Membrane Stabilization: Effects of Hydrocortisone Sodium Succinate on Phospholipase C-Treated Sheep Erythrocytes[1] (34337)

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The effects of steroids on biological membranes are well recognized (1–4) and model membranes made of lipid mixtures (spherules or liposomes) have been reported to respond to the effects of steroids (5). In the present study, we used sheep erythrocytes treated with phospholipase C (PLC) as a testing model for the effect of hydrocortisone sodium succinate (HSS).

Unlike human and rabbit erythrocytes, which lyse readily after treatment with PLC, sheep erythrocytes remain intact until the temperature is lowered. This characteristic was first observed by Van Heyningen (6) and was further documented by MacFarlane (7). We took advantage of this property of the PLC-modified sheep erythrocytes to observe the effects of HSS on membrane stability. It was found that HSS stabilized the PLC-treated cells, as well as the untreated cells. Since PLC removes phosphoryl amines from phospholipids, it appears that the phosphoryl amine moiety of the membrane phospholipids may not participate in the action of the steroid. Our experiments further showed that in addition to its effect on the stability of erythrocyte membrane, HSS inhibits the hydrolytic activity of PLC. These novel observations are described below.

Materials and Methods. Defibrinated sheep blood was purchased from Brown Laboratory. The HSS used in these experiments was Solu-Cortef of Upjohn Co. The PLC was *Clostridium welchi* α-toxin, purchased from Calbiochem. Gas gangrene antitoxin was a product of Parke, Davis Co.

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The erythrocytes were separated from the defibrinated sheep blood by centrifugation at 1000*g* for 15 min at 22°, washed 3 times with isotonic borate buffer (pH 7.6), and then suspended in the same buffer (1.5%, v/v). The method used to modify the erythrocyte membrane by PLC was that of Ikezawa and Murath (8). The erythrocyte suspensions were gently shaken in a water bath at 37° with PLC (5 μg/ml) in the presence of 0.004 *M* $CaCl_2$ to allow removal of the phosphoryl amines from the membrane phospholipids by action of the enzyme. The reaction was arrested by the addition of gas gangrene antitoxin (50 units/ml). The activity of PLC was followed by assaying the remaining lipid phosphate, which was extracted with a mixture of ethanol:ether, 1:2, as described by MacFarlane (7). Phosphate determination was according to the method of Bartlett (9).

Stability of the cells was determined by the amount of hemoglobin liberated into the medium. The samples were centrifuged at 1000*g* for 15 min to remove the nonhemolyzed cells, and the hemoglobin in the supernatant solutions was measured by optical density at 540 mμ. The degree of hemolysis was expressed as a percentage of complete hemolysis effected by dilution of the sample with 3 vol of distilled water.

Results. Dose response. In a preliminary experiment, suspensions of erythrocytes (25 ml) were incubated with various concentrations of HSS for 30 min at 22°, and then gently shaken at 37° with PLC (5 μg/ml) for 1 hr. The enzymic reaction was then stopped by addition of the antitoxin. To cause lysis of the modified cells, the sample was chilled in an ice-water bath for 30 min as described by MacFarlane (7), (our previous experiments

showed that lysis of the PLC-modified cells essentially ended within 15 min), then the non hemolyzed cells were removed by centrifugation at 1000*g* for 15 min. Absorbance of the supernatant fluid at 540 mμ showed that HSS prevented hemolysis of the erythrocytes with an optimal concentration of 1 mg of HSS/ml (Fig. 1). Since the HSS preparation used in these studies contains 0.8 mg of sodium biphosphate/100 mg of HSS, control experiments were carried out in which the erythrocytes were incubated with equivalent amounts of sodium biphosphate and sodium succinate. No effect on the stability of the erythrocytes by the salts could be detected.

Time course. The erythrocyte suspension (25 ml) was put into each of four flasks: A, B, C, and D. To assure equal content of erythrocytes in these flasks, a 1-ml aliquot was taken from each flask and added to 3 ml of distilled water to effect complete hemolysis; the resulting solutions had equal optical density at 540 mμ. The HSS (2 mg/ml) was added to flasks A and C. After being maintained at 22° for 30 min, the flasks were gently shaken at 37° for 15 min. PLC (5 μg/ml) was then added to flasks A and B. At various time intervals up to 75 min after the addition of PLC, 3-ml aliquots were taken from each flask and placed in test tubes containing gas gangrene antitoxin (50 units/ml sample) to stop the enzymic reaction. The modified cells were lysed by chilling at 0° for 30 min. Absorbance of the supernatant fluids at 540 mμ showed less hemolysis in A, which contained HSS and PLC than in B, which contained PLC but no HSS. The results from A and B are shown in Fig. 2. Hemolysis increased with time of incubation with PLC and the protection due to HSS was clearly demonstrated. It can be calculated from these data that the inhibition afforded by HSS after 1 hr of incubation with PLC was 64%. The supernatant fluids of control flasks C and D showed almost no hemolysis.

