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THE EFFECTS OF SOME NON-STEROIDAL ANTI-INFLAMMATORY AGENTS ON MEMBRANE-ASSOCIATED CALCIUM IN RABBIT PERITONEAL NEUTROPHILS

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Abstract—Several non-steroidal anti-inflammatory agents (NSAIAs) (flufenamate, flurbiprofen, indomethacin, phenylbutazone, piroxicam and salicylate), one anti-inflammatory steroid (hydrocortisone) and three compounds known to affect cellular calcium metabolism [nifedipine, calcium ionophore A23187, 8-(diethylamino)octyl 3,4,5,-trimethoxy benzoate hydrochloride (TMB-8)] were tested for their effects on membrane-associated calcium in rabbit peritoneal neutrophils using the fluorescent probe chlortetracycline (CTC). The NSAIAs reduced the level of fluorescence attained by incubating neutrophils with CTC, and caused an immediate reduction in the fluorescence of CTC-loaded neutrophils, both effects being concentration-related. Nifedipine, A23187 and TMB-8 also reduced the fluorescence of CTC-loaded cells. However, these measured reductions in fluorescence were due in whole or in part to a drug-induced drop in the autofluorescence of the neutrophils. After applying a correction factor which took account of this effect there was still an immediate concentration-related reduction in CTC-dependent fluorescence after the addition of all the NSAIAs. A23187 also caused a reduction in CTC fluorescence, but nifedipine, TMB-8 and hydrocortisone were inactive. Flufenamate, indomethacin and piroxicam reduced the drop in fluorescence brought about by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). These findings suggest that NSAIAs and A23187 displace membrane-associated calcium. Electron microscopical findings confirm that indomethacin and salicylate both affect membrane-associated calcium.

Using the fluorescent probe Quin 2, an attempt was made to determine whether or not the displacement of membrane-associated calcium by NSAIAs was accompanied by changes in cytoplasmic calcium ion concentration. This was unsuccessful, however, since the effects of the NSAIAs on the fluorescence of Quin 2-loaded neutrophils was accounted for almost entirely by their effects on the autofluorescence of the cells.

Indomethacin reduces the binding of calcium to various tissues [1] and reduces the amount of intracellular calcium detectable in neutrophils with an oxalate/antimonate staining method [2]. An attempt has been made in the present paper to determine whether indomethacin and other NSAIAs have a specific action on membrane-associated calcium. CTC has been used as a calcium probe since it forms a complex with calcium which fluoresces more strongly in non-aqueous situations, such as cell membranes, than in aqueous situations such as cytoplasm [3]. A second fluorescent probe, Quin 2, which purports to measure cytoplasmic free calcium concentration [4] has also been used. The effects of indomethacin and salicylate on the amount of antimonate precipitate in neutrophils have been investigated further.

MATERIALS AND METHODS

Reagents. FMLP and A23187 (both from Sigma Chemical Co., St. Louis, MO) were dissolved in dimethylsulphoxide (DMSO). Nifedipine was dissolved in ethanol. All were diluted with 0.9% saline solution immediately before use. CTC HCl was obtained from Sigma Chemical Co., St. Louis, MO), hydrocortisone succinate (Efcortelan injection) from Glaxo Laboratories Ltd. (Greenford, U.K.), TMB-8 from Aldrich Chemical Co. (Milwaukee, WI), phenylbutazone (Butazolidin injection) from Geigy Pharmaceutical Co. Ltd. (Manchester, U.K.), and sodium salicylate from Hopkin & Williams, Chadwell Heath, (U.K.). Flufenamic acid (Parke-Davis Co. Ltd.), sodium flurbiprofen (Boots Co. Ltd., Nottingham, U.K.), indomethacin (Merck, Sharpe & Dohme Ltd., Hoddesdon, U.K.), nifedipine (Bayer, Leverhusen, F.R.G.) and piroxicam (Pfizer, Sandwich, U.K.) were donated by the companies indicated. Quin 2AM was kindly donated by Dr. T. J. Rink (Physiological Laboratory, University of Cambridge). Test compounds were added in a volume of 0.05 ml.

