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MODE OF STABILIZING ACTION OF NON-STEROID ANTI-INFLAMMATORY DRUGS ON ERYTHROCYTE MEMBRANE

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Abstract—This study was designed to test a working hypothesis that the stabilizing effect of non-steroid anti-inflammatory drugs on the erythrocyte membrane is due to a stabilizing effect of the drugs on certain proteins in the cell membrane. Inhibitory potencies of 0·5 mM anti-inflammatory and other drugs on heat-induced canine erythrocyte lysis and heat coagulation of bovine serum albumin showed a good correlation except with basic anti-inflammatory drugs. In most cases 0·5 mM phenylbutazone, indomethacin, benzydamine and aminopyrine protected canine erythrocytes from lysis induced not only by heating at pH 7·4 and 6·2 but also by possible protein denaturants such as low pH, mechanical stress and Na laurylsulfate. However, they did not protect the cells from haemolysis induced by saponin, phospholipase C and lysolecithin which interact directly with the lipid components of the membrane. Canine erythrocytes treated with trinitrobenzene sulfonic acid (TNBS) which denatures proteins were no longer stabilized adequately against heat by 0·5 mM phenylbutazone, indomethacin and 3 mM aminopyrine, while the erythrocytes treated with phospholipase C or lysolecithin were strongly stabilized by the drugs. The stabilizing effect of 0·5 mM phenylbutazone both on canine erythrocytes and serum albumin against heat was observed immediately after mixing the cells or the protein with the drugs. Phenylbutazone at concentrations of 5, 10 and 20 mg% inhibited to the same degree both heat-induced erythrocyte lysis and heat denaturation of serum albumin in an identical experimental situation. Phenylbutazone, flufenamic acid and salicylate at therapeutic concentrations, which stabilize some proteins in whole serum, stabilized also canine erythrocytes against heat in a solution containing 3% bovine albumin. These results provide strong support for the view that the stabilizing effect of acidic non-steroid anti-inflammatory drugs on canine the erythrocyte membrane is due to the stabilizing effect of the drugs on certain proteins in the membrane.

BROWN, Mackey and Riggilo[1] and Inglot and Wolna[2] found that non-steroid anti-inflammatory drugs such as phenylbutazone and indomethacin protected canine and human erythrocyte membranes from heat-induced and hypotonic haemolysis. This effect of anti-inflammatory drugs is interesting, since stabilization of the cell membrane is considered to be connected with a decrease in inflammatory reactions, and erythrocyte and lysosomal membranes are thought to have common properties.

As reported previously acidic non-steroid anti-inflammatory drugs protect proteins from heat denaturation.[3–6] Both results suggest that the stabilizing effect of non-steroid anti-inflammatory drugs on erythrocyte membrane may be due to a stabilizing effect of the drugs on certain proteins in the membrane.

MATERIALS AND METHOD

Drugs. All drugs were dissolved in saline to a proper concentration and neutralized with 0·1 N NaOH or HCl.

Procedure of heat-induced haemolysis. Blood was removed from mongrel dogs of either sex in a heparinized syringe. When the blood removed on the preceding day was used, the blood was mixed with Alsver Solution (1·32g Na citrate, 0·48 g citric acid, 1·33 g glucose in 100 ml) in a ratio of 4:1 and stored at 4°. The plasma was discarded and erythrocytes were washed three times with saline.

Unless otherwise stated, the procedure of haemolysis was as follows. To 0·2 ml of the drug solution or saline was added 1·8 ml of 5% (v/v) suspension of the erythrocytes in 1/8 M phosphate buffer at pH 7·4. After standing for 10 min at room temperature (20–25°) the mixtures were heated in a water bath at 49–51° for 20 min. A temperature producing proper haemolysis in each experiment was determined beforehand. The tubes were shaken gently several times during the heating. Samples were cooled with running water and promptly centrifuged for 10–15 min at 2700 *g*. The haemoglobin content of the clear supernatant was measured with Beckman spectrophotometer at 540 mμ. The heated control tubes showed a reading of the spectrophotometer at −log transmission 0·7–1·8 in the method used. When the haemolysis was too strong the supernatant was diluted before measurement. Since the unheated control tubes kept in ice water showed minimal haemolysis, the values were neglected in this study. The mean haemoglobin content of the heated control tubes was expressed as 100 as a relative amount of haemolysis in each experiment. The relative amount of haemolysis of each tested tube was calculated and the average values are shown in the table with the respective standard errors.

