Alamethicin-Induced Pore Formation in Biological Membranes

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Abstract. The effects of alamethicin on the membrane barrier function of rabbit erythrocytes, human platelets and sarcoplasmic reticulum vesicles, as well as on that of brain microsomes and liver mitochondria of the rat were compared. An upset of the barrier function was observed for plasma membranes of brain microsomes as well as for erythrocyte and platelet membranes at alamethicin concentrations ranging between 25–80 μg/ml. The membrane barrier functions of sarcoplasmic reticulum vesicles, of endoplasmic reticulum vesicles of rat brain microsomes, and of liver mitochondria were disturbed at 3–7 μg/ml alamethicin. The different sensitivities of plasma and intracellular membranes to alamethicin were supposed to be due to the presence of considerable quantities of cholesterol in plasma membranes as well as to peculiarities of their protein compositions.

Key words: Alamethicin — Erythrocytes — Platelets — Mitochondria — Sarcoplasmic reticulum — Endoplasmic reticulum — Brain microsomes

Introduction

The naturally occurring antibiotic, alamethicin, possesses an ability to form aqueous pores or channels in model and biological membranes, thus increasing their permeability for ions and some low molecular weight compounds (Latorre and Donovan 1980; Mathew and Balaram 1983; Sakmann and Boheim 1979). The concentration of the antibiotic needed for such channels to be formed depends on the value of the membrane potential as well as on the ionic strength of the incubation medium (Hall et al. 1984; Rizzo et al. 1987). Studies with bilayer lipid membranes revealed that the electrical properties of alamethicin channels depend critically on the lipid composition of biomembranes (Vodyanoy et al. 1983). Also, the affinity of the antibiotic for liposomes was found to decrease in the presence of cholesterol (Stankowski et al. 1988).

The aim of the present paper was to compare alamethicin-induced pore for-

mation in cholesterol-rich plasma membranes with that in intracellular membranes which are practically devoid of cholesterol.

Materials and Methods

Erythrocytes were isolated from rabbit blood. The pellet was suspended in 0.9 % w/w NaCl and used immediately for hemolytic assays. Sarcoplasmic reticulum (SR) membranes were isolated from white skeletal muscles of rabbit hind limb (Ritov et al. 1985). Platelets were isolated from freshly collected human blood (Hallam and Rink 1985); the pellets were suspended in Tyrode solution and used immediately. The microsomal fraction was prepared from rat brain homogenates after removal of the cerebellum (Menshikova et al. 1989). Rat liver mitochondria were isolated as described previously (Johnson and Lardy 1967).

Alamethicin was isolated from the fungus *Trichoderma viride* by the method developed earlier in our laboratory; the major component is alamethicin I (F_{30}) (Vodyanoy et al. 1982).

The hemolytic activity of alamethicin was measured spectrophotometrically (λ = 546 nm) in a thermostatted cell with continuous stirring. The assay mixture contained 10 mmol.l^{-1} HEPES, 20 mmol.l^{-1} KCl, 130 mmol.l^{-1} NaCl, 3 mmol.l^{-1} $MgCl_2$ and 5 mmol.l^{-1} NaN_3 (pH 7.2, 37 °C). The erythrocyte hemoglobin content in the sample corresponded to $6.4.10^7$ human erythrocytes in 1 ml. Alamethicin was dissolved in 60% ethanol to a concentration of 20 mg/ml. The resulting alamethicin solution was added to erythrocyte suspension (0.1-4 μl/ml suspension).

ATPase activities of human platelet and rat brain microsomal membranes were measured spectrophotometrically from NADH oxidation in a coupled enzymatic system (Fischer et al. 1985). All measurements were made in a thermostatted cell with continuous stirring. The incubation mixture contained 130 mmol.l^{-1} NaCl, 20 mmol.l^{-1} KCl, 5 mmol.l^{-1} NaN_3, 3 mmol.l^{-1} $MgCl_2$, 10 mmol.l^{-1} HEPES, 0.2 mmol.l^{-1} NADH, 0.25 mmol.l^{-1} phosphoenolpyruvate, 3 mmol.l^{-1} ATP, pyruvate kinase (2 I.U.) and lactate dehydrogenase (6 I.U.) (pH 7.2, 37 °C). The medium for platelet ATP assay contained additionally 1 mmol.l^{-1} $CaCl_2$ and 1 mmol.l^{-1} EGTA; that used for measuring the brain microsomal ATPase activity was supplemented with 1 mmol.l^{-1} EGTA.

