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STUDIES OF ($Na^+ + K^+$)-SENSITIVE ATPase ACTIVITY IN PIG LYMPHOCYTES

EFFECTS OF CONCANAVALIN A

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Summary

($Na^+ + K^+$)-ATPase activity is demonstrated in plasma membranes from pig mesenteric lymph nodes. After dodecyl sulfate treatment plasma membranes have an 18-fold higher ($Na^+ + K^+$)-ATPase activity, while their ouabain-insensitive Mg^{2+}-ATPase is markedly lowered. A solubilized ($Na^+ + K^+$)-ATPase fraction, obtained by Lubrol WX treatment of the membranes, has very high specific activity (21 μmol P_i/h per mg protein). Concanavalin A has no effect on these partially purified ($Na^+ + K^+$)-ATPase, while it inhibits (40%) this activity in less purified fractions which still contain Mg^{2+}-ATPase activity.

The intracellular concentrations of Na^+ and K^+ in mammalian cells are different from their corresponding values in the extracellular fluid, the intracellular K^+ and the extracellular Na^+ concentrations being much higher. It is generally agreed that these concentration gradients are maintained by the 'Na-K' pump, the energy of which derives from the hydrolysis of ATP by a membrane ($Na^+ + K^+$)-ATPase. This ouabain-inhibited ATPase is found on the surface of all mammalian cells and is often used as a marker for the characterization of plasma membrane fractions. However, in a few types of cells, and specially in lymphocytes, the presence of this enzymatic activity is hard to demonstrate [1, 2] on account of its very low value [1—3]. It is impossible to detect this activity in whole lymphocytes [4], while in untreated lymphocyte plasma membranes ($Na^+ + K^+$)-ATPase accounts only for 5—10% of the total Mg^{2+}-stimulated ATPase activity [3]. As ($Na^+ + K^+$)-ATPase activity is deter-

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Abbreviation: EGTA, ethyleneglycol bis(α-aminoethylether)-*N,N'*-tetraacetic acid.

mined by measuring the difference between total Mg^{2+}-ATPase activity and Mg^{2+}-ATPase activity in the absence of Na^+ and K^+ (or in the presence of ouabain), the relative error (about 5%) in these determinations makes any accurate measurement of ($Na^+ + K^+$)-ATPase activity impossible, under normal experimental conditions.

Nevertheless ($Na^+ + K^+$)-ATPase appears to play an important role in lymphocyte blastic transformation. Indeed, Quastel and Kaplan [5] showed that its inhibition by ouabain prevented the transformation of stimulated lymphocytes. More recent work [6—8] showed that ouabain pretreatment of lymphocytes strongly affected their immune responses. The importance of monovalent ion transport through plasma membranes during the initiation of blastogenesis by lectins or specific antigens was widely investigated. The effects of lectins on intracellular K^+ level are very controversial. Negendank and Collier [9] claimed a decreased K^+ level while Quastel and Kaplan [10] and Averdunk [11, 12] observed an increased level, during the same period. However, Segel et al. [13] showed that one of the early effects of mitogenic stimulation was to render the lymphocyte membrane leaky, such that K^+ would be exchanged for other ions in the medium during washing before determination of internal ions, and this could lead to factitious interpretation of data. Indeed, more accurate determinations of intracellular K^+ levels by Segel et al. [14] and Hamilton and Kaplan [15] showed recently that mitogenic lectins increased K^+ active influx and K^+ passive efflux to the same extent, keeping constant the potassium intracellular concentration.

To know if active potassium transport was involved in the mitogenic action it was important to see if these mitogens had a direct effect on ($Na^+ + K^+$)-ATPase activity. Here again published data present many discrepancies, mainly because of the above-mentioned difficulties to determine this enzymatic activity, especially in the presence of mitogenic lectins which strongly enhanced ouabain-insensitive Mg^{2+}-ATPase [3, 12, 16]. We intended to obtain lymphocyte plasma membranes with high specific ($Na^+ + K^+$)-ATPase activity, depleted as much as possible from Mg^{2+}-ATPase, and to solubilize ($Na^+ + K^+$)-ATPase in order to investigate a possible direct effect of lectins.