Haemolysis by other membrane labilizers: To 0·2 ml of the drug solution or saline was added 1·6 ml of 5·6% (v/v) suspension of washed erythrocytes in 1/8 M phosphate buffer at pH 7·4. After leaving the mixture for 10 min at room temperature, 0·2 ml of a proper concentration of membrane labilizers in saline was added followed by gentle shaking and further incubation for 30 min at 37° in the experiment using phospholipase C and for 60 min at room temperature in the other experiments. Sodium azide (3 mg%) was added to the solution of saponin. Further procedures were as described above.

Modification of erythrocytes by drugs: Canine erythrocytes were modified by 2,4,6-trinitrobenzene sulfonic acid (TNBS), phospholipase C (Sigma, U.S.A.) or lysolecithin (from egg, Sigma, U.S.A.) in this study. Modification by TNBS: Four ml of 40 mM TNBS in saline was added to 36 ml of 5% suspension of washed erythrocytes in 1·39% bicarbonate buffer at pH 8·1. After incubation for 90 min at room temperature the suspension was centrifuged. The treated erythrocytes were washed with saline. Modification by phospholipase C: Four ml of 1·5–2 mg% of phospholipase C in saline was added to 36 ml of 5% suspension of erythrocytes in 1/8 M phosphate buffer at pH 7·4. After incubation for 60 min at 37° the mixture was centrifuged and the treated erythrocytes were washed with saline. Modification by lysolecithin: Four ml of 5 mg% of lysolecithin in saline was added to 36 ml of 5% suspension of the erythrocytes in 1/8 M phosphate buffer at pH 7·4. After incubation for 60 min at room temperature the mixture was centrifuged and the treated erythrocytes were washed with saline.

About one twentieth of the erythrocytes were lyzed during the incubation with

phospholipase C and lysolecithin, while minimal haemolysis was observed in the control tubes without the labilizers. This suggested that nonlyzed erythrocytes were also modified by phospholipase C and lysolecithin.

Protein coagulation. To 0·2 ml of 5×10^{-3} M drug solutions was added 1·8 ml of 1% bovine serum albumin (Armour) in 1/8 M phosphate buffer at pH 6·0. After standing for 10 min at room temperature the mixtures were heated for 3 min in a water bath at 72°. The heated tubes were cooled in water and the turbidity of the solution was measured spectrophotometrically at 660 mμ. Relative coagulation (denaturation) was calculated by the same method described in the erythrocyte lysis test.

RESULTS

1. *Inhibitory effect of anti-inflammatory and miscellaneous drugs on heat-induced erythrocyte lysis and albumin denaturation*

Effect of 0·5 mM anti-inflammatory and miscellaneous drugs on heat-induced erythrocyte lysis and albumin coagulation is summarized in Table 1. As shown in the Table inhibitory potency of the drugs on heat-induced erythrocyte lysis and albumin coagulation showed a good correlation except with basic anti-inflammatory drugs.

TABLE 1. INHIBITORY EFFECT OF ANTI-INFLAMMATORY AND OTHER DRUGS ON HEAT-INDUCED ERYTHROCYTE LYSIS AND ALBUMIN DENATURATION

	Drugs	Relative erythrocyte lysis*	Relative albumin coagulation†
	Control	100 ± 1·1	100 ± 2·5
	Indomethacin	11 ± 1·6	22 ± 1·7
	Flufenamic acid	19 ± 1·3	11 ± 0·3
	Phenylbutazone	26 ± 5·0	12 ± 0·7
A	Oxyphenbutazone	20 ± 3·3	35 ± 1·7
	Ibufenac	38 ± 2·7	11 ± 0·7
	Bucolome	35 ± 4·7	28 ± 1·9
	Salicylic acid	56 ± 3·3	72 ± 1·9
	Aspirin	69 ± 2·4	79 ± 1·6
	Benzydamin	135 ± 8·4	226 ± 3·4
B	Aminopyrine	49 ± 3·4	101 ± 3·6
	K 2673	58 ± 4·6	174 ± 3·9
	Acetylanthranilic acid	69 ± 3·6	75 ± 3·3
C	Capric acid	26 ± 3·9	6 ± 0·3
	Others	>90	>90

* Average values of ten samples from five animals.
† Average values of four samples.
A: acidic, B: basic, C: non-anti-inflammatory drugs.
Others: Histamine hydrochloride, Serotonin creatine sulfate, Diphenhydramine hydrochloride, Atropine sulfate, Dopamine hydrochloride, Cocaine hydrochloride, Dihydrocodeine phosphate, Meprobamate, Thiamine hydrochloride, Folic acid, *m*-hydroxy benzoic acid, 2,3-dihydroxy benzoic acid, 2,6-dihydroxy benzoic acid, Acetanilide.

