Biochemical Pharmacology, Vol. 28, pp. 2739–2746

0006-2952/79/0915-2739 $02.00/0

THE GUINEA PIG NEUTROPHIL CALCIUM-DEPENDENT LYSOSOMAL ENZYME SECRETORY PROCESS

INHIBITION BY NONSTEROID ANTI-INFLAMMATORY AGENTS

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(*Received* 7 *October* 1978, *accepted* 23 *March* 1979)

Abstract—A selective release of lysosomal granule-associated β-glucuronidase and acid protease, but not cytoplasmic lactate dehydrogenase, occurs during contact between zymosan-treated serum and cytochalasin B-treated neutrophils. The calcium-dependent aspect of this secretory process is demonstrated by the absence of lysosomal enzyme discharge from neutrophils incubated with zymosan-treated serum in calcium-free Krebs-Ringer phosphate medium containing 7.5 mM glucose, pH 7.4, at 37°. The capacity of the divalent cation ionophore, A23187, to induce lysosomal enzyme release also implies a requirement of calcium for secretion to occur. Further, zymosan-treated serum and ionophore-induced release of β-glucuronidase was accompanied by a marked association of $^{45}CaCl_2$ with neutrophils. Indomethacin, diftalone, naproxen, ketoprofen and suprofen impeded A23187-stimulated extracellular extrusion of lysosomal enzymes from, and $^{45}CaCl_2$ association with, neutrophils. Acetylsalicylic acid and flazalone were inactive. The inhibitory effects of these nonsteroid anti-inflammatory agents could be reversed by increasing the extracellular calcium ion concentration. The specificity of calcium in reversing the effects of these agents was indicated by the observation that supplementing the incubation medium with magnesium would not attenuate the action of these nonsteroid agents. Therefore, the regulation of the selective secretion of lysosomal enzymes from neutrophils by nonsteroid anti-inflammatory agents may be related to their capacity to modulate the association of calcium with these cells.

Recent reports from this laboratory have demonstrated the ability of certain nonsteroid anti-inflammatory agents (NSAIA) to inhibit the selective release of lysosomal enzymes from guinea pig polymorphonuclear neutrophils [1–4]. However, the mechanism(s) by which these therapeutic agents exert their effects on this cellular process remains to be elucidated. Perhaps the most frequently cited mode of action of these agents is lysosomal membrane stabilization whereby these agents alter the configuration of the lysosomal membrane in such a way as to impair the release of the enzymatic contents of these organelles. Indeed, NSAIA have been demonstrated to retard the discharge of enzymes from isolated lysosomal granule preparations of liver [5, 6], as well as from lysosomes of rabbit [7] and guinea pig neutrophils [8].

In addition to the lysosome granule, another integral component of the lysosomal enzyme secretory process in neutrophils is calcium. In this regard, Ignarro and George [9] and Smith and Ignarro [10] have shown the inability of human neutrophils to release their lysosomal constituents in a calcium-free environment. Furthermore, a divalent cation ionophore, which is known to stimulate calcium influx into various cells, was demonstrated to induce the selective secretion of lysosomal enzymes from human neutrophils [10]. Therefore, the purpose of the present investigation was to evaluate the mechanism by which NSAIA regulate lysosomal enzyme secretion from guinea pig neutrophils with particular reference to the calcium-dependent aspect of this cellular function.

METHODS

Isolation of guinea pig neutrophils. Neutrophils were harvested from guinea pigs (male, Hartley, albino, 400–750 g) according to a modification of the method of Michell *et al.* [11] as described previously [2]. Final cell suspensions contained a minimum of 95% neutrophils. Viability of the neutrophils was always greater than 97 per cent, as determined by trypan blue exclusion.

Preparation of zymosan-treated serum. Zymosan-treated serum (ZTS) was prepared according to a modification of the procedure of Ignarro *et al.* [12]. Zymosan particles measuring 0.5 to 3 μm in diameter were suspended in fresh autologous serum (5 mg/ml) and incubated with shaking at 37° for 30 min. Epsilon aminocaproic acid (250 mM) was added to the ZTS in order to impede the inactivation of the activated serum lysosomal enzyme-releasing activity [13]. The ZTS was centrifuged at 3500 *g* for 15 min in order to remove the zymosan.

Incubation conditions and quantitation of calcium association with neutrophils. Neutrophils (5×10^6) in 2 ml of Krebs–Ringer phosphate medium, pH 7.4, containing 7.5 mM glucose (w/v) and 0.2 ml of ZTS were incubated at 37° in a Dubnoff shaker set at 120 excursions/min, according to the various procedures described under "Results". After incubation the samples were centrifuged at 750 *g* for 10 min and the supernatant fractions were assayed for β-glucuronidase, acid protease and lactate dehydrogenase activities. In those

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experiments where $^{45}CaCl_2$ was utilized, the methodology employed has been described previously [10]. The data signify the association of extracellular calcium with neutrophils and are expressed as nmoles of calcium/5 × 10^6 cells.

