

EFFECT OF INDOMETHACIN AND RELATED DRUGS ON THE CALCIUM ION-DEPENDENT SECRETION OF LYSOSOMAL AND OTHER ENZYMES BY NEUTROPHIL POLYMORPHONUCLEAR LEUCOCYTES *In vitro*

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- 1 Rabbit isolated peritoneal neutrophil polymorphonuclear leucocytes were depleted of calcium by exposure for 1 h to calcium-free bathing fluid at 4°C.
- 2 Addition of calcium ions to the previously calcium-depleted cells during incubation at 37°C stimulated the release of β -glucuronidase and of lysozyme but not of lactate dehydrogenase.
- 3 Low concentrations of indomethacin, flufenamate or salicylate, such as those which occur in the blood plasma after therapeutic doses of these drugs, selectively inhibited the calcium-induced release of β -glucuronidase. The slight release of this enzyme which occurred in the absence of added calcium ions was not altered by these drugs, neither was the release of lactate dehydrogenase.
- 4 Release of lysozyme was inhibited by low concentrations of salicylate, amidopyrine or oxyphenbutazone, independent of the presence or absence of calcium ions.
- 5 Chloroquine, hydrocortisone or colchicine did not alter the release of leucocyte enzymes.

Introduction

Indomethacin and related drugs inhibit many types of inflammation but the pharmacological basis of this effect is still obscure. One possibility is that these drugs inhibit the secretion of lysosomal enzymes by the neutrophil polymorphonuclear leucocytes (neutrophils) which attach themselves at an early stage of inflammation to the walls of the small blood vessels. Selective secretion by the neutrophils of their lysosomal contents occurs in response to a variety of physiological and pathological stimuli but in most cases stimulus-secretion coupling ceases under conditions of calcium depletion (Woodin & Wieneke, 1963; Henson, 1972; Becker & Showell, 1974; Goldstein, Horn, Kaplan & Weissmann, 1974; Ignarro, 1974b; Ignarro & George, 1974; Goldstein, Hoffstein & Weissmann, 1975; Smith & Ignarro, 1975). Indomethacin and related drugs deplete the membrane-bound stores of calcium in smooth muscle cells and vascular endothelial cells (Northover, B.J., 1971, 1972, 1973, 1975a, b; Northover, A.M., 1975). Thus, it was of interest to determine whether anti-inflammatory drugs would modify the calcium-dependent secretion of lysosomal and other enzymes by the neutrophils *in vitro*.

Methods

Rabbits of the New Zealand White strain weighing 2 to 3 kg were injected intraperitoneally through a 0.8 mm external diameter stainless steel needle with 100 ml of sterile thioglycollate medium (United States Pharmacopoeia, 18th revision, 1970; supplied by Oxoid Ltd.). Between 17 and 22 h later each rabbit was lightly anaesthetized by inhalation of ether vapour and injected intraperitoneally with 250 ml of sterile 0.15 M sodium chloride solution containing heparin (4 iu/ml). After massaging the abdomen for 10 s the peritoneal fluid was withdrawn by gentle suction via a 1.5 mm external diameter plastic catheter the end of which was multiply perforated. The cells in the peritoneal fluid were sedimented at 22°C by centrifugation in 100 ml plastic tubes at 200 g for 4 minutes. The sedimented cells were washed twice by resuspension and recentrifugation at 200 g for 4 min at 4°C, each time using 100 ml of suspending medium having the following composition (mM): NaCl 150, KCl 3, glucose 10, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid 5, adjusted to pH 7.4 with NaOH. In some experiments 1.0 mM sodium 1,2-bis-2-aminoethoxyethane-*NNN'*-tetra-acetate (EGTA) was added to the suspending medium. When EGTA-

containing medium was used as the washing fluid the cells were suspended in this fluid for the remainder of the experiment. Otherwise, the cells were washed with and suspended in EGTA-free medium throughout. The suspension of washed cells was adjusted to a total count of 5×10^6 cells/ml, of which 94 to 98% were neutrophils, the remainder being various types of mononuclear cells. Each rabbit served as a source of neutrophils on four occasions separated by intervals of a week or longer. Preliminary experiments showed that the proportion of neutrophils in the peritoneal washings decreased if animals were used more than four times or more frequently than once a week. The total time which elapsed between the withdrawal of the peritoneal fluid from a rabbit and the start of the incubation of the neutrophils as part of an experiment was 60 minutes.