2. *Effect of anti-inflammatory drugs on erythrocyte lysis induced by several techniques*

Effects of 0·5 mM anti-inflammatory drugs on erythrocyte lysis induced by heating at pH 6·2, low pH and mechanical stress and by membrane labilizers such as Na laurylsulfate, saponin, phospholipase C and lysolecithin are summarized in Table 2. The methods used particularly in this experiment are as follows.

Heating (pH 6·2). The same method as heating (pH 7·4) except the use of 1/8 M phosphate buffer at pH 6·2 and incubation at 48°.

Low pH (5·5). To 0·2 ml of the drug solution was added 1·8 ml of 5% erythrocyte suspension in 1/8 M phosphate buffer at pH 5·5 and the mixture was incubated for 30 min at 37°.

Mechanical stress. A mixture of 5% erythrocyte suspension in 1/8 M phosphate buffer and the drug solution was shaken vigorously for 2–3 min in a KM-model Shaker, Iwaki K.K. Tokyo to produce haemolysis.

TABLE 2. EFFECT OF 0·5 mM ANTI-INFLAMMATORY DRUGS ON ERYTHROCYTE LYSIS INDUCED BY SEVERAL CONDITIONS

	Relative haemolysis ± standard error				
Labilizer	Control	Phenylbutazone	Indomethacin	Benzydamin	Aminopyrine
Heat (pH 7·4)	100 ± 1·1	26 ± 5·0	11 ± 1·1	135 ± 8·4*	49 ± 3·4
Heat (pH 6·2)	100 ± 2·3	40 ± 5·6	55 ± 1·6	20 ± 2·0	92 ± 2·7
Low pH (5·5)	100 ± 1·1	80 ± 4·9	169 ± 21·0	63 ± 4·7	96 ± 3·1
Mechanical stress	100 ± 1·0	187 ± 18·4	30 ± 4·6	166 ± 18·7	59 ± 2·9
Na lauryl-sulfate	100 ± 4·3	83 ± 5·5	65 ± 7·9	55 ± 4·8	88 ± 5·6
Saponin	100 ± 3·2	108 ± 3·8	218 ± 11·0*	304 ± 66·7†	100 ± 3·7
Phospholipase C	100 ± 1·9	119 ± 14·0	108 ± 17·4	277 ± 52·6	97 ± 5·4
Lysolecithin	100 ± 2·4	97 ± 3·4	101 ± 10·3	102 ± 5·9	99 ± 5·2

Average values of at least eight samples from four animals.
* No inhibition at lower concentration.
† No inhibition at pH 6·2.

As 15 mg% of Na laurylsulfate, 20–30 mg% of saponin, 5 mg% of phospholipase C and 20 mg% of lysolecithin was used the final concentration of the drugs was 1·5 mg%, 2–3 mg%, 0·5 mg% and 2 mg% respectively.

As indicated in the Table, some, or all of the tested anti-inflammatory drugs protected erythrocyte membranes not only from heat at pH 7·4 and 6·2 but also from low pH, mechanical stress and Na laurylsulfate. However, they did not stabilize erythrocytes against haemolysis induced by saponin, phospholipase C and lysolecithin.

TABLE 3. STABILIZING EFFECT OF SOME ANTI-INFLAMMATORY DRUGS ON MODIFIED ERYTHROCYTES AGAINST HEAT-INDUCED HAEMOLYSIS

	Relative haemolysis ± standard error			
Modification:	No	TNBS	Phospholipase C	Lysolecithin
Control	100 ± 0·9	100 ± 0·7	100 ± 1·4	100 ± 0·7
Phenylbutazone	22 ± 4·7	83 ± 1·8	34 ± 2·9	21 ± 2·6
Indomethacin	14 ± 4·0	87 ± 7·4	15 ± 2·3	14 ± 2·5
Aminopyrine	28 ± 0·7	101 ± 2·4	61 ± 3·1	39 ± 2·1

Canine erythrocytes treated with and without TNBS, phospholipase or lysolecithin were heated with and without 0·5 mM phenylbutazone, indomethacin or 3 mM aminopyrine. Average values of fifteen samples from five animals.