Enzyme assays. Acid protease activity was measured according to a modification of the method of Barrett [14]. Aliquots (1.0 ml) of supernatant fractions, obtained as described above, were incubated in 1.0 ml of 1.0 M sodium formate, pH 3.5, together with 0.5 ml of 5% hemoglobin (w/v) at 37° for 18 hr. Reactions were terminated by the addition of 3.0 ml of 7.5% trichloroacetic acid to the reaction mixture. After a 10 min incubation reaction the product was determined according to Lowry *et al.* [15]. Data are expressed as μg of tyrosine equivalents/18 hr/5 10^6 cells.

β-Glucuronidase and lactate dehydrogenase activities were determined as described previously [2].

Drug solutions and sources. All solutions of test agents were prepared fresh and used within 15 min. The drugs employed in this study were: acetylsalicylic acid (Sigma Chemical Co. St. Louis, MO.); diftalone (Gruppo Lepetit, Milan, Italy); flazalone (Riker Laboratories, Inc., Northridge, CA); indomethacin (Merck, Sharp & Dohme, West Point, PA); ketoprofen (Ives Laboratories, Inc., New York, NY); naproxen (Syntex Laboratories, Inc., Palo Alto, CA); suprofen (Janssen R and D, Inc., New Brunswick, NJ); and cytochalasin B (Aldrich Chemical Co., Milwaukee, WI).

All agents tested were dissolved in dimethylsulfoxide. All agents were soluble under the defined incubation conditions, and they produced no alteration of the pH of the incubation media. The small amounts of dimethylsulfoxide (final concentration of 0.1%) employed as vehicles did not alter cell viability or enzyme release.

RESULTS

Release of enzymes from cytochalasin B-treated guinea pig neutrophils in the presence of zymosan-treated serum. Neutrophils (5 × 10^6) preincubated with cytochalasin B demonstrated selective secretion of the lysosomal enzymes, β-glucuronidase and acid protease (Fig. 1), following 5 min of exposure to ZTS. The rate of release was linear for 25 min and appeared to reach a maximum between 30 and 60 min. Lactate dehydrogenase, a cytoplasmic enzyme, was not released significantly during 60 min of incubation which is indicative of selective lysosomal enzyme extrusion during cell contact with ZTS. Serum which had not been previously incubated with zymosan did not demonstrate lysosomal enzyme-releasing activity.

Kinetics of β-glucuronidase and acid protease from cytochalasin B-treated guinea pig neutrophils incubated with zymosan-treated serum in the presence and absence of calcium. The data in Fig. 2 demonstrate the release of acid protease and β-glucuronidase, as a function of time, from cytochalasin B-treated neutrophils incubated with ZTS with calcium present in the extracellular medium. However, in the absence of extracellular calcium the selective release of lysosomal enzymes associated with neutrophil contact with ZTS failed to occur.

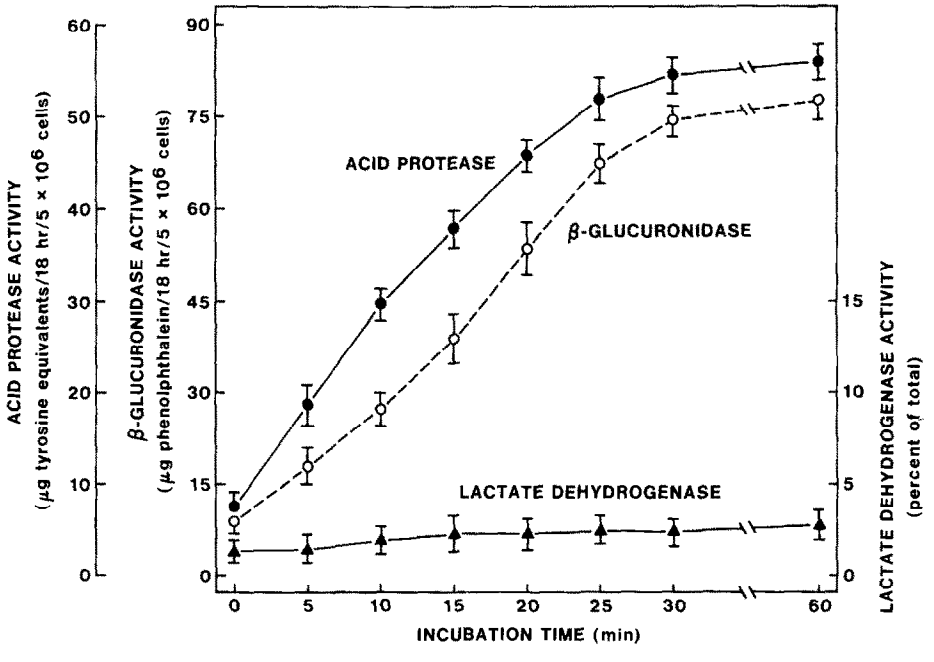

Fig. 1. Secretion of β-glucuronidase, acid protease and lactate dehydrogenase from cytochalasin B-treated guinea pig neutrophils in the presence of zymosan-treated serum (ZTS). Neutrophils (5 × 10^6) were preincubated with cytochalasin B (5 μg/ml) for 10 min at 37°. The cells were then incubated in 2.0 ml of Krebs-Ringer phosphate medium containing ZTS (10%, v/v). Values for total cell enzyme activities, determined after incubating cells in 0.2% Triton X-100–0.04 M Tris acetate, pH 7.4, for 15 min were: 212 ± 19.5 μg phenolphthalein/18 hr/5 × 10^6 cells for β-glucuronidase, 192 ± 17.6 μg tyrosine equivalents/18 hr/5 × 10^6 cells for acid protease, and 244 ± 23.5 absorbancy units/min/5 × 10^6 cells for lactate dehydrogenase. Data represent the means ± S.E.M. of four separate experiments.

