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SHORT COMMUNICATIONS

Lack of effect of ouabain on creatine phosphokinase efflux from skeletal muscle*

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Plasma creatine phosphokinase (CPK) (EC 2.7.3.2) activity is elevated in a variety of human and animal skeletal and cardiac muscle diseases, among them Duchenne muscular dystrophy [1], hamster muscular dystrophy [2], myocardial infarction [3], malignant hyperpyrexia [4], hypothyroidism [5] and alcoholic myopathy [6]. Since CPK activity is confined almost exclusively to skeletal muscle, cardiac muscle and brain, only these tissues need be considered as sources of serum CPK [7]. Since brain-type CPK is rarely present in serum, only skeletal and cardiac muscle need be considered as sources of CPK activity in serum under most circumstances. There are probably a variety of mechanisms for the exit of CPK from muscle and its entry into serum. Release from necrotic cells, though important in such diseases as rhabdomyolysis [8] and myocardial infarction [9], may not be the major one in most conditions with increased serum CPK activity. It has been suggested that proteins such as CPK can passively diffuse through the cell membrane [10] or can be transported across the cell membrane [11]. It has also been proposed that diminished activity of Na^+,K^+-adenosine triphosphatase (Na^+,K^+-ATPase), which is part of the sarcolemma [12], and which mediates Na^+–K^+ ion flux, is causally related to the efflux of CPK from skeletal muscle and its subsequent entry into plasma in malignant hyperpyrexia [13]. This was based on the finding of decreased erythrocyte Na^+,K^+-ATPase activity in 8 of 13 people at high risk for malignant hyperpyrexia [13]. It was proposed that in malignant hyperpyrexia a decrease in Na^+,K^+-ATPase activity and anesthetic agents might interact to produce uncoupling of oxidative phosphorylation, which would lead to muscle breakdown. Further evidence for this theory is the observation that massive, prolonged increases in serum CPK activity occur in volunteers given small doses of digoxin [14]. This led to the proposal that digoxin, an inhibitor of Na^+,K^+-ATPase, had a direct effect on cell membrane permeability [14]. The low temperature-induced efflux of aldolase and lactic dehydrogenase (LDH) from erythrocytes *in vitro* has been attributed to the inactivation of Na^+,K^+-ATPase [15]. The activity of "membrane" ATPase (Mg^{2+}-ATPase activity) in the erythrocytes and skeletal muscle of patients and laboratory animals with various types of muscle dystrophies has been reported to be enhanced rather than inhibited by ouabain [16, 17]. However, these results have been disputed by other investigators [18–20]. Increased Na^+,K^+-ATPase [21] activity and Mg^{2+}-ATPase activity [22] have also been found in the red cell ghosts of patients with "functional" psychoses, many of whom have increased serum CPK activity in the acute stages [23].

Ouabain is an effective inhibitor of Na^+,K^+-ATPase activity [24]; thus, ouabain can be used to test the thesis that diminished activity of Na^+,K^+-ATPase activity promotes enzyme efflux. Ouabain at 0·01 mM did not enhance the efflux of five soluble enzymes from the chicken biceps muscle *in vitro*, CPK release was not studied [25]. We have previously reported that ouabain at 1 mM did not enhance the release of CPK from rat platelets or LDH from human platelets *in vitro* [26]. Ouabain did not effect the release of acid phosphatase from rat liver lysosomes *in vitro* [27]. Ouabain slightly enhanced the release of LDH and aldolase from human erythrocytes which had been chilled at 1·5° for 2 hr and then incubated in plasma for 24 hr [15].

We have now studied the effect of ouabain on enzyme release from skeletal muscle in a variety of ways. Male Sprague–Dawley rats, 150–175 g, and male Duncan–Hartley guinea-pigs, weighing 450–500 g, were studied. They were fed Purina chow and water *ad lib*. Experimental and control groups consisted of three animals per group. The method of obtaining blood from the animals and of determining CPK activity has been previously described [28].

