was 1.01 ± 0.13, 1.83 ± 0.18 and 4.40 ± 0.60, respectively.

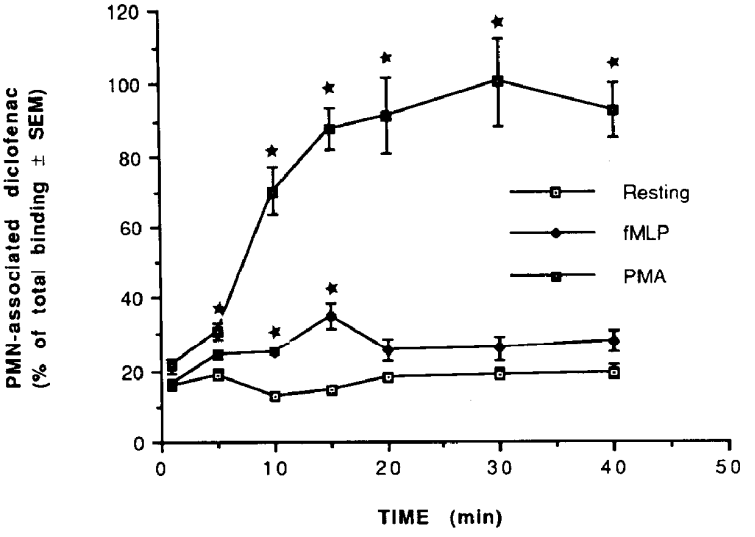

Fig. 1. Time course of diclofenac association with resting and stimulated PMNs. Cells were treated at 37° in the presence of 25 μg/mL (78 μM) labeled diclofenac for 2 min and then incubated in the absence (resting) or presence of 0.16 μM PMA or 10 μM fMLP. Data represent the total amount of diclofenac associated with 2×10^6 PMNs after cells were washed twice (means of four experiments). Results are expressed as percentages of the maximal binding obtained in the presence of PMA (i.e. 1309 ± 157 cpm). Total counts were approximately 83,000 cpm, representing 5 μg of diclofenac. Statistically significant differences between results of experiments performed with and without stimulant are designated by * ($P < 0.05$).

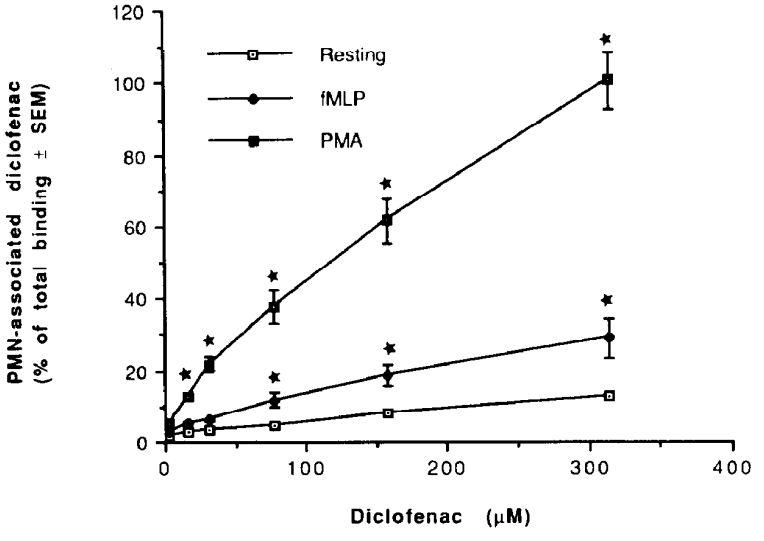

Fig. 2. Effect of drug concentration on diclofenac uptake by resting and stimulated PMNs. Cells were pretreated at 37° for 2 min with various concentrations of labeled diclofenac ranging from 3.1 to 310 μM and then incubated for 30 min at the same temperature without (resting) or with 10 μM fMLP or 0.16 μM PMA. Data represent the total amount of diclofenac per 2×10^6 PMNs (means of four experiments) and are expressed as per cent of the maximal measured binding obtained with PMA (i.e. 2996 ± 240 cpm). Statistically significant differences between results of experiments performed in the absence and presence of stimulant are designated by * ($P < 0.05$).