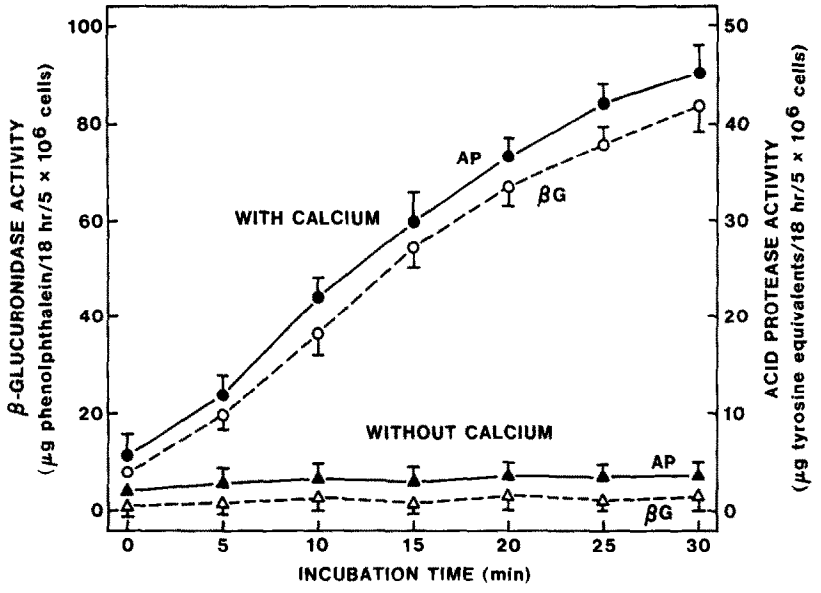

Fig. 2. Effects of calcium on β-glucuronidase (βG) and acid protease (AP) secretion from cytochalasin B-treated guinea pig neutrophils in the presence of zymosan-treated serum (ZTS). Neutrophils (5 × 10^6) were preincubated with cytochalasin B for 10 min at 37°. The cells were then incubated with ZTS (10%, v/v) in Krebs-Ringer phosphate medium with or without calcium (1.27 mM) for the time periods indicated. Total cell enzyme activities were: 237 ± 21.4 μg phenolphthalein/18 hr/5 × 10^6 cells for β-glucuronidase; and 205 ± 20.1 μg tyrosine equivalents/18 hr/5 × 10^6 cells for acid protease. Data represent the means ± S.E.M. of five separate experiments.

Acid protease and β-glucuronidase secretion from cytochalasin B-treated guinea pig neutrophils in contact with zymosan-treated serum as a function of the extracellular calcium concentration. The effects of increasing concentrations of extracellular calcium on the discharge of lysosomal enzymes from neutrophils is shown in Table 1. A calcium concentration between 0.10 and 1.25 mM stimulated a graded enhancement of β-glucuronidase and acid protease release from cytochalasin B-treated neutrophils in the presence of ZTS. No further increase in enzyme secretion was noted with a calcium concentration above 1.25 mM. It should be noted that calcium failed to induce lysosomal enzyme release from neutrophils in the absence of ZTS.

Kinetics of zymosan-treated serum-induced release of β-glucuronidase from, and calcium association with, cytochalasin B-treated guinea pig neutrophils. Neutrophil contact with ZTS resulted in the selective discharge of β-glucuronidase (Fig. 3); ZTS also provoked calcium association with neutrophils which was linear from 1 to 15 min. β-glucuronidase release demonstrated a lag period from 0 time to approximately 5 min and was linear through 20 min of incubation with ZTS. Serum which was not reacted with zymosan, as com-

Table 1. Effects of calcium ion concentration on acid protease (AP) and β-glucuronidase (βG) release from guinea pig neutrophils in the presence of zymosan-treated serum (ZTS)*

