EFFECTS OF 2-{4-(2-IMIDAZO[1,2-a]PYRIDYL)PHENYL} PROPIONIC ACID (Y-9213) AND ANTI-INFLAMMATORY DRUGS ON ERYTHROCYTES, POLYMORPHONUCLEAR LEUKOCYTES AND LYPOSOMES IN VITRO*

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Abstract—2-{4-(2-Imidazo[1,2-a]pyridyl)phenyl}propionic acid (Y-9213) with analgesic, antipyretic and anti-inflammatory activities significantly inhibited hemolysis of rat erythrocytes. Activity of Y-9213 (100–500 μM) on hemolysis was more potent than that of phenylbutazone, and less potent than that of indomethacin. The spontaneous release of enzymes from rat liver lysosomes by incubation alone was significantly inhibited by Y-9213 (1–100 μM) to the same degree as phenylbutazone or tinoridine hydrochloride. Release of enzymes from the lysosomes by addition of phospholipase C (PLC, 0.03 units/ml) was slightly inhibited by Y-9213 (10–100 μM) and phenylbutazone (100 μM). Dexamethasone, prednisolone, hydrocortisone and tinoridine hydrochloride (1–10 μM) inhibited more potently the PLC-induced release than the spontaneous release. Y-9213 (1–100 μM) inhibited considerably the release of enzymes from intact lysosomes of rabbit polymorphonuclear (PMN) leukocytes. The release of enzymes from the PMN leukocyte lysosomes preincubated at 37°C for 15 min was strongly inhibited by dexamethasone, prednisolone and hydrocortisone (1–100 μM), but not by Y-9213, phenylbutazone and indomethacin (100 μM). Y-9213 (0.1–10 μM) also inhibited significantly the phagocytic secretion of lysosomal enzymes from PMN leukocytes without affecting phagocytosis of the particles. Activity of this agent was similar to that of phenylbutazone, and less active than that of indomethacin, dexamethasone or prednisolone. Our results suggest that Y-9213 may stabilize membranes of erythrocytes and lysosomes and inhibit phagocytic secretion of lysosomal constituents from PMN leukocytes.

The mechanism of action of several anti-inflammatory drugs such as indomethacin is suggested to be the inhibition of prostaglandin biosynthesis (1, 2) and the stabilization of lysosome-membranes (3, 4). In a previous paper we showed that both steroidal and non-steroidal anti-inflammatory drugs inhibit the release and secretion in vitro of lysosomal enzymes from polymorphonuclear (PMN) leukocytes. The inhibitory activities of these drugs appeared to be correlated with the protection of local Shwartzman reaction in rabbits by the treatment with anti-inflammatory drugs (5). In addition, the recent findings using the model of acute inflammation in rats (6, in preparation) have demonstrated that the oral treatment with steroidal and nonsteroidal anti-inflammatory drugs results in the inhibition of the secretion in vivo of lysosomal enzymes from the emigrated PMN leukocytes and

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an increased vascular permeability.

The present studies were carried out to examine the properties of a new compound, 2-\{-4-(2-imidazo[1,2-a]pyridyl)phenyl\}-propionic acid (Y-9213), with potent analgesic, antipyretic and anti-inflammatory activities (7, in preparation) on biomembranes such as erythrocytes, PMN leukocytes and lysosomes.

MATERIALS AND METHODS

Agents and preparation of test solution

Y-9213, 2-(5H-[1]benzopyrano[2,3-b]pyridin-7-yl)propionic acid (pranoprofen) (6), indomethacin, phenylbutazone, mefenamic acid, acetylsalicylic acid and tinoridine hydrochloride (4) were products of our own laboratories. Dexamethasone, prednisolone, hydrocortisone, 6-mercaptopurine, colchicine, propranolol hydrochloride, isoproterenol hydrochloride, theophylline and papaverine hydrochloride were purchased from Sigma Chemical Co. Ltd. In the experiments using rat erythrocytes, all agents were dissolved or suspended in 0.015 M phosphate buffer (pH 7.4). In the experiments using PMN leukocytes or lysosomes, all agents were dissolved in ethanol. A small amount of ethanol (less than 1%) used as solvent had no influence on the stability of PMN leukocytes or lysosomes.

Animals

Male Wistar rats, weighing 250-300 g, and male albino rabbits, weighing 3-4 kg, were used.

Assay of erythrocyte-membrane stability

Effects of the agents tested on the hyperthermic-hypotonic hemolysis of the rat erythrocytes were measured by a modification of the method of Glenn and Brown (8). Briefly, 2.0 ml of the test solution or solvent as the control was added to a test tube containing 2.0 ml of the 2% (v/v) erythrocyte suspension in 0.15 M phosphate buffer (pH 7.4) (9). The mixtures were kept at 25°C for 10 min. As the blank test, the mixtures containing 2.0 ml of the erythrocyte suspension and 2.0 ml of 0.15 M phosphate buffer (pH 7.4) were centrifuged at 3,000 rpm for 10 min. The other mixtures were incubated at 53°C for 20 min. After cooling in chilled water, the mixtures were centrifuged under the same conditions. Hemoglobin released in the supernatant was measured at 540 nm. The results are shown as % inhibition compared with the control.