Substances to be tested for their effect upon the release of enzymes from the neutrophils were added in a volume of 0.2 ml to 1.0 ml aliquots of leucocyte suspension in 10 mm diameter polypropylene tubes. The tubes were rotated at 30 rev/min in a rack inclined at 45° to the vertical in an incubator room at 37°C . After incubation for a pre-arranged time (40 min unless otherwise specified) the tubes were cooled in melting ice and then centrifuged at 3,000 g for 6 min at 4°C . Enzyme assays were performed in 1 cm cuvettes in a Unicam SP 500 spectrophotometer. In experiments where a drug was added to the leucocytes during incubation or the ionic composition of the suspending medium was altered, the drug or ion involved was added to all tubes, including the controls, during the subsequent enzyme assays. This precaution was necessary since some anti-inflammatory drugs have been reported to inhibit certain lysosomal enzymes such as β -glucuronidase and acid phosphatase (Anderson, 1968; 1970).

The results of all enzyme assays were expressed as a percentage of the activity of an aliquot of the supernatant obtained by centrifuging a sample of leucocyte suspension to which Triton X-100 (Rhone & Has, Philadelphia) had been added in a final concentration of 0.2%. It was assumed that Triton released the entire leucocytic content of enzymes into the suspending medium.

β -Glucuronidase activity was determined by pipetting a sample of the supernatant fluid into a glass test tube containing 1.5 ml of 0.2 M sodium acetate buffer at pH 4.5 and 0.2 ml of a 6 mM solution of phenolphthalein glucuronide (Sigma). The mixture was incubated at 56°C for 3 h after which the pH was adjusted to 11.0 by adding 2 ml of a 0.2 M solution of 2-amino-2-methyl-1-propanol, and the optical extinction measured at 550 nm. The red colour of the liberated phenolphthalein was used as a measure of the glucuronidase activity, as suggested by Fishman (1955).

Lysozyme activity was determined by pipetting a sample of the supernatant fluid into a glass test tube

containing 2.5 ml of a freshly prepared suspension of 0.5 mg of freeze-dried *Micrococcus luteus* (Boehringer Mannheim) in 0.27 M sodium orthophosphate buffer at pH 6.0. The optical extinction at 510 nm was measured immediately after mixing and again after exactly 20 min incubation at 22°C . The reduction in optical extinction during this time was considered to be due to bacterial lysis and was used as a measure of lysozyme activity, as proposed by Smolelis & Hartsell (1949).

Lactate dehydrogenase activity was determined by reducing pyruvate with the reduced form of nicotinamide adenine dinucleotide. A sample of the supernatant fluid was pipetted into a glass test tube containing 50 μl of a 9 mM solution of the dinucleotide (BDH) and 3 ml of a 50 mM sodium orthophosphate buffer at pH 7.5 containing sodium pyruvate 0.3 mM. The optical extinction at 366 nm was measured immediately after mixing and again after exactly 5 min incubation at 22°C . The reduction in optical extinction during this time was taken as a measure of the dehydrogenase activity, as proposed by Wróblewski & La Due (1955).

Amidopyrine and colchicine were used as freshly prepared aqueous solutions. Chloroquine was dissolved in water in the form of its sulphate salt. Indomethacin, flufenamate, oxyphenbutazone, phenylacetate, gentisate and salicylate were dissolved in water as their respective sodium salts and the pH adjusted to 7.4. Hydrocortisone was dissolved in water as its sodium succinate derivative. The quoted concentrations of these drugs refer to their respective free acids, bases or alcohol.

