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PHYSICO-CHEMICAL PROPERTIES OF POTENT NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

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Abstract—The stabilizing action of some nonsteroidal anti-inflammatory drugs on erythrocyte membranes and serum albumin and the affinity of these drugs for erythrocytes were studied. Most of these potent anti-inflammatory drugs inhibited intensively the heat-induced erythrocyte lysis, and there was a rough correlation between the stabilizing activity of the drugs and their anti-inflammatory activity in a series of three phenylalkanoic acids. Most of the anti-inflammatory drugs strongly stabilized serum albumin and had a high affinity for erythrocytes. These results are in conformity with our previous suggestion that the stabilizing action of the drugs is due to an effect of the drugs on certain proteins in the membrane. The stabilizing activity of some potent drugs was not much influenced by the addition of a carrier protein. The relation of these physico-chemical activities of the drugs to their anti-inflammatory effects was discussed.

Though several theories [1], including the inhibition of prostaglandin synthesis [2], have been proposed for the mode of action of nonsteroidal anti-inflammatory drugs, membrane stabilization is one of the most important actions of the drugs [1]. Having this property, anti-inflammatory drugs protect several cell membranes and lysosomes, and inhibit platelet aggregation [3]. Since lysosomal and erythrocyte membranes are thought to have common properties [4, 5], erythrocyte lysis has been used as a model to test the membrane-stabilizing action of the drugs [6].

Our previous study was compatible with the suggestion that the membrane-stabilizing action of nonsteroidal anti-inflammatory drugs on erythrocytes was due to a stabilizing effect of the drugs on certain proteins in the membrane [7]. In this study the stabilizing action of the drugs on erythrocyte membranes and serum albumin, and the affinity of the drugs with erythrocytes were measured. These experiments were performed in order to obtain further support for our conclusions and to find out what is the most important property of a potent nonsteroidal drug in determining its anti-inflammatory characteristics.

MATERIALS AND METHODS

Drugs. Drugs were dissolved in 0·15 M phosphate buffer at pH 7·4 in the experiments on erythrocyte lysis and in water for the albumin denaturation test. Less soluble drugs were dissolved in the same buffer or in water by heating below 50°. Ibufenac, ibuprofen and flurbiprofen were supplied from The Boots Co., Ltd., and *d*- and *l*-naproxen were from Syntex Co., Ltd.

Heat-induced hemolysis. A slightly modified method of a procedure described elsewhere [7] was used. Briefly, 2 ml of a 7·5% (v/v) suspension of canine erythrocytes in 0·15 M phosphate buffer at pH 7·4 was mixed with 1 ml of drug solution. After leaving for 10 min at room temperature, the suspension was heated for 20 min at 49·5 ± 0·5°. The samples were cooled with running water and promptly centrifuged. The hemoglobin content of the supernatant was measured with a Beckman spectrophotometer at 540 nm. A series of experiments was carried out with the erythrocyte suspension containing 0·1 or 1% bovine serum albumin (BSA, Armour).

Albumin denaturation (coagulation). To 1 ml of drug solution was added 2 ml of 0·75% BSA in 0·225 M phosphate buffer at pH 6·0. After standing for 10 min at room temperature, the samples were heated for 2·5 min at 72°. The tubes were cooled in water and the turbidity of the solution was measured spectrophotometrically at 660 nm.

Measurement of erythrocyte-bound drug. To 3 ml of a drug solution was added 3 ml of a 10% suspension of canine erythrocytes in the phosphate buffer or 2·7 ml of buffer alone. The mixtures were incubated for 10 min at 37° and promptly centrifuged at 37°. Five ml of the supernatant and the solution without erythrocytes were used for the determination of drug concentration. The amount of erythrocyte-bound drug was calculated from the difference in drug concentration of both samples.