Preparation of rat liver lysosomes

The 700-3500 x g liver fraction suspended in 0.25 M sucrose-0.04 M Tris acetate buffer (pH 7.4) was used as a lysosome suspension (10).

Preparation of rabbit PMN leukocytes

The suspension containing over 90% PMN leukocytes was collected from rabbit peritoneal exudates induced by glycogen. The PMN leukocytes were washed carefully (4) and suspended in Hanks' solution (pH 7.4) (5) or 0.25 M sucrose-0.04 M Tris acetate buffer (pH 7.4) to adjust the leukocyte counts to 5 x 10^7/ml or 1 x 10^9/ml, respectively.
Preparation of PMN leukocyte lysosomes

The 600 x g supernatant fraction was prepared from the PMN leukocyte suspension (1 x 10^9/ml) in 0.25 M sucrose-0.04 M Tris acetate buffer (pH 7.4) as a lysosome suspension (4).

Assay of lysosome-membrane stability

Effects of test agents on the lysosome-membrane were studied by determining the release of acid phosphatase or aryl sulfatase from the lysosomes. The test solution (30 µl) or solvent as the control was added to a 10-ml conical flask containing 3.0 ml of the liver lysosome suspension. After being kept at 25°C for 5 min, 1.0 ml of the mixtures was removed to determine the initial activity of free lysosomal enzymes in the 27,000 x g supernatant. The residual mixtures were incubated at 37°C for 30 min with shaking at an agitation cycle of 75/min. In certain experiments, 20 µl of 0.25 M sucrose-0.04 M Tris acetate buffer containing phospholipase C (PLC, Sigma, Type I) was added to the conical flask containing 2.0 ml of the residual mixtures. The above mixtures were incubated at 37°C for 15, 30 or 60 min with shaking. The mixtures removed before and after the incubation were centrifuged at 27,000 x g for 15 min at 4°C. The resulting supernatants were used for the measurement of the lysosomal enzymes released.

Effects of test agents on the release of enzymes from the PMN leukocyte lysosomes or lysosomes preincubated at 37°C for 15 min were also studied by our method (4).

The total activities of the marker enzymes were assayed using the 27,000 x g supernatant obtained by centrifuging the lysosome suspension incubated at 37°C for 30 min in 0.2% (v/v) Triton X-100-buffer solution.

The results are shown as % of the total activity (the free activity/the total activity) or % inhibition compared with the control.

Assay of release of lysosomal enzymes from PMN leukocytes during phagocytosis of particles

Lysosomal enzymes are released from the PMN leukocytes (5 x 10^7/ml) suspended in Hanks' solution during phagocytosis of the serum-treated zymosan particles (0.5-3 µ in diameter, Sigma) by our method (5). The total activities of the enzymes were assayed using the supernatant obtained by the same procedure described above. The results are shown as % of the total activity or % inhibition compared with the control.

Assay of phagocytosis

The method of Stossel et al. (11) was slightly modified. Briefly, Oil Red O (Chroma) was dissolved in the paraffin oil (Merck) by heating at 100°C for 60 min. The mixture was kept at 25°C for several days, and then centrifuged at 300 x g for 10 min. Emulsions of paraffin oil containing Oil Red O were prepared by sonic dispersion of 1.0 ml of the supernatant added to 3.0 ml of Hanks' solution containing 2% (w/v) bovine serum albumin (Sigma, Fraction V) for 1.5 min at a setting of 40 W (Branson B-12 sonifier).

The test solution (15 µl) or 50% (v/v) ethanol-Hanks' solution as the control was added to a siliconized 10-ml conical flask containing 2.0 ml of the PMN leukocyte suspension in Hanks' solution. The mixtures were preincubated at 37°C for 10 min with shaking at an
agitation cycle of 100/min. Immediately after adding 1.0 ml of the above paraffin oil emulsion to the mixtures, samples of 0.5 ml were removed, and added to 3.0 ml of ice-cold 0.15 M NaCl solution containing 1 mM N-ethylmaleimide in a siliconized 10-ml test tube. The residual mixtures were incubated for another 10 min, and then samples of 0.5 ml were added to the same solution to assay the rate of phagocytosis. The cell pellets obtained after the centrifugation at 150 × g for 15 min were washed carefully four times with the same solution. The test tubes containing the cell pellets were finally drained by inversion. Oil Red O was extracted from the washed pellets at 25°C for 15-16 hr in 2.5 ml of dioxane (Merck). The extracts were centrifuged at 1,000 × g for 15 min, and the content of Oil Red O ingested by the PMN leukocytes was determined at 524 nm. The results are shown as % inhibition compared with the control.