A single dose of ouabain, 25 mg/kg, i.p., did not increase plasma CPK activity in the rat at 1, 3, 6 or 24 hr (Table 1). A single 20 mg/kg dose of ouabain s.c. did increase plasma CPK activity at 2 hr (Table 1). Ouabain, 25 mg/kg, i.p., for 3 days, also did not raise plasma CPK activity in the rat.

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Table 1. Effect of ouabain on plasma CPK activity in the rat

Treatment (N)	Route	Dose (mg/kg)	Sacrifice (hr)	Plasma CPK activity (mU/ml)*
Saline (6)	i.p.		1	105 ± 16
Ouabain (3)	i.p.	25	1	97 ± 22
Ouabain (3)	i.p.	25	3	109 ± 18
Ouabain (3)	i.p.	25	6	119 ± 22
Ouabain (3)	i.p.	25	24	103 ± 16
Ouabain (6)	s.c.	20	2	97 ± 26

* Mean ± S. D.

Table 2. Effect of ouabain on plasma CPK activity in the guinea-pig

Treatment (N)	Dose (mg/kg)	Time of sacrifice (hr)	Plasma CPK activity (mU/ml)	P
Saline (3)		2	154 ± 45	
Ouabain (3)	0·2	2	161 ± 37	NS*
Ouabain (3)	0·2	4	145 ± 42	NS

* NS, not significant.

The LD_{50} for ouabain in the guinea-pig is 0·25 mg/kg [29]. Ouabain, 0·2 mg/kg, i.p., did not increase plasma CPK levels in the guinea pig (Table 2).

The effect of ouabain pretreatment on the increase in plasma CPK activity in known myotoxic treatments was also studied. We have previously reported that serotonin, 20 mg/kg, s.c., raises plasma CPK activity in the rat [30].

Table 4. Efflux of CPK from extensor digitorum longus muscle *in vitro**

Group (N)	Time (min) 0	30	60
	CPK activity (mU/ml)		
Control (4)	37 ± 5	1590 ± 45	2410 ± 150
1 mM (4)	65 ± 25	1565 ± 40	2270 ± 190
0·1 mM (4)	47 ± 5	1680 ± 75	2150 ± 205
	LDH activity (mU/ml)		
Control (4)	4 ± 2	69 ± 19	155 ± 35
1 mM (4)	2 ± 2	72 ± 19	163 ± 12
0·1 mM (4)	4 ± 2	74 ± 17	172 ± 27

* The effects of the drug at different times were tested by means of a two-way analysis of variance. There was no significant difference in efflux at any time for either concentration of ouabain.

Table 3. Effect of ouabain on increases in plasma CPK activity produced by myotoxic procedures

Pretreatment (N)	Treatment	Sacrifice	Plasma CPK activity (mU/ml)*
Saline (6)	Serotonin, 20 mg/kg, s.c.	2 hr	344 ± 62
Ouabain, 25 mg/kg, i.p. (6)	Serotonin, 20 mg/kg, s.c.	2 hr	288 ± 45
Saline (6)	Restraint at 2°	30 min	563 ± 40
Ouabain, 10 mg/kg, i.p. (6)	Restraint at 2°	30 min	504 ± 106
Saline (6)	Restraint at 24°	30 min	310 ± 56
Ouabain, 10 mg/kg, i.p. (6)	Restraint at 24°	30 min	366 ± 101

* Mean ± S. D.

Similarly, restraint at 2° or at room temperature produced increases in plasma CPK activity in the rat [31]. Pretreatment with ouabain, 25 mg/kg i.p. or 10 mg/kg, i.p., did not significantly change the increase in plasma CPK activity produced by serotonin, 20 mg/kg, s.c., or by 30 min of restraint at 2° or room temperature (Table 3).

The effect of ouabain on the efflux of CPK and LDH from the isolated extensor digitorum longus muscle *in vitro* was studied by methods described elsewhere [27]. Ouabain at 1 and 0·1 mM did not enhance the efflux of CPK or LDH from the extensor digitorum longus muscle *in vitro* at 15, 30 or 60 min of incubation (Table 4).

