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Variability in ouabain-induced inhibition of human erythrocyte membrane (Na^+-K^+)-ATPase

Part of the evidence for the relationship between the transport of Na^+ and K^+ across erythrocyte membranes and the (Na^+-K^+)-activated membrane-bound ATPase rests on the observation that concentrations of cardiac glycosides inhibiting the active cation exchange also inhibit the stimulatory effect of Na^+ and K^+ on the enzyme-catalyzed hydrolysis of ATP[1]. Half maximal inhibition with the most commonly used cardiac glycoside, ouabain, occurs at concentrations of 0.1 μM with approx. 95 % inhibition at concentrations of 1–100 μM[2]. Therefore, when ouabain failed to regularly inhibit (Na^+-K^+)-ATPase in erythrocyte membranes which we were studying, we tested the inhibitory effect of ouabain against the (Na^+-K^+)-stimulated ATPase of 11 consecutive erythrocyte membrane preparations. Human erythrocyte membranes were prepared from outdated blood stored in acid–citrate–dextrose by the technique of stepwise osmotic lysis[3]. In membrane Preps. 1 and 2, a 5-step lysis was used and yielded pink-red material, while in Preps. 3–11, a 6-step lysis was used and yielded essentially white membranes. Erythrocyte membrane ATPase was assayed using the method of POST *et al.*[2]. For "total" ATPase approx. 1 mg of membrane protein was incubated for 1 h in a 3.0-ml volume with 3 mM ATP, 3 mM $MgCl_2$, 80 mM NaCl, 33 mM KCl and 20 mM Tris buffer (pH 7.4); the P_i released was measured[4]. Mg^{2+}-ATPase activity was determined in parallel tubes incubated without addition of NaCl or KCl; however, the actual concentrations of Na^+ and K^+ in these tubes were 7.5–9.0 mM and 0.1 mM, respectively, as measured by flame photometry. The difference between the total ATPase and Mg^{2+}-ATPase represented (Na^+-K^+)-ATPase. Protein was determined using Armour's recrystallized bovine plasma albumin as the standard[5]. Ouabain, strophanthidin and oligomycin were added to tubes containing the constituents for the total ATPase assay to determine their inhibitory action against erythrocyte membrane (Na^+-K^+)-ATPase.

The results of the studies on ouabain inhibition appear in Table I. Complete ouabain inhibition of (Na^+-K^+)-ATPase was observed in Preps. 1 and 2 which were reddish. Ouabain inhibition of (Na^+-K^+)-ATPase of hemoglobin-free membranes (Preps. 3–11) was incomplete and erratic with a mean inhibition of 28 % in the last 9 preparations at ouabain concentrations of 0.5–1.0 mM.

Two other known inhibitors of erythrocyte membrane (Na^+-K^+)-ATPase were tested. Strophanthidin, an aglycone, has been reported to inhibit erythrocyte membrane (Na^+-K^+)-ATPase at concentrations of 0.1 mM[6]. However, even with 1.0 mM of strophanthidin, a complete inhibition of (Na^+-K^+)-ATPase was not observed (Table II). In contrast, oligomycin, an antibiotic chemically unrelated to either ouabain or strophanthidin, completely inhibited (Na^+-K^+)-ATPase, and the inactivation curve obtained was essentially parallel to that obtained by BLAKE *et al.*[7] (Table II). It has been reported that when human erythrocyte membranes are gently disrupted by controlled sonication[8] or by treatment with low concentrations of sodium dodecyl sulfate[9], there is a partial loss of the ouabain inhibition of (Na^+-K^+)-ATPase, along with the appearance of a stimulation of Mg^{2+}-ATPase by addition of either Na^+ or K^+. Two membrane preparations were studied to determine whether their Mg^{2+}-ATPase could be stimulated by either Na^+ or K^+ (Table III). The (Na^+-K^+)-

ATPase of membrane Preps. 10 and 11 (Table I) were inhibited by ouabain to the same degree; however, Prep. 10 exhibited a partial stimulation by K^+ addition, while Prep. 11 followed the classical pattern and required the simultaneous presence

TABLE I

ATPase activity expressed as μmoles P_i per h per mg protein.

