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Proline at position 14 of alamethicin is essential for hemolytic activity, catecholamine secretion from chromaffin cells and enhanced metabolic activity in endothelial cells

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Abstract

Alamethicin is known to lyse different biological cells and to induce voltage dependent ion channels in lipid bilayers. A set of analogs with proline shifted from position 14 in the native peptide towards the N- and C-terminus was used to investigate the role of proline in: (i) alamethicin induced hemolysis of human red blood cells, (ii) stimulation of catecholamine secretion from bovine adrenal chromaffin cells and (iii) induction of metabolic activity in bovine aortic endothelial cells. Half maximal hemolytic activity was found at 30 μM alamethicin concentration, complete lysis occurred at 100 μM. The stimulation of catecholamine secretion in the presence of extracellular Ca^{2+} was concentration dependent up to 50 μM alamethicin. At this high concentration mild secretion was also found in the absence of Ca^{2+} indicating cell membrane damage. Alamethicin transiently stimulated the metabolic rate of endothelial cells in a concentration dependent mode up to 20 μM while the inhibition of metabolism at higher concentrations pointed to a toxic effect. The alamethicin analogs were completely inactive in all the biological assays. The effects correlated with a loss of dye release inducing activities on phosphatidylcholine vesicles and reduction of channel forming properties in lipid bilayers and were associated with modifications of membrane affinity rather than conformational changes of the peptides. The results indicate that proline at position 14 of the native peptide is essential for the interaction with different membrane systems. © 1998 Elsevier Science B.V.

Keywords: Alamethicin; Hemolytic activity; Catecholamine secretion; Metabolic activity; Conformation; Dye release

1. Introduction

Trichoderma are widespread soil fungi characterized by the production of cytolytic enzymes and antibiotic peptides. The peptides exhibit a broad spectrum of bio-activity, including growth inhibition of Gram positive bacteria induced by alamethicin [1,2] and saturnisporins [3] and inhibition of amoebae cell multiplication by trichorzianines [4]. Hemolysis caused by alamethicin [5] and paracelsin [6], lysis of leukocytes exposed to alamethicin, suzukacillin and

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trichotoxin [7,8], catecholamine release from adrenal chomaffin cells stimulated by trichosporins [9,10] and trichorcellins [11] and uncoupling of oxidative phosphorylation in mitochondria caused by alamethicin [12] and trichosporins [13] are examples of their actions on mammalian cells. These effects have all been related to a reduction of the barrier function of the cell membranes. Voltage induced ion conductance in lipid bilayers by alamethicin [14,15], trichorzianins [16], trichosporins [9] and trichocellins [11] and the release of dye entrapped in lipid vesicles in the presence of trichorzianins [4], saturnisporins [3] and harzianin [17] have been observed in model membrane systems, confirming the membrane permeabilizing activity of the peptides.

Numerous papers have tried to rationalize the relationship between the structure of the peptaibols with their ion-channel forming ability in bilayers or their capability to disorder the lipid matrix of membranes [9,16,18–20].

Alamethicin, originally isolated from the fungus *Trichoderma viride* is one of the best characterized peptaibols. It is a linear peptide consisting of 20 amino acid residues with an acetylated N-terminus and an aminoalcohol at the C-terminus. In common with the other peptaibols, alamethicin contains highly hydrophobic residues with a high proportion of the helicogenic α-methylalanine and a proline at position 14 inducing a kink in the largely helical arrangement of the peptide chain and conferring flexibility to the C-terminal half. The resulting amphipathic bent-rod structure, highly hydrophobic in the N-terminus and rich in polar residues at the C-terminus has been suggested to play a decisive role in the function of the peptaibol. Thus, using an alamethicin analog Duclohier et al. [21] showed that the substitution of proline 14 by alanine did not prevent voltage induced conductivity in lipid bilayers but distinctly reduced single channel formation. Also proline 14 substitution in trichosporin-Bs by α- methylalanine did not alter the voltage activation of ion channels but seriously reduced the probability of channel formation, the number of levels and their open times [20].

In our recent studies [22], alamethicin peptides with proline in positions different to 14 were found to display reduced conductivity inducing properties. Although voltage dependent macroscopic current flow remained detectable, single channel events were found only for analogs with proline at positions 15, 16 or 13 while further shifts towards the N- and C-terminus abolished channel formation.

This paper describes the effect of the change of proline 14 position in alamethicin on biological activities such as lysis of human red blood cells and catecholamine secretion from bovine adrenal chromaffin cells. In addition, a novel approach, namely determination of changes of the metabolic rate of bovine endothelial cells has been used to obtain further information concerning alamethicin peptides/mammalian cell membrane interactions. Peptide induced permeabilization of model membranes was studied by monitoring the dye release from phosphatidylglycerol liposomes. The biological effects have been related to the channel forming properties of the peptides and their lytic effect on lipid vesicles to contribute to an understanding of the general mechanistic principles of the membrane activity of alamethicin.