Preparation of neutrophils. Rabbit peritoneal neutrophils were obtained and washed as described previously [5], using a physiological salt solution of composition (mM):- NaCl 150, KCl 3, glucose 10, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid (Hepes) 5, adjusted to pH 7.4 with NaOH, and containing either $CaCl_2$ 1 (Ca Hepes solution) or Na 1,2-bis-2-aminoethoxyethane-*NNN'N'*-tetraacetate (EGTA) 1 (EGTA Hepes solution).

CTC fluorescence measurements. Rabbit neutrophils, obtained as above, were washed in Ca Hepes solution and stored as a suspension of 4×10^7 cells/ml at 4°. When required, 0.5 ml of neutrophil suspension was diluted with 1.1 ml Ca Hepes solution or 1.05 ml Ca Hepes solution plus 0.05 ml

of solution of NSAIA in 0.9% saline in a 1 cm cuvette and incubated at 37° for 15 min. Then 0.4 ml of a 50 μM solution of CTC HCl in Ca Hepes solution was added, giving a final concentration of 10 μM CTC and 10^7 cells/ml. The cuvette was shaken and placed in a spectrofluorimeter (Aminco-Bowman J4 8202G). The cell suspension was stirred using a small magnetic "flea" and an inverted magnetic stirrer mounted over the cuvette housing, which was maintained at 37° by circulating water through a surrounding jacket. Fluorescence readings were recorded at 1-min intervals from the time of addition of CTC, using an excitation λ of 390 nm, an emission λ of 520 nm and slit widths of 27.5 nm. The position of the slits was adjusted so that the "flea" did not interfere with the light path. The fluorescence of the neutrophil/CTC mixture reached near-equilibrium at about 40 min. Where the effects of added compounds on CTC uptake were being followed, the reading at 2 min was taken as unity and all subsequent readings were expressed as a ratio of this figure. When compounds were being tested for their effects on already CTC-loaded neutrophils the reading at 40 min was taken as unity and all other readings expressed as a ratio of this figure.

For the investigation of the effects of NSAIAs on responses to FMLP, neutrophils were loaded with CTC, the NSAIA was added, fluorescence readings were continued and after 5 min FMLP was added in a volume of 0.05 ml. The fluorescence reading immediately prior to the addition of FMLP was taken as unity and other readings expressed as a ratio of this figure.

Some of the compounds were tested for their effects on the efflux of CTC from previously loaded neutrophils. Neutrophils were incubated with CTC for 40 min, spun at 6000 *g* for 5 min, and then resuspended in 2 ml Ca Hepes solution. The subsequent decline in fluorescence was monitored, taking the reading at 2 min from the start of efflux as unity. Compounds to be tested were added 10 min after the start of efflux.

A minimum of three experiments using neutrophils from different rabbits was performed in all fluorescence experiments.

Quin 2 fluorescence experiments. Following the method of Tsien *et al.* [6] rabbit neutrophils (10^8/ml) were suspended at 37° in Ca Hepes solution, to which 50 μM Quin 2 AM was added. After 20 min the suspension was diluted 10 times with Ca Hepes solution and incubated for a further 1 hr. The neutrophils were then centrifuged at 1500 *g* for 5 min and resuspended in fresh Ca Hepes solution and kept at room temperature. Aliquots of this suspension were spun briefly at 6000 *g* and resuspended in 2 ml Ca Hepes solution in a 1 cm cuvette to give a final count of 10^7 cells/ml. The spectrofluorimeter was modified so that test compounds could be added to the cuvette directly, and fluorescence was recorded with a chart recorder (Bryans 60000). An excitation λ of 339 nm, an emission λ of 492 nm and slit widths of 11 and 22 nm respectively were used.