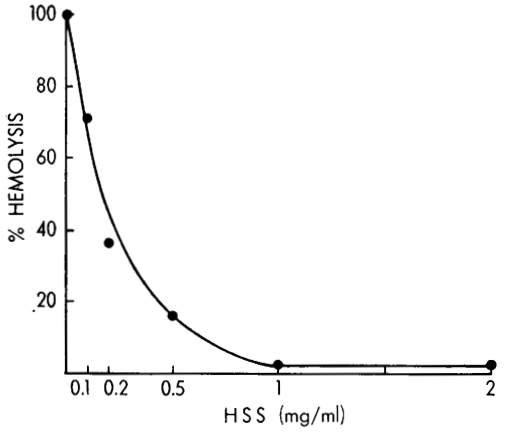

FIG. 1. Dose response of sheep erythrocytes to HSS; the erythrocytes were incubated with PLC and various amounts of HSS at 37° for 1 hr. Hemolysis was induced at 0° and determined by absorbency at 540 mμ. The extent of hemolysis was expressed as a percentage of complete hemolysis effected by dilution with 3 vol of distilled water.

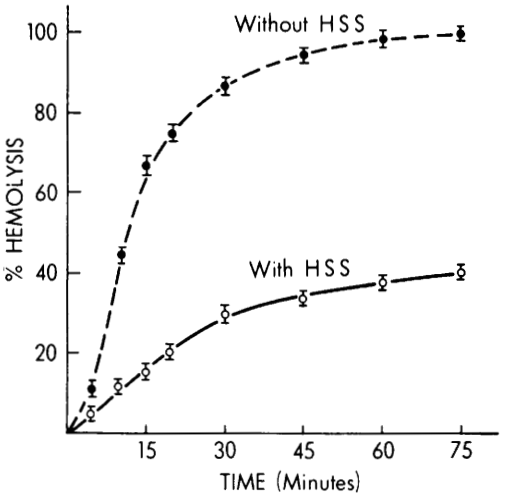

FIG. 2. Time course of hemolysis; sheep erythrocytes were treated with PLC in the presence and absence of HSS. Hemolysis was effected by chilling the cells at 0°, and measured by absorbency at 540 mμ. The data are expressed as percentages of the complete hemolysis. Each point shown is the average of triplicate determinations.

The diminished hemolysis in the presence of HSS demonstrated in the above experiments could be due to stabilization of the membrane; it could also be due to an inhibition of the hydrolytic activity of PLC by HSS. These possibilities were further examined in the following experiments.

Effect of HSS on PLC modified erythrocytes. Sheep erythrocytes were treated with PLC (5 μg/ml of suspension) at 37° for 1 hr. Determination of lipid phosphate showed

that 88% of the lipid phosphate had been removed within 1 hr and no more phosphate was released from the erythrocytes in longer incubations. After the enzymic reaction was stopped by the antitoxin (50 units/ml), the cells were sedimented by centrifugation at 1000*g* at room temperature for 15 min (this procedure caused approximately 8% hemolysis), and then gently resuspended in isotonic borate buffer. Three batches of the modified erythrocytes were subjected to hemolysis at 0° with and without HSS (2 mg/ml). The results shown in Table I indicated that 34–56% less hemolysis of the modified cells in the presence of HSS.

Hypotonic hemolysis of erythrocytes. To further examine the effect of HSS on sheep erythrocytes, hemolysis was induced in hypotonic solutions so that the effect on the PLC-treated and the untreated cells could be compared. Erythrocyte suspensions were diluted with various proportions of distilled water. After 30 min at 37°, the intact cells were removed by centrifugation, and the percentage hemolysis was determined by absorbence of the supernatant fluid at 540 mμ (Fig. 3).

TABLE I. Effect of HSS on Hemolysis of Sheep Erythrocytes after Treatment with Phospholipase C.[a]

Batch		O.D. at 540 mμ Without HSS	With HSS	Protection by HSS (%)
1		0.602	0.394	
		0.614	0.401	
		0.609	0.411	
	Av	0.606	0.402	34
2		0.593	0.237	
		0.595	0.284	
		0.590	0.259	
	Av	0.592	0.260	56
3		0.653	0.399	
		0.647	0.408	
		0.642	0.413	
	Av	0.647	0.406	37

[a] Sheep erythrocytes were treated with PLC so that 88% of the lipid phosphate was removed. The modified cells were suspended in isotonic borate buffer (pH 7.6) with and without HSS (2 mg/ml). Hemolysis was induced by chilling in ice, and determined by absorbance at 540 mμ.

