Biochemical Pharmacology, Vol. 22, pp. 879–886. Pergamon Press, 1973. Printed in Great Britain.

TEMPERATURE-DEPENDENT REACTION OF FLUFENAMIC ACID WITH RAT ERYTHROCYTE MEMBRANE

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(*Received* 31 *July* 1972; *accepted* 25 *October* 1972)

Abstract—(1) Effect of flufenamic acid on hypotonic hemolysis of rat erythrocytes was investigated with special reference to temperature at which hemolysis took place. At low temperature such as 0 or 12°, flufenamic acid exhibited a dose-dependent inhibition of hemolysis at relatively low concentrations. This protective effect of flufenamic acid was, however, diminished by increase of temperature of hypotonic medium and finally it caused acceleration of hemolysis at 37°. Chlorpromazine was, in contrast with flufenamic acid, found to be protective at any temperature between 0 and 37°.

(2) Flufenamic acid molecules were found to be adsorbed by rat erythrocyte either at 0 or 37°, but the manner of adsorption at 0° was quite different from adsorption at 37°. Kinetically, adsorption at 37° seems to be only passive, whereas some active binding-sites are assumed at the surface of erythrocyte membrane at 0°.

(3) Effect on hypotonic hemolysis of a number of clinically useful drugs known as the membrane stabilizer were tested with respect to their temperature-dependency and they were found to be classified into two types; flufenamic acid type and chlorpromazine type. Drugs classified as the former were mostly acidic in the chemical nature and only anti-inflammatory drugs were found in this group. On the other hand, drugs of the latter were basic or non-ionic compounds and most of the tested tranquilizers, anti-histamines, local anesthetics and non-ionic anti-inflammatory drugs belonged to this group.

IT IS well known that a variety of clinically useful drugs protect or stabilize erythrocyte membrane from hypotonic or hyperthermic hemolysis when drugs are used at low concentrations. The evidence so far obtained may be valuable to analyze the action mechanism of drugs such as local anesthetics,[1] tranquilizers[2,3] and anti-inflammatory acids.[4,5]

The changes produced by one type of drugs are, however, not likely to be common to several different types of drugs. It may then be pertinent to introduce to the hypotonic hemolysis system an additional experimental condition, "temperature", by which the differentiated activities of flufenamic acid and chlorpromazine, for instance, are able to be elucidated to each other.

The present paper deals with the findings that flufenamic acid and some other anti-inflammatory acids react with rat erythrocyte membrane in a quite different manner from chlorpromazine with respect to the dependency on reaction temperature.

MATERIALS AND METHODS

Preparation of erythrocyte suspension. Blood was obtained by the cardiac puncture with heparinized syringes from Sprague–Dawley male rats weighing about 250 g anesthesized with chloroform. Plasma was discarded and the erythrocytes were washed

twice and resuspended in isotonic saline-buffer to make a final concentration of 10^9 cells/ml and kept at 0° unless otherwise specified. The isotonic saline-buffer was composed of 154 mM NaCl in 10 mM sodium phosphate buffer, pH 7·0.

Hypotonic hemolysis. Procedures were modified from those reported by Seeman and Weinstein.[6] At a designated temperature (one of 0, 12, 24 and 37°), 0·1 ml of the stock suspension of erythrocytes was mixed with 1·1 ml of the test solution and incubated for 10 min. After the incubation, mixture was centrifuged at 600 *g* for 3 min at the same temperature and the resultant supernatant was subjected to measurement of adsorption at 541 mμ in Hitachi–Perkin–Elmer spectrophotometer model 139.

The test solution was composed of about 50–80 mM NaCl in 10 mM sodium phosphate buffer, pH 7·0, containing the drug at a concentration between 10^{-6} M to 10^{-3} M and 1·1% ethanol as the solvent for the drug.