3. *Stabilizing effect of some anti-inflammatory drugs on modified erythrocytes against heat-induced haemolysis*

The stabilizing effect of phenylbutazone, indomethacin and aminopyrine against heat on erythrocytes treated with trinitrobenzene sulfonic acid (TNBS), phospholipase C or lysolecithin is summarized in Table 3. The tested drugs stabilized erythrocytes treated with phospholipase C and lysolecithin as well as non-treated erythrocytes, while the erythrocytes treated with TNBS were minimally or not stabilized by the drugs, as briefly reported in the previous paper.[7] The stabilizing effect of the drugs, especially of indomethacin, on TNBS-treated erythrocytes differed considerably according to the animal used.

4. *Preincubation time needed for phenylbutazone to stabilize erythrocytes and serum albumin*

The preincubation time of erythrocyte suspensions or albumin solutions with 0·5 mM phenylbutazone was varied from 0 min to 60 min, followed by heating for a few minutes as indicated in Table 4. As shown in the Table, the stabilizing effect of phenylbutazone both on erythrocytes and albumin was observed immediately or at least within a few minutes after phenylbutazone reacted with them.

TABLE 4. INHIBITORY EFFECT OF 0·5 mM PHENYLBUTAZONE ON HEAT-INDUCED ERYTHROCYTE LYSIS AND ALBUMIN DENATURATION

Incubation time before heating	Relative erythrocyte lysis	Relative albumin coagulation
without phenylbutazone	100	100
0 min	54	66
3	52	63
6	52	62
10	51	64
20	51	63
60	53	63

Erythrocyte lysis: 58°, 3·5 min, pH 6·2.
Albumin coagulation: 80°, 2·0 min, pH 6·2.

TABLE 5. INHIBITORY EFFECT OF PHENYLBUTAZONE ON HEAT-INDUCED ERYTHROCYTE LYSIS AND HEAT DENATURATION OF ALBUMIN IN AN IDENTICAL EXPERIMENTAL CONDITION

Concentration of phenylbutazone	Relative erythrocyte lysis	Relative albumin coagulation
0 mg %	100 ± 1·2	100 ± 0·8
5	100 ± 2·6	97 ± 5·5
10	83 ± 1·7	85 ± 3·8
20	64 ± 2·3	73 ± 1·6

Average values of six samples from three animals.
Experimental condition: 5% erythrocytes and 1% serum albumin in phosphate buffer, pH 6·2 with and without phenylbutazone.
Heating: 49°, 20 min for haemolysis, 80°, 2·5 min for coagulation of the supernatant.

5. *Inhibitory effect of phenylbutazone at different concentrations on heat-induced erythrocyte lysis and protein denaturation in an identical situation*

Since concentration of free and active phenylbutazone is related to the concentration of protein in a solution, the following experiment was devised. Two tubes were prepared, both of which contained 4·5% erythrocytes and 1% bovine serum albumin in phosphate buffer at pH 6·2 with or without phenylbutazone. One tube was heated for 20 min at 49° to produce erythrocyte lysis. The other tube was centrifuged. The supernatant was then heated for 2·5 min at 80° to produce protein coagulation.

As indicated in Table 5, phenylbutazone of each concentration inhibited to the same degree both heat-induced erythrocyte lysis and heat denaturation of albumin in an identical situation.

6. *Stabilization of erythrocytes by therapeutic concentration of anti-inflammatory drugs*

Since acidic anti-inflammatory drugs combine mostly with serum proteins, especially albumin, in the plasma and the tissue fluid and albumin concentration in the plasma from rheumatoid patients is usually below 3g/100 ml, the following experiment was performed. A 1·8 ml of the mixture containing 5% erythrocytes and 3·3% bovine serum albumin in 1/8 M phosphate buffer at pH 7·4 was added to 0·2 ml of ten times the therapeutic concentration of anti-inflammatory drugs. After standing for 10 min at room temperature, the mixture was heated for 20 min at 52°. The results shown in Table 6 indicate that the tested anti-inflammatory drugs at a therapeutic concentration protected the erythrocytes from heat-induced haemolysis even in the solution with a high albumin concentration.

TABLE 6. INHIBITORY ACTION OF ANTI-INFLAMMATORY DRUGS AT A THERAPEUTIC CONCENTRATION ON HEAT-INDUCED HAEMOLYSIS OF CANINE ERYTHROCYTES IN A SOLUTION CONTAINING 3% BOVINE SERUM ALBUMIN

Drugs	Final concentration	Relative haemolysis ± Standard error
No		100 ± 1·1
Phenylbutazone	12 mg %	73 ± 1·9
Flufenamic acid	3 mg %	92 ± 1·6
Salicylic acid	20 mg %	59 ± 2·2

Average values of six samples.