These studies suggest that inhibition of Na^+,K^+-ATPase by ouabain does not promote the efflux of CPK from either normal rats or rats treated with chemical agents that promote the efflux of CPK from skeletal muscle or from normal skeletal muscle in guinea pigs. These results make it unlikely that diminished activity of Na^+,K^+-ATPase is a significant factor in enzyme efflux from skeletal muscle.

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Inhibitory effects by anti-inflammatory drugs on enzyme release from rabbit polymorphonuclear leukocyte lysosomes*

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In 1967, Weissmann [1] reviewed the important roles of polymorphonuclear (PMN) leukocyte lysosomes in inflammation. In our previous paper [2], it was suggested that nonsteroidal anti-inflammatory drugs such as phenylbutazone had a membrane-stabilizing activity on intact lysosomes, for they inhibited the release of enzymes from rabbit PMN leukocyte lysosomes *in vitro*.

This paper deals with the influence of certain anti-inflammatory agents on the release of enzymes from the rabbit PMN leukocyte lysosomes. The method of preparation of lysosomes reported by Ignarro [3] was modified in order to obtain the intact lysosomes. Namely, heparinized Ca^{2+}-free Hanks' solution containing glucose was injected before harvesting the leukocyte suspension, and the lysosomes were liberated from the leukocytes suspended in 0·25 M sucrose–0·04 M Tris–acetate buffer (pH 7·4). Anti-inflammatory drugs exhibited a membrane-stabilizing activity on these intact lysosomes, which were prepared by the method described below.

Preparation of PMN leukocytes and their lysosomes. Male albino rabbits (3 to 3·5 kg) were injected intraperitoneally with 200 ml of 0·1% (w/v) glycogen in sterile saline, and 4 hr later, with 100 ml of Ca^{2+}-free Hanks' solution containing 0·1% glucose and 10 units/ml of heparin. Immediately after the latter injection, the exudate containing suspended PMN leukocytes (2–6 × 10^6 cells/ml, 150–200 ml/animal) was withdrawn from the abdominal cavity through a needle.

This suspension was stored in ice water until the next operation. The leukocyte suspension, contaminated with a small number of erythrocytes, was filtered through cheese cloth. The filtrate containing over 90 per cent PMN leukocytes was centrifuged at 250 *g* for 5 min. The cell pellet was resuspended in 0·25 M sucrose–0·04 M Tris–acetate buffer (pH 7·4). This suspension was centrifuged again under the same conditions.

To the cell pellet was added the same buffer or 0·34 M sucrose–0·04 M Tris–acetate buffer (pH 7·4) to adjust the leukocyte counts to 1 × 10^8/ml, followed by vigorous uptake and expulsion from a 10-ml pipette five or six times, to liberate the lysosomes from PMN leukocytes. The suspension was centrifuged at 600 *g* for 10 min to separate lysosomes from intact cells, nuclei and other cellular debris. The supernatant was mainly used as a suspension of lysosomes (600 *g* supernatant fraction), which contained mitochondria and other organelles besides the lysosomes. This lysosome suspension was further centrifuged at 8200 *g* for 15 min. To the pellet was added the same volume of 0·25 M sucrose–0·04 M Tris–acetate buffer (pH 7·4). This was also used as the suspension of lysosomes (600–8200 *g* fraction). These procedures were carried out as quickly as possible at a temperature of 4° or less.

Drugs and preparation of test solution. Phenylbutazone, acetylsalicylic acid, ibuprofen, indomethacin and tinoridine hydrochloride were supplied by our Laboratories. Hydrocortisone and prednisolone, which were not in salt form, were purchased from Sigma Chemical Co., Ltd. These drugs were dissolved in ethanol solution and diluted with sucrose buffer in such a way that the concentration of ethanol finally became 10% (v/v) in the test solution.

Assay procedure. Effects of test drugs on lysosome membranes were studied by determining the release of acid phosphatase or aryl sulfatase as a marker enzyme. None of the agents tested inhibited directly the activities of the marker enzymes. The test solution (200 μl) or 10% (v/v) ethanol–

* This paper corresponds to "Studies on Anti-inflammatory Agents—XXVII".