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Alamethicin as a permeabilizing agent for measurements of Ca^{2+}-dependent ATPase activity in proteoliposomes, sealed membrane vesicles, and whole cells

Vladimir B. Ritov, Maira K. Murzakhmetova, Irene L. Tverdislova, Elizaveta V. Menshikova, Andrey A. Butylin, Tatiana Yu. Avakian and Leonid V. Yakovenko

Lomonosov Moscow State University, Moscow (Russian Federation)

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The channel-forming antibiotic peptide alamethicin was used in measurements of Ca-ATPase activity in sarcoplasmic reticulum (SR) vesicles, proteoliposomes containing Ca^{2+}-ATPase from SR, and native human platelets. Alamethicin was used as a permeabilizing agent providing for a free access of the whole cells or sealed vesicles interiors for ions, ATP, and other reactants. The experiments were carried out with the use of alamethicin preparations obtained in our laboratory and that purchased from the Upjohn Company (antibiotic U-22,324). A comparative study of the effects of Ca^{2+}-ionophore A23187 and alamethicin was performed on native SR vesicles containing Ca^{2+}-ATPase molecules with right orientation and SR vesicles treated with cholate in order to randomize Ca^{2+}-ATPase molecules orientation in the membrane. It was found out that alamethicin, like A-23187, prevents the ATP-dependent Ca^{2+} accumulation by the vesicles and therefore activates the Ca^{2+}-ATPase. Maximal specific activities of the Ca^{2+}-ATPase in native SR vesicles in the presence of either alamethicin, or A23187, or both of them, are equal in all cases to 20 activity units (μmol P_i per min per mg protein). The operative range of alamethicin concentrations is 5–25 μg/ml and is a little wider than that for A23187. The ATPase activity of the SR vesicles treated with cholate reached 20 units in the presence of alamethicin while in the presence of A23187 it was only 10 units. These data suggest that alamethicin unlike A23187 allows ATP to reach the ATPase's active centers from the inside of the SR vesicles with 'randomized' membranes, the ATP transport through the membrane not being the rate-limiting stage of ATP hydrolysis. It was shown that diffusion flux of ATP through a BLM in the presence of alamethicin may reach 10% of the flux through the hole without the BLM. With the use of alamethicin it was found out that the quality of randomization of the ATPase molecules orientation in the membrane depends on the proteoliposome preparation technique. The ATP transport through the alamethicin pores makes possible the use of alamethicin in accurate measurements of Ca^{2+}-ATPase activity in whole cells. A method was developed for determination of the Ca^{2+}-ATPase activity of whole platelets. The membrane-bound Ca^{2+}-ATPase activity of human platelets was found to be 90–100 nmol P_i per min per mg protein.

1. Introduction

Measurement of a cytosolic enzyme activity usually doesn't constitute a serious problem. But in the case of membrane-bound enzymes it may be difficult to provide free accesses of substrates and activators to their binding sites. Cell desintegration does not solve the problem because it results usually in the membrane vesiculation with unknown probabilities of right and inside-out orientations of the membrane and enzymes. One of the approaches to the evaluation of the actual activities of membrane-bound enzymes consists in use of permeabilizing agents.

Jones et al. [1] were first who reported the use of alamethicin as a permeabilizing agent in measurements of activities of membrane-bound enzymes, Na^+/K^+-ATPase and adenylate cyclase in cardiac sarcolemmal vesicles from the dog myocardium. Alamethicin (antibiotic U-22,324) has a bactericidal effect on Gram-positive bacteria and as a membranotropic agent it belongs to the class of channel-forming peptides. It forms aqueous pores in model and biological mem-

Correspondence to: V.B. Ritov, School of Biology, Lomonosov Moscow State University, Moscow 119899, Russian Federation.

branes [2,3]. The pore formation at low concentrations is voltage dependent [2,4].

The objective of our work was to investigate the possibility of the use of alamethicin as a permeabilizing agent for measurements of Ca^{2+}-ATPase activity in proteoliposomes, sealed vesicles, and intact human platelets. Besides, alamethicin-induced transfer of ATP through planar bilayer lipid membranes (BLMs) was studied directly.