Assay of viability of cells

The integrity of the PMN leukocytes was assayed by the method of Zurier et al. (12). Viability of the PMN leukocytes before and after the incubation was always greater than 97% of the total cells (eosin Y exclusion).

Enzyme assays

Aryl sulfatase (EC 3.1.6.1, aryl sulfate sulfohydrolase), β-glucuronidase (EC 3.2.1.31, β-glucuronide glucuronohydrolase) and acid phosphatase (EC 3.1.3.2, orthophosphoric monoester phosphohydrolase) activities were assayed using dipotassium p-nitrocatechol sulfate (13), p-nitrophenyl glucuronide (14) and sodium p-nitrophenyl phosphate (13) as substrate, respectively. SH-Protease activity which is activated by the addition of cysteine was assayed in 0.05 M phosphate buffer (pH 7.1) using casein (Merck) as substrate by a modification of method of Hayashi et al. (15). Non SH-protease and acid protease activities were assayed respectively by the modified method using 0.15 M NaCl instead of cysteine and 0.05 M citrate buffer (pH 3.0) instead of 0.05 M phosphate buffer (pH 7.1). The arbitrary unit of proteolytic activity was defined as the amount of the enzyme that produced an increase of 0.001/min in the absorbancy at 276 nm (15). LDH (EC 1.1.1.27, L-lactate: NAD oxidoreductase) activity was assayed according to the method of Cabaud and Wroblewski (16).

None of the agents tested inhibited directly the activities of the above enzymes at concentrations tested.

RESULTS

Effects of agents on hyperthermic-hypotonic hemolysis

Table 1 shows that Y-9213 inhibited significantly the hemolysis at concentrations from 100 to 500 μM. The inhibitory effect of this agent was more potent than that of phenylbutazone or acetylsalicylic acid, and less potent than that of indomethacin or mfenamic acid. Of the agents tested, only prednisolone showed no effect at 500 μM.

Effects of agents on the stability of rat liver lysosomes

Table 2 indicates that Y-9213 inhibited the spontaneous release of acid phosphatase
TABLE 1. Effect of test agents on hyperthermic-hypotonic hemolysis

| Compound          | Final concn. (µM) | % inhibition±SE (N=4) |
|-------------------|-------------------|----------------------|
|                   |                  |                      |
| Y-9213            | 50                | 10.6±1.1             |
|                   | 100               | 19.4±0.4**           |
|                   | 250               | 45.6±0.2**           |
|                   | 500               | 61.7±0.4**           |
| Phenylbutazone    | 100               | 16.8±1.8**           |
|                   | 250               | 35.8±2.9**           |
|                   | 500               | 53.9±1.6**           |
| Indomethacin      | 1                 | 26.0±4.0**           |
|                   | 2.5               | 35.6±3.2**           |
|                   | 5                 | 47.9±1.3**           |
|                   | 10                | 65.2±0.7**           |
|                   | 25                | 77.0±1.6**           |
|                   | 50                | 81.0±0.8**           |
| Mefenamic acid    | 10                | 28.0±2.0**           |
|                   | 25                | 45.0±0.7**           |
|                   | 50                | 64.7±1.4**           |
|                   | 100               | 75.2±0.9**           |
|                   | 250               | 81.9±0.6**           |
| Acetylsalicylic acid | 1,000             | 13.3±0.4**           |
|                   | 2,500             | 25.5±0.1**           |
|                   | 5,000             | 40.5±0.8**           |
| Prednisolone      | 100               | -4.1±0.6             |
|                   | 250               | -3.4±0.3             |
|                   | 500               | -4.6±1.5             |

The erythrocyte suspension (2.0 ml) in 0.15 M phosphate buffer (pH 7.4) was incubated with each test solution (2.0 ml) in 0.015 M phosphate buffer (pH 7.4) at 53°C for 20 min. Hemoglobin released in controls; 0.6~0.7 (OD540). **p<0.01 (significant).

from the lysosomes by the incubation at concentrations from 1 to 100 µM. The activity of Y-9213 was similar to that of phenylbutazone or tinoridine hydrochloride. Steroidal anti-inflammatory drugs such as dexamethasone, however, inhibited significantly the enzyme release only at a high concentration of 100 µM.

Table 3 shows that the release of acid phosphatase and β-glucuronidase from the lysosomes was accelerated by the addition of PLC (0.01~0.1 units/ml). The release of the enzymes was time-dependent for periods up to 60 min (Table 4). Tinoridine hydrochloride and prednisolone inhibited the PLC-induced release in a different manner. The % inhibition by the former on the enzyme release was maximum during the first 15-min incubation period, and that by the latter was constant for periods up to 60 min.