The accumulation and release of Ca^{2+} from SR vesicles and rat brain microsomes were measured by fluorescence of Quin 2 (Rink 1983) using a Hitachi M-850 spectrofluorimeter; the assay medium contained 100 mmol.l^{-1} KCl, 1.5 mmol.l^{-1} $MgCl_2$, 2 mmol.l^{-1} ATP, 5 mmol.l^{-1} creatine phosphate, creatine kinase (3 I.U.), 20–25 μmol.l^{-1} Ca^{2+}, 25–30 μmol.l^{-1} Quin 2, 5 mmol.l^{-1} NaN_3 and 10 mmol.l^{-1} HEPES (pH 6.8, 28 °C). All measurements were performed in a thermostatted cell under continuous stirring (Menshikova and Ritov 1986).

Mitochondrial respiration was measured polarographically with the help of rotating platinum electrodes. The assay medium contained 0.12 mmol.l^{-1} sucrose, 75 mmol.l^{-1} KCl, 2.5 mmol.l^{-1} $MgCl_2$, 4 mmol.l^{-1} succinate, 3 μmol.l^{-1} rotenone and 10 mmol.l^{-1} HEPES (pH 7.4, 20 °C). Protein was determined with the biuret raction.

Results and Discussion

The effects of alamethicin on the barrier function of plasma membranes were stud-

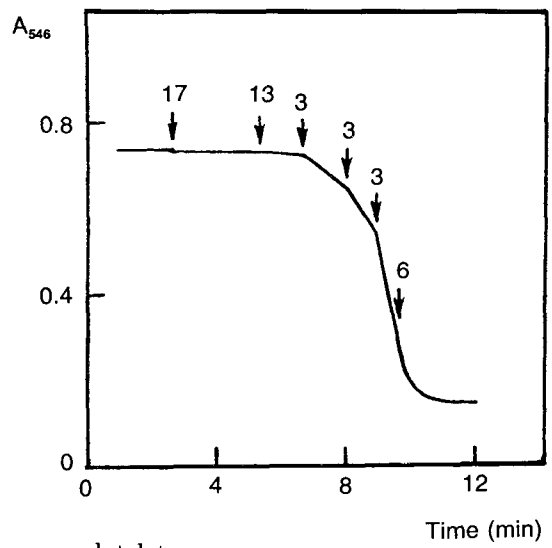

Figure 1. Rabit erythrocyte lysis induced by alamethicin. Alamethicin additions (μg/ml) are marked by the arrows.

ied using rabbit erythrocytes and human platelets.

Erythrocyte lysis under isotonic conditions was used to determine the barrier function disturbances induced by alamethicin; the results of these experiments are illustrated in Fig. 1. At concentrations below 20 μg/ml alamethicin did not change the optical density of the erythrocyte suspensions, i.e., no hemolysis occurred. Erythrocyte lysis started at 33 μg/ml alamethicin concentration and full lysis at 40–50 μg/ml.

The changes in the barrier properties of human platelet membranes were followed by the activation of ATP hydrolysis in platelet suspensions. Earlier alamethicin has been found to reveal latent ATPase activity in closed membrane vesicles and proteoliposomes (Besch et al. 1977; Ritov et al. 1982). This was said to be due to the ATP passage through alamethicin-formed pores to the ATPase active centers located at the inner side of membrane vesicles (Jones et al. 1980). Hence, the alamethicin-induced activation of ATP hydrolysis in platelet suspensions supports the view that ATP penetrates into the inside of the cells, i.e. that the barrier function of platelet plasma membranes is impaired. The rate of ATP hydrolysis in platelet suspensions was measured spectrophotometrically as the decrement of NADH in the presence of a coupled enzymatic system. The kinetics of NADH oxidation in platelet suspensions is shown in Fig. 2.

As can be seen from the Figure, there was no ATP hydrolysis in platelet suspensions containing no alamethicin. Alamethicin added at a concentration sufficient to induce full erythrocyte lysis, i.e., 50 μg/ml, did not induce ATP hydrolysis. In-