Procedures for adsorption experiment. Adsorption experiments of flufenamic acid by erythrocytes were carried out under the conditions as those used in the hypotonic hemolysis test mentioned above, except that an isotonic saline-buffer, pH 7·0 was used instead of the hypotonic one. After an incubation for 10 min, the reaction mixture was centrifuged at 600 *g* for 3 min at the same temperature and separated into two fractions: the supernatant and the concentrated cell fraction. Aliquots of these fractions were then subjected to estimation of flufenamic acid by fluorometry, while per cent cell volume in the concentrated cell fraction was determined by microhematocrit technique. Using these values, the amounts of flufenamic acid in both forms, free and cell-bound, were calculated.

Fluorometric determination of flufenamic acid. Content of flufenamic acid in a sample was determined according to the method of Hattori *et al.*[7] as follows: a sample containing 0·25–2·5 μg of flufenamic acid was extracted with ethyl acetate at pH 4·5. After evaporation of solvent, the residue was dissolved in 5 ml of absolute ethanol and a fluorescence-complex was obtained by the subsequent addition of 0·1 ml of 0·5% aluminium chloride in ethanol. The fluorescence at 440 mμ (activated at 358 mμ) was measured in Hitachi fluorescence spectrophotometer model MPF-2A.

RESULTS

Temperature-dependent inhibition or acceleration of hypotonic hemolysis by flufenamic acid. Effect of flufenamic acid and chlorpromazine on hypotonic hemolysis of rat erythrocytes was investigated at various temperatures. In these experiments, extent of hemolysis was controlled always to keep at around 50 per cent in the absence of drug. Therefore, the difference in temperature was necessarily accompanied by the change in concentration of NaCl of the medium, since osmotic fragility of erythrocytes was enhanced by decrease of temperature.[4,8] It was found that concentrations of NaCl required for 50 per cent hemolysis at 0, 12, 24 and 37° were obtained as 79·3 ± 0·61, 73·1 ± 0·95, 70·6 ± 0·39 and 66·4 ± 0·49 mM as mean values from several rats, respectively.

It is clear as shown in Fig. 1, that effect of flufenamic acid on hemolysis is quite different from that of chlorpromazine with respect to the temperature-dependency. Namely, flufenamic acid protected erythrocytes from hypotonic hemolysis at low temperatures such as 0 or 12,° but this protective effect was diminished by increase of temperature and it was even hemolytic at 37°. In contrast with flufenamic acid, chlorpromazine was protective at any temperature between 0 and 37°. In the dose–response

relationship, both the inhibitory and stimulatory effect of these drugs was revealed to be dose-dependent, as shown in Table 3.

The inhibitory effect of flufenamic acid on hypotonic hemolysis at low temperature was also affected by the temperature at pre-incubation period, when erythrocytes were, prior to the transfer to hypotonic medium, kept in an isotonic state for 10 min (Table 1). Namely, a high temperature at this pre-incubation period caused significant

TABLE 1. INHIBITORY EFFECT OF FLUFENAMIC ACID ON HYPOTONIC HEMOLYSIS AS A FUNCTION OF TEMPERATURE OF PREINCUBATION PERIOD

Temperature (°)		
Pre-incubation (isotonic)	Incubation (hypotonic)	% Inhibition of hemolysis by flufenamic acid (3×10^{-4} M)
0	0	49·0 ± 3·02
12	0	48·3 ± 2·07
24	0	60·6 ± 7·57
37	0	70·0 ± 3·35
0	12	−1·4 ± 2·50
12	12	9·7 ± 3·80
24	12	12·1 ± 4·05
37	12	30·0 ± 3·02
0	24	−23·9 ± 2·98
12	24	−5·7 ± 1·70
24	24	−1·7 ± 5·04
37	24	5·5 ± 4·95
0	37	−26·6 ± 6·37
12	37	−18·0 ± 8·28
24	37	−15·1 ± 4·16
37	37	−18·4 ± 5·82

Erythrocytes (10^9/ml) were pre-incubated in isotonic saline-buffer (pH 7·0) for 10 min, then transferred to hypotonic NaCl buffer medium. Final concentration of erythrocytes, $8·3 \times 10^7$/ml.

potentiation of inhibition by flufenamic acid, in so far as temperature in hypotonic state was kept low. In other words, the more the temperature decreases during transferring process, the more prominent is the effect of this drug.

