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DETERGENT-LIKE EFFECTS OF ALAMETHICIN ON LYMPHOCYTE PLASMA MEMBRANES*

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SUMMARY Alamethicin enhanced adenylate cyclase and Na^+-K^+-ATPase activities in microsomes and purified plasma membranes from pig lymphocytes. As this stimulation was also found in inside-out vesicles obtained from these membranes and as we showed that lymphocyte membrane vesicles are not impermeable to 5'AMP, ATP and concanavalin A, it appears clearly that alamethicin effects are not related to its channel-forming properties, but rather to its detergent-like character. Indeed sodium dodecylsulfate and Lubrol PX mimicked alamethicin effects. Moreover alamethicin treatment of plasma membranes induced protein and phospholipid solubilization.

INTRODUCTION The problem of membrane sidedness is a very important one in studies of membrane-bound enzymatic activities. It is tightly related to the problem of membrane vesicle leakage. Indeed it would be impossible to measure the hormonal activation of adenylate cyclase in sealed vesicles, as the catalytic site is on the inner face and the hormonal receptor on the outer face : so in right-side out (RSO) vesicles the substrate ATP could not reach its catalytic site, while in inside-out (IO) vesicles the receptor site could be reached only by molecules able to cross the membrane barrier. Na^+-K^+-ATPase has its catalytic site on the inner membrane surface and could be measured only in IO vesicles, while 5'nucleotidase which is an ectoenzyme (1) could be measured only in RSO vesicles. As membrane preparations are generally a mixture of RSO and IO vesicles, one would expect a latent activity for these enzymes if vesicles were not leaky. This latent activity would be unmasked by membrane treatments allowing the substrate to enter the vesicles : channel formation or partial solubilization.

In a recent paper (2) we described the unmasking of latent Na^+-K^+-ATPase activity in lymphocyte plasma membranes by mild SDS treatment. The magnitude of the activity enhancement (18 times) demonstrated unambiguously that latent ATPase activity is much larger than RSO vesicle activity as for our preparation the ratio of RSO over IO vesicles is about 50:50 (3). Therefore the role of SDS is not restricted to opening channel in membrane vesicles.

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Alamethicin is a channel-forming antibiotic ionophore. Jones *et al* (4) showed that alamethicin prevented Ca^{2+} uptake by cardiac membrane vesicles, while stimulating the Ca^{2+}-ATPase activity of these membranes 65 % above control. For these authors, alamethicin, like A 23187, exerts its effects by increasing the Ca^{2+} permeability of membrane vesicles. More recently Besch *et al* (5) reported that alamethicin markedly increased adenylate cyclase and Na^{+}-K^{+}-ATPase activities of cardiac membrane vesicles in a concentration-dependent manner and that the non-ionic detergent Lubrol PX mimicked the activation produced by alamethicin. They interpreted these results by the formation of channels large enough to allow penetration of ATP and NaF and thus eliminating effectively the sidedness of adenylate cyclase associated with these vesicles.

In this paper we show that alamethicin strongly increases Na^{+}-K^{+}-ATPase and adenylate cyclase activities of lymphocyte plasma membranes and probably acts as a detergent since it removes phospholipids and proteins from these membranes. Even if we cannot exclude that alamethicin can form channels in the membranes (a property also exhibited by some detergents (6)), this possibility alone cannot account for the magnitude of its effect in lymphocyte membranes, the more so as these membrane vesicles are shown to be leaky in the absence of alamethicin.

MATERIALS AND METHODS Preparation of lymphocyte plasma membranes from pig mesenteric lymph nodes was reported previously (7) as well as membrane characterization by electron microscopy and enzymatic markers. Microsomes were obtained by 30,000×g centrifugation (1h) after elimination of cell debris, nuclei and mitochondria.

Enzymatic activities were determined under conditions reported earlier : 5' nucleotidase (1,8), Na^{+}-K^{+}-ATPase and Mg^{+}-ATPase (2,7). Adenylate cyclase was assayed according to Salomon *et al* (9) : the reaction mixture contained 25 mM Tris,HCl, pH 7.5, 25 units per ml creatine phosphokinase, 15 mM creatine phosphate, 2.5 mM $MgCl_2$, 50 µM GTP, 1 mM cyclic AMP, 0.5 mM ATP and [α-^{32}P]-ATP ($1.5\text{-}3\times10^{6}$ cpm per assay). The contamination of our preparations by Golgi vesicles was checked by measuring their thiamine pyrophosphatase activity, as described by Monneron and d'Alayer (10). Protein concentrations were measured by Lowry's method (11).