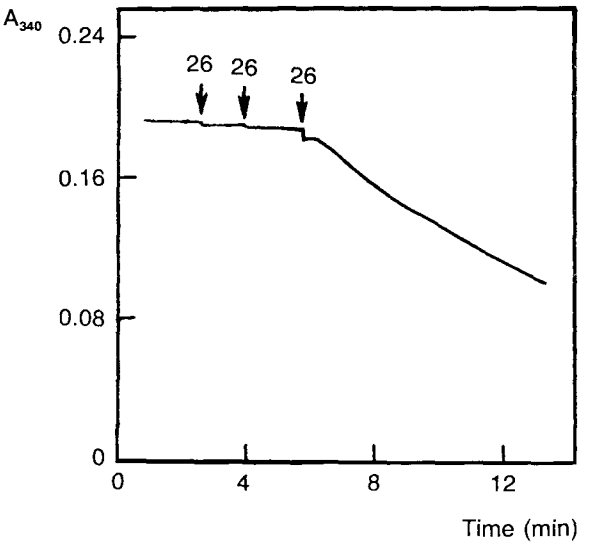

Figure 2. ATP hydrolysis in human platelet suspensions as measured spectrophotometrically by NADH oxidation. Alamethicin additions (μg/ml) are marked by the arrows.

creasing the alamethicin dose up to 80 μg/ml resulted in ATP hydrolysis in platelet suspensions.

The effect of alamethicin on barrier function of intracellular membranes was studied in preparations of rabbit skeletal muscle SR; more than 90% of this SR consist of vesicles formed from intracellular membranes (Martonosi 1984). The major protein component of the SR vesiculate fraction is Ca-ATPase responsible for the active influx of Ca^{2+}; this process utilizes the energy of ATP hydrolysis. SR membranes have been found to contain no cholesterol (Drabikowski et al. 1972). The changes in the permeability of the SR vesiculate membranes induced by alamethicin were followed by Ca^{2+} release from membrane vesicles after Ca^{2+} accumulation via ATP hydrolysis. The accumulation and release of Ca^{2+} in SR vesicles were measured by Quin 2 fluorescence. The effect of the antibiotic on the Ca^{2+} content in SR vesicles is shown in Fig. 3. Addition of SR membranes to an incubation medium containing Mg-ATP, Ca^{2+} and Quin 2 resulted in a time-dependent decrease of fluorescence due to the active transport of Ca^{2+} into SR vesicles. Further addition of alamethicin induced Ca^{2+} release from SR vesicles (Fig. 3a). Alamethicin concentrations able to induce a 100% release of Ca^{2+} from SR vesicles ranged between 3–5 μg/ml.

The different sensitivities of the plasma and the intracellular membrane to the antibiotic were also seen in experiments with Ca^{2+} release from brain microsomes as well as during the analysis of alamethicin-induced activation of plasma membrane Na,K-ATPase in rat brain microsomes. The kinetics of ATP-dependent Ca^{2+}

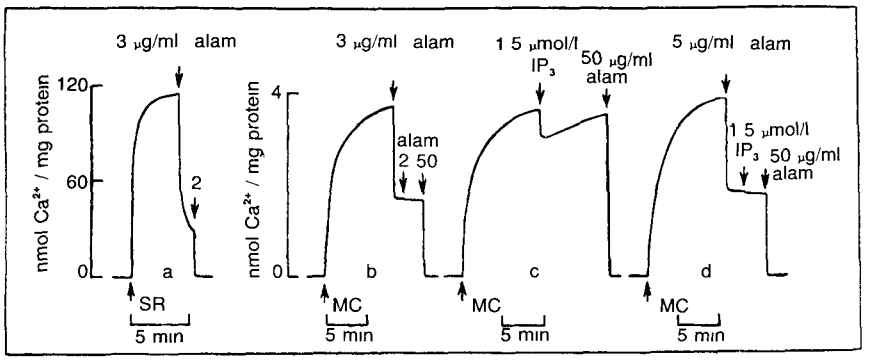

Figure 3. Ca^{2+} transport in rabbit skeletal muscle sarcoplasmic reticulum (SR) vesicles (a) and rat brain microsomes (MC) (b,c,d) as measured by Quin 2 fluorescence. Ca^{2+} release was induced by addition of alamethicin or inositol-1,4,5-triphosphate (IP_3).

accumulation in rat brain microsomes, as measured from Quin 2 fluorescence, is shown in Fig. 3 b,c,d. Alamethicin added to the incubation medium after Ca^{2+} accumulation in microsomes stimulated the release of these cations from the vesicles. Under these conditions two Ca^{2+} pools can be distinguished, which become filled in the course of the ATP-dependent reaction and which differ in their sensitivities to the antibiotic (Fig. 3b). From pool I, which makes up approx. 50% of the total microsomal pool of Ca^{2+}, calcium ions are released at alamethicin concentrations of 3–5 μg/ml. In order to induce the release of the remaining Ca^{2+} (pool II), the alamethicin concentration had to be no less than 20–50 μg/ml. One may infer from these data that the existence of two Ca^{2+} pools is due to the presence in the microsomal fraction of two vesiculate fractions that are capable of ATP-dependent accumulation of Ca^{2+}: the endoplasmic reticulum (ER) and plasma membrane vesicles. As can be seen from Fig. 3a, only one Ca^{2+} pool is distinguishable after alamethicin-induced Ca^{2+} release from skeletal muscle SR. Taking 5 μg/ml as alamethicin concentration able to induce complete efflux of Ca^{2+} from SR vesicles, one may assume that this Ca^{2+} pool corresponds to pool I of the brain microsomal fraction.