2. Materials and Methods

2.1. Chemicals. Imidazole, Tris, EGTA, Hepes, dioleoylphosphatidylcholine (DOPC), glycerol, NaN_3, cholic acid Na-salt (analytical grade) were purchased from Serva; creatin kinase, pyruvate kinase, lactate dehydrogenase, histidine, ATP and NADH were obtained from Reanal (Hungary); sucrose, KCl and sodium oxalate were from Merck; phospholipase A_2 (bee venom), BSA (fatty acid free), phosphocreatine, phosphoenolpyruvate, asolectin, egg phosphatidylcholine (EPC), Lubrol PX, quin2, A23187, sodium oxalate, NaCl, $MgCl_2$ and $CaCl_2$ were from Sigma. EDTA was from Chemapol (Czechoslovakia). Alamethicin (antibiotic U-22,324) was from the Upjohn Company. Alamethicin A was isolated from the fungus *Trichderma viridae* with the use of a technique developed earlier in our laboratory. Alamethicin was dissolved in 60% ethanol to the concentration of 20 mg/ml. The resulting alamethicin solution was added to the assay medium (0.1–2.5 μl per ml of the assay medium).

2.2. Methods. Sarcoplasmic reticulum membranes (SR) were isolated from white skeletal muscles of rabbit hind limbs [5]. Platelets were obtained from freshly collected human blood [6], the pellets suspended in Tyrode solution and used immediately. The microsomal fraction was obtained from rat brains after removal of the cerebellums and homogenization [7].

Membrane vesicles with random orientation of Ca^{2+}-ATPase molecules in the membrane were prepared by means of treatment of SR membranes with cholate at a protein-to-cholate concentration ratio 1:0.6 which was followed by dialysis [8]. Proteoliposomes with Ca^{2+}-ATPase from SR and DOPC or EPC were prepared either chromatographically [9] or by the dilution technique [10].

Ca^{2+}-ATPase activities of human platelets, rat brain microsomal membranes, and SR membranes, were measured spectrophotometrically in the coupled enzymatic system with NADH oxidation [11] at constant temperature and under continuous stirring. The incubation mixture contained 130 mM NaCl, 20 mM KCl, 5 mM NaN_3, 3 mM $MgCl_2$, 10 mM Hepes, 0.2 mM NADH, 0.25 mM phosphoenolpyruvate, 50 μM of 3 mM ATP, pyruvate kinase (2 I.U.), and lactate dehydrogenase (6 I.U.), at pH 7.2 and 37°C. The medium for platelet Ca^{2+}-ATPase assay contained additionally 0.5 mg/ml BSA, 1 mM $CaCl_2$ and 1 mM EGTA, and that one for microsomal Na^+/K^+-ATPase contained additionally only 1 mM EGTA.

The Ca^{2+}-ATPase activity of SR and proteoliposomes was measured pH-metrically [12] in the medium of the following composition: 100 mM NaCl, 4 mM $MgCl_2$, 5 mM sodium oxalate, 30 μM $CaCl_2$, 2 mM ATP, 2.5 mM imidazole (pH 7.1–7.2 at 37°C).

The uptake and release of Ca^{2+} by SR vesicles were measured by quin2 fluorescence [13] in a medium (2 ml) containing 100 mM KCl, 1.5 mM $MgCl_2$, 2 mM ATP, 0.1 mM KH_2PO_4, 7 mM phosphocreatine, 2–3 I.U. creatine kinase, 20 μM Ca^{2+} (including contaminant Ca^{2+}), 25 μM quin2, 50–90 μg/ml of SR membrane protein and 10 mM Hepes (pH 6.8) (28°C). The measurements were performed on a Hitachi 850 spectrofluorimeter in a thermostated cell supplied with a vibrator.

The bilayer lipid membranes (BLM) were formed from 40 mg/ml asolectin (total lipid fraction of soybeans) solution in n-heptane on a 0.5 mm hole in the partition between the two compartments of the conventional teflon cell. The bathing solution contained 0.1 M KCl, 50 mM Tris-HCl (pH 7.3 at 20°C). Alamethicin was added to the final concentration of 2.4 μg/ml. AgCl electrodes were used for conductance and capacitance measurements. The ATP transmembrane transfer was monitored spectrophotometrically