Preparation of IO vesicles was achieved by filtration of either microsomes or purified plasma membranes on concanavalin A - Sepharose 4B : the unretarded fraction is constituted of IO vesicles. RSO + IO vesicles are the fraction obtained by filtration on Sepharose 4B under the same conditions (3).

Phospholipids were determined following Gurd's method (12).

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RESULTS

5'nucleotidase activity of IO vesicles. IO vesicles are not retained on Con A-Sepharose 4B and can easily be separated from RSO vesicles. As several authors (3) we were unable to obtain RSO vesicles by this method. Therefore we could only compare the properties of IO vesicles with those of a mixture IO : RSO vesicles. When purified plasma membranes were filtered on Con A-Sepharose, the amount of eluted proteins represented about

Table 1 Con A inhibition of 5'nucleotidase activity in fractionated lymphocyte plasma membrane vesicles

Membrane Preparations	Con A concentration µg per ml	5'nucleotidase activity µmoles Pi . mg protein^{-1} per h	
		RSO + IO vesicles	IO vesicles
Microsomes	0	4.3	4.3
	10	2.5	2.2
	20	1.4	1.4
Purified Plasma Membranes	0	17.3	16.9
	10	7.9	7.2
	20	1.6	1.9

half of the starting material and was entirely unretained on a second Con A-Sepharose column, which indicated that our membrane preparations contained a 50 : 50 mixture of IO and RSO vesicles, in agreement with the findings of Walsh *et al* (3). Golgi contamination of our preparations were very low, as compared with Monneron and d'Alayer's results (10) : thiamine pyrophosphatase activity was only 0.40 µmole Pi.h^{-1} per mg protein for membranes and 0.72 µmole Pi.h^{-1} per mg protein for microsomes; moreover the ratio 5'nucleotidase versus thiamine pyrophosphatase was quite high and unchanged in unretained fractions : 8.6 for microsomes and 9.2 for eluted microsomes, 23 for membranes and 19 for unretained membranes.

Table 1 shows the 5'nucleotidase activity of these vesicle populations obtained either from microsomes or from purified plasma membranes, and its inhibition by concanavalin A (1,8). The activity of microsome vesicles was determined in order to use the same material as for adenylate cyclase measurements. In both preparations there is absolutely no difference between IO and RSO + IO vesicles. This means that our membrane vesicles are not sealed : 5'AMP, the 5'nucleotidase substrate, and Con A which both have their binding site on the outer membrane surface can reach this site in IO vesicles.

Effects of alamethicin on lymphocyte adenylate cyclase and Na^+-K^+-ATPase activities

In adenylate cyclase studies, microsomal fractions were used instead of purified membranes because of the enzyme instability (13). Na^+-K^+-ATPase activity was determined both in microsomes and in purified plasma membranes.

In the presence of alamethicin (30 min preincubation at 4°C), adenylate cyclase activity was strongly enhanced (Table 2). Basal and PGE_1-stimulated activities were enhanced about 2 times with an alamethicin : membrane protein ratio (w:w) 0.083 and about 4 times with a 0.13 ratio. Fluoride-stimulated adenylate cyclase appeared a little

Table 2 Alamethicin effects on adenylate cyclase activity in lymphocyte microsomes

Experiment	Alamethicin mg per mg protein		Adenylate cyclase activity pmoles cAMP . mg protein^{-1} per 15 min		
			Basal	10^{-5} PGE_1 stimulated	10 mM NaF stimulated
1	0	activity	53	82	232
		%	100	100	100
	0.13	activity	240	373	822
		%	453	458	354
2	0	activity	129	189	631
		%	100	100	100
	0.083	activity	275	366	782
		%	214	194	124

less affected by alamethicin, specially at low antibiotic concentration. On the same microsomal fraction 0.5 % Lubrol PX induced a similar increase of adenylate cyclase activities : 3.30, 2.52 and 4.61-fold for basal, PGE_1 and fluoride respectively.