Autofluorescence measurements. Rabbit neutrophils in a suspension of 10^7 cells/ml were placed in a spectrofluorimeter cuvette as described above. The excitation and emission λ_{max} were determined by recording excitation/emission spectra on a chart recorder. The effects of varying concentrations of NSAIAs on fluorescence of neutrophils at these excitation/emission wavelengths were then recorded. To determine the effect of a test compound on neutrophil autofluorescence at the excitation/emission λs (wavelengths) used for CTC and Quin 2 measurements, suspensions of neutrophils (10^7 cells/ml) were placed in a 1 cm cuvette in the fluorimeter at 37°, fluorescence readings were recorded at either 390/520 nm or 339/492 nm for several minutes, the test compound was added, and readings were continued. The difference in fluorescence readings before and after the addition of a test compound was divided by the reading before the addition. This quotient (q) was used to determine a correction factor ($cf1$) which was the product of q and the autofluorescence of neutrophils measured before the addition of CTC. An adjustment ($cf2$) was also made for the effect of test compounds on the fluorescence of CTC (10 μM) in Ca Hepes, measured in the absence of cells. Thus, in one experiment, the fluorescence reading of neutrophils plus CTC after 40 min incubation was 70 scale division (sd). After the addition of 50 μM indomethacin the fluorescence dropped to 62 sd, giving a ratio of 0.89. After adding $cf1$ (2 sd) and $cf2$ (0.5 sd) the adjusted fluorescence value became 64.5 sd, giving a corrected ratio of 0.92 (see Table 2). Correction factors were also determined for the effects of FMLP on the autofluorescence of NSAIA-treated neutrophils.

Electron microscopy. Details of the technique have been described previously [7]. Briefly, suspensions of neutrophils were washed in EGTA Hepes solution, incubated at 37° for 3 min, fixed for 2 hr in 3% glutaraldehyde solution at pH 7.4, gelled in 1.5% agar, and post-fixed in a solution of 1% osmium tetroxide and 2% potassium antimonate in 0.01 M potassium acetate at pH 7.4. The resulting gelled suspensions of neutrophils contained approx. 2×10^7 cells/ml. Where appropriate, indomethacin (final concentration 5×10^{-5} M) or salicylate (5×10^{-3} M) was added at the beginning of the 3-min incubation period. Electron micrographs of 30 neutrophils from two samples of each treatment group from each of three rabbits were scored independently by two people on a scale 0–5 for cytoplasm- and plasmalemma-associated calcium antimonate precipitate.

RESULTS

CTC fluorescence

Effects on loading with CTC. Neutrophils incubated with NSAIAs before the addition of CTC attained a lower level of fluorescence than non-NSAIA-treated cells (Table 1, Fig. 1). The drug effects were concentration-related.

Effects on CTC-loaded neutrophils. When NSAIAs were added to CTC-loaded neutrophils there was an immediate drop in fluorescence, which was also concentration-related. However, the NSAIAs and most of the other compounds tested caused a reduction in the autofluorescence of the neutrophil suspensions and some also quenched the fluorescence of CTC in Ca Hepes solution. At the

Table 1. Effects of varying concentrations of NSAIAs on the increase in fluorescence of rabbit neutrophils incubated with CTC

Compound	Number of observations	Concentration (M)	Relative fluorescence at 40 min†
Control	39	—	1.52 ± 0.03
Flurbiprofen	4	1×10^{-7}	1.40 ± 0.07
Flurbiprofen	4	1×10^{-6}	1.31 ± 0.03*
Flurbiprofen	4	1×10^{-5}	1.18 ± 0.02*
Flurbiprofen	4	5×10^{-5}	1.19 ± 0.06*
Indomethacin	3	2.5×10^{-5}	1.32 ± 0.09
Indomethacin	3	5×10^{-5}	1.23 ± 0.06*
Indomethacin	3	1×10^{-4}	1.24 ± 0.03*
Phenylbutazone	3	5×10^{-5}	1.35 ± 0.05
Phenylbutazone	3	2×10^{-4}	1.31 ± 0.06*
Phenylbutazone	3	5×10^{-4}	1.19 ± 0.10*
Piroxicam	3	2×10^{-5}	1.39 ± 0.07
Piroxicam	4	5×10^{-5}	1.28 ± 0.04*
Piroxicam	4	7.5×10^{-5}	1.24 ± 0.08*
Salicylate	3	1×10^{-3}	1.42 ± 0.05
Salicylate	3	5×10^{-3}	1.24 ± 0.04*
Salicylate	3	1×10^{-2}	1.15 ± 0.02*

* There is a significant difference ($P < 0.05$, Student's t-test) compared with control value.