Table 5 shows that the PLC-induced release of acid phosphatase during the 30-min incubation period was inhibited moderately by Y-9213 (10~100 µM), slightly by indomethacin (100 µM), and markedly by tinoridine hydrochloride (0.1~10 µM) and steroidal
anti-inflammatory drugs (0.1–100 μM).

**Effects of agents on the stability of PMN leukocyte lysosomes of rabbits**

Y-9213 (1–100 μM) inhibited most potently the release of aryl sulfatase and \( \beta \)-glucuronidase from the intact lysosomes among the agents tested (Table 6). The enzyme release from the lysosomes preincubated at 37°C for 15 min without the test solution, however, was not inhibited by Y-9213, indomethacin and phenylbutazone (Table 7). In contrast, steroidal anti-inflammatory drugs tested inhibited more markedly the enzyme release from the preincubated lysosomes than that from the intact lysosomes. 6-Mercap-

### Table 2. Effect of test agents on acid phosphatase release from rat liver lysosomes

| Compound          | % inhibition±SE (N=3) |
|-------------------|-----------------------|
|                   | 1 μM                  | 10 μM                 | 100 μM                |
| Y-9213            | 18.9±2.0**            | 28.7±0.9**            | 42.6±1.3**            |
| Phenylbutazone    | 15.5±1.8**            | 30.1±1.6**            | 49.6±2.1**            |
| Indomethacin      | 7.2±0.5               | 13.2±1.1**            | 18.9±2.0**            |
| Mefenamic acid    | 7.9±1.3               | 9.6±0.6**             |                       |
| Acetylsalicylic acid | 6.8±2.1          | 18.3±1.5**            |                       |
| Tinoridine HCl    | 11.7±2.6**            | 24.5±2.6**            | 40.4±1.1**            |
| Dexamethasone     | 6.2±0.5               | 13.0±1.4**            |                       |
| Prednisolone      | 7.4±0.6               | 19.8±1.4**            |                       |
| Hydrocortisone    | 7.2±0.6               | 18.7±2.7**            |                       |

The lysosome suspension (3.0 ml) was incubated with each test solution (30 μl) in 0.25 M sucrose-0.04 M Tris acetate buffer (pH 7.4) at 37°C for 30 min. Enzyme activity in controls: before incubation, 3.5±0.4; after incubation, 14.2±0.9; total activity, 129.1±4.7 μg p-nitrophenol formed. **P<0.01 (significant).

### Table 3. Phospholipase C-induced lysosomal enzyme release from rat liver lysosomes

| Compound          | Marker enzymes\(^a\) | Free activity (%±SE (N=3) in the presence of phospholipase C |
|-------------------|----------------------|-------------------------------------------------------------|
|                   |                      | 0               | 0.01             | 0.03             | 0.1 (unit)   |
| Control           | AP                   | 11.6±1.0        | 14.1±1.2        | 24.0±1.2        | 34.4±1.4     |
|                   | \( \beta \)G         | 9.3±0.8         | 12.9±1.0        | 21.2±1.0        | 28.5±1.8     |
| Tinoridine HCl    | AP                   | 8.3±0.4 \( (38.4) \) | 9.8±0.8 \( (38.7) \) | 17.4±1.0 \( (31.4) \) | 27.3±1.2 \( (22.6) \) |
| \( (30 \mu M)     | \( \beta \)G         | 6.6±0.4 \( (32.5) \) | 8.6±0.8 \( (36.1) \) | 16.0±1.2 \( (25.7) \) | 24.5±1.2     |
| Prednisolone      | AP                   | 10.4±0.6 \( (13.9) \) | 11.9±1.0 \( (19.8) \) | 13.9±1.6 \( (48.1) \) | 17.0±1.8     |
| \( (30 \mu M)     | \( \beta \)G         | 7.9±0.4 \( (16.9) \) | 9.8±1.0 \( (26.0) \) | 11.4±1.9 \( (48.5) \) | 14.5±1.4     |

The lysosome suspension (3.0 ml) containing each test solution (30 μl) was incubated with or without phospholipase C in 0.25 M sucrose-0.04 M Tris acetate buffer (pH 7.4) at 37°C for 30 min. Figures in parentheses show the percent inhibition. \( a\) AP, acid phosphatase. \( \beta \)G, \( \beta \)-glucuronidase. Free activity before incubation: 3.9±0.2 and 0.8±0.1; total activity: 128.6±1.2 and 79.3±5.4 μg p-nitrophenol formed for AP and \( \beta \)G respectively. \( *P<0.05, **P<0.01 \) (significant).
topurine and propranolol hydrochloride showed no effect on both the lysosomes at 100 μM.