Distribution of diclofenac in resting and stimulated PMNs

The above results indicate that fMLP and PMA enhanced diclofenac uptake by PMNs at 37°. We determined the distribution of the incorporated drug in the soluble and particulate fractions of sonicated cells (Fig. 5). The soluble fraction of PMNs treated with 78 μM labeled diclofenac in the absence and presence of 10 μM fMLP or 0.16 μM PMA contained, respectively, 73 ± 10, 78 ± 10 and 76 ± 7% (means ± SD) of the total cellular diclofenac. However, the amount of diclofenac in the soluble fraction of fMLP or PMA-treated cells was significantly

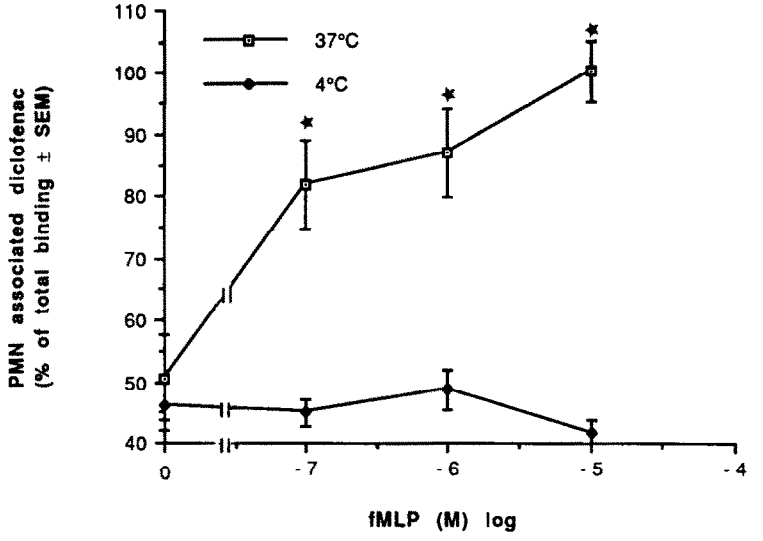

Fig. 3. Effect of temperature on fMLP-induced diclofenac uptake. PMNs were incubated with 25 μg/mL labeled diclofenac for 2 min at 37° or 4° and then treated for 30 min with various doses of fMLP. Data represent the total amount of diclofenac per 2×10^6 PMNs and are expressed as per cent of the maximal measured binding i.e. 424 ± 28 cpm (means of four experiments). Statistically significant differences between results of experiments performed at 4° and 37° are designated by * ($P < 0.05$).