† Mean values ± S.E. For details of experimental protocol see Methods section.

excitation/emission λs used for CTC, namely 390/520 nm, however, salicylate and A23187 fluoresced weakly. The results given in Tables 2 and 3 have been corrected, therefore, to permit an estimation of changes in the CTC-dependent fluorescence alone.

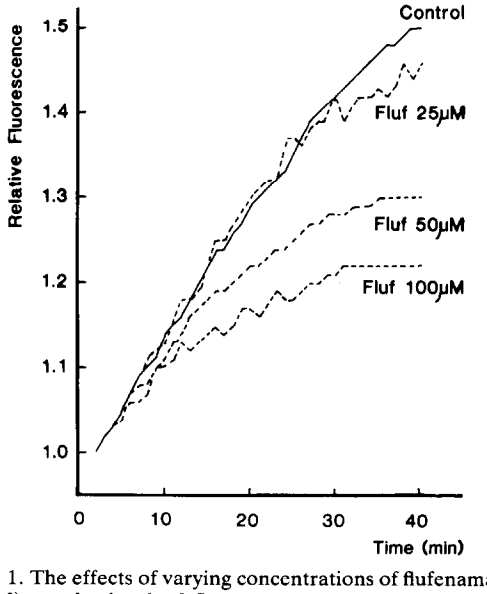

Fig. 1. The effects of varying concentrations of flufenamate (Fluf) on the level of fluorescence attained when CTC (10 μM) was added to rabbit peritoneal neutrophils. At concentrations of 5×10^{-5} M and 1×10^{-4} M, flufenamate caused a significant reduction in the fluorescence achieved over a 40-min period ($P < 0.05$, Student's t-test). The autofluorescence of neutrophils represented 75–80% of the fluorescence at time 2 min.

Uncorrected values are included for comparison. After these corrections had been applied all the NSAIAs tested and A23187 still caused an immediate drop in the fluorescence of CTC-loaded neutrophils, although the differences did not always reach statistical significance. At concentrations lower than those quoted in Table 2 the fluorescence returned to unity within 15 min, while at the higher drug concentrations the fluorescence declined even further, but more slowly. These subsequent slow changes were not statistically significant.

FMLP caused an initial fall in the fluorescence of CTC-loaded neutrophils which, at concentrations of 10^{-7} and 10^{-8} M, was followed by a return to unity. After correcting for the effects on autofluorescence it was found that flufenamate, indomethacin and piroxicam almost completely blocked the fall caused by 10^{-9} M FMLP (Table 4), but were less effective against 10^{-7} and 10^{-8} M FMLP.

Hydrocortisone (2×10^{-3} M) caused a significant decline in CTC fluorescence over 15 min, although its initial effect was only slight. Nifedipine (5×10^{-6} M) and TMB-8 (2×10^{-4} M) both caused an immediate small drop in fluorescence, but this could be attributed to their effects on the autofluorescence of neutrophils. DMSO (0.5%) and lignocaine HCl (0.0025%, present in Butazolidin injection) were also inactive.

Efflux of CTC from loaded neutrophils. When neutrophils were loaded with CTC and then resuspended in CTC-free Ca Hepes solution, the addition of flufenamate or indomethacin caused a rapid drop in fluorescence (Table 3).