Calcium (mM)	Lysosomal enzyme secretion (% of total activity)† Cells + ZTS AP	Cells + ZTS βG	Cells alone AP	Cells alone βG
0.00	1.3 ± 0.12‡	2.3 ± 0.33	0.8 ± 0.07	1.1 ± 0.13
0.10	2.5 ± 0.27	3.1 ± 0.38	0.9 ± 0.08	0.9 ± 0.08
0.25	4.7 ± 0.51	4.2 ± 0.29	1.2 ± 0.09	1.3 ± 0.12
0.50	5.3 ± 0.37	6.4 ± 0.63	1.5 ± 0.14	1.6 ± 0.15
0.75	11.1 ± 0.94	12.5 ± 0.88	1.4 ± 0.13	0.9 ± 0.07
1.00	17.6 ± 1.53	18.8 ± 1.75	1.4 ± 0.14	1.5 ± 0.14
1.25	23.5 ± 1.94	25.6 ± 2.14	1.6 ± 0.15	1.2 ± 0.12
1.50	23.1 ± 2.33	26.1 ± 2.43	1.3 ± 0.18	1.5 ± 0.13
2.00	22.7 ± 2.49	25.2 ± 1.99	1.5 ± 0.16	0.9 ± 0.09

* Neutrophils (5 × 10^6) were preincubated with cytochalasin B (5 μg/ml) for 10 min followed by a 15-min incubation either alone or with zymosan-treated serum (10% v/v) in Krebs–Ringer phosphate medium containing various concentrations of calcium.

† Total cell enzyme activities were: 223 ± 22.4 μg phenolphthalein/18 hr/5 × 10^6 cells for β-glucuronidase, and 189 ± 17.9 μg tyrosine equivalents/18 hr/5 × 10^6 cells for acid protease.

‡ Each value represents the mean ± S.E.M. of three separate experiments.

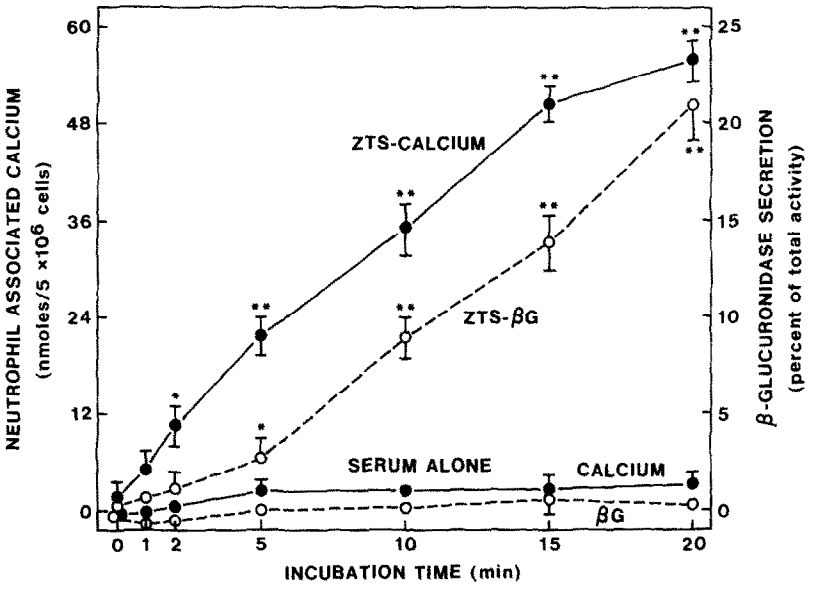

Fig. 3. Effects of zymosan-treated serum (ZTS) on calcium association with, and β-glucuronidase (βG) secretion from, cytochalasin B-treated guinea pig neutrophils. Neutrophils (5×10^6) were preincubated with cytochalasin B for 10 min at 37° and then incubated with ZTS or serum alone for the time periods indicated. $^{45}CaCl_2$ was added to the cell suspensions at zero time (0 min) and neutrophil-associated calcium was determined as described in the text. Total cell enzyme activity for β-glucuronidase was 241 ± 22.3 μg phenolphthalein/18 hr/5×10^6 cells. Data represent the means ± S.E.M. of three separate experiments. The asterisk (*) indicates significance at $P < 0.05$ vs cells + serum alone. The double asterisk (**) indicates significance at $P < 0.01$ vs cells + serum alone.