Results

Effect of calcium ions and of EGTA

The release of enzymes from the neutrophils depended upon the duration of incubation at 37°C and upon the composition of the fluid in which the cells were suspended. Cells washed with and suspended in calcium-free suspending medium released very little of their enzyme content during incubation (Figure 1). Addition of calcium ions during incubation caused a time-dependent increase of the release of glucuronidase and lysozyme but not of lactate dehydrogenase (Figures 1 and 2). The effect of added calcium ions during incubation was more consistent when the leucocytes had been treated with EGTA than when they had not been, and hence EGTA-containing suspensions were employed for the remaining experiments. As would be expected, more added calcium was required in the presence of EGTA than in its absence to produce a 10% release of glucuronidase and lysozyme (Figure 2). Added calcium ions failed to modify the release of enzymes

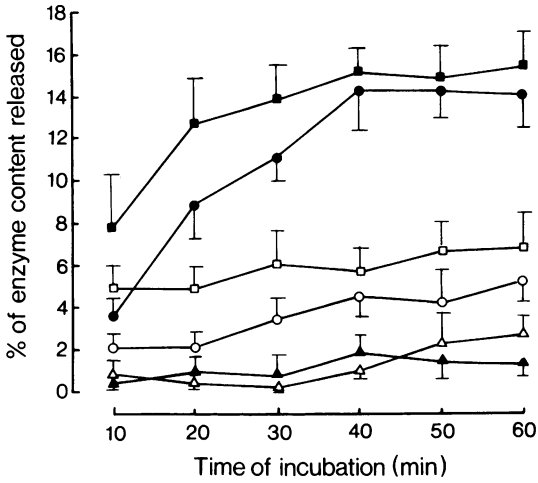


Figure 1 Release of glucuronidase (○ and ●), lysozyme (□ and ■) and lactate dehydrogenase (△ and ▲) from neutrophils incubated for varying lengths of time in an EGTA-containing calcium-free suspending medium. Open symbols represent cells to which no calcium was added during incubation and closed symbols represent cells to which CaCl_2 (2 mM) was added. Each point is the mean of between 12 and 20 observations. Vertical bars represent one s.e.

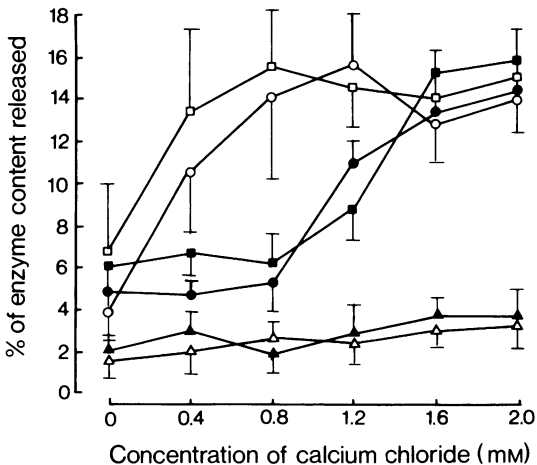


Figure 2 Effect of calcium concentration on the release of glucuronidase (○ and ●), lysozyme (□ and ■) and lactate dehydrogenase (△ and ▲) from neutrophils incubated for 40 min in EGTA-free (open symbols) or in EGTA-containing (closed symbols) suspending medium. Each point is the mean of between 12 and 20 observations. Vertical bars represent one s.e.

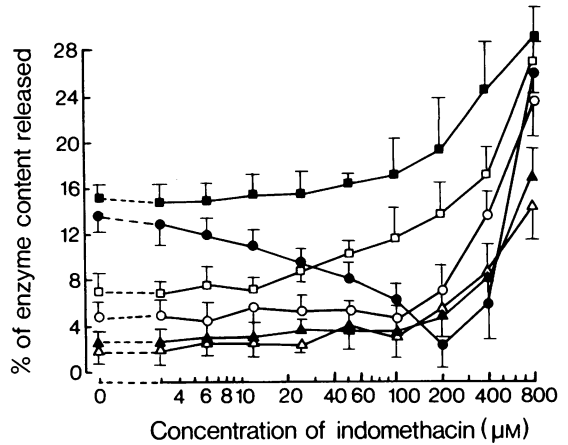


Figure 3 Release of glucuronidase (○ and ●), lysozyme (□ and ■) and lactate dehydrogenase (△ and ▲) from neutrophils incubated for 40 min in EGTA-containing suspending medium to which various concentrations of indomethacin were added. Open symbols represent cells to which no calcium was added and closed symbols represent cells to which CaCl_2 (2 mM) was added during incubation. Each point is the mean of between 12 and 20 observations. Vertical bars represent one s.e.

when the neutrophils had been kept at 4°C for up to 1 hour.