Adsorption of flufenamic acid molecule by erythrocyte membrane. The temperature-dependent capricious property of flufenamic acid on the erythrocyte membrane prompted us to study the possible adsorption of flufenamic acid molecules by rat erythrocytes at both 0 and 37°.

In these experiments, isotonic saline-buffer was used as the incubation medium, instead of hypotonic one, in order to avoid confusion which might be caused by the possible affinity of flufenamic acid either with hemoglobin or erythrocyte ghost membrane. In the preliminary trials, the amount of adsorption of flufenamic acid molecules by a definite number of erythrocytes ($8·3 \times 10^7$ cells/ml) was found to reach a plateau within several minutes either at 0 or 37°, when the incubation was carried out with a

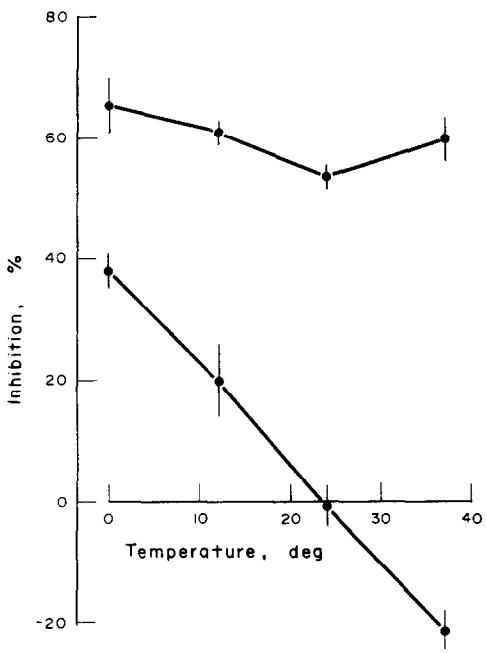

FIG. 1. Temperature-dependency of inhibitory effect of flufenamic acid and chlorpromazine on hypotonic hemolysis. Erythrocytes were kept at 0° in isotonic saline-buffer (pH 7·0) until transfer to hypotonic buffer. Erythrocyte concentration, $8{\cdot}3 \times 10^7$/ml; top curve, chlorpromazine, 10^{-4} M; lower curve, flufenamic acid, 10^{-4} M.

high level of flufenamic acid (3×10^{-4} M). Consequently, an incubation for 10 min was assumed to be a sufficient reaction period to get an equilibrium between flufenamic acid and erythrocyte membrane.

The adsorbed amount of flufenamic acid per a definite number of erythrocytes were found to increase with increasing concentration of flufenamic acid up to 3×10^{-4} M at both temperatures (Table 2). However, if the same concentration of flufenamic acid was used, the adsorbed amount was always found to be greater at 0° than the corresponding one at 37° and this tendency was more prominent when lower concentration of this drug was used.

Inhibition or acceleration of hypotonic hemolysis by various clinically useful drugs. From the results mentioned above, a question arises whether the temperature-dependent effect of flufenamic acid on hypotonic hemolysis could be generalized to the other membrane-stabilizing drugs. Thus, ten anti-inflammatory drugs, four tranquilizers, four antihistamines and two local anesthetics were tested for effect on hypotonic hemolysis of rat erythrocytes at both 0 and 37° using their low concentrations. Results are listed in Table 3.

Compounds to be inhibitory at 37° are found to be either basic or nonionic in their chemical nature and most of the tested tranquilizers, antihistamines or antiserotonines, local anesthetics and nonionic anti-inflammatory drugs (bimetopyrol[9–11] and indoxole[12]) belong to this group. In addition, they are also inhibitory at 0°, too, suggesting that

TABLE 2. ADSORPTION OF FLUFENAMIC ACID BY ERYTHROCYTES AT 0 AND 37°

Incubation (°)	Flufenamic acid content (μg/g/ml of initial cell suspension)				
			Found in		
	Initial	(M)	Cell	Supernatant	Ratio (cell/sup.)
0°	2·8	10^{-5}	1·00	1·57	0·64
	14·1	5×10^{-5}	4·10	8·67	0·47
	28·1	10^{-4}	6·13	20·12	0·30
	56·2	2×10^{-4}	8·38	46·08	0·18
	84·4	3×10^{-4}	10·27	72·05	0·14
37°	2·8	10^{-5}	0·29	2·39	0·12
	14·1	5×10^{-5}	1·30	11·72	0·11
	28·1	10^{-4}	3·46	25·91	0·13
	56·2	2×10^{-4}	4·56	47·94	0·10
	84·4	3×10^{-4}	6·27	75·13	0·08