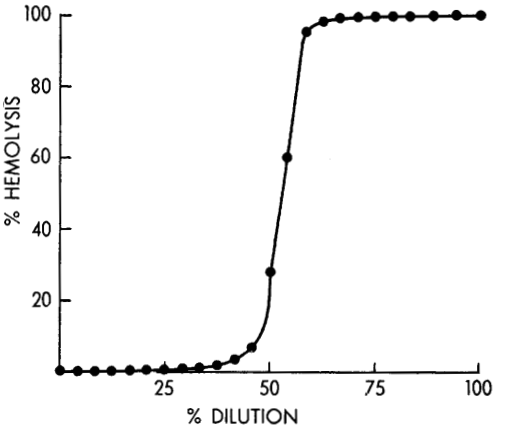

FIG. 3. Hypotonic hemolysis of erythrocytes; hemolysis of the erythrocytes was effected by dilution with various proportions of water [e.g., 50% dilution is 1:1 (v/v) dilution of the suspension with water], and incubation at 37° for 30 min.

It was found that a 1:1 dilution of the cell suspension with water-allowed hemolysis to occur at a slow but measurable rate (28% in 30 min). The effect of HSS on the rate of hemolysis was tested in the next experiment. Two batches of a suspension of erythrocytes, one with HSS (2 mg/ml) and one without HSS, were hemolyzed by 1:1 dilution with distilled water at 37°. At various time intervals, aliquots were removed and the percentage hemolysis was determined. It was found that in the presence of HSS, 13% hemolysis occurred in 3 hr, while the control cells, (without HSS) were 98% hemolyzed. This experiment was repeated using cells which had been treated with PLC for 12 and 60 min; such treatments removed 44 and 88% of the lipid phosphate, respectively. These cells were washed, resuspended with and without HSS, and subjected to hypotonic hemolysis (1:1 dilution). Figure 4 shows that removal of the phosphoryl amine caused decreases in the stability of the erythrocytes, so that the initial rate of hemolysis was greater for the PLC-treated cells than the untreated cells; and the greatest rate of hemolysis was observed with cells from which 88% of the phosphate was removed. But removal of the phosphate did not alter the protection afforded by HSS, as the rates of hemolysis of the three types of cells in the presence of HSS

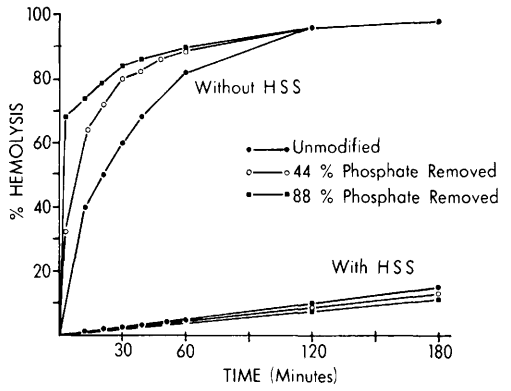

FIG. 4. Rates of hypotonic hemolysis of PLC-modified and unmodified sheep erythrocytes; hemolysis was effected in hypotonic solution as described in the text. The initial rates of hemolysis of the PLC-modified erythrocytes were greater, but the protection afforded by HSS to the modified cells and the unmodified cells was similar.

were similar, and at the end of 3 hr, only 13–16% were hemolyzed.

Inhibition of PLC by HSS. The erythrocytes were incubated with PLC (5 μg/ml) in the presence and absence of HSS (2 mg/ml). The activity of PLC was determined by measuring the remaining lipid phosphate, and the progress of the reaction was followed for 60 min. The results are shown in Fig. 5. The inhibiton of PLC by HSS is evident. In another experiment the phospholipids were extracted from the erythrocytes with ethanol:ether, 1:2, and used as the substrate for PLC in borate buffer (pH 7.6) containing 0.004 *M* $CaCl_2$. The reaction was compared in the presence and absence of HSS. The data presented in Fig. 6 show inhibition of PLC by HSS, independent of the cell membrane.