Evidence for the localization of rat brain microsomal pool I in ER vesicles can be derived from the results of experiments, in which the effect of alamethicin on Ca^{2+} release was studied in the presence of inositol-1,4,5- triphosphate (IP_3). This compound is known to stimulate Ca^{2+} release from intracellular depots of ER in cells of different types (Abdel-Latif 1986). Earlier it has been shown that IP_3 induces Ca^{2+} release from rat brain microsomes (Menshikova et al. 1989; Shah and Pant 1988). As can be seen from Fig. 3c, IP_3 used at 1.5 μmol.l^{-1} releases 0.4–0.6 nmol Ca^{2+} per mg protein, i.e. up to 10–15% of the total amount of

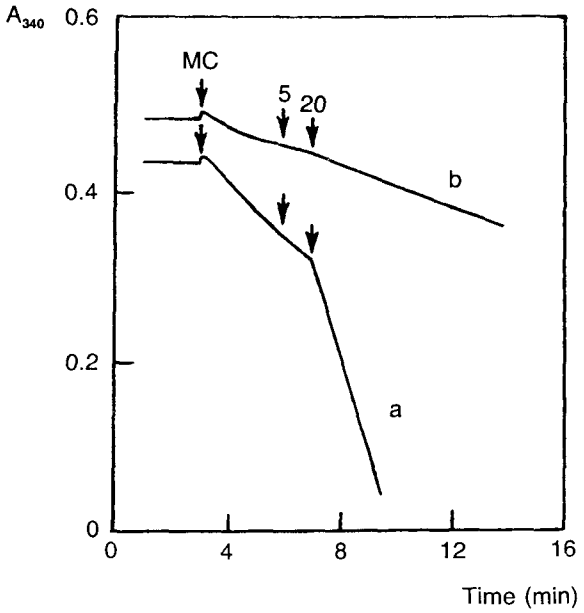

Figure 4. ATP hydrolysis in rat brain MC as measured spectrophotometrically by NADH oxidation in the absence (a) and presence (b) of ouabain (0.5 mmol.l^{-1}). Alamethicin additions (μg/ml) are marked by the arrows.

Ca^{2+} accumulated in microsomal fraction during ATP-dependent hydrolysis. After alamethicin-induced depletion of pool I, IP_3 was unable to induce Ca^{2+} release from pool II (Fig. 3d). Assuming that the IP_3-activated system of Ca^{2+} release is localized in intracellular membranes, one may conclude that pool I pertains to the vesiculate fraction of ER.

As for pool II, it seems to be localized in plasma membrane vesicles. This hypothesis can be substantiated by experimental evidence of the alamethicin effect on the activity of brain microsomal Na,K-ATPase. The kinetics of ATP hydrolysis in the microsomal fraction of rat brain in a coupled enzymatic system is shown in Fig. 4. The incubation medium contained Na^+ and K^+ at ratios necessary for the maximal Na,K-ATPase activity to be manifested, as well as EGTA and NaN_3 used to inhibit Ca-ATPase and mitochondrial ATPase. Moreover, alamethicin added at a concentration causing Ca^{2+} release from microsomal pool I (5 μg/ml) did practically not affect the rate of ATP hydrolysis (Fig. 4a). At the alamethicin concentration causing Ca^{2+} release from pool II (25 μg/ml) ATP was markedly activated (Fig. 4a). The observed stimulating effect of alamethicin on ATP hydrolysis is due to the activation of Na,K-ATPase, and is highly suppressed in ouabain-containing media (Fig. 4b). The "unmasking" effect of the antibiotic on Na,K-ATPase was reported previously for heart microsomes (Besch et al. 1977) and was interpreted by the authors as being due to two reasons: the levelling off of the Na-pump generated ionic gradients formed across the vesiculate membrane, eventually resulting in the activation of ATP hydrolysis, and the increased per-