Measurements of flurbiprofen, ibuprofen, ibufenac, flufenamic acid, naproxen and phenylbutazone were as follows. To 5 ml of sample or standard drug solution in buffer were added 1 ml of 2·5 N HCl and 10 ml of ethylene dichloride (EDC). The mixture was shaken vigorously for 30 min and centrifuged. To 6 ml of the EDC layer was added 5 ml of 1 N NaOH; this was shaken again for 30 min, then centrifuged. To 4 ml of the NaOH layer were added 1 ml of 8 N HCl and 5 ml *n*-heptane. The mixture was shaken vigorously and centrifuged. The heptane layer was used for measurement of drugs. The drug concentration was determined with a Beckman spectrophotometer at appropriate ultraviolet wavelengths. For the determination of indomethacin, 0·6 ml of 1 N HCl and 5 ml ether were added to 5 ml of sample. The mixture was

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Table 1. Inhibitory effect of some anti-inflammatory drugs on heat-induced erythrocyte lysis

Drugs	Inhibition (% ± S. E.)* Drug concn (μM) 100	30	10	3	Anti-inflammatory activity (hindpaw) X phenylbutazone†
Flurbiprofen	78 ± 1·0	74 ± 1·9	55 ± 1·8	28 ± 1·0	20
Ibuprofen	45 ± 4·2	25 ± 2·8	19 ± 1·4	15 ± 0·5	2
Ibufenac	43 ± 2·3	21 ± 0·9	17 ± 2·4	6 ± 2·9	0·5
d-Naproxen	43 ± 2·1	32 ± 1·2	16 ± 1·7	13 ± 1·2	6
l-Naproxen	35 ± 2·1	16 ± 4·0	14 ± 1·6	10 ± 3·4	1
Indomethacin	84 ± 1·7	74 ± 2·0	51 ± 2·5	27 ± 1·9	12
Flufenâmic acid	83 ± 2·3	82 ± 1·8	73 ± 2·5	49 ± 6·3	2
Phenylbutazone	52 ± 1·8	31 ± 2·3	26 ± 3·2	13 ± 1·1	1
Salicylic acid	26 ± 1·5	12 ± 1·6	7 ± 2·6	1 ± 0·7	0·2

* Average of ten samples.
† Summarized from the data by Glenn [9], Tomlinson [10], and Masumoto and Takase [11].

shaken for 10 min and the ether layer was evaporated and dried. The dry sample obtained was dissolved in 10 ml ethanol and used for the spectrophotometric determination of indomethacin. Salicylic acid was measured by the usual method [8].

RESULTS

Table 1 shows the stabilizing effect of nonsteroidal anti-inflammatory drugs on heat-induced erythrocyte lysis and anti-inflammatory activity of each drug. Most of the potent drugs gave a strong stabilizing effect. The stabilizing activity of the drugs was roughly correlated with their anti-inflammatory activity in a series of phenylalkanoic acids (flurbiprofen > ibuprofen > ibufenac).

Inhibitory effects of some anti-inflammatory drugs on heat-induced albumin denaturation are given in Table 2. Flufenamic acid showed an intensive stabilizing effect on both erythrocyte and serum albumin, while naproxen and salicylic acid gave weak results in both tests. However, indomethacin and flurbiprofen, potent drugs in the anti-inflammatory and erythrocyte lysis tests, had less of a stabilizing effect on serum albumin.

The affinity of nonsteroidal anti-inflammatory drugs for erythrocytes is illustrated in Table 3. Weak stabilizers (salicylic acid, naproxen and ibufenac) in the erythrocyte lysis test had a low affinity for erythrocytes. On the other hand, potent drugs (flufenamic acid, indomethacin, phenylbutazone and flurbiprofen) had a higher affinity for the cells.

To determine whether the drugs added to the erythrocyte suspensions combined mostly with the cell membrane, the influence of incubation time on the affinity of drugs for erythrocytes was studied. The method was almost the same as that described above. The drug solution and an 8% erythrocyte suspension were heated separately in a water bath at 37°. The drug solution and the cell suspension were then mixed and incubated further at 37°. At 3 or 10 min after mixing, the suspensions were centrifuged and the drug concentration of the supernatant was measured. A series of experiments were carried out at 20°. The results (Table 4) indicate not only that the association of the drugs with the cell occurred immediately after mixing the drugs and the cell, and did not increase after 3 or 10 min of incubation, but also that the association rate was high at the lower temperature (20°).