Effect of indomethacin

Addition of low concentrations of indomethacin to neutrophils produced a concentration-dependent inhibition of the calcium-induced release of glucuronidase (Figure 3). In contrast, the small release of glucuronidase observed in the absence of added calcium ions was not affected by low concentrations of the drug (Figure 3). The release of lysozyme both in the absence and in the presence of added calcium ions either was not altered or was slightly enhanced by low concentrations of indomethacin (Figure 3). Low concentrations of indomethacin did not alter the release of lactate dehydrogenase either in the presence or in the absence of added calcium. High concentrations of the drug caused a more or less equally enhanced release of all three enzymes, irrespective of the addition of calcium (Figure 3).

Effect of other anti-inflammatory drugs

The effects of added flufenamate were similar to those of indomethacin (Table 1). In contrast, salicylate inhibited the release of both glucuronidase and

lysozyme induced by the addition of calcium ions, although the release of lactate dehydrogenase was not altered by low concentrations of salicylate either in the presence or in the absence of added calcium ions, and higher concentrations of the drug caused a generalized enhancement of the release of all three enzymes (Table 1). In the absence of calcium, low concentrations of salicylate failed to alter the release of glucuronidase but did inhibit the release of lysozyme (Table 1). Both amidopyrine and oxyphenbutazone in low concentrations inhibited the release of lysozyme but failed to alter the release of glucuronidase or lactate dehydrogenase (Table 1). These actions of both drugs were independent of the presence of calcium ions. Chloroquine, hydrocortisone and colchicine each display anti-inflammatory activity *in vivo* under appropriate circumstances. Nevertheless, all three drugs failed to alter the release of leucocytic enzymes both in the presence and in the absence of calcium ions, although they were tested at concentrations at least as high as the plasma concentrations associated with anti-inflammatory effects *in vivo* (Table 1).

Discussion

Effect of calcium ions on the release of enzymes

The present investigation has shown that low concentrations of calcium ions, such as those which normally occur in the blood plasma, increase the release of both glucuronidase and lysozyme but not of lactate dehydrogenase from rabbit neutrophils previously depleted of calcium. Using human neutrophils, Goldstein *et al.* (1974) found that the addition of calcium ions substantially increased the release of lysozyme, whereas the release of glucuronidase was increased only slightly and lactate dehydrogenase release was unaltered.

Effects of indomethacin, flufenamate and salicylate on the release of enzymes

In the present experiments low concentrations of indomethacin, flufenamate and salicylate inhibited the calcium-induced release of glucuronidase in a

Table 1 Effect of drugs on the release of enzymes from neutrophils incubated in EGTA-containing suspending medium with and without added calcium chloride