The effects of alamethicin and SDS on the ATPase activities of the same microsomes are shown in Table 3. Na^+-K^+-ATPase was stimulated about 10 times by alamethicin (0.5 mg per mg protein). As shown previously (2) SDS (20 min preincubation at 4°C) enhanced also Na^+-K^+-ATPase, while inhibiting Mg^{2+}-ATPase. The inhibition of Mg^{2+}-ATPase by alamethicin was much lower than by SDS, for doses giving approximatively the same Na^+-K^+-ATPase enhancement.

The effects of various alamethicin doses on both ATPase activities of purified plasma membranes are also presented in Table 3. The maximum of Na^+-K^+-ATPase enhancement was reached with 0.75 mg alamethicin per mg protein. Under these conditions the Na^+-K^+-ATPase specific activity was 8.3 µmoles $Pi.h^{-1}$ per mg protein, a value very close from that found with 0.043 % SDS treatment (8.5 µmoles $Pi.h^{-1}$ per mg protein).

Effects of alamethicin on adenylate cyclase and Na^+-K^+-ATPase activities of IO vesicles

In IO vesicles the catalytic site of adenylate cyclase and Na^+-K^+-ATPase is on the outer face of the membrane vesicles, in contact with the extravesicular fluid. If vesicles were impermeable to ATP, one should expect an increased activity for Na^+-K^+ ATPase and at least basal adenylate cyclase in IO vesicles, as compared with RSO + IO vesicles. On the contrary we found in IO vesicles an identical value for Na^+-K^+-ATPase and a very low adenylate cyclase activity. This lack of adenylate cyclase activity is

Table 3 Effects of alamethicin and SDS on lymphocyte ATPase activities

Membrane Preparation	Alamethicin mg per mg protein	SDS %	ATPase activities µmoles Pi.mg protein^{-1} per h	
			Mg^{2+}-ATPase	Na^+-K^+-ATPase
Microsomes	0	0	8.0	0.2
	0.5	0	6.4	2.2
	0	0.043	1.1	2.0
Purified Plasma Membranes	0	0	8.0	1.2
	0.25	0	7.8	3.2
	0.5	0	7.3	6.1
	0.75	0	7.1	8.3
	1.0	0	6.2	6.4
	0	0.043	2.6	8.5

not the result of increased contamination (by Golgi for instance) of the unretained fractions but is probably due to the loss of some activator during Con A-Sepharose filtration : the study of this activator and of adenylate cyclase restoration is under way in our laboratory (14). Anyhow Table 4 shows that this low adenylate cyclase activity of IO vesicles was enhanced by alamethicin treatment, which means that alamethicin action is not related to membrane sidedness. An identical result was found for Na^+-K^+-ATPase (Table 4). Lubrol PX (not represented) and SDS mimicked alamethicin effects on adenylate cyclase and Na^+-K^+-ATPase, respectively.

Membrane solubilization by alamethicin. After alamethicin or SDS treatment, lymphocyte plasma membranes (2 mg protein) were centrifuged 1 h at 100,000×g. Protein and phospholipid contents, ATPase and 5'nucleotidase activities were determined in each supernatant and pellet. It is clear from Table 5 that non-negligible protein and phospholipid fractions were solubilized by alamethicin treatment : 0.720 mg protein for instance as compared with 0.710 mg solubilized by SDS.

Na^+-K^+-ATPase activity is largely enhanced in alamethicin and SDS pellets (P_3 and P_2 respectively) as compared with controls (P_1 from untreated membranes). Under the same conditions, supernatants S_1, S_2, S_3 had practically no 5'nucleotidase activity, while total 5'nucleotidase was unchanged.