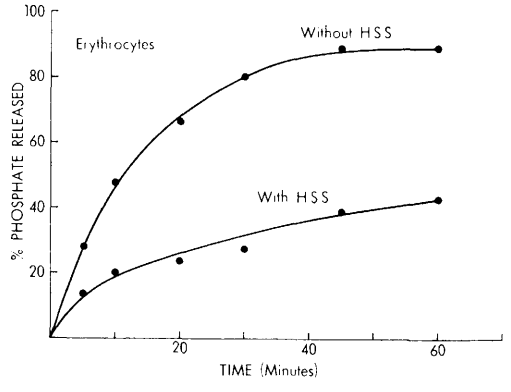

FIG. 5. Inhibiton of PLC activity on erythrocytes by HSS; the erythrocytes were incubated with PLC, with and without HSS. The activity of the enzyme was assayed by measuring phosphate release using the methods of MacFarlane (7) and Bartlett (9).

Discussion. Although the stabilizing effect of anti-inflammatory steroids on biological membranes is well recognized, its mode of action has not been elucidated. The results of the present study show that HSS prevents hemolysis of the PLC modified erythrocytes

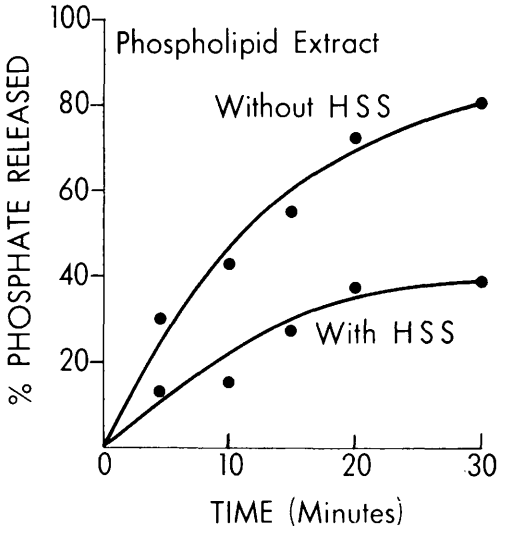

FIG. 6. Inhibition of PLC by HSS using lipid phosphate extracted from sheep erythrocytes as the substrate; the experimental conditions were similar to those described in Fig. 5, except that the lipid phosphate extracted from the erythrocytes was used as the substrate.

(Table I and Fig. 4). Since the enzyme releases up to 88% of the phosphoryl amine moiety of membrane phospholipids, the stabilizing effect of the steroid apparently does not require that portion of the phosphoryl amine. Whether or not the remaining 12% of the lipid phosphate is a crucial requirement of steroid action remains to be resolved. Previous work by Weissman *et al.* (5) indicated that the stabilizing effect of steroids could be demonstrated in the absence of membrane proteins. Weissman and Sessa (10) also showed the membrane labilizing steroids,

etiocholanolone and deoxycorticosterone, to be active on spherules made of phospholipids free of cholesterol. It would be interesting to test in further experiments if the glyceride moiety of membrane lipids or cholesterol is involved in the stabilizing action of the steroids.

It was also noticed in these experiments that the stabilizing effect alone could not account totally for the inhibition of hemolysis by HSS as observed in our initial experiments (Figs. 1 and 2). For instance, the inhibition afforded by HSS after 1 hr of incubation was 64% (calculated from data in Fig. 2), and was more than the protection due to membrane stabilization (34–56%, Table I). The data suggest that in addition to the stabilizing effect of HSS on the modified cell membrane, the steroid may interfere with the hydrolytic activity of the lipase. The inhibition of PLC activity by HSS was indeed confirmed in subsequent experiments (Figs. 5 and 6).

As in most *in vitro* studies of membrane stabilizing and labilizing steroids, the concentrations of HSS used in the present studies were far greater than the physiologic concentrations of corticosteroids. The effects of steroids in such concentrations may represent instances of pharmacologic rather than physiologic action. However, in living tissues the stabilizing steroids, cortisol, cortisone, their acetates, and other derivatives, antagonize the effects of labilizing agents such as etiocholanolone, vitamin A, endotoxins, and streptolysin S (4). These facts suggest that the stabilizing action of these steroids observed *in vitro* may be partially responsible for their biological activity.

Summary. The stabilizing effect of hydrocortisone sodium succinate (HSS) on sheep erythrocytes was demonstrated after the erythrocytes were modified with phospholipase C (PLC). Since this enzyme removes the phosphoryl amine moiety of the membrane phospholipids, and the modified cells had 88% of the lipid phosphate removed, the stabilizing effect of the steroid apparently does not require the participation of the phosphoryl amines. The stability of intact as well as PLC-modified erythrocytes was tested by hypotonic hemolysis in the presence and absence of HSS. Removal of the lipid phosphate by the enzyme labilized the cell membrane, but the stabilizing effect of HSS on the cell membrane was not altered by treatment with PLC. In additon to stabilizing the cell membrane, inhibiton of PLC activity by HSS was also demonstrated.

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