The preparation and characterization of lymphocyte plasma membranes from mesenteric lymph nodes of young pigs have been described elsewhere [3]. ($Na^+ + K^+$)-ATPase activity was determined by calculating the difference between total ATPase activity measured in the presence of 3 mM ATP (Sigma, Grade I), 3 mM $MgCl_2$, 120 mM NaCl, 30 mM KCl, 0.1 mM ethyleneglycol bis(α-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), 60 mM Tris·HCl (pH 7.5) and ouabain-insensitive Mg^{2+}-ATPase measured in the same medium supplemented with $5 \cdot 10^{-4}$ M ouabain. The above ionic concentrations were previously shown to give optimal activities. ATP hydrolysis was determined by measuring the amount of released inorganic phosphate (P_i) as described elsewhere [3]. In the presence of Lubrol WX ($Na^+ + K^+$)-ATPase activity was measured according to Nakao et al. [17]. Specific activities were expressed as μmol P_i/h per mg protein. Protein concentrations were determined by the method of Lowry [18] using bovine serum albumin as a standard.

($Na^+ + K^+$)-ATPase specific activity of crude membranes is 0.7 μmol P_i/h per mg protein and accounts for 9% of total ATPase activity. NaI treatment is

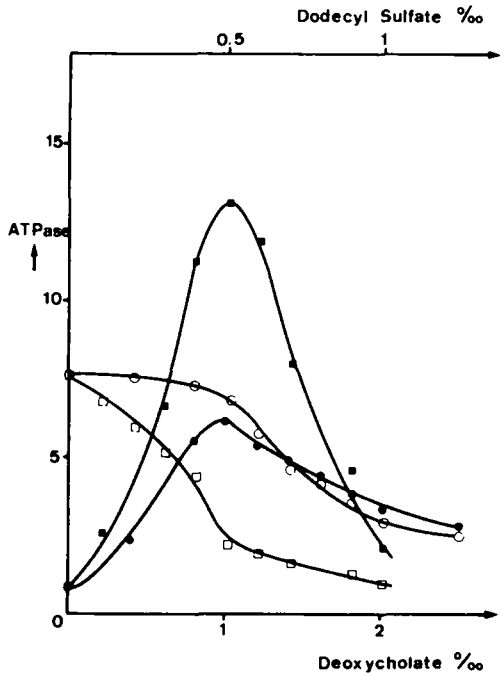

Fig. 1. Detergent effects on ATPase specific activities (μmol P_i/h per mg protein). Lymphocyte plasma membranes (1.2 mg protein/ml) in 2 ml 60 mM Tris · HCl/3 mM EDTA buffer (pH 7.5) were incubated 20 min with stirring in the presence of various detergent concentrations at 4°C. After 1 h centrifugation at 40 000 × *g*, ATPase activities of the pellet were determined: ○—○, Mg^{2+}-ATPase; ●—●, $(Na^+ + K^+)$-ATPase in the case of deoxycholate treatment; □—□, Mg^{2+}-ATPase; ■—■, $(Na^+ + K^+)$-ATPase in the case of dodecyl sulfate treatment.

generally used to inhibit Mg^{2+}-ATPase and determine $(Na^+ + K^+)$-ATPase more easily; this method is not suitable for lymphocytes as NaI inhibits both ATPase activities. After treatment with increasing concentrations of ionic detergent (sodium deoxycholate or dodecyl sulfate) and 1 h centrifugation at 40 000 × *g*, $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase activities in the membrane pellet are as presented in Fig. 1. 0.1% deoxycholate concentration gives a maximum value for $(Na^+ + K^+)$-ATPase (6.2 μmol P_i/h per mg protein) while Mg^{2+}-ATPase is slightly inhibited (10%). Sodium dodecyl sulfate treatment is much more selective: $(Na^+ + K^+)$-ATPase reaches a maximum specific activity (13.2 μmol P_i/h per mg protein) with 0.05% detergent; this value is 18 times higher than the initial one and represents 85% of the total ATPase activity. Under these conditions Mg^{2+}-ATPase has only 30% of its initial specific activity. Higher detergent concentrations lead to a complete inactivation of both ATPase activities, very likely by excessive delipidation of plasma membranes. The dodecyl sulfate concentration giving maximum $(Na^+ + K^+)$-ATPase activity depends on protein concentration: 0.03, 0.05 and 0.07% dodecyl sulfate for 0.6, 1.2 and 2.4 mg protein per ml, respectively. Dodecyl sulfate treatment at 4°C and pH 7.5 gives the best specific activity; EDTA has no effect, even when 3 mM Mg^{2+} and 5 mM ATP were added to protect the ATPase active site [19]. Under these last conditions only the Mg^{2+}-ATPase inhibition is decreased (40%).

TABLE I

7 mg membrane proteins were treated with 0.05% dodecyl sulfate, as described in Fig. 1. Then the 40 000 × *g* pellet was treated with 0.45% Lubrol WX (pH 7.5) at room temperature for 20 min and centrifuged at 100 000 × *g*. Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities and protein concentrations were determined in these various fractions.