Autofluorescence

Under the present experimental conditions the autofluorescence of neutrophils was strongest at an

Table 2. Effects of adding varying concentrations of NSAIAs and other compounds to CTC-loaded rabbit neutrophils

Compound	Number of observations	Concentration (M)	Relative fluorescence at 41 min‡
Saline 0.9%	4	—	1.00 ± 0.03 (0.98)
Flufenamate	3	1.75×10^{-5}	0.96 ± 0.02 (0.95)
Flufenamate	3	2.5×10^{-5}	0.97 ± 0.02 (0.92)
Flufenamate	4	5×10^{-5}	0.95 ± 0.02† (0.88)
Flufenamate	3	1×10^{-4}	0.88 ± 0.03* (0.81)
Flurbiprofen	4	1×10^{-7}	0.99 ± 0.003 (0.94)
Flurbiprofen	4	1×10^{-6}	0.95 ± 0.03 (0.96)
Flurbiprofen	4	1×10^{-5}	0.91 ± 0.02* (0.90)
Flurbiprofen	4	5×10^{-5}	0.87 ± 0.03* (0.85)
Indomethacin	3	5×10^{-6}	0.99 ± 0.05 (0.93)
Indomethacin	3	2.5×10^{-5}	0.97 ± 0.03 (0.93)
Indomethacin	3	5×10^{-5}	0.92 ± 0.01* (0.89)
Indomethacin	3	1×10^{-4}	0.90 ± 0.001* (0.85)
Phenylbutazone	3	2×10^{-5}	0.98 ± 0.03 (0.96)
Phenylbutazone	3	5×10^{-5}	0.97 ± 0.02 (0.95)
Phenylbutazone	3	2×10^{-4}	0.93 ± 0.04† (0.92)
Phenylbutazone	3	5×10^{-4}	0.90 ± 0.06† (0.86)
Piroxicam	4	1×10^{-5}	0.93 ± 0.01* (0.87)
Piroxicam	4	2×10^{-5}	0.97 ± 0.01† (0.87)
Piroxicam	4	5×10^{-5}	0.90 ± 0.04* (0.73)
Piroxicam	4	7.5 ± 10^{-5}	0.89 ± 0.04* (0.69)
Salicylate	3	5×10^{-4}	0.97 ± 0.02 (0.97)
Salicylate	3	1×10^{-3}	1.01 ± 0.02 (0.99)
Salicylate	4	5×10^{-3}	0.91 ± 0.04† (0.91)
Salicylate	4	1×10^{-2}	0.92 ± 0.04† (0.96)
TMB-8	3	2×10^{-4}	1.01 ± 0.03 (0.97)
Nifedipine	3	5×10^{-6}	1.02 ± 0.03 (0.99)
Hydrocortisone	3	2×10^{-3}	1.01 ± 0.01 (0.99)
A23187	4	1×10^{-6}	0.90 ± 0.04* (0.89)

There is a significant difference compared with the fluorescence of 0.9% saline-treated neutrophils (*$P < 0.05$, †$P < 0.1$, Student's t-test).

‡ Mean values corrected to allow for the effects of test compounds on autofluorescence and aqueous CTC fluorescence ± S.E. Uncorrected means of the same experiments are given in parentheses. For details of experimental protocol see Methods section.

Table 3. Effects of adding varying concentrations of NSAIAs to CTC-loaded rabbit neutrophils which have been resuspended in CTC-free Ca Hepes solution

Compound	Number of observations	Concentration (M)	Relative fluorescence at 11 min‡
Saline 0.9%	4	—	0.84 ± 0.02 (0.80)
Flufenamate	3	1.75×10^{-5}	0.82 ± 0.01 (0.79)
Flufenamate	3	5×10^{-5}	0.78 ± 0.02† (0.73)
Flufenamate	3	1×10^{-4}	0.79 ± 0.02† (0.73)
Indomethacin	3	5×10^{-6}	0.89 ± 0.002 (0.84)
Indomethacin	3	5×10^{-5}	0.81 ± 0.01 (0.79)
Indomethacin	3	1×10^{-4}	0.73 ± 0.01* (0.71)
Salicylate	3	5×10^{-4}	0.87 ± 0.02 (0.87)
Salicylate	3	5×10^{-3}	0.86 ± 0.06 (0.94)
Salicylate	3	1×10^{-2}	0.84 ± 0.03 (0.82)

There is a significant difference compared with the fluorescence of 0.9% saline-treated neutrophils (*$P < 0.05$, †$P < 0.1$, Student's t-test).