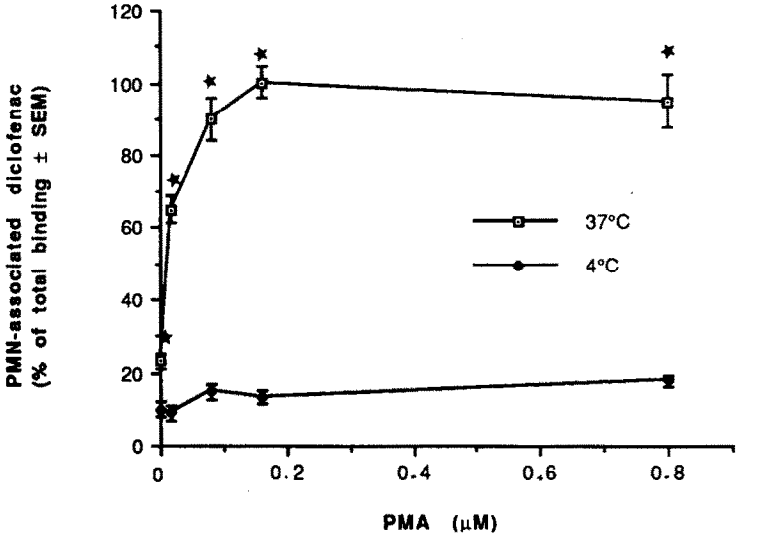

Fig. 4. Effect of temperature on PMA-induced diclofenac uptake. PMNs were pretreated with 25 μg/mL labeled diclofenac for 2 min at 37° or 4° and then with various doses of PMA for 30 min at the same temperature. Results represent the total amount of diclofenac per 2×10^6 PMNs, expressed as per cent of maximal measured binding i.e. 1409 ± 65 cpm (means of four experiments). Statistically significant differences between result of experiments performed at 4° and 37° are designated by * ($P < 0.05$).

enhanced (i.e. 19.5 ± 2.1 and 50.4 ± 4.5 ng, respectively), as compared to that of resting cells (10.8 ± 1.6 ng). The amount of diclofenac in the particulate fraction of resting and fMLP-treated cells was similar (4.0 ± 0.8 and 5.42 ± 0.8 ng, respectively) whereas in PMA-treated cells, the amount of diclofenac associated with particulate fractions was significantly greater (17.3 ± 2.77 ng). These data support the possibility that activatable membrane processes induced by fMLP and PMA mediate diclofenac accumulation into the cytosol of PMNs.

Influence of cellular diclofenac on PMN migration

To determine whether the incorporated diclofenac affected PMN functions, cells were treated at 37° for 30 min without (control) and with 78 and 156 μM diclofenac either in the absence (resting) or presence of 10 μM fMLP. PMNs were then washed and their

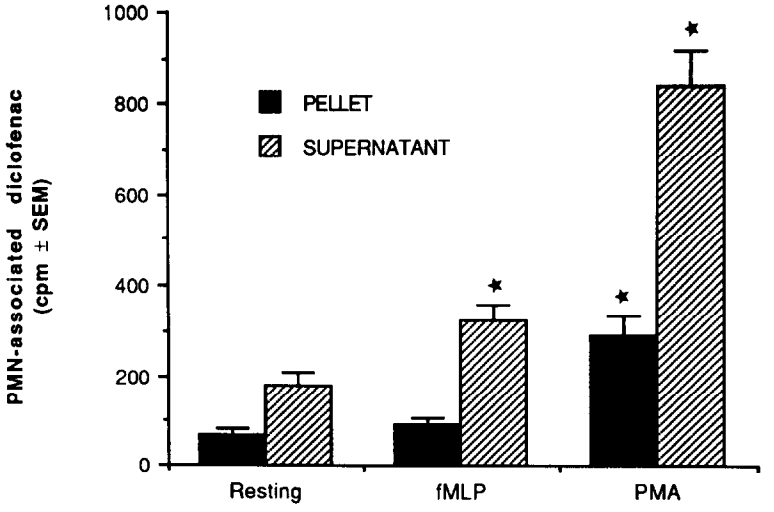

Fig. 5. Distribution of diclofenac in the particulate and soluble fractions of resting and stimulated PMNs. PMNs were treated with 78 μM labeled diclofenac and then incubated in the absence (control) or presence of 10 μM fMLP or 0.16 μM PMA for 30 min at 37°. Cells were washed and disrupted by sonication. Homogenates were spun down and supernatants and pellets were carefully separated. Results represent the total amount of diclofenac in the pellet and supernatant derived from 2×10^6 PMNs (means of four experiments). Statistically significant differences between cell fractions obtained with resting and stimulated PMNs are designated by * ($P < 0.05$).