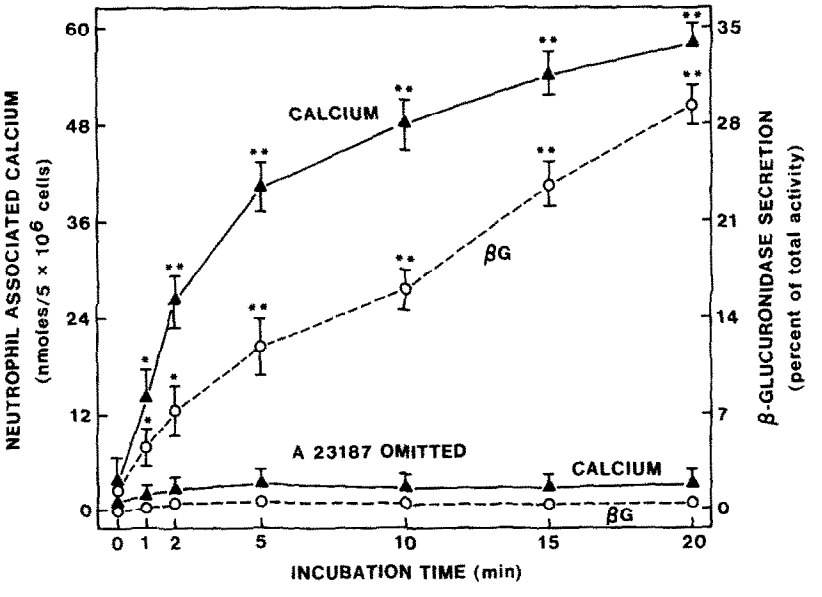

Fig. 4. Effects of ionophore A23187 on calcium association with, and β-glucuronidase (βG) secretion from, guinea pig neutrophils. Neutrophils (5×10^6) were incubated with (0.5 μM) or without ionophore A23187 for the time periods indicated. $^{45}CaCl_2$ was added to the cell suspensions at zero time (0 min) and neutrophil-associated calcium was determined as described in the text. Total cell enzyme activity for β-glucuronidase was 193 ± 18.4 μg phenolphthalein/18 hr/5×10^6 cells. Data represent the means ± S.E.M. of three separate experiments. The asterisk (*) indicates significance at $P < 0.05$ vs cells without A23187. The double asterisk (**) indicates significance at $P < 0.01$ vs cells without A23187.

Table 2. Effect of zymosan-treated serum and A23187 on viability of guinea pig neutrophils

Experimental condition*	Lactate dehydrogenase release (% of total activity)†	Per cent of neutrophils with trypan blue uptake
5×10^6 Neutrophils (N)	1.5 ± 0.13	2.2 ± 2.13
N + Zymosan-treated Serum	2.4 ± 0.18	1.7 ± 0.11
N + A23187 (0.5 μM)	1.9 ± 0.16	2.5 ± 0.21

* Neutrophils were incubated alone, with A23187 or with zymosan-treated serum at 37° for 30 min. Incubation with zymosan-treated serum was preceded by a 10 min incubation with cytochalasin B (5 μg/ml). Data express the means ± S.E.M. of five separate experiments.

† Total cell enzyme activity for lactate dehydrogenase, determined after incubating cells in 0.2% triton × 100–0.04M tris acetate, pH 7.4, for 15 min, was 237 ± 25.7 absorbancy units/min/5×10^6 cells.

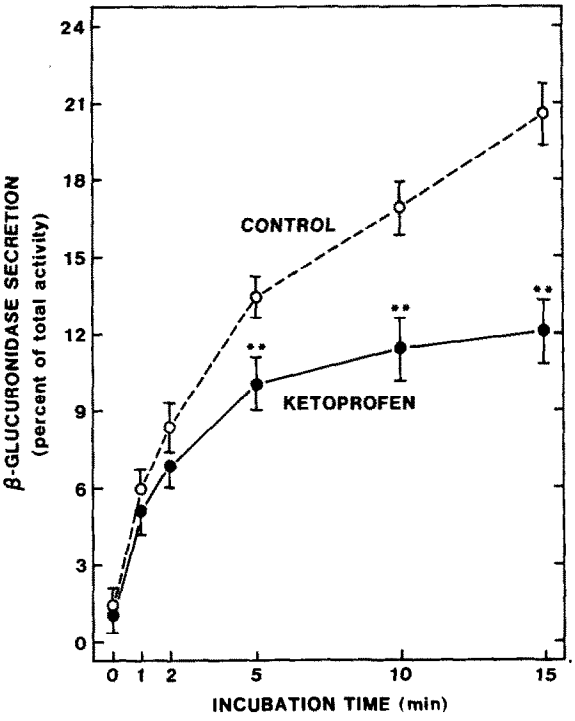

Fig. 5. Effects of ketoprofen on the initial rate of ionophore A23187-induced secretion of β-glucuronidase from guinea pig neutrophils. Neutrophils were preincubated with ketoprofen (100 μM) in 2.0 ml of Krebs–Ringer phosphate medium for 10 min followed by incubation with A23187 (0.5 μM) for the time periods indicated. Total cell enzyme activity for β-glucuronidase was 224 ± 21.1 μg phenolphthalein/18 hr/5×10^6 cells. Data represent the means ± S.E.M. of four separate experiments. The double asterisk (**) indicates significance at $P < 0.01$ vs control.

Table 3. Effects of nonsteroid anti-inflammatory agents on ionophore A23187 (0.5 μM)-induced calcium association with and β-glucuronidase secretion from guinea pig neutrophils*

Agent	Percent inhibition of Cell-associated calcium	Percent inhibition of β-Glucuronidase release
Acetylsalicylic acid		
100 μM	12 ± 0.9	10 ± 0.9
10 μM	8 ± 0.7	5 ± 0.4
1 μM	2 ± 0.2	3 ± 0.3
Diftalone		
100 μM	22 ± 1.8†	27 ± 2.1‡
10 μM	16 ± 1.5	20 ± 1.6†
1 μM	7 ± 0.7	13 ± 0.9
Flazalone		
100 μM	4 ± 0.4	8 ± 0.7
10 μM	2 ± 0.1	6 ± 0.5
1 μM	0	2 ± 0.2
Indomethacin		
100 μM	22 ± 1.3†	21 ± 1.8†
10 μM	9 ± 0.8	13 ± 1.2
1 μM	3 ± 0.2	6 ± 0.5
Ketoprofen		
100 μM	39 ± 3.3‡	49 ± 3.1‡
10 μM	26 ± 2.5‡	35 ± 2.8‡
1 μM	14 ± 1.3	19 ± 1.4
Naproxen		
100 μM	20 ± 1.5†	29 ± 2.2†
109 μM	17 ± 1.1	20 ± 1.8†
1 μM	3 ± 0.2	9 ± 0.8
Suprofen		
100 μM	30 ± 2.7‡	38 ± 3.2‡
10 μM	21 ± 1.9†	27 ± 2.4‡
1 μM	7 ± 0.5	14 ± 1.1