‡ Mean values corrected to allow for the effects of test compounds on autofluorescence and aqueous CTC fluorescence ± S.E. Uncorrected means of the same experiments are given in parentheses. For details of experimental protocol see Methods section.

Table 4. Effects of adding FMLP 10^{-9} M to CTC-loaded rabbit neutrophils with or without 5 min prior incubation with NSAIAs

NSAIA	Number of observations	Concentration (M)	Relative fluorescence at 1 min†
—	3	—	0.95 ± 0.01 (0.91)
Flufenamate	3	5×10^{-5}	0.98 ± 0.01* (0.98)
Indomethacin	3	1×10^{-4}	0.99 ± 0.01* (0.99)
Piroxicam	3	5×10^{-5}	0.99 ± 0.01* (0.99)

* There is a significant difference ($P < 0.1$, Student's t-test) compared with control values.

† Mean values corrected to allow for the effects of test compounds on autofluorescence and aqueous CTC fluorescence ± S.E. Uncorrected means of the same experiments are given in parentheses. For details of experimental protocol see Methods section.

emission λ of 330 nm. This probably represents the combined fluorescence of tyrosine and tryptophane, since these two amino acids (along with phenylalanine, which only fluoresces weakly) are mainly responsible for the fluorescence of proteins [8]. At excitation/emission λs of 275/303 nm (tyrosine), 287/348 nm (tryptophane) and 285/330 nm (neutrophils) flufenamate, indomethacin, phenylbutazone and piroxicam all had a concentration-related quenching effect on the fluorescence of solutions of these two amino acids (1 μg/ml) and on neutrophils. Flurbiprofen and salicylate, however, themselves fluoresced strongly. At the excitation/emission λs used for CTC measurements (390/520 nm) the quenching effects on the autofluorescence of neutrophils were all considerably reduced; the effect of flurbiprofen changed to a weak quenching effect, but salicylate still fluoresced slightly. FMLP caused a concentration-related small reduction in the autofluorescence of untreated neutrophils. In the presence of NSAIAs the reduction was smaller, and at 10^{-9} M FMLP was completely prevented (Table 4). Under the conditions used for measuring Quin 2 fluorescence, the quenching effects of flufenamate, indomethacin, phenylbutazone and piroxicam were more marked than at the λs used for CTC.

Quin 2 fluorescence

Rabbit neutrophils incubated with Quin 2 AM showed a readily measurable increase in fluorescence over and above their autofluorescence. However, with the exception of salicylate which fluoresced so strongly at the excitation/emission λs used for Quin 2 that it could not be used, the NSAIAs tested caused an immediate concentration-related fall in fluorescence. This was almost fully accounted for by a quenching effect on autofluorescence.

The increase in fluorescence brought about by adding A23187 (10^{-6} M) to Quin 2-loaded neutrophils in the present experiments was fully accounted for by the innate fluorescence of A23187, since there was a similar rise in fluorescence when the same concentration of A23187 was added to Ca Hepes solution or to neutrophils which had not been loaded with Quin 2.

Electron microscopy

Neutrophils fixed in glutaraldehyde and post-fixed in osmium tetroxide/antimonate solution showed copious calcium antimonate precipitate in association with the plasmalemma. This does not occur with primary fixation in glutaraldehyde containing

Table 5. Effects of adding indomethacin or salicylate to rabbit neutrophils before fixation in glutaraldehyde and post-fixation in osmium tetroxide/antimonate solution

Treatment	Concentration (M)	Antimonate precipitate Mean score (scale 0–5)†	
		Cytoplasm	Plasmalemma
Control	—	2.27 ± 0.07$^{\pi}$	2.48 ± 0.12*
Indomethacin	5×10^{-5}	2.24 ± 0.09	0.77 ± 0.06**
Salicylate	5×10^{-3}	2.06 ± 0.10$^{\pi\pi}$	0.14 ± 0.2**

There is a significant difference ($P < 0.05$) between groups marked $^{\pi}$ and $^{\pi\pi}$, and between groups marked * and **: Significant differences have been calculated using a χ^2 test.