ability to locomote in the presence or absence of activated serum was examined (Fig. 6). This chemoattractant was preferred to fMLP since PMNs treated with 10 μM fMLP for 30 min failed to display any directed migration in the presence of the same tripeptide (results not shown) due to homologous deactivation [20]. When PMNs were incubated with diclofenac in the absence of any stimulant, they displayed normal random and directed locomotion, as compared to untreated controls, although a non-significant decrease of 18% in the migration of PMNs pretreated with 156 μM diclofenac was observed (Fig. 6A and B). Cell pretreatment with fMLP alone enhanced random locomotion of PMNs without affecting their directed migration induced by activated serum. When PMNs were treated with 78 or 156 μM diclofenac in the presence of fMLP, they displayed a decrease of 54 and 55% in random migration, respectively. Their directed locomotion induced by activated serum was also reduced by 44 and 49% of locomotion, respectively, as compared to appropriate controls, i.e. PMNs treated with fMLP in the absence of diclofenac. PMNs pretreated in the presence or absence of diclofenac and stimulated with 0.16 μM PMA failed to exhibit any spontaneous or directed locomotion (data not shown). These results are in agreement with previous data showing inhibitory effects of PMA on chemo-attractant-induced PMN functions [21].

DISCUSSION

Diclofenac is widely used in the treatment of certain rheumatoid inflammatory disorders. In previous studies, we showed that this drug binds to PMNs non-specifically [12] and interferes with biological functions of chemoattractant-stimulated cells [5]. The diclofenac-induced alteration of PMN functions is reversible, since normal cell functions are recovered when diclofenac is washed out [5, 12]. To gain insight into the mechanism of action of this drug, we characterized the association of diclofenac with resting and stimulated PMNs and its potential effect on chemotaxis. The results show that diclofenac is spontaneously taken up by resting PMNs and that stimulation of PMNs by fMLP or PMA potentiates this uptake at 37° but not at 4°. Moreover, stimulated diclofenac uptake induced by fMLP more potently impaired the ability of PMN to locomote, as compared to controls.

The characteristics of diclofenac uptake described here appear to be different from those previously reported in PMNs with other members of the NSAID family [18, 23] or antibiotics [15–17, 23–27]. Thus, in non-stimulated PMNs, diclofenac uptake occurred rapidly, resulting in C/E of 1.01. This factor varies from 1 to 5 for other NSAID such as acetylsalicylic acid and sodium salicylate [18] and from 20 to 90 for indomethacin [22]. Other drugs, such as clindamycin [16], erythromycin [24], ofloxacin [25] or josamycin [24] are rapidly incorporated, with a resulting C/E of above 5, suggesting that, in contrast to diclofenac, these drugs are spontaneously concentrated in PMNs. PMA, which appears here to be a potent stimulant of diclofenac uptake (Figs 1, 2 and 3), does not enhance the incorporation of antibiotics such as rifampicin [28] or azythromycin [17] but does potentiate the uptake of clindamycin [29] and acetylsalicylic acid [18]. Thus, accumulation of drugs by PMNs is mediated through both passive and activatable processes. Enhanced diclofenac incorporation in the presence of fMLP and PMA occurred at 37° but not at 4° (Figs 3 and 4) and was mainly located in the soluble fraction of disrupted PMNs (Fig. 5),