Erythrocytes ($8·3 \times 10^7$/ml) in isotonic saline-buffer (pH 7·0) were incubated for 10 min with drug. Each value is a mean from three or four experiments.

they might have the same property as chlorpromazine in hypotonic hemolysis system.

Drugs to be stimulatory at 37° are mostly acidic except chloroquine, and only the anti-inflammatory drugs are seen in this group. Some of them are inhibitory at 0° but the others do not show any appreciable activity at this temperature.

DISCUSSION

The data presented in this paper suggested that fluefenamic acid reacts with rat erythrocyte membrane in a different manner from chlorpromazine.

At low concentrations, flufenamic acid protects rat erythrocytes from rapid hypotonic hemolysis, when erythrocytes are incubated at a cold medium. This protective effect was, however, reduced by the increase in temperature and finally lytic activity was observed at 37°. Chlorpromazine was, in contrast with this anti-inflammatory drug, found to be protective at any temperature from 0 to 37°.

According to Seeman,[13] the antihemolytic activity of alcohol anesthetics was known to be reduced at higher temperature. But, these drugs were never lytic at low concentration, so that the property of flufenamic acid reported here may be distinguished from them, too.

Effect of flufenamic acid on hypotonic hemolysis of erythrocytes was also affected by the temperature-change during the transfer of erythrocytes from an isotonic to a hypotonic state. In general, it seems likely that the more the temperature decreased during this process, the more prominent is the protective effect of this drug. According to Inglot and Wolna,[4] they failed to demonstrate the erythrocyte stabilization by mefenamic acid when erythrocytes were preincubated at 4°. This may be at least partly due to the "warming up procedure" during transfer of erythrocytes, because they carried out the hypotonic test at room temperature.

Adsorption study indicated that the adsorbed amount of flufenamic acid by a

TABLE 3. INHIBITION OF HYPOTONIC HEMOLYSIS OF RAT ERYTHROCYTE BY VARIOUS CLINICALLY USEFUL DRUGS

Drug	Concentration (M)	% Inhibition at 0°	% Inhibition at 37°
Flufenamic acid	5×10^{-5}	20	−15
	10^{-4}	38	−20
	6×10^{-4}	68	−30
Indomethacin	10^{-4}	17	−8
	5×10^{-4}	52	−15
Mefenamic acid	5×10^{-5}	22	−20
	5×10^{-4}	24	−33
Ibufenac	5×10^{-4}	10	−10
	10^{-3}	19	−8
Phenylbutazone	5×10^{-5}	5	−11
	5×10^{-4}	1	−22
Oxyphenbutazone	5×10^{-4}	0	−14
Acetylsalicylic acid	5×10^{-4}	−2	−8
Chloroquine diphosphate	10^{-4}	−4	−15
	10^{-3}	−4	−40
Bimetopyrol	10^{-4}	25	21
	5×10^{-4}	27	24
Indoxole	5×10^{-5}	16	25
	5×10^{-4}	27	41
Chlorpromazine *p*-hydroxybenzoyl benzoate	5×10^{-6}	37	20
	10^{-5}	43	31
	10^{-4}	66	60
Benzodiazepinium halide A*	5×10^{-5}	44	54
Benzodiazepinium halide B†	5×10^{-5}	22	24
Reserpine	5×10^{-4}	5	4
Cyproheptadine–HCl	5×10^{-5}	52	43
Methysergide bimaleate	5×10^{-4}	21	7
Chlorpheniramine maleate	5×10^{-4}	4	19
Diphenhydramine–HCl	5×10^{-4}	24	45
Dibucaine–HCl	10^{-4}	36	47
Lidocaine–HCl	10^{-3}	17	27

Erythrocytes were kept at 0° in isotonic saline-buffer until transfer to hypotonic medium. Final concentration of erythrocytes, $8{\cdot}3 \times 10^7$/ml.