Drug	Concentration (M)	Percentage release of enzymes*					
		β -Glucuronidase		Lysozyme		Lactate dehydrogenase	
		Without calcium	With calcium†	Without calcium	With calcium	Without calcium	With calcium
Control	—	5 ± 1	14 ± 2	7 ± 2	15 ± 2	2 ± 1	2 ± 1
Flufenamate	4 × 10 ⁻⁶	5 ± 2	13 ± 2	6 ± 1	15 ± 2	3 ± 1	3 ± 1
Flufenamate	8 × 10 ⁻⁶	4 ± 1	10 ± 1‡	7 ± 2	14 ± 2	2 ± 1	1 ± 1
Flufenamate	1.5 × 10 ⁻⁵	4 ± 1	8 ± 2‡	8 ± 2	16 ± 2	2 ± 2	2 ± 1
Flufenamate	3 × 10 ⁻⁵	6 ± 2	5 ± 1‡	7 ± 2	15 ± 3	3 ± 1	3 ± 2
Flufenamate	6 × 10 ⁻⁵	5 ± 2	2 ± 1‡	9 ± 2	18 ± 2	3 ± 1	4 ± 2
Flufenamate	1.2 × 10 ⁻⁴	17 ± 2‡	10 ± 2	11 ± 3	29 ± 2‡	8 ± 2‡	12 ± 3‡
Flufenamate	2.4 × 10 ⁻⁴	20 ± 2‡	23 ± 2‡	36 ± 4‡	34 ± 3‡	14 ± 2‡	28 ± 3‡
Salicylate	1.2 × 10 ⁻³	5 ± 1	12 ± 2	6 ± 1	10 ± 2	2 ± 1	3 ± 1
Salicylate	2.5 × 10 ⁻³	5 ± 2	6 ± 1‡	3 ± 1‡	8 ± 1‡	3 ± 1	3 ± 1
Salicylate	5 × 10 ⁻³	6 ± 1	5 ± 1‡	4 ± 1‡	11 ± 2	2 ± 1	6 ± 2‡
Salicylate	1 × 10 ⁻²	12 ± 2‡	23 ± 2‡	17 ± 2‡	28 ± 3‡	10 ± 2‡	22 ± 3‡
Amidopyrine	5 × 10 ⁻⁴	6 ± 1	13 ± 3	4 ± 1	9 ± 2	3 ± 1	2 ± 1
Amidopyrine	1 × 10 ⁻³	7 ± 1	16 ± 2	3 ± 1‡	7 ± 2‡	3 ± 2	1 ± 1
Amidopyrine	2 × 10 ⁻³	19 ± 2‡	25 ± 2‡	20 ± 3‡	27 ± 3‡	15 ± 2‡	11 ± 2‡
Oxyphenbutazone	3 × 10 ⁻⁴	4 ± 1	15 ± 2	6 ± 1	11 ± 1	3 ± 1	1 ± 1
Oxyphenbutazone	6 × 10 ⁻⁴	7 ± 1	16 ± 2	2 ± 1‡	5 ± 1‡	2 ± 1	4 ± 1
Oxyphenbutazone	1.2 × 10 ⁻³	23 ± 3‡	17 ± 2	16 ± 2‡	20 ± 2‡	10 ± 2‡	13 ± 2‡
Chloroquine	2 × 10 ⁻⁵	6 ± 1	17 ± 2	10 ± 2	19 ± 2	3 ± 1	5 ± 2
Colchicine	2.4 × 10 ⁻⁷	3 ± 1	12 ± 1	5 ± 1	13 ± 2	1 ± 1	2 ± 1
Colchicine	2.4 × 10 ⁻⁶	7 ± 1	16 ± 2	9 ± 1	18 ± 2	2 ± 1	4 ± 1
Hydrocortisone	3 × 10 ⁻⁴	5 ± 1	13 ± 1	8 ± 2	14 ± 2	2 ± 1	3 ± 1
Phenylacetate	1 × 10 ⁻²	4 ± 1	16 ± 2	3 ± 1‡	6 ± 1‡	3 ± 1	2 ± 1
Gentisate	1 × 10 ⁻²	4 ± 1	18 ± 2	2 ± 1‡	5 ± 1‡	4 ± 1	1 ± 1

* The mean of at least 12 observations. † The concentration of calcium added was 2 mM.

Values marked ‡ are significantly different (Student's *t* test, *P* < 0.05) from their corresponding control values.

concentration-dependent manner. In the case of indomethacin and flufenamate the inhibition disappeared in the absence of calcium ions in the suspending medium. The release of lactate dehydrogenase was unaffected by low concentrations of all three drugs. Drug concentrations which selectively inhibited the release of glucuronidase are comparable to those which are attained in the blood plasma in therapeutic use (Hucker, Zacchei, Cox, Brodie & Cantwell, 1966; Rothermich, 1966; Paulus, Siegel, Mongan, Okun & Calabro, 1971; Huidberg, Lausen & Jansen, 1972). On the other hand, the higher concentrations required to produce an unselective release of all three enzymes are probably not attained in the blood plasma after therapeutic doses.