Effects of agents on the release of lysosomal enzymes from PMN leukocytes during phagocytosis

Table 8 shows that the incubation of the PMN leukocytes with serum-treated zymosan particles resulted in the release of aryl sulfatase, β-glucuronidase, SH-protease and acid protease without a concomitant release of acid phosphatase, non SH-protease and LDH. Table 9 indicates that the release of aryl sulfatase from the PMN leukocytes during phagocy-

**TABLE 4.** Time course of phospholipase C-induced lysosomal enzyme release from rat liver lysosomes

| Compound          | Marker enzymes | Free activity (%) ± SE (N=3) | 0    | 15    | 30    | 60 (min) |
|-------------------|----------------|-----------------------------|------|-------|-------|----------|
| None              | AP, βG         | 3.5±0.2                     | 8.7±0.4 | 12.0±1.0 | 19.0±1.0 |
| None+PLC (control)| AP, βG         | 3.5±0.2                     | 16.7±1.6 | 24.4±1.9 | 42.0±1.8 |
| Tino-rin HCl      | AP, βG         | 3.3±0.3                     | 10.7±0.6** (43.9) | 17.7±1.2** (31.3) | 34.0±1.9* (20.3) |
| (30 μM)+PLC       | βG             | 1.1±0.1                     | 6.9±0.8** (43.7) | 14.3±1.6* (31.2) | 32.9±1.2* (14.5) |
| Prednisolone      | AP, βG         | 3.1±0.2                     | 9.5±0.4** (51.5) | 13.6±0.3** (49.8) | 21.2±1.2** (53.0) |
| (30 μM)+PLC       | βG             | 1.1±0.1                     | 5.2±0.2** (60.2) | 9.4±1.2** (56.8) | 16.5±1.4** (38.6) |

Experimental details except for incubation time are in Table 3. Final concentration of phospholipase C (PLC) added was 0.03 units/mL. Figures in parentheses show the percent inhibition. Total activity in controls: 134.2±1.8 for AP, 85.5±3.7 for βG.

a) See Table 3. b) Incubation time. *P<0.05, **P<0.01 (significant).

**TABLE 5.** Effect of test agents on phospholipase C-induced acid phosphatase release from rat liver lysosomes

| Compound         | % inhibition ±SE (N=6) |
|------------------|------------------------|
|                  | 0.1 μM     | 1 μM       | 10 μM      |
| Y-9213           | 7.3±0.6    | 19.2±0.4** | 22.5±0.4** |
| Phenylbutazone   | 5.0±0.9    | 12.5±0.7** |
| Indomethacin     | 6.2±2.2    | 15.2±1.1** |
| Mefenamic acid   | −3.9±1.1   | −10.0±0.7  |
| Acetylsalicylic acid | 5.1±1.6 | 1.4±1.9  |
| Tino-rin HCl     | 18.3±1.5** | 26.4±1.9** | 35.3±1.8** |
| Dexamethasone    | 30.9±1.3** | 36.5±1.8** | 45.5±2.3** |
| Prednisolone     | 23.2±1.2** | 31.9±1.5** | 40.7±0.9** |
| Hydrocortisone   | 19.3±1.3** | 26.5±0.9** | 33.9±1.6** |

Experimental details are in Table 3. Final concentration of phospholipase C used was 0.03 units/mL. Enzyme activity in controls: before incubation, 4.7±0.5; after incubation, 32.3±1.7; total activity, 127.8±8.9 μg p-nitrophenol formed. **P<0.01 (significant).
TABLE 6. Effect of test agents on release of enzymes from intact lysosomes of rabbit PMN leukocytes

| Compound   | Marker enzyme | 1 µM     | 10 µM     | 100 µM    |
|------------|---------------|----------|-----------|-----------|
| Y-9213     | AS            | 25.6±2.1**| 43.5±0.9**| 55.8±1.6**|
|            | 3G            | 28.7±1.9**| 42.9±1.2**| 50.7±1.8**|
| Phenylbutazone | AS        | 17.7±2.5**| 38.3±2.7**| 50.7±2.0**|
| Indomethacin | AS          | 10.1±3.1  | 20.1±3.6**| 36.8±2.1**|
| Dexamethasone | AS         | 12.6±1.6  | 18.5±2.1**| 25.7±1.1**|
|            | 3G           | 13.7±1.3  | 20.6±2.8**| 27.2±1.0**|
| Prednisolone | AS         | 13.5±1.4  | 20.2±1.0**| 27.8±1.3**|
|            | 3G           | 16.5±0.7  | 22.3±1.8**| 28.7±1.9**|
| Hydrocortisone | AS       | 18.0±1.2**| 23.0±2.1**| 30.8±0.6**|
|            | 3G           | 20.5±1.9**| 25.4±1.8**| 33.5±1.2**|
| 6-Mercaptopurine | AS        | 6.5±0.4   | 11.6±2.3  |           |
|            | 3G           | 1.4±2.4   | 8.7±2.9   |           |
| Propanolol HCl | AS    | 2.9±1.8   | 1.8±2.5   |           |
|            | 3G           | 1.6±2.9   | 0.5±2.7   |           |