* 7-Chloro-5-(*o*-chlorophenyl)-4-(1-methyl-2-hydroxyethyl)-2-oxo-1,3-dihydro-2H-1,4-benzodiazepinium chloride.

† 7-Chloro-5-(*o*-chlorophenyl)-4-(2-hydroxyethyl)-2-oxo-1,3-dihydro-2H-1,4-benzodiazepinium chloride.

All drugs were assayed at least two times in every dose.

definite number of erythrocyte at 0° was always greater than those observed at 37°. Moreover, the most striking aspect brought by the change of temperature was the difference in manner of adsorption. Namely, when the incubation was carried out at 37°, the adsorbed amount was clearly as a linear function of the initial concentration of this drug in the medium, suggesting that adsorption may take place in a simple and passive manner at this high temperature.

However, at 0°, a more complicated mechanism of adsorption was suggested. After an incubation at this low temperature, the ratio of the adsorbed amount of flufenamic acid by cells to that remained in supernatant, was varied with varying concentration

of flufenamic acid used. Namely, the ratio increased with the decrease of the initial concentration. Consequently, adsorption at 0° seems to be active in the manner and it may allow an assumption that a new type of binding-site appears at the surface of erythrocyte membrane when they are exposed to such a low temperature. We already observed that flufenamic acid protected erythrocytes from hypotonic hemolysis only at low temperature. Taking this into account, the present data strongly suggest that flufenamic acid protects erythrocyte by means of binding to membrane at these sites.

The protective effect of chlorpromazine[2] and anesthetics[1,14] on hypotonic hemolysis of human erythrocytes was reported to be due to the increase of the critical volume of erythrocyte and it did not alter by change of temperature of medium in which erythrocytes were incubated.[8] In our preliminary trials at cold temperature, increase of the critical volume of rat erythrocytes was found to be only little, if any, by the addition of flufenamic acid. Thus, so far as the effect of flufenamic acid on the critical volume of erythrocyte is concerned, it is still premature to reach any conclusion.

A variety of clinically useful drugs have been reported to protect erythrocyte membrane from hypotonic hemolysis, although correlation between pharmacological activity and the effect on erythrocyte membrane has not yet been fully elucidated.[15,16] Among them are nonsteroidal anti-inflammatory drugs,[4,15] tranquilizers,[6,17–19] anesthetics[1,14] and antihistamines.[6] Thus, a survey test for the temperature-dependency of these drugs has been done in the hypotonic hemolysis system. Results indicate that a number of anti-inflammatory drugs are stimulatory at 37° and inhibitory or without effect at 0°, being similar to the property of flufenamic acid, whereas most of tested tranquilizers, antihistamines and local anesthetics are inhibitory at both 37 and 0°, in agreement with the characteristic of chlorpromazine.

However, two nonionic anti-inflammatory drugs, bimetopyrol and indoxole were classified as chlorpromazine type, while the pharmacological activities of them were very similar to those of flufenamic acid, such as high potency in carrageenin-induced rat paw edema,[9,12] rat adjuvant arthritis,[10,12] ultraviolet-induced erythema of guinea-pig[9] and, to a lesser degree, in granuloma pouch inflammation of rat.[9,12] In addition, basic drugs were not always classified as chlorpromazine type. Chloroquine is the example for this. In this connection, the "polyvalency" of nonsteroidal anti-inflammatory drugs as proposed by Glenn[15] may be valid here, too. Nevertheless, acceleration of hypotonic hemolysis at high temperature seems likely to be one of the characteristic aspects of anti-inflammatory acids, in differentiating themselves from the other mostly basic or nonionic membrane stabilizers.

Acknowledgements—The authors wish to express their sincere thanks to Dr. B. Berde of Sandoz Ltd. for supplying us with a sample of methysergide bimaleate. Thanks are also due to Dr. N. Yoshida and Dr. R. Tachikawa of this Company for the synthesis and supply of bimetopyrol and indoxole and benzodiazepinium halides, respectively.

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