Fractions	Proteins (mg)	Proteins (%)	Specific activities (μmol P_i/h per mg protein)		Specific activity $(Na^+ + K^+)$-ATPase / Specific activity Mg^{2+}-ATPase	Ratio
			Mg^{2+}-ATPase	$(Na^+ + K^+)$-ATPase		
Untreated membranes	7	100	7.6	0.72	0.095	
Dodecyl sulfate-treated membranes	7	100	2.50	8.53	3.40	
40 000 × *g* pellet after dodecyl sulfate treatment	3.2	45.7	2.30	13.2	5.80	
40 000 × *g* supernatant after dodecyl sulfate treatment	3	42.8	0.32	0.78	2.43	
100 000 × *g* pellet after Lubrol WX treatment	0.9	12.8	1.50	4	2.66	
100 000 × *g* supernatant after Lubrol WX treatment	1.15	16	2.1	21	10	

TABLE II

DETERMINATION OF ATPase ACTIVITIES

ATPase activities are shown of the 40 000 × *g* pellet obtained after 0.05% dodecyl sulfate treatment of membranes (under conditions of Fig. 1) and of the 100 000 × *g* supernatant after Lubrol WX treatment of this pellet (under conditions of Table I), in the presence of various metal ions and ouabain.

Incubation media	Specific activity (μmol P_i/h per mg protein)	
	40 000 × *g* pellet after dodecyl sulfate treatment	100 000 × *g* supernatant after Lubrol WX treatment
3 mM Mg^{2+}/0.1 mM EGTA	2.30	2.10
3 mM Mg^{2+}/120 mM Na^+/0.1 mM EGTA	2.29	2.20
3 mM Mg^{2+}/30 mM K^+/0.1 mM EGTA	2.35	2.20
3 mM Mg^{2+}/120 mM Na^+/30 mM K^+/0.1 mM EGTA	15.50	23.10
3 mM Mg^{2+}/120 mM Na^+/30 mM K^+/$5 \cdot 10^{-4}$ M ouabain/0.1 mM EGTA	2.32	2.15

The 18-fold enrichment in $(Na^+ + K^+)$-ATPase activity of lymphocyte plasma membranes by dodecyl sulfate treatment cannot be explained only by the disruption of inside-out vesicles (about 50% of total vesicle population [20]) as this disruption would make only twice as many enzymatic sites accessible for the substrate. Active site unmasking by dodecyl sulfate must result from removing membrane components. Table I shows that 54% of membrane proteins were solubilized under conditions giving maximal $(Na^+ + K^+)$-ATPase activity.

$(Na^+ + K^+)$-ATPase can be further purified by a 20 min treatment of the high activity membrane fraction with a non-ionic detergent, 0.45% Lubrol WX (pH 7.5) at room temperature. After 1 h centrifugation at 100 000 × *g* $(Na^+ + K^+)$-ATPase activity is found in the supernatant fraction, as shown by Nakao et al. [17] for pig brain membranes. The specific activity (21 μmol P_i/h per mg protein) of $(Na^+ + K^+)$-ATPase and the ratio $(Na^+ + K^+)$-ATPase/Mg^{2+}-ATPase are 30- and 100-fold higher, respectively, than in untreated membranes. However, it must be noticed that the recovery of $(Na^+ + K^+)$-ATPase is low, probably because of denaturation induced by the detergent.

As for the 0.1% deoxycholate treatment [3], we confirmed the synergy between Na^+ and K^+ of the ouabain-sensitive ATPase activity in the 40 000 × *g* pellet after dodecyl sulfate treatment and in the 100 000 × *g* supernatant after Lubrol WX treatment. Results in Table II confirm that the ouabain-sensitive ATPase activity corresponds to the $(Na^+ + K^+)$-ATPase.