* Neutrophils (5×10^6) were preincubated with the respective NSAIA for 10 min. at 37° followed by a 15-min incubation with A23187. $^{45}CaCl_2$ was added to the cell suspensions with the ionophore. Neutrophil-associated calcium, was determined as described in the text. Control incubations yielded a value of 37.4 ± 2.9 μg phenolphthalein/18 hr/5×10^6 cells (21.3 per cent of total cell activity) for release of β-glucuronidase. Data represent the means ± S.E.M. of three separate experiments.

† Significant at $P < 0.05$ vs control.

‡ Significant at $P < 0.01$ vs control.

pared to ZTS, induced significantly less calcium association with, and lysosomal enzyme release from, cytochalasin B-treated neutrophils.

Kinetics of ionophore A23187-induced discharge of β-glucuronidase from, and calcium association with, guinea pig neutrophils. The data in Fig. 4 demonstrate the capacity of A23187 to stimulate the release of β-glucuronidase from guinea pig neutrophils in a linear fashion from 0 through 20 min of incubation. A rapid association of calcium with neutrophils was shown when these cells were incubated with A23187. The association of calcium with, and secretion of β-glucuronidase from, neutrophils in the absence of ionophore were negligible.

Viability of guinea pig neutrophils treated with A23187 or zymosan-treated serum. The data in Table 2 indicate that A23187 and ZTS were exerting a specific

Table 4. Effects of calcium ion concentration on the capacity of nonsteroid anti-inflammatory agents to inhibit ionophore A23187(0.5 μM)–induced secretion of β-glucuronidase from guinea pig neutrophils*

Agent	β-Glucuronidase released (% of total activity) (Calcium)			
	0.60 mM	1.2 mM	1.8 mM	2.4 mM
Control (no agent)	12 ± 0.8	23 ± 2.1	25 ± 1.9	24 ± 2.2
Diftalone				
100 μM	10 ± 0.5†	17 ± 1.3†	18 ± 1.7†	21 ± 1.5
10 μM	11 ± 1.2	19 ± 1.8	21 ± 2.2	23 ± 2.2
Indomethacin				
100 μM	10 ± 0.9†	18 ± 1.5†	19 ± 1.5†	22 ± 1.7
10 μM	11 ± 1.2	21 ± 1.8	23 ± 2.1	23 ± 2.1
Ketoprofen				
100 μM	8 ± 0.7‡	11 ± 0.7‡	16 ± 1.8‡	21 ± 1.8
10 μM	10 ± 0.6†	14 ± 1.2	21 ± 1.7	22 ± 2.3
Suprofen				
100 μM	9 ± 0.8‡	13 ± 1.1‡	18 ± 1.3‡	22 ± 1.4
10 μM	11 ± 1.3	16 ± 0.9†	22 ± 2.2	24 ± 2.5
Naproxen				
100 μM	10 ± 1.1†	17 ± 1.3†	22 ± 1.5	23 ± 2.3
10 μM	12 ± 0.4	20 ± 1.8	24 ± 2.5	24 ± 1.8

* Neutrophils (5×10^6) were preincubated with the respective NSAIA at 37° for 10 min in Krebs–Ringer phosphate medium containing the designated concentrations of calcium and then further incubated with A23187 for 15 min. Control incubations yield values of 190 ± 18.2 μg phenolphthalein/18 hr/5×10^6 cells (19.5 per cent of total cell activity) for release of β-glucuronidase. Data represent the means ± S.E.M. of three separate experiments.

† Significant at $P < 0.05$ vs control.

‡ Significant at $P < 0.01$ vs control.

effect on the lysosomal enzyme secretory process in that there was no appreciable release of cytoplasmic lactate dehydrogenase from these cells nor did the neutrophils demonstrate a significant uptake of trypan blue during the respective incubation periods.

Effect of ketoprofen on the time course of A23187-induced release of β-glucuronidase from guinea pig neutrophils. The rate of A23187-induced secretion of β-glucuronidase was linear through 15 min of incubation. Ketoprofen significantly curtailed enzyme release from 5 through 15 min of cell contact with the ionophore (Fig. 5). The maximum effect of this nonsteroid anti-inflammatory agent occurred at the 15-min time interval. The concentration of this agent had been shown previously to have no effect on cell viability, as determined by trypan blue exclusion and release of lactate dehydrogenase [2, 4].