Previous workers have shown that indomethacin, salicylate and some pharmacologically related drugs inhibit the release of glucuronidase from human neutrophils exposed either to phagocytosable or to opsonized solid materials (Andrews & Phelps, 1971; Perper & Oronsky, 1974). The present work indicates that the release of glucuronidase from rabbit neutrophils in response to a soluble stimulus may also be inhibited by these drugs. Nevertheless, at least two previous groups of workers have failed to show any inhibition of lysosomal enzyme release from phagocytosing neutrophils after treatment with salicylate in the concentration range used here (Wright & Malawista, 1973; Hawkins, 1974). Stimulus-secretion coupling mechanisms probably vary with different types of stimuli and may not be inhibited equally by drugs.

In general, the present findings lend support to the suggestion of Perper & Oronsky (1974) that part of the anti-inflammatory action of indomethacin, flufenamate and salicylate may be due to inhibition of the release of lysosomal enzymes from neutrophils in inflamed tissues. This is made more probable by the fact that phenylacetate and gentisate, non-anti-inflammatory analogues of indomethacin and salicylate respectively (Northover, 1963; 1964; Durant, Smith, Spickett & Szarvasi, 1965) both failed to alter the release of glucuronidase in the present experiments (Table 1).

Effects of other anti-inflammatory drugs on the release of enzymes

In concentrations comparable with those occurring in the blood plasma during effective anti-inflammatory

therapy, no inhibition of glucuronidase release was found in the present experiments with hydrocortisone, colchicine, chloroquine, amidopyrine or oxyphenbutazone. Previous workers, using phagocytosable or opsonized solid materials as the stimulus for release, have demonstrated an inhibition of lysosomal enzyme release from neutrophils with many of these drugs (Rajan, 1966; Andrews & Phelps, 1971; Oronsky, Ignarro & Perper, 1973; Weissmann, 1973; Wright & Malawista, 1973; Hawkins, 1974; Ignarro, 1974a; Perper & Oronsky, 1974; Ackerman & Beebe, 1975; Goldstein, 1975; Lewis & Day, 1975; Malawista, 1975; Oronsky & Perper, 1975). As noted previously, stimulus-secretion coupling mechanisms may vary with the nature of the stimulus involved, and consequently may vary in their capability of being inhibited by drugs. Perhaps the only anti-inflammatory drugs which inhibit the release of glucuronidase under the conditions of the present experiments are those that prevent the entry of calcium ions into the leucocyte.

Intra-leucocytic distribution of enzymes

Rabbit neutrophils possess at least two morphologically distinct types of enzyme-containing structures derived from the Golgi apparatus, known as the azurophil and the specific granules respectively (Bainton & Farquhar, 1966). The azurophil granules store almost all of the glucuronidase whilst the specific granules contain a majority of the lysozyme (Baggiolini, Hirsch & DeDuve, 1969; 1970). The azurophil granules are typical of lysosomes in other types of cells and possess a full complement of acid hydrolases (Bainton & Farquhar, 1968a, b; Dewald, Rindler-Ludwig, Betz & Baggiolini, 1975). The specific granules are not typical lysosomes in their enzymatic content and their physiological and pathological significance is uncertain. In contrast to these two granule-bound enzymes, lactate dehydrogenase is a soluble cytoplasmic enzyme. Douwes (1973) has reported that indomethacin and some related drugs promote the release of lysozyme from phagocytosing neutrophils but inhibit the release of acid phosphatase, a typical lysosomal enzyme. A rather similar disparity between the effect of indomethacin on the release of glucuronidase and lysozyme was demonstrated in the present work. Since the majority of the lysozyme of the rabbit neutrophil is stored in the non-lysosomal specific granules this is readily understandable.

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