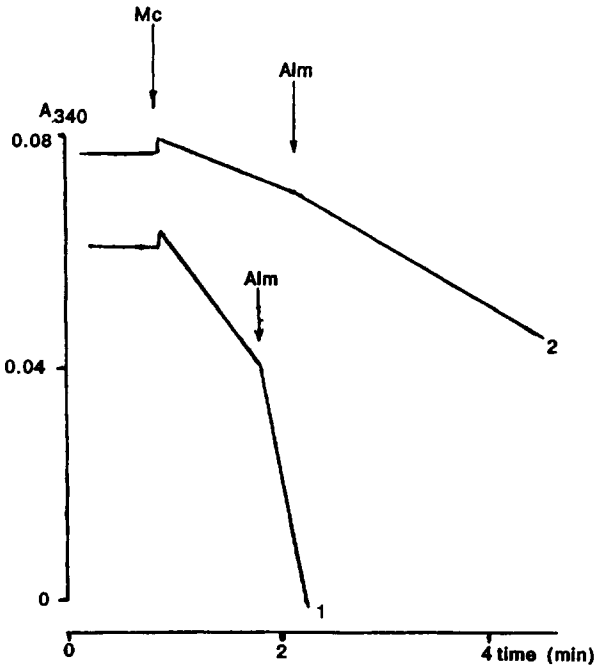

Fig. 1. Activation of brain microsomes (Mc) Na^+/K^+-ATPase induced by alamethicin in the absence (1) and in the presence (2) of 0.5 mM oubain. Alamethicin (Alm), 25 μg/ml; ATP, 3 mM; total protein, 3.5 μg/ml.

at $\lambda = 259$ nm. Protein was assayed by the biuret reaction.

3. Results and Discussion

Alamethicin A as well as the Upjohn Company preparation both are able to unmask latent Na^+/K^+-ATPase activity of the membrane vesicles.

Fig. 1 shows the kinetics of ATP hydrolysis by the microsomal fraction of rat brain under optimal conditions for Na^+/K^+-ATPase reaction and with both Ca^{2+}-ATPase and the ATPase of mitochondrial fragments inhibited. Alamethicin at 25 μg/ml sharply activates the reaction, which was not the case if oubain was present. Jones et al. suggested that the activation is due to involvement of some latent Na^+/K^+-ATPase in the reaction. This latent enzyme is that fraction of Na^+/K^+-ATPase which has its active sites inside the vesicles. In the presence of alamethicin ATP can penetrate into the vesicles through the induced pores and be hydrolised by that normally idle enzyme [1]. Such possibility follows also from our data on alamethicin-induced ATP transfer through planar BLMs.

Direct permeability of the alamethicin channels for ATP was estimated after ATP was added to the solution in one of the cell compartments (the cis-compartment) to the final concentration of 34.6 mM. ATP concentrations were measured in 5-μl aliquotes taken from the trans-compartment. Acceleration of the diffusion was achieved by application of a DC voltage to the electrodes. Without the membrane, the ATP concentration in the trans-compartment which was held under positive electric potential increased from 0 to up to 6.5 mM for 50 min (see Fig. 2). Unmodified membranes

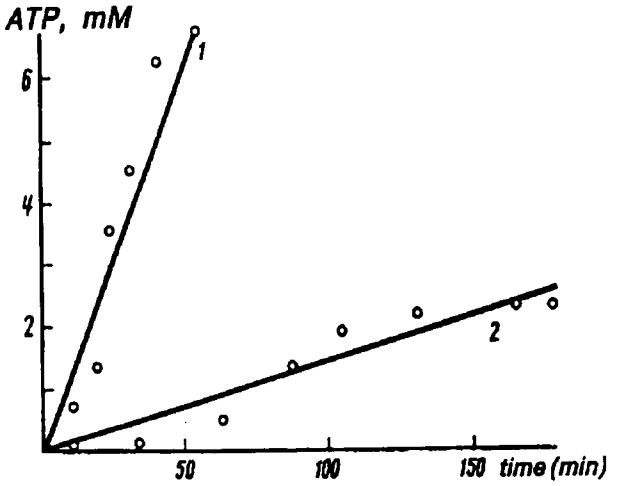

Fig. 2. Time dependence of the ATP concentration in the trans-compartment for free diffusion of ATP through the hole in the partition (1) and for the diffusion through a BLM in the presence of 2.4 μg/ml alamethicin (2).

were impermeable for ATP. In the presence of 2.4 μg/ml alamethicin the membrane conductance increased from 25 πS to 50 μS and ATP transfer from cis- into trans-compartment was observed. Estimation of the diffusion of ATP through the alamethicin pores gives 10% of that for the hole without the membrane.

The data given here evidence that ATP molecules can penetrate through the alamethicin pores in the BLM. Probably, they would as well penetrate through the bilayer areas in isolated or reconstituted biological membranes which are usually obtained as sealed vesicles.