DISCUSSION Results presented in this paper show unambiguously that latent Na^+-K^+-ATPase and adenylate cyclase activities of lymphocyte plasma membranes are not related

Table 4 Alamethicin and SDS effects on adenylate cyclase and ATPase activities in fractionated membrane vesicles prepared from lymphocyte microsomes

	RSO + IO vesicles						IO vesicles					
Alamethicin mg per mg protein	0	0	0.12	0.4	0.5	0.75	0	0	0.125	0.42	0.5	0.75
SDS %	0	0.043	0	0	0	0	0	0.043	0	0	0	0
Mg^{2+}-ATPase µmoles Pi.mg protein^{-1} per h	12	2.8	nd	nd	8.2	5.3	12.2	2	nd	nd	8	4.8
Na^{+}-K^{+}-ATPase µmoles Pi.mg protein^{-1} per h	<0.2	3.2	nd	nd	2.7	2.7	<0.2	2.9	nd	nd	2.4	2.2
Adenylate cyclase pmoles cAMP.mg protein^{-1} per 15 min												
Basal	102	nd	261	290	nd	nd	4	nd	16	12	nd	nd
10 mM NaF stimulated	518	nd	1632	2391	nd	nd	22	nd	88	71	nd	nd

Table 5 5'nucleotidase and ATPase activities of supernatant (S) and pellet (P) obtained by 1 h 100,000 × g centrifugation of lymphocyte plasma membranes (2 mg protein) after alamethicin or SDS treatment

Treatment	Fraction	Protein mg	Phospholipids mg	Mg^{2+}-ATPase SA[a]	Mg^{2+}-ATPase Total[b]	Na^{+}-K^{+}-ATPase SA[a]	Na^{+}-K^{+}-ATPase Total[b]	5'Nucleotidase SA[a]	5'Nucleotidase Total[b]
None	S_1	0.160	≈0	≈0	≈0	≈0	≈0	0.025	0.004
	P_1	1.650	0.950	8.6	14.2	1.4	4.0	14.6	24.0
SDS 0.043 %	S_2	0.710	0.450	≈0	≈0	≈0	≈0	0.015	≈0
	P_2	0.890	0.520	3.0	2.7	15.3	13.6	27.4	24.4
Alamethicin 0.7 mg per mg protein	S_3	0.720	0.394	1.7	1.2	1.7	1.2	0.015	≈0
	P_3	1.200	0.610	9.0	10.8	13.8	16.6	22.2	23.0

a. specific activity in μmoles $Pi.h^{-1}$ per mg protein

b. μmoles $Pi.h^{-1}$

to membrane sidedness but rather to the presence of masking components (phospholipids or proteins). Removing these components by a detergent, like SDS for Na^+-K^+-ATPase or Lubrol PX for adenylate cyclase enhances these enzymatic activities. Alamethicin acts exactly as a detergent and the increase in Na^+-K^+-ATPase or adenylate cyclase activity elicited by this antibiotic is not correlated with its channel-forming properties, as we showed that our vesicles are leaky and allow penetration of 5'AMP and Con A (Table 1); ATP can also enter membrane vesicles as evidenced by the Mg^{2+}-ATPase activity of IO vesicles (Table 4) : we showed earlier that the catalytic site of this enzyme is on the outer membrane surface (7,15) and should be masked in IO vesicles. On the other hand the enhancement of adenylate cyclase and Na^+-K^+-ATPase activities is much larger than expected if "opening" of RSO vesicles was the only reason of this enhancement; as the ratio RSO : IO is about 50 : 50 for lymphocyte plasma membranes, one should expect a two-fold increase in enzymatic activity in alamethicin-treated membranes. This result is corroborated by the fact that alamethicin elicited the same enhancement of Na^+-K^+-ATPase and adenylate cyclase activities in unfractionated vesicles and in IO vesicles where the catalytic site is in contact with the reaction medium.

The detergent-like activity of alamethicin is not surprising if one considers the amphipathic character of this molecule. This detergent-like activity is emphasized by the results presented in Table 5 : proteins and phospholipids are solubilized by alamethicin treatment. Alamethicin acts as a mild detergent as compared with SDS : only 14 % Mg^{2+}-ATPase activity was lost during alamethicin treatment, and part of this activity was solubilized (10 %), while only 20 % Mg^{2+}-ATPase activity remained after SDS treatement, with no activity in the supernatant.

We cannot exclude the possibility that alamethicin forms channels in our lymphocyte membranes but, since our vesicles are already leaky, this effect could not account for the magnitude of the observed increases in enzymatic activities, even at the lowest concentrations used by other authors (16,17). We found a 3-fold increase in NaF-

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