DISCUSSION

Erythrocytes[1, 8, 9] and serum proteins[3] are stabilized against heat-induced haemolysis or heat denaturation by non-steroid anti-inflammatory drugs such as phenylbutazone and indomethacin but not by antihistamine, chloroquine, a low concentration of anti-inflammatory steroid and other drugs. Brown, Mackey and Riggilo[1] already mentioned a good correlation between the relative potencies of anti-inflammatory drugs in stabilizing canine erythrocytes and serum albumin against heat. The good correlation except with basic anti-inflammatory drugs was confirmed on many drugs in this study (Table 1). These results suggest that the stabilizing effect of non-steroid anti-inflammatory drugs on erythrocytes may be due to a stabilizing

effect of the drugs on certain proteins in the cell membrane, as briefly discussed in the previous communication.[7]

The membrane stabilizing action of non-steroid anti-inflammatory drugs is easily detectable in the experiment of heat-induced lysis of canine erythrocytes. However, some or most of them stabilize other membranes such as those of the human erythrocyte[2] and rat lysosome.[10] As shown in this study (Table 2) non-steroid anti-inflammatory drugs protected canine erythrocytes from haemolysis induced by low pH, mechanical stress and detergent as well as by heat. Therefore the stabilizing action of the anti-inflammatory drugs on canine erythrocytes against heat is considered as one of the models for analysing the stabilizing action of the drugs on cell membrane. Furthermore it is of interest that erythrocyte and lysosomal membranes are considered to have common properties[1, 11, 12] and that a disruption of lysosome may cause inflammation.[13, 14]

As shown in Table 2 some or most anti-inflammatory drugs protected erythrocyte membranes from haemolysis induced by heat, low pH, mechanical stress and Na laurylsulfate but not from lysis induced by saponin, phospholipase C and lysolecithin. This discrepancy of drug action may be explained by the action of the labilizers used. The former group seems to modify or denature certain proteins in the cell membrane, while the latter group is considered to affect the lipid layer of the membrane. Table 2 also illustrates that the membrane stabilizing action of each non-steroid anti-inflammatory drug differs slightly in quality as well as in quantity.

A similar difference in drug action was observed in the experiment using modified erythrocytes. Erythrocytes treated with TNBS which has a property of denaturing proteins or binding with proteins—probably competitively with acidic anti-inflammatory drugs at the site of ϵ-amino-group[15]—were no longer stabilized adequately by anti-inflammatory drugs. We suggested in a previous paper[7] that the effect of TNBS was probably due to the competitive binding of TNBS to the drugs. However, the denaturation of certain proteins in the membrane by TNBS seems to be more important in this experiment, since the stabilizing effect of aminopyrine which is not bound to ϵ-amino-groups also disappeared after treatment with TNBS. The membrane stabilizing action of anti-inflammatory drugs did not change in cells modified by phospholipase C or lysolecithin which probably do not affect proteins in the cell membrane. Inhibition of anti-inflammatory steroids on the uptake of glucose into the cells was observed in intact cells but not in cells treated with phospholipase C.[16]

Release of histamine from cells by histamine liberators was inhibited by some non-steroid anti-inflammatory drugs.[17, 18] This inhibition of the drugs is thought to be due to a biochemical effect of the drugs as uncouplers of oxidative phosphorylation. However, the stabilizing effect of a low concentration of an anti-inflammatory drug on canine erythrocytes was observed immediately after mixing the cells with the drug (Table 4). Moreover, no active transport was found in the canine erythrocyte.[19] The stabilizing action of the drug on canine erythrocytes, therefore, could not be explained through a biochemical process. On the other hand a physico-chemical action of the drugs such as the stabilization of proteins is observed within a few minutes, as shown in Table 4.

If the stabilizing action of acidic non-steroid anti-inflammatory drugs on the cell membrane and proteins is through a similar mechanism, they must be stabilized by a similar concentration of the drugs. It was proved also in this experiment as shown in

Table 5. It was found that phenylbutazone,[20] flufenamic acid[21] and salicylate[22] at therapeutic concentrations which stabilize some proteins in the whole serum[4] also stabilized canine erythrocytes in a solution with a high protein concentration (Table 6).

These results strongly suggest the view that the stabilizing effect of acidic non-steroid anti-inflammatory drugs on cell membranes, at least on the canine erythrocyte membrane, is due to a stabilizing effect of the drugs on certain proteins in the membrane. The mode of membrane stabilizing action of basic anti-inflammatory drugs such as benzydamine is still unknown.

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