The influence of concentration of a carrier protein in the erythrocyte suspension on the membrane-stabilizing activity of anti-inflammatory drugs is shown in Table 5. The erythrocyte-stabilizing activity of flurbiprofen, ibufenac and flufenamic acid decreased

Table 2. Inhibitory effect of some anti-inflammatory drugs on heat-induced albumin denaturation*

Drugs	Inhibition (% ± S. E.)† Drug concn (μM) 300	100	30
Flurbiprofen	75 ± 3·2	42 ± 0·9	18 ± 1·7
Ibuprofen	74 ± 1·7	54 ± 3·2	23 ± 0·9
Ibufenac	89 ± 0·3	62 ± 1·2	20 ± 1·7
d-Naproxen	47 ± 2·0	17 ± 0·6	10 ± 0·6
l-Naproxen	12 ± 3·1	6 ± 0·9	2 ± 1·5
Indomethacin	82 ± 1·5	49 ± 1·5	13 ± 1·2
Flufenamic acid	94 ± 1·9	78 ± 1·8	36 ± 1·5
Phenylbutazone	53 ± 1·5	20 ± 1·9	4 ± 1·5
Salicylic acid	21 ± 0·3	11 ± 1·9	7 ± 2·7

* An albumin solution was heated with or without drugs, and the turbidity of the solution was measured.
† Average of six samples.

Table 3. Per cent of cell-bound anti-inflammatory drugs in a 5% erythrocyte suspension*

Drugs	Per cent of drug bound to cells (% ± S. E.)† Drug concn (μM) 100	30
Flurbiprofen	23 ± 0·2	20 ± 0·9
Ibuprofen	8 ± 0·4	7 ± 0·3
Ibufenac	6 ± 1·0	4 ± 0·4
d-Naproxen	11 ± 0·6	9 ± 0·2
l-Naproxen	9 ± 1·0	9 ± 0·7
Indomethacin	27 ± 0·4	29 ± 0·3
Flufenamic acid	65 ± 0·7	83 ± 0·8
Phenylbutazone	32 ± 1·0	35 ± 2·3
Salicylic acid	6 ± 0·3	

* A 5% cell suspension was incubated with drugs and centrifuged. The per cent of cell-bound drug was calculated from the drug concentration in the supernatant.
† Average of six samples.

Table 4. Influence of incubation time on the affinity of anti-inflammatory drugs for erythrocytes*

Drugs†	Per cent of drug bound to cells (% ± S. E.)‡ Incubation time (min) 1§	1§,‖	3	10
Flurbiprofen	18·9 ± 0·55	28·4 ± 1·21	18·7 ± 0·58	18·6 ± 0·38
Ibuprofen	5·9 ± 0·74	13·5 ± 0·39	5·6 ± 0·40	5·9 ± 0·49
Indomethacin	25·4 ± 1·37	31·1 ± 0·14	25·0 ± 0·80	26·9 ± 0·33
Flufenamic acid	44·9 ± 0·17	70·1 ± 0·17	43·7 ± 1·40	44·1 ± 0·87
Phenylbutazone	23·1 ± 0·64	32·6 ± 0·59	22·8 ± 0·24	23·1 ± 0·29

* A 4% suspension of erythrocytes was mixed with drugs and incubated further for 1, 3 or 10 min. All experiments were carried out at 37°.
† Concentration of drugs was 100 μM.
‡ Average of three samples.
§ Approximate time.
‖ Experiments were performed at 20°.

when the concentration of BSA in the suspension increased. Though the data are not given in Table 5, similar results were obtained with indomethacin, phenylbutazone and salicylic acid. The erythrocyte-stabilizing effect was not much influenced by the addition of serum albumin in the case of *d*-naproxen and ibuprofen.