Table 5. Effects of magnesium on ionophore A23187 (0.5 μM)-induced secretion of β-glucuronidase from guinea pig neutrophils

Experimental condition*	β-Glucuronidase release (% of total activity)†
A23187 (A)	24 ± 2.1
A + Mg^{2+} (0.6 mM)	22 ± 1.8
A + Mg^{2+} (1.2 mM)	21 ± 1.5
A + Mg^{2+} (2.4 mM)	15 ± 1.2‡
A + Mg^{2+} (4.8 mM)	11 ± 0.7‡

* Neutrophils (5×10^6) were incubated with A23187 in Krebs–Ringer phtsphate medium in the presence of various concentrations of magnesium at 37° for 15 min. Total cell enzyme activity for β-glucuronidase was 199 ± 18.9 μg phenolphthalein/18 hr/5×10^6 cells.

† Each value represents the mean ± S.E.M. of four separate experiments.

‡ Significant at $P < 0.01$ vs control.

Effects of nonsteroid anti-inflammatory agents on lysosomal enzyme extrusion from, and calcium association with, guinea pig neutrophils treated with A23187. Diftalone, indomethacin, ketoprofen, naproxen and suprofen demonstrated a dose-related curtailment of β-glucuronidase release from, and calcium association with neutrophils stimulated with A23187 (Table 3). The relative effects of these NSAIA show that ketoprofen > suprofen > naproxen = diftalone > indomethacin. Acetylsalicylic acid and flazalone had no effect on ionophore-induced lysosomal enzyme discharge or calcium flux.

Influence of extracellular calcium concentration on the capacity of nonsteroid anti-inflammatory agents to modulate A23187-induced secretion of lysosomal enzymes from guinea pig neutrophils. Curtailment of A23187-induced release of β-glucuronidase from guinea pig neutrophils by diftalone, indomethacin, naproxen, suprofen and ketoprofen was demonstrated with an extracellular calcium concentration of 0.60 to 1.2 mM (Table 4). Increasing the calcium concentration of the Krebs–Ringer phosphate medium to 1.8 or 2.4 mM significantly attenuated the capacity of the

NSAIA to retard A23187-stimulated lysosomal enzyme release.

Effect of magnesium on A23187-induced discharge of β-glucuronidase from guinea pig neutrophils. The data in Table 5 indicate that, while an extracellular magnesium concentration of 0.6 and 1.2 mM had no effect on β-glucuronidase release, increasing the concentration to 2.4 or 4.8 mM significantly inhibited the secretion of this lysosomal granule-associated enzyme.

DISCUSSION

The lysosomal constituents of polymorphonuclear neutrophils play a significant role in the tissue injury which characterizes the pathogenesis of numerous inflammatory reactions [16–18]. Indeed, the lysosome granule-associated enzymes which are selectively extruded from neutrophils appear to destroy such tissues as collagen [19] and the mucopolysaccharide matrix of articular cartilage [20, 21]. The capacity of certain clinically effective NSAIA to inhibit the release of lysosomal enzymes from inflammatory cells, such as neutrophils, has been reported from several laboratories [2, 4, 22–24], and it may be this action which accounts for part of the therapeutic effect observed with these agents. Therefore, we investigated the mechanism by which certain NSAIA influence the guinea pig neutrophil lysosomal enzyme secretory process.

The calcium-dependent aspect of secretory mechanisms associated with numerous biological systems is well documented [25–27]. We report here that contact between cytochalasin B-treated guinea pig neutrophils and the soluble immune reactant-zymosan-treated serum (ZTS) results in the selective release of the lysosomal hydrolases, β-glucuronidase and acid protease, when calcium is present in the extracellular medium. However, this cellular activity is lost in the absence of calcium. These data agree with that of Ignarro and George [9] and Smith and Ignarro [10] concerning human neutrophils. We have also shown a requirement of cell contact with an immune reactant, such as ZTS, in order for lysosomal enzyme secretion to occur, in that cells alone in the presence of extracellular calcium will not discharge their β-glucuronidase or acid protease. Goldstein *et al.* [28] showed that calcium, in the absence of other stimuli, had the capacity to induce the release of lysozyme but not β-glucuronidase from human neutrophils. However, it should be noted that, while β-glucuronidase and acid protease are localized in azurophil granules [29], lysozyme is distributed in both azurophil and specific granules [30]. Therefore, the capacity of calcium to stimulate the secretion of lysozyme but not β-glucuronidase indicates that two mechanisms may be responsible for enzyme secretion from the respective granules. Thus, our data agree with that of Goldstein *et al.* [28] in that calcium alone will not induce the discharge of β-glucuronidase and acid protease from guinea pig neutrophils.