The lysosome suspension (2.0 ml) was incubated with each test solution (0.20 ml) in 0.25 M sucrose-0.04 M Tris-acetate buffer (pH 7.4) at 37°C for 30 min. *AS, aryl sulfatase. Activity in controls: before incubation, 0.6±0.2; after incubation, 2.4±0.8; total activity, 18.4±2.6 µg p-nitroanisole formed. 3G, 3-glucuronidase. Activity in controls: before incubation, 0.2±0.1; after incubation, 1.3±0.4; total activity, 3.3±0.8 µg p-nitrophenol formed. **P<0.01 (significant).

TABLE 7. Effect of test agents on release of enzymes from preincubated lysosomes of rabbit PMN leukocytes

| Compound   | Final concn. (µM) | Aryl sulfatase | 3-Glucuronidase |
|------------|-------------------|----------------|----------------|
| Y-9213     | 100               | -33.4±1.7**    | -15.4±1.8**    |
| Phenylbutazone | 100          | -15.4±0.8**    | -12.8±0.4      |
| Indomethacin | 100         | -6.5±4.1       | -6.8±1.4       |
| Dexamethasone | 1             | 30.6±2.4**     | 29.2±2.7**     |
|            | 10              | 38.8±2.4**     | 37.8±1.8**     |
|            | 100             | 46.2±3.2**     | 48.8±2.4**     |
| Prednisolone | 1              | 15.8±3.1       | 15.0±2.6       |
|            | 10              | 25.4±2.8**     | 23.7±2.4**     |
|            | 100             | 40.3±2.3**     | 42.3±2.1**     |
| Hydrocortisone | 1             | 10.4±2.3       | 12.2±3.0       |
|            | 10              | 21.2±1.8**     | 20.5±1.7**     |
|            | 100             | 41.5±2.8**     | 37.5±2.3**     |
| 6-Mercaptopurine | 100         | 0.0±4.8        | 0.4±0.9        |
| Propanolol HCl | 100        | 0.4±2.4        | -1.2±0.8       |

After the lysosome suspension (2.0 ml) in 0.25 M sucrose-0.04 M Tris-acetate buffer (pH 7.4) was preincubated at 37°C for 15 min, it was further incubated with each test solution (0.20 ml) at 37°C for 30 min. Enzyme activity in controls: before preincubation, 0.6±0.1; after preincubation, 1.5±0.5; after incubation, 3.8±0.2; total activity, 15.5±1.1 µg p-nitroanisole formed for aryl sulfatase. before preincubation, 0.6±0.2; after preincubation, 0.9±0.2; after incubation, 3.2±0.5; total activity, 12.2±1.2 µg p-nitrophenol formed for 3-glucuronidase. **P<0.01 (significant).
### Table 8. Effect of zymosan on release of enzymes from rabbit PMN leukocytes

| Serum-treated zymosan | Marker enzyme* | Percent of total activity | Total activity$\dagger$ |
|-----------------------|----------------|--------------------------|------------------------|
|                       |                | 0            | 60 min      |                        |
| -                     | AS             | 2.5±0.7      | 8.5±0.7     | 74.9±3.1               |
| -                     | AS             | 2.2±0.2      | 26.5±0.4**  |                         |
| -                     | $\beta$G       | 1.2±0.8      | 4.8±1.0     | 27.0±2.1               |
| -                     | $\beta$G       | 0.9±0.5      | 24.9±0.6**  |                         |
| -                     | SH-P           | 6.7±1.0      | 10.7±0.9    | 11.3±1.0               |
| -                     | SH-P           | 6.4±0.5      | 22.9±0.9*** |                         |
| -                     | Acid P         | 9.3±0.5      | 20.0±2.0    | 18.8±1.0               |
| -                     | Acid P         | 9.7±0.2      | 32.0±1.9**  |                         |
| -                     | AP             | 3.4±0.6      | 7.6±1.0     | 28.6±3.7               |
| -                     | AP             | 3.6±0.2      | 8.7±1.2     |                         |
| -                     | Non SH-P       | 9.3±0.5      | 10.9±2.4    | 9.6±1.3                |
| -                     | Non SH-P       | 8.8±0.2      | 12.6±0.6    |                         |
| -                     | LDH            | 9.1±1.0      | 10.7±1.6    |                         |
| -                     | LDH            | 9.2±1.2      | 11.1±1.4    | 240±23                 |

The leukocyte suspension (5×10⁷ cells/ml, 2.0 ml) in Hanks’ solution was incubated with (−) or without (−) serum-treated zymosan particles (50 μl) at 37°C for 60 min. Results are shown as the mean±SE of four separate experiments. *AS: aryl sulfatase, $\beta$G: $\beta$-glucuronidase; SH-P: SH-protease, Acid P: acid protease, AP: acid phosphatase, Non SH-P: Non SH-protease, LDH: lactate dehydrogenase. $\dagger$See the text. **P<0.01, significant v.s. no addition (−) of zymosan particles.