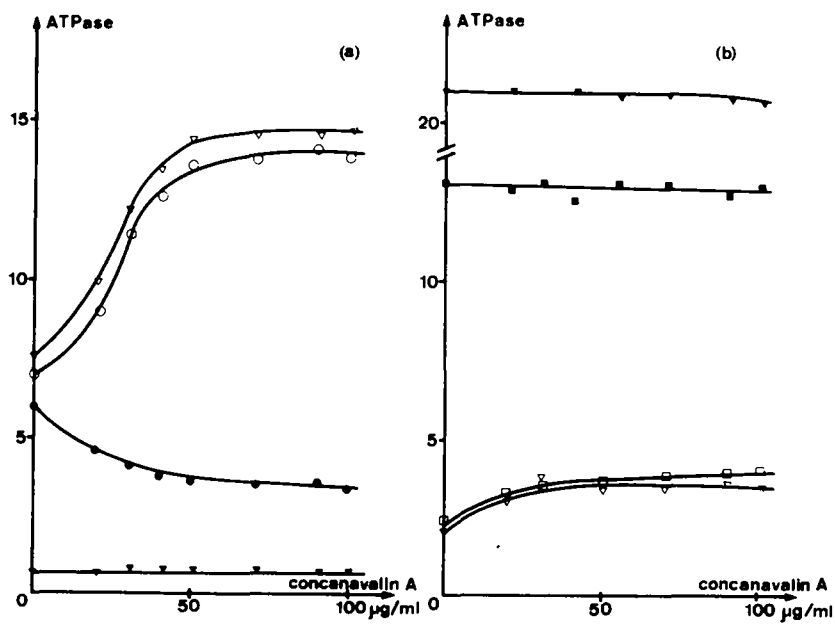

Fig. 2. Concanavalin A effects on ATPase specific activities (μmol P_i/h per mg protein). Various plasma membrane fractions (50 μg protein/ml) were preincubated 20 min at 37°C in the buffer used for ATPase determination in the presence of concanavalin A. ATPase activities were then measured. a. Mg^{2+}-ATPase (▽—▽) and $(Na^+ + K^+)$-ATPase (▼—▼) activities of untreated membranes; Mg^{2+}-ATPase (○—○) and $(Na^+ + K^+)$-ATPase (●—●) activities of the 40 000 × *g* pellet after deoxycholate treatment. b. Mg^{2+}-ATPase (□—□) and $(Na^+ + K^+)$-ATPase (■—■) activities of the 40 000 × *g* pellet after dodecyl sulfate treatment; Mg^{2+}-ATPase (▽—▽) and $(Na^+ + K^+)$-ATPase (▼—▼) activities of the 100 000 × *g* supernatant after Lubrol WX treatment.

The various membrane fractions displaying ($Na^+ + K^+$)-ATPase activity were studied by electrophoresis on polyacrylamide gel (10%) in the presence of 0.1% dodecyl sulfate, after solubilization in 2% dodecyl sulfate plus 5% 2-mercaptoethanol, according to Chavin et al. [21]. Although the Lubrol-solubilized fraction had the highest ($Na^+ + K^+$)-ATPase specific activity (21 μmol P_i/h per mg protein), the number of protein components on the polyacrylamide gel was not notably reduced, and a further purification would be necessary.

However, with the various treatments of lymphocyte plasma membranes we are able now to determine high ($Na^+ + K^+$)-ATPase activity almost free from ouabain-insensitive ATPase activity, and to investigate the effects of concanavalin A, a potent mitogenic lectin, after a 20 min incubation at 37°C. With untreated membranes, as already stated [3], concanavalin A markedly enhanced Mg^{2+}-ATPase and had no apparent effect on ($Na^+ + K^+$)-ATPase activity (Fig. 2a). On deoxycholate-treated membranes which still have a high Mg^{2+}-ATPase activity, concanavalin A again stimulates Mg^{2+}-ATPase and inhibits ($Na^+ + K^+$)-ATPase. This inhibition reaches a maximum (40%) for a lectin concentration (50 μg/ml) which corresponds to the maximum of Mg^{2+}-ATPase stimulation; at higher concanavalin A concentration no further increase of Mg^{2+} ATPase, nor decrease of ($Na^+ + K^+$)-ATPase, are evidenced (Fig. 2a). Both effects of concanavalin A are prevented or reversed by methyl-α-D-mannopyranoside which specifically binds the lectin. On membrane fractions with high ($Na^+ + K^+$)-ATPase and low Mg^{2+}-ATPase activities (40 000 × *g* pellet after dodecyl sulfate treatment and 100 000 × *g* supernatant after Lubrol WX treatment) concanavalin A has no effect on ($Na^+ + K^+$)-ATPase while it still enhances the residual Mg^{2+}-ATPase activity (Fig. 2b).

Based on these studies, we concluded that a ($Na^+ + K^+$)-ATPase activity can be evidenced in lymphocyte plasma membranes from pig mesenteric lymph nodes, after detergent treatment. This activity is not directly affected by concanavalin A. The inhibition observed (Fig. 2a) when ouabain-insensitive ATPases are present simultaneously in the membranes might result from interactions between membrane proteins during the Mg^{2+}-ATPase stimulation by the lectin.

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