The studies of Goldstein *et al.* [31, 32] and Henson *et al.* [33] indicate that the lysosomal enzyme-releasing activity of ZTS can be attributed to C5a, a low molecular weight product of the fifth component of complement. Goldstein *et al.* [34] reported that C5a induced the selective release of lysosomal enzymes from human neutrophils in the absence of calcium. However, we have shown that ZTS-stimulated release of β-glucuronidase and acid protease is accompanied by a marked association of calcium with cells, and that enzyme release from guinea pig neutrophils requires calcium in the extracellular medium. One possible explanation for this difference is that Goldstein *et al.* [34] employed NaCl–HEPES * buffer as their cell suspension medium whereas we utilized Krebs–Ringer phosphate buffer. C5a, when purified, may also have a different mode of action than ZTS. A possibility in this regard is that C5a might induce the mobilization of previously sequestered intracellular calcium as opposed to ZTS, which, in addition to C5a, consists of other complement components that may act to stimulate lysosomal enzyme release in a calcium-dependent fashion.

It is important to note that C5a [31–34] and to a lesser degree ZTS, in order to be effective, require that neutrophils be pretreated with cytochalasin B. Weissmann *et al.* [35] have demonstrated that cytochalasin B, via its capacity to inhibit phagocytosis, converts the neutrophil into a pure secretory cell. However, besides its effect on phagocytosis, cytochalasin B has the ability to modulate other cellular activities [36–38]. Therefore, the mechanism(s) by which C5a and ZTS stimulate the lysosomal enzyme secretory process may be a function of the action of cytochalasin B on the neutrophil, prior to being exposed to the stimuli. It also appears that the requirements for secretion depend, at least in part, upon the stimulus employed. In this regard Becker and Showell [39] reported that a bacterial chemotactic factor, which has activities similar to C5a, required calcium in order to induce β-glucuronidase and lysozyme secretion from cytochalasin B-treated rabbit neutrophils. This observation, together with our findings, indicates that C5a-stimulated-release of lysosomal enzymes from guinea pig, and rabbit, but not human, neutrophils requires extracellular calcium.

In view of the knowledge that calcium is required for allergic histamine release from cells [40] and that divalent cation ionophores provoke mast cell degranulation only in the presence of calcium [40], we investigated the effects of the divalent cation ionophore A23187 on the release of lysosomal enzymes from guinea pig neutrophils. The experimental data presented here indicate that A23187 stimulated a specific time-dependent release of lysosomal enzymes from guinea pig neutrophils. In addition, A23187 caused a rapid and sustained association of calcium with neutrophils which, on a time basis, paralleled lysosomal enzyme discharge. In the absence of ionophore, guinea pig neutrophils neither released their lysosomal contents nor demonstrated an association with calcium. ZTS also stimulated an association of calcium with cytochalasin B-treated neutrophils which correlated with the capacity of this immune reactant to induce β-glucuronidase release. These observations, together with the finding that serum alone failed to induce an association of calcium with neutrophils and lysosomal enzyme secretion, suggest that agents such as ZTS may elicit the extracellular extrusion of lysosomal enzymes from guinea pig neutrophils via an ionophore-like

* HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

action on these cells. Therefore, calcium may indeed function as the link between the stimulus for (immune reactant) and secretion of lysosomal enzymes from neutrophils, as suggested by the "stimulus-secretion coupling" mechanism described for neutrophils [9, 10, 41, 42].

NSAIA modulate the discharge, of lysomal enzymes from neutrophils induced by various agents, [1–4, 22–24]. However, the precise mechanism of action of these agents remains to be elucidated. Relative to this point it is important to note that NSAIA have been reported to influence the calcium concentrations of various tissues [43, 44]. We have shown that diftalone, indomethacin, ketoprofen, naproxen and suprofen, in addition to curtailing A23187-induced secretion of β-glucuronidase from neutrophils, also impeded calcium association with these cells. Further, these effects could be reversed by increasing the extracellular calcium concentration. The specificity of calcium in counteracting the inhibitory influence of NSAIA was demonstrated by the finding that increasing the magnesium concentration of the incubation medium retarded the release of lysosomal enzymes. These data are in agreement with that of Goldstein *et al.* [28] who demonstrated that magnesium concentrations in excess of 2.5 mM inhibited the human neutrophil lysosomal enzyme secretory process. The fact that ketoprofen, employing a very short preincubation (10 min) period, had the capacity to curtail the initial rate of β-glucuronidase release suggests that this agent is interfering with an early stage of the lysosomal enzyme secretory mechanism. Because we have shown an immediate association of calcium with neutrophils in the presence of ZTS or A23187, we would suggest that this early effect of ketoprofen indicates that this agent is impeding the association of calcium with neutrophils.

In conclusion, the data presented here show the requirement of calcium for the selective discharge of lysosomal enzymes from guinea pig neutrophils. The fact that cell contact with a substance possessing lysosomal enzyme-releasing activity (e.g. ZTS) is necessary for enzyme secretion to occur suggests that these substances may function to stimulate calcium association with neutrophils. This is supported by the observations that ZTS provoked the association of calcium with neutrophils, and a divalent cation ionophore, A23187, induced selective lysosomal enzyme release. The capacity of certain NSAIA to impede ionophore-induced calcium association with neutrophils represents a possible mechanism by which these clinically effective agents curtail the extrusion of lysosomal enzymes. This action may explain, in part, the mechanism by which these therapeutic agents retard the development of various inflammatory diseases.

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