### Table 9. Effect of test agents on phagocytic release of lysosomal enzymes from rabbit PMN leukocytes

| Compound      | Marker enzyme* | 0.01 μM | 0.1 μM | 1 μM | 10 μM |
|---------------|----------------|---------|--------|------|-------|
| Y-9213        | AS             | 8.8±1.2 | 15.7±3.0** | 27.6±2.4** | 44.6±2.1*** |
|               | SH-P           |         |        | 29.7±1.3** | 48.9±2.4** |
| Phenylbutazone| AS             | 9.0±1.0 | 21.8±2.1** | 36.8±2.8** |
|               | SH-P           |         |        | 15.4±2.3 | 37.8±1.4** |
| Indomethacin  | AS             | 20.0±1.7** | 34.8±5.5** | 49.9±3.3** | 69.3±3.3** |
|               | SH-P           |         |        | 31.0±2.6** | 47.5±2.9** |
| Dexamethasone | AS             | 20.3±1.7** | 33.0±0.3** | 52.7±0.4** | 69.6±4.0** |
|               | SH-P           | 18.0±2.3** | 31.6±2.4** |        |       |
| Prednisolone  | AS             | 18.8±1.7** | 24.9±0.6** | 41.6±0.6** | 54.3±1.9** |
|               | SH-P           | 23.9±2.0** | 44.6±1.1** |        |       |
| Hydrocortisone| AS             | 13.7±1.9 | 20.8±2.9** | 29.2±2.0** | 42.7±0.6** |
|               | SH-P           |         |        | 24.0±2.7** | 39.9±3.1** |
| 6-Mercaptopurine| AS           | 1.2±1.6 |        | -7.2±9.8 |       |
| Propranolol HCl| AS            |         |        | 8.9±2.4 |       |

The leukocyte suspension (5×10⁷ cells/ml, 2.0 ml) containing each test solution (20 μl) was incubated with serum-treated zymosan particles (50 μl) in Hanks’ solution (pH 7.4) at 37°C for 60 min. *AS, aryl sulfatase. Activity in controls: before incubation, 2.7±0.2; after incubation, 18.1±2.9; total activity, 85.3±4.3 μg p-nitrocatechol formed. SH-P, SH-protease. Activity in controls: before incubation, 0.4±0.1; after incubation, 2.9±1.0; total activity, 13.4±1.8 units. **P<0.01 (significant).
tosis of the particles was inhibited by Y-9213 and anti-inflammatory drugs tested at low concentrations from 0.1 to 10 μM, but not by 6-mercaptopurine and propranolol hydrochloride at 10 μM. The release of S1-I-protease was also inhibited by these anti-inflammatory drugs.

**DISCUSSION**

Since the constituents of PMN leukocyte lysosomes released in the tissues can provoke acute inflammation and tissue injury (17, 18), inhibition of the release of lysosomal enzymes may be of value in attenuating the severity of inflammation and tissue destruction. The two mechanisms have been considered to account for the release of the lysosomal constituents from the PMN leukocytes. One mechanism is the release of the enzymes from the lysosomes, and such is attributed to the autolysis of the PMN leukocytes or to the labilization of the lysosome-membranes. Another mechanism is the selective release of the lysosomal constituents from viable PMN leukocytes during phagocytosis or through reverse endocytosis (19).
Nonsteroidal anti-inflammatory drugs have been shown to stabilize rat liver lysosomes in vitro (10, 14, 20, 21). Because the property of the lysosome-membranes is considered to resemble that of the erythrocyte-membranes (22, 23), the membrane-stabilizing action of anti-inflammatory drugs has been assayed using erythrocytes (8, 9, 24-26). Glenn and Brown (8) reported that nonsteroidal anti-inflammatory drugs result in inhibition of the hyperthermic-hypotonic hemolysis. The results in Table 1 suggest that Y-9213 as well as nonsteroidal anti-inflammatory drugs may stabilize the erythrocyte-membranes. In addition, Y-9213 was found to stabilize the intact lysosomes obtained from rat liver (Table 2). In these experimental conditions, steroidal anti-inflammatory drugs such as prednisolone were found to stabilize to some extent the intact lysosomes of the rat liver only at 100 μM, and not the erythrocytes. The relative order of the stabilizing activity of these agents on the erythrocytes, however, is not in accord with that on the liver lysosomes.