It was found out that alamethicin at 5 μg/ml blocks completely the ATP-dependent Ca^{2+} accumulation by

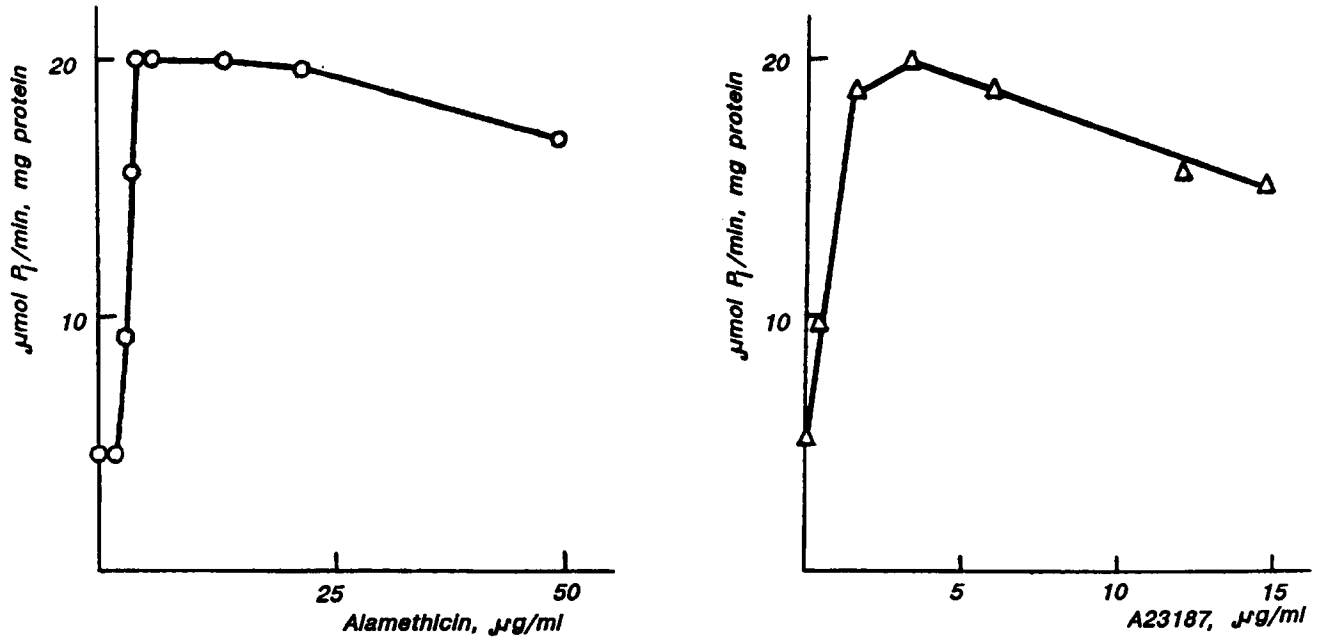

Fig. 3. Effects of alamethicin and A23187 on Ca^{2+}-ATPase activity of sarcoplasmic reticulum (SR) vesicles at 2 mM ATP, 5 μg/ml protein.

TABLE I

Influence of alamethicin on the Ca^{2+}-ATPase activity of membranes of the sarcoplasmic reticulum (SR) and membranes SR treated with cholate

Preparations	Ca^{2+}-ATPase activity (μmol P_i per min per mg protein)			
	control	alamethicin (5 μg/ml)	A23187 (3 μg/ml)	A23187 + alamethicin (5 μg/ml)
Membranes SR	5.4	19.8	19.7	19.9
Membranes SR treated with cholate	–	–	10.8	19.6

SR vesicles and induced release of Ca^{2+} deposited in them (data not shown).

Fig. 3 shows that alamethicin at 5 μg/ml activates Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) membranes. The ATPase active sites are on the outer surface of the membrane therefore the activating effect of alamethicin is not related to the alamethicin-induced of passage ATP through the SR membranes. It is due to an increase of Ca^{2+}-permeability of the SR membranes induced by alamethicin. As follows from the data shown in Fig. 3 and Table I same effect as alamethicin had Ca^{2+}-ionophore A23187. Moreover, in the presence of A23187 alamethicin had no additional effect of activation. The ATPase activation at an increase of Ca^{2+}-permeability is due to prevention of the ATPase inhibition by the intravesicular calcium [14]. Perhaps, in applications to ATPase activity determination alamethicin has that advantage in comparison with A23187 that its range of optimal concentrations is wider. Besides, adsorption of alamethicin on the glass walls of the cell in which the ATPase activity is being measured is less than that of A23187. Some decrease of Ca^{2+}-ATPase activity found at high alamethicin concentrations (50 μg/ml) might result from the perturbation of molecular organization of the lipid bilayer [15]. Liposomes bound alamethicin with molar ratio greater than 0.4 (per mole of lipid) at saturating ionophore concentrations [16]. The binding of alamethicin must be taken into account any time alamethicin is used as a permeabilizing agent.