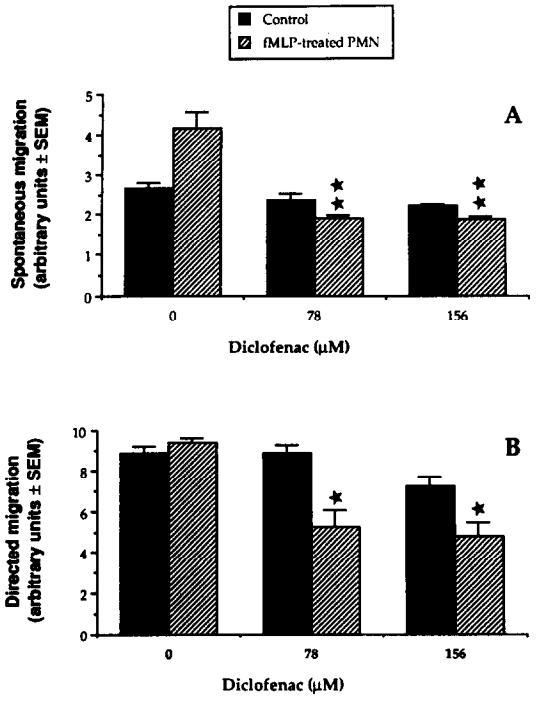

Fig. 6. Effect of diclofenac uptake on PMN random and directional migration. PMNs were first treated for 2 min at 37° without (control) or with 78 and 156 μM diclofenac and then incubated for 30 min in the absence (resting) or presence of 10 μM fMLP. PMNs were washed twice and allowed to migrate in the absence of any chemoattractant (part A) or in the presence of activated serum (part B) during 2 hr. Results are expressed in arbitrary units and represent the mean of three experiments. One unit represents 156 μM. Significant differences between control and assay values are indicated by * ($P < 0.05$) and by ** ($P < 0.01$).

suggesting that activatable transmembrane processes induced by these two stimuli mediate diclofenac accumulation in the cytosol. It is possible that a proportion of the diclofenac measured in the soluble PMN fraction was artificially released from particulate components during PMN disruption, since diclofenac weakly binds to the PMN membrane [7].

The mechanisms whereby diclofenac enters PMNs were not studied here but some explanations can be inferred from studies with other NSAID. Cell association with the tested drugs can occur through various processes including absorptive or fluid-phase endocytosis, passive diffusion, carrier-mediated uptake, ligand trapping or permeation and the activation of sodium/hydrogen antiport [22]. PMA and fMLP are active stimulators of pinocytosis, a process that could explain diclofenac uptake. In the case of PMA, this process is probably mediated through the activation of protein kinase C, since PMA potently stimulates this enzyme [30] and the internalization of membrane proteins [31]. However, PMA may also operate through other mechanisms than pinocytosis since it fails to enhance the uptake of certain drugs [17, 28]. Internalization of fMLP receptors [32] may also potentially account for diclofenac entry into PMNs. This hypothesis is suggested by the observation that diclofenac behaves like a competitive inhibitor of fMLP binding to its specific sites [12], which indicates that diclofenac may interact with fMLP binding sites or cellular components involved in the receptor activities of this peptide. Interestingly, 5 to 30 μg/mL cytochalasin B, an agent known to depress chemotaxis and to potentiate the respiratory burst of fMLP-stimulated PMNs [33, 34], enhanced the amount of diclofenac in fMLP-stimulated cells by approximately 50% without affecting that induced by PMA (results not shown). These data suggest that diclofenac uptake in fMLP- and PMA-stimulated PMNs are regulated through different mechanisms.

The diclofenac associated with resting PMNs did not interfere with the primary stimulation of PMNs, as examined by measuring fMLP- or activated serum-induced migration (Fig. 6). The diclofenac incorporated by fMLP-stimulated PMNs significantly depressed the capacity of PMNs to locomote (Fig. 6). This inhibition, which may result from the rise in the amount of cellular diclofenac, similarly affected both random and stimulated migration, suggesting that diclofenac primarily alters the PMN locomotor apparatus. The increased sensitivity of activated PMNs to drug effects is likely to be important in NSAID effects *in vivo*, particularly with regard to exudative PMNs since these cells are more reactive to chemoattractants than are blood PMNs [35, 36]. Supporting this assumption is the observation that therapeutic doses of some NSAIDs induce *in vivo* modification of the functions of pleural PMNs *in vivo*, an effect which persists after cell washing [4, 37], whereas alterations of the functions of blood PMNs by these drugs *in vitro* usually require non-pharmacologic NSAID doses and can be reversed when the drugs are washed out [7, 12].

In summary, the stimulation of PMNs by fMLP or PMA induces intracellular diclofenac accumulation. This uptake is dependent on incubation time and diclofenac and stimuli concentrations. The stimulated diclofenac uptake induced by fMLP potentiated diclofenac impairment of random and stimulated PMN migration. These data suggest that diclofenac may potentially attenuate inflammatory properties of PMNs partly by reducing secondary stimulation of these cells.

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