Lewis et al. (27) have shown that the labilization of the liver lysosomes induced by PLC is inhibited by steroidal anti-inflammatory drugs such as prednisolone at 500 μM. In the preliminary experiments, the release of lysosomal enzymes induced by addition of PLC was found to be dose-dependent (Table 3) and time-dependent (Table 4). The relative order of the inhibitory activity on the release of the lysosomal enzyme by PLC is dexamethasone > prednisolone > hydrocortisone (Table 5), and is consistent with that of their anti-inflammatory activities. However, Y-9213 and nonsteroidal anti-inflammatory drugs tested were 100 times less active than the above steroidal anti-inflammatory drugs in the present experiments. The same results were obtained in the experiment using PMN leukocytes instead of the liver lysosomes (28). Therefore, this experimental method may be useful for testing the lysosome-stabilizing activity of steroidal anti-inflammatory drugs. Tinoridine hydrochloride which prevents the CCl₄-induced labilization of the liver lysosomes in vivo (29) also inhibits the labilization of the liver lysosomes induced by PLC. The action of tinoridine hydrochloride on the lysosomes seems to be somewhat different from that of other nonsteroidal anti-inflammatory drugs such as Y-9213, phenylbutazone and indomethacin, which inhibit more markedly the spontaneous release of lysosomal enzymes than the PLC-induced release of these enzymes.

More recently, certain steroidal and nonsteroidal anti-inflammatory drugs were shown to stabilize human leukocyte lysosomes in vitro (30). The lysosomes of rabbit PMN leukocytes are also stabilized by drugs such as indomethacin and hydrocortisone (Table 6). In this system, Y-9213 stabilized most markedly the intact lysosomes of the PMN leukocytes, while Y-9213 failed to prevent the labilization of the preincubated lysosomes (Table 7). These findings suggest that the action of Y-9213 on the lysosome-membranes may be similar to that of nonsteroidal anti-inflammatory drugs. In contrast, steroidal anti-inflammatory drugs have the ability to stabilize more markedly the unstable rather than the intact lysosomes (Table 7). These findings suggest that the agents tested except for tinoridine hydrochloride (29) have the capacity to stabilize biomembranes only under specific experimental conditions. Therefore, these activities of anti-inflammatory drugs tested may partially affect the inflammatory process. As the order of the membrane-stabilizing activities of these drugs,
however, is not necessarily consistent with that of their anti-inflammatory activities, additional experiments were carried out to determine the capacity of their inhibitory effects on the secretion of lysosomal enzymes.

SH-Protease as well as aryl sulfatase and β-glucuronidase were found to be secreted from the PMN leukocytes during phagocytosis of the particles (Table 8). The phagocytic secretion of SH-protease and aryl sulfatase was also confirmed to be inhibited by steroidal and nonsteroidal anti-inflammatory drugs tested (Table 9). The relative order of the inhibitory activity of three steroidal anti-inflammatory drugs on the enzyme secretion from PMN leukocytes is in accord with that on the enzyme release from the preincubated lysosomes of PMN leukocytes and from the PLC-added liver lysosomes. Y-9213 and phenylbutazone inhibit the enzyme secretion from the leukocytes (Table 9) and the enzyme release from the intact lysosomes (Table 6) in the same degree at the same concentration. Indomethacin, however, is effective at lower concentrations in inhibiting the enzyme secretion rather than the enzyme release. The relative order of the inhibitory activity on the enzyme secretion of Y-9213, phenylbutazone and indomethacin is consistent with that of their anti-inflammatory activities (in preparation). In the in vivo experiment (6, in preparation), the phagocytic secretion of aryl sulfatase or SH-protease is prevented by oral treatment with steroidal and nonsteroidal anti-inflammatory drugs at the same dose showing the anti-inflammatory activity.

Ignarro et al. (31) reported that isoproterenol hydrochloride could prevent both the secretion of lysosomal enzymes and phagocytosis of the zymosan particles. Similarly, colchicine and theophylline were reported to inhibit the secretion of lysosomal enzymes (12), and phagocytosis (11). In the present experiment (Table 10), colchicine, isoproterenol, theophylline and papaverine inhibited phagocytosis of the particles. Y-9213 and anti-inflammatory drugs tested, however, failed to inhibit phagocytosis at lower concentrations which prevented the enzyme secretion. In addition, these agents inhibit the nonphagocytic secretion of the lysosomal enzymes from the PMN leukocytes (28). These findings suggest that Y-9213 and other anti-inflammatory drugs tested may inhibit independently the secretion of the lysosomal enzymes from and phagocytosis by the PMN leukocytes.

The conclusion drawn from these results is that a new anti-inflammatory agent, Y-9213, can inhibit secretion of lysosomal enzymes during phagocytosis by the leukocytes. Therefore, this activity of Y-9213 may play a role in the anti-inflammatory action of this agent.

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