† Mean values of 180 observations ± S.E.

For details of experimental protocol see Methods section.

oxalate [2, 7]. The addition of indomethacin (5×10^{-5} M) or salicylate (5×10^{-3} M) before fixation in the absence of oxalate significantly reduced the amount of this plasmalemma-associated precipitate (Table 5). Micrographs illustrating this effect have been published previously [9].

DISCUSSION

CTC has been used for several years as a probe for membrane-associated calcium in various tissues including human and rabbit neutrophils [10–16], although the need for caution in the interpretation of the results has also been expressed [17–20]. Quin 2 is being used increasingly as a probe for cytoplasmic free calcium [21–24]. Unfortunately, the present results indicate that the use of both fluorescent probes is somewhat limited by the effects of test compounds on the autofluorescence of cells. Some compounds also have an innate fluorescence which interferes with CTC measurements [13].

Using CTC several workers have shown that various stimulants of neutrophil function such as FMLP, the fifth component of complement (C5a), concanavalin A (con A), phorbol myristate acetate (PMA) and leukotriene B_4 each release calcium from membrane-associated stores [10–17, 24, 25]. It has been shown also that indomethacin inhibits FMLP-induced lysosomal enzyme release [26, 27] and superoxide (O_2^-) generation [26, 28]; that flurbiprofen reduces the release of β-glucuronidase and lysozyme from FMLP-stimulated neutrophils [27]; that phenylbutazone reduces FMLP-induced cellular hyperadhesiveness and lysosomal enzyme release [29], O_2^- generation [28, 29], hexose monophosphate shunt activity [29, 30] and hydrogen peroxide production [31]; and that piroxicam inhibits the release of membrane-associated calcium brought about by FMLP, con A and PMA [12], and inhibits aggregation, O_2^- generation and degranulation in response to FMLP [15, 32]. The anti-FMLP actions of indomethacin [33], piroxicam [15] and phenylbutazone [29, 30] have been attributed to reduction of the binding of FMLP to neutrophil membranes, although indomethacin has also been reported to be inactive in this respect [15]. At the present time, therefore, a mechanistic interpretation of these inhibitory actions of NSAILAs remains uncertain, although the displacement of membrane calcium stores is one possibility.

Neutrophils can be stimulated in ways additional to those quoted above and are but one of many types of cell involved in the inflammatory process. Others include vascular endothelium and smooth muscle, platelets and mast cells. It seems more than a coincidence that the regulation of function in all these cells is calcium ion-dependent and can be disrupted by NSAIAs [9]. This suggests that NSAIAs in some general way may alter the handling of cellular calcium, a view put forward by Tanaka *et al.* [32] for the action of piroxicam.

The present work indicates that NSAIAs release calcium from membrane-associated sites in neutrophils, thus rendering the ion unavailable to act as an intracellular messenger when a stimulus is applied. The usual responses to stimulation, such as lysosomal enzyme release and O_2^- generation, therefore, will not occur.

Two possible intracellular mediators of the activation of the respiratory burst have been suggested, namely calmodulin and protein kinase C, both of which are calcium ion-dependent [34]. Therefore, it is possible that NSAIAs exert an anti-inflammatory action in part by removing the calcium necessary for the action of these mediators.

A similar suggestion has been advanced for the enzyme phospholipase A_2, which is also calcium ion-dependent [35, 36]. Indomethacin inhibits the phospholipase A_2 activity of rabbit neutrophils [37], and indomethacin [35, 36, 38] and flufenamate [35] have both been shown to inhibit platelet phospholipase A_2.

NSAIAs also inhibit other calcium ion-dependent features of the inflammatory process in neutrophils such as granule exocytosis [9], although the mechanism of calcium ion involvement in these processes is not fully understood.

However, NSAIAs also interfere with non-calcium-dependent systems, such as cyclooxygenase activity [39]. Further experiments are necessary to determine the relative importance of these various actions.

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