DISCUSSION

As reported previously, we have suggested that the stabilizing effect of nonsteroidal anti-inflammatory drugs on the erythrocyte is due to a stabilizing effect of the drugs on certain proteins in the cell membrane [7]. However, the protein-stabilizing and membrane-stabilizing activity are not correlated in some cases. For example, flurbiprofen, ibuprofen and ibufenac, a series of phenylalkanoic acids, have similar protein-stabilizing activity, as shown in Table 2. Flurbiprofen is strongly anti-inflammatory [11, 12] and is a potent erythrocyte stabilizer (Table 1). Ibufenac is weakly anti-inflammatory and is not a potent stabilizer. Therefore, we speculated that the affinity of each drug for erythrocytes differs. As shown in Table 3, the affinity of drugs for erythocytes is well correlated with the membrane-stabilizing activity in the three phenylalkanoic acids. Indomethacin, flufenamic acid and phenylbutazone, potent erythrocyte-stabilizing agents, have a high affinity for the cells. It is suggested, therefore, that these potent anti-inflammatory drugs may stabilize the cells with the help of high affinity for the cell membrane, although their protein-stabilizing activity is not very strong (Table 2).

As shown in Table 4, the association of nonsteroidal anti-inflammatory drugs with erythrocytes occurred immediately after mixing the drugs with the cells and did not increase further. Moreover, the rapid association was high when the experiments were performed at a lower temperature. The intracellular uptake by red cells of drugs (for example, thiamine disulfides) was reported to depend upon incubation time and temperature, i.e. uptake increases with longer incubation and higher temperature [13]. On the other hand, the association of anti-inflammatory drugs with proteins is considered to be independent from incubation time, and the association of salicylate and warfarin with proteins does not increase, but rather decreases at a higher temperature [14, 15]. These facts and the results of the present experiments suggest that the nonsteroidal anti-inflammatory drugs combined mostly with the cell membrane and did not enter into the cells. Tanaka *et al.* [16] also reported the high association of flufenamic acid with erythrocytes at a low temperature.

When serum albumin, an inactive carrier protein, was added to the erythrocyte suspension, the membrane-stabilizing activity of most nonsteroidal anti-inflammatory drugs decreased. However, the stabilizing activity of *d*-naproxen and ibuprofen was not much influenced by the addition of protein.

It is concluded from this study that potent nonsteroidal anti-inflammatory drugs have the property of

Table 5. Influence of carrier protein on the erythrocyte-stabilizing activity of anti-inflammatory drugs*

Drugs	Concn (μM)	Inhibition (% ± S. E.)† BSA in solution (%) 0	0·1	1·0
Flurbiprofen	30	75 ± 2·7	42 ± 3·6	28 ± 2·8
Ibuprofen	30	25 ± 2·8	25 ± 2·7	22 ± 2·7
Ibufenac	100	46 ± 1·8	39 ± 3·2	25 ± 3·0
d-Naproxen	30	32 ± 1·2	33 ± 3·1	28 ± 3·3
Flufenamic acid	10	73 ± 2·5	43 ± 3·0	26 ± 4·0

* A 5% erythrocyte suspension containing drugs and BSA was heated and the stabilizing activity of the drugs was measured.
† Average of five samples.

stabilizing proteins and may have a high affinity for certain membranes. Both properties may be important in stabilizing certain proteins in the membranes. However, there are a few results not explainable by this hypothesis. For example, the erythrocyte-stabilizing activity of *d*- and *l*-naproxen was similar; the anti-inflammatory activity of the former is definitely superior to that of the latter [10]. Therapeutic blood levels of the drugs used in this study and the activity of their metabolites have not been well studied, and we do not know the site of action of the drugs. Moreover, it is impossible to test the drugs *in vitro* in a situation similar to that in the body. Therefore, no definite correlation was obtained between various effects *in vitro* and anti-inflammatory activities *in vivo* [9]. In this study canine erythrocytes were used as a model of membrane, and bovine serum albumin was used as a model of protein and carrier of the drugs. Some experiments were performed in an acidic solution. We chose these materials and conditions mainly for ease of experimentation.

Nonsteroidal anti-inflammatory drugs have a variety of chemical structures and broad activity profiles. We think that the membrane- and protein-stabilizing action may be the most important property of nonsteroidal anti-inflammatory drugs. By virtue of this property, the drugs stabilize lysosomes, inhibit platelet aggregation [3], and might inhibit prostaglandin biosynthesis [17].

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