Membrane Prep. No.	*Total ATPase*	*Mg^{2+}-ATPase*	*Total ATPase with ouabain addition*	*Ouabain concentration (mM)*	*Ouabain inhibition of (Na^+-K^+)-ATPase (%)**
1	2.60	1.21	1.18	1.0	102
2	1.61	1.04	1.02	1.0	104
3	1.25	0.87	1.18	1.0	18
	0.96**	0.52	0.82	1.0	32
			0.85	0.1	25
			0.91	0.01	11
4	1.82	1.03	1.97	0.1	0
5	2.68	2.00	2.54	0.5	21
6	2.53	1.88	2.21	0.5	49
7	2.03	1.43	1.93	0.5	17
8	2.02	1.36	2.00	0.5	3
9	2.99	1.33	2.31	1.0	41
10	3.13	2.11	2.57	1.0	55
11	2.12	1.30	1.75	1.0	45

* Ouabain inhibition of (Na^+-K^+)-ATPase =

$$\frac{\text{total ATPase} - \text{total ATPase with added ouabain}}{\text{total ATPase} - Mg^{2+}\text{-ATPase}} \times 100.$$

** Same preparation reassayed after being stored for 2 weeks in a frozen state.

TABLE II

ATPase activity expressed as μmoles P_i per h per mg protein.

Membrane Prep. No.	*ATPase with addition*		*ATPase activity*	*Inhibition of (Na^+-K^+)-ATPase (%)*
7	*Total ATPase		1.07	—
	Mg^{2+}-ATPase		0.50	—
	Total ATPase + strophanthidin,	1.0 mM	0.69	67
		0.5 mM	0.69	67
		0.1 mM	0.80	47
	Total ATPase + oligomycin,	40 μg/ml	0.50	100
		20 μg/ml	0.54	93
		8 μg/ml	0.58	86
		4 μg/ml	0.69	67
10	Total ATPase		3.13	—
	Mg^{2+}-ATPase		2.11	—
	Total ATPase + oligomycin,	30 μg/ml	2.13	98

* Membrane Prep. 7 had been stored in the frozen state for 5 days.

of both cations for stimulation. The ouabain preparations used were tested for their ability to inhibit influx of K^+ into rabbit erythrocytes[10] (Table IV). Since there is some passive influx of K^+ into erythrocytes, as well as some exchange diffusion with Na^+ (ref. 11), the ouabain-induced inhibition of the active K^+ influx is probably greater than 87% and indicates that we were using a biologically active material.

TABLE III

ATPase activity expressed as μmoles P_i per h per mg protein.

Membrane Prep. No.	*Mg^{2+}-ATPase*	*Mg^{2+}-ATPase + 30 mM K^+*	*Mg^{2+}-ATPase + 100 mM Na^+*	*Total ATPase (Mg^{2+}-ATPase + 30 mM K^+ + 100 mM Na^+)*	*Total ATPase + 1 mM ouabain*
10	2.11	2.64	2.13	3.13	2.57
11	1.30	1.22	1.22	2.12	1.75

TABLE IV

	Influx of K^+ (μmoles K^+ per min per ml erythrocytes)
Control	$6 \cdot 10^{-8}$
Ouabain, 1.0 mM	$0.8 \cdot 10^{-8}$

Biologically active ouabain incompletely inactivates (Na^+-K^+)-ATPase in erythrocyte membranes, while oligomycin inhibition of (Na^+-K^+)-ATPase is preserved. The fact that the effectiveness of ouabain inhibition varies among different membrane preparations (seemingly more effective in less rigorously prepared membranes) suggests that in certain preparations a ouabain-responsive site is impaired. However, this loss of ouabain responsiveness is not correlated with loss of Na^+-K^+ activation of the enzyme system nor with oligomycin inhibition. The results suggest that ouabain does not act directly on the (Na^+-K^+)-ATPase enzyme.

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Department of Medicine,
Stanford University Medical Center,
Stanford, Calif. 94305 (U.S.A.)

STANLEY L. SCHRIER*

Department of Biophysics,
Weizmann Institute of Science,
Rehovoth (Israel)

ELDAD GIBERMAN
EPHRAIM KATCHALSKI

* Please send reprint requests to Dr. Stanley L. Schrier.

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