To make clear whether it is possible to use alamethicin for quantitative evaluation of Ca^{2+}-ATPase activity in membrane vesicles which contain the enzyme with some molecules having 'wrong' orientation we studied the influence of alamethicin on the SR membrane Ca^{2+}-ATPase treated with cholate with following dialysis for the detergent removal.

Fleischer et al. [17,18] have shown with the use of electron microscopy that cholate treatment of SR membranes resulted in random orientation of the ATPase in them. Therefore, if alamethicin increases the SR membrane permeability for ATP to such an extent that it ceases to limit the ATPase reaction rate then in the case of cholate-treated membranes alamethicin should increase the ATPase activity to the level characteristic of the untreated membranes. As may be seen from Table I SR membranes incubated with cholate (0.6 mg per 1 mg of protein) and subsequently dialysed retain exactly half of the activity which should be the case if half of the enzyme molecules are oriented with their active sites inside the vesicles. In the presence of alamethicin the enzyme activity increases by a factor of two, i.e., alamethicin restores the original activity of the enzyme. If membranes were treated with cholate at a lower concentration (0.25 mg per 1 mg of protein) than alamethicin did not affect the enzyme activity. This implies that alamethicin may be used for quantative determination of the Ca^{2+}-ATPase activity in proteoliposomes and in combination with the ionophore A23187 it may be used in determination of the fraction of 'wrongly' oriented Ca^{2+}-ATPase molecules in the vesicle membranes.

We tried to find out dependence of Ca^{2+}-ATPase orientation on the way the proteoliposomes were obtained. Two methods were used for the proteoliposomes preparation: the dilution method [10], when the ATPase plus lipid solution in cholate was diluted 200–400 times (and the ATPase activity was then determined), and the chromatographic method [9], when the mixture of the enzyme and lipids in cholate solution was passed through a small column filled with Dowex 2X4 in the chloride form. The results of these experiments are given in Table II. All measurements were performed in the presence of A23187. As may be seen from the Table II the ATPase activity of the proteoliposomes obtained by the dilution method increases by a factor of about two in the presence of

TABLE II

Effects of alamethicin on the Ca^{2+}-ATPase activity of proteoliposomes

Procedure	Preparations [a]	Protein/lipid/cholate in reconstitution mixture (mg/ml)	Ca^{2+}-ATPase activity (μmol P_i per min per mg protein)	
			A23187 (3 μg/ml)	A23187 (3 μg/ml) + alamethicin (5 μg/ml)
Chromatography	DOPC-ATPase	2:2:3	26.2	31.2
	EPC-ATPase	2:2:3	24.7	28.7
	EPC-ATPase	0.2:2:3	18.0	30.0
	EPC-ATPase	1:10:5	8.9	31.2
Dilution	DOPC-ATPase	2:2:3	15.4	29.8

[a] Preparations of DOPC-, EPC-ATPases are obtained by reconstruction of delipidated ATPase in proteoliposomes with dioleoylphosphatidylcholine or egg phosphatidylcholine chromatographically [9] or by the dilution technique [10].

alamethicin, i.e., such preparations have equal amounts of right side and inside-out oriented enzyme. The effect of alamethicin on the Ca^{2+}-ATPase activity of proteoliposomes prepared by means of chromatography depends on the lipid-to-protein concentration ratio and on the concentration of the lipid. At the lipid concentration not exceeding 2 mg/ml and lipid-to-protein ratio 1 : 1, proteoliposomes formed contained about 20% of Ca^{2+}-ATPase molecules with 'wrong' orientation. When lipid-to-protein ratio was 10 : 1 and the concentration of lipid was 10 mg/ml then Ca^{2+}-ATPase activity of the proteoliposomes increased 3–4-times in the presence of alamethicin. Most plausible seems the explanation that at high lipid concentrations multilayer proteoliposomes were formed, and alamethicin induced an increase in their permeability for ATP. It is very important that the Ca^{2+}-ATPase specific activity in the presence of alamethicin didn't depend on the way of preparation of the proteoliposomes. This allows for the use of alamethicin for measurements of the Ca^{2+}-ATPase activity of proteoliposomes prepared by different methods as well as for estimation of the Ca^{2+}-ATPase (and, perhaps, other enzymes) active centres accessability for water soluble substances. It was shown earlier that alamethicin may also be used for the permeabilization of proteoliposomes containing Na^+/K^+-ATPase [19]. Alamethicin-induced permeabilization of proteoliposomes made with the single phospholipid DOPC gives evidence that alamethicin as permeabilizing agent doesn't require specific lipids. This is a distinctive property of alamethicin incomparison with polyenic antibiotics such as nystatin the effects of which depend on the presence of steroles [20–22].