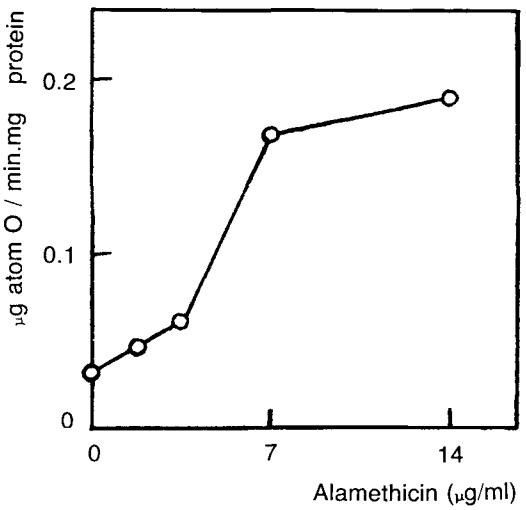

Figure 5. The dependence of succinate oxidation rate in rat liver mitochondria on alamethicin concentration. Mitochondrial protein concentration was 2 mg/ml.

meability of vesiculate membranes for ouabain and ATP; as a result of the latter, the vesicles with correct orientation, i.e., those with the active center of ATPase facing the cell interior, begin hydrolysing ATP. In this case ouabain acquires an ability to inhibit ATP hydrolysis in inside-out vesicles with an inward orientation of the ouabain-binding centers. This process culminates in the activation of Na,K-ATPase and its complete inhibition by ouabain. Taking account of the fact that Na,K-ATPase is a marker enzyme for plasma membranes, the upset of the barrier function of rat brain microsomal plasma membranes supposedly occurs at the alamethicin concentration of 25 μg/ml.

We studied the effect of alamethicin on the barrier function of inner mitochondrial membranes containing no cholesterol. Alamethicin, added to aerobic suspensions of tightly coupled rat liver mitochondria, activated succinate oxidation. The activity of mitochondrial succinate oxidase as a function of the antibiotic concentration is shown in Fig. 5. It shows that the rate of succinate oxidation is maximal with 7 μg/ml alamethicin. Hence, the sensitivity of mitochondrial membranes to alamethicin compares with that of ER membranes. The effects of alamethicin on the barrier function of biological membranes are listed in Table 1. Alamethicin induced the formation of pores in membranes for ion transfer (lysis of erythrocytes, Ca^{2+} release from microsomes and succinate oxidation in mitochondria) or for ATP transfer ("unmasking" of ATPase activity in platelets and microsomes). At the same time, the plasma membranes showed a lower sensitivity to alamethicin than did intracellular membranes and mitochondria. The observed discrepancies in the sensitivities of intracellular and plasma membranes to the antibiotic seem to be

Table 1. Effects of alamethicin on the barrier function of different types of biological membranes

Membrane	Parameter	Alamethicin concentration able to induce maximal effect, μg/ml
Rabbit erythrocyte membranes	Hemolysis under isotonic condition (permeability for ions)	40–50
Human platelet plasma membranes	ATPase activity (permeability for ATP)	80
Rat brain microsomal plasma membrane vesicles	Activity of Na,K-ATPase (permeability for ATP)	25
Rat brain microsomal endoplasmic reticulum vesicles	Ca^{2+} release (permeability for Ca^{2+})	5
Rabbit skeletal muscle sarcoplasmic reticulum vesicles	Ca^{2+} release (permeability for Ca^{2+})	3–5
Rat liver mitochondria	Succinate oxidation rate (permeability for H^+)	7

due, at least partially, to the presence in plasma membranes of considerable quantities of cholesterol which attenuated alamethicin affinity for liposomes (Stankowski et al. 1988). The intramembrane content of cholesterol is not the sole factor which influences the sensitivity of plasma membranes to the antibiotic. A comparative analysis of the data presented in Table 1 suggests that the platelet membranes are less sensitive to alamethicin than are erythrocyte membranes. Taking account of the similarity of lipid compositions of platelet and erythrocyte plasma membranes (Fauvel et al. 1986; Mitchell et al. 1986), it can be inferred that membrane proteins influence, to a certain extent, the membrane interactions with the antibiotic, the intramembrane protein concentration being presumably the main factor determining alamethicin interaction with biological membranes. Peripheral proteins can impede alamethicin incorporation into the membrane by screening the membrane by screening the membrane surface, whereas the intrinsic proteins binding the membrane lipids can diminish the area of free bilayer zones within the membrane and thus hamper the formation of alamethicin pores.

The discrepancies observed in sensitivities of intracellular and plasma mem-

branes to alamethicin can be used to develop techniques which would allow the assessment of the relative contributions of intracellular and plasma membranes in the mixed membrane functions to membrane-linked processes of any kind, e.g. the active Ca^{2+} transport in microsomes (Menshikova et al. 1989).

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