Alamethicin can also be used in accurate measurements of the Ca^{2+}-ATPase activity of intact human platelets. The platelets were obtained from healthy donors, and the ATPase activity was measured spectrophotometrically with the use of NADH oxidation-coupled enzymatic system. It was found out that permeabilizing concentration of alamethicin in this case was 70–80 μg/ml which is three times that for rat brain microsomes and 15–20 times that for SR vesicles [23]. This high concentration of alamethicin would have inhibited Ca^{2+}-ATPase but we have found out that in the presence of Lubrol PX at a low concentration (25 μg/ml) same permeabilizing effects could be achieved with only 20 μg/ml of alamethicin.

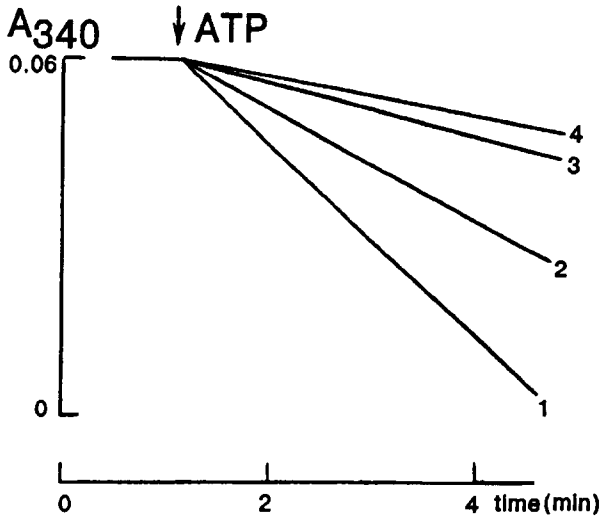

Fig. 4. ATP hydrolysis by whole human platelets in the presence 1 mM $CaCl_2$, 1 mM EGTA (1, 3) or 1 mM EGTA (2, 4) at 50 μM ATP, 20 μg/ml alamethicin, 25 μg/ml Lubrol PX, 100 μg/ml platelets protein. Platelets were preincubated for 5 min in the absence (1, 2) and in the presence (3, 4) of phospholipase A_2 (1 μg/ml).

The medium with alamethicin and Lubrol PX did not inhibit the enzyme activity as may be seen from the data in Table III. Rat brain microsomes or sarcoplasmic reticulum vesicles retain their specific Ca^{2+}-ATPase activity upon injection into the suspension of platelets under experimental conditions.

The platelets specific Ca^{2+}-ATPase activity was about 90–100 nmol P_i per min per mg protein or per $0.5 \cdot 10^8$ cells. This activity is provided by the membrane bound enzyme. Hydrolysis of the cell membrane phospholipids by phospholipase A_2 from the been venom with concurrent binding of the hydrolysis products (fatty acids and lysophospholipids) by bovine serum albumin results in practically complete loss of the Ca^{2+}-ATPase activity (Fig. 4). This shows that the contribution of non-membrane Ca^{2+}-ATPases (such as actomyosin) into the total activity is insignificant. As it is known that the transport Ca^{2+}-ATPases demonstrate their activity only in the presence of lipids [24–26].

The results obtained suggest that alamethicin as a permeabilizing agent could be recommended for accurate measurements of membrane bound Ca^{2+}-ATPases activities.

TABLE III

The Ca^{2+}-ATPase of the sarcoplasmic reticulum vesicles and the Ca-ATPase of the rat brain microsomes used as 'internal standard', in human platelet suspensions

Incubation medium; 50 μM ATP, platelets at 100 μg of protein/ml, SR at 0.2 μg of protein/ml, brain microsomes at 5 μg of protein/ml. The ATPase activities of SR and brain microsomes in the absence of platelets were both measured without additions of Lubrol PX and with alamethicin concentrations 5 and 25 μg/ml, respectively.

Preparations	Ca^{2+}-ATPase activity (μmol P_i per min per mg protein)
SR vesicles	14.6
SR vesicles in platelet suspension	16.7
Rat brain microsomes	0.6
in platelet suspensions	0.58

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