2. Materials and methods

2.1. Peptide synthesis

Alamethicin F 30 and the analogs were synthesised by means of Fmoc amino acid fluorides using the multiple peptide synthesis technique [23]. Fmoc amino acid fluorides were prepared according to the procedure described recently [24]. All peptides were analyzed using reversed-phase high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry. The peptide content was determined by quantitative amino acid analysis. Table 1 summarizes the sequences used.

2.2. Hemolytic activity

The hemolytic activity of the peptides was determined using human red blood cells as described previously [25]. In brief, 150 μl of cell suspension containing 1.2×10^9 cells/ml and varying amounts of peptide stock solution [concentration usually 10^{-4} mol/l in Tris buffer] and buffer were pipetted into Eppendorf tubes to give a final volume of 1 ml. The suspensions containing 1.8×10^8 cells/ml were incubated for 30 min at 37°C. After cooling in ice

Table 1
Alamethicin F 30 analogs tested in this study

Sequence	Abbreviation
Ac–Aib–Pro–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–**Pro**–Gly–Leu–Aib–Val–Aib–Aib–Glu–Gln–Pheol	Alam-P11
Ac–Aib–Pro–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–**Pro**–Leu–Aib–Val–Aib–Aib–Glu–Gln–Pheol	Alam-P12
Ac–Aib–Pro–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–**Pro**–Aib–Val–Aib–Aib–Glu–Gln–Pheol	Alam-P13
Ac–Aib–Pro–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–**Pro**–Val–Aib–Aib–Glu–Gln–Pheol	**Alam-P14**
Ac–Aib–Pro–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–Val–**Pro**–Aib–Aib–Glu–Gln–Pheol	Alam-P15
Ac–Aib–Pro–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–Val–Aib–**Pro**–Aib–Glu–Gln–Pheol	Alam-P16
Ac–Aib–Pro–Aib–Ala–Aib–Ala–Gln–Aib–Val–Aib–Gly–Leu–Aib–Val–Aib–Aib–**Pro**–Glu–Gln–Pheol	Alam-P17

[a] Aib denotes α- methylalanine.

water and centrifugation at $2000 \times g$ for 5 min 200 μl of supernatant was combined with 1.8 ml of 0.5% NH_4OH and the optical density was measured at 540 nm. Peptide concentrations causing 50% hemolysis [EC_{50}] were derived from the dose-response curves.

2.3. Catecholamine secretion from chromaffin cells

Bovine adrenal chromaffin cells were isolated by collagenase digestion as described elsewhere [26]. After four days of culturing in Eagle's minimum essential medium, the chromaffin cells (2×10^6 cells/dish) were washed twice with Krebs-Ringer-Hepes-buffer (KRH) and pre-incubated in KRH solution at 37°C for 10 min. After washing again, the cells were incubated with or without the alamethicin peptides at 37°C for 7 min. The reaction was terminated by transferring the incubation medium to tubes in ice-cold water. The catecholamines secreted into the medium were extracted with 0.4 M perchloric acid, adsorbed on aluminium hydroxide and quantified by the ethylenediamine condensation method using a fluorescence spectrometer (650-10S, Hitachi, Tokyo, Japan) at an excitation wavelength of 420 nm and an emission wavelength of 540 nm [27]. The amount of secreted catecholamines was expressed as percentage of the total cellular catecholamines. A Ca^{2+} free medium was prepared by omitting Ca^{2+} and adding 0.2 mM ethylene glycero bis [ß-aminoethyl ether]-*N*, *N*, *N′*, *N′*,-tetraacetic acid (EGTA).

2.4. Metabolic stimulation of endothelial cells

Bovine aortic endothelial cells (line BKEz-7) were cultivated in minimum essential medium (MEM) + 10% fetal calf serum (FCS). For the micro-physiometric experiments the cells were seeded into 12 mm diameter disposable polycarbonate cell capsules (Molecular Devices, Menlo Park, CA) at 10^5 cells/well in MEM + 10% FCS. The cultures were incubated at 37°C in a humidified atmosphere of 97% air/3% CO_2 for 48 h to attach to the membrane.

The silicon micro-physiometer, a semiconductor-based instrument, detects the extrusion of the acidic metabolic products of glycolysis, respiration and ATP hydrolysis, including lactic acid, CO_2, and protons [28]. To measure the rate of acidification, the cultures in the capsules were loaded into the chambers of the micro-physiometer Cytosensor (Molecular Devices) as described in detail elsewhere [28]. Briefly, the chambers were perfused with the culture medium lacking bicarbonate and with low buffering capacity (1 mM sodium phosphate), adjusted with sodium chloride to obtain isotonic conditions. This medium also contained alamethicin or its analogs as indicated in the experiments. The flow rate in the micro-physiometer was 50 μl/min. Cells were perfused for 110 s and the extracellular acidification rate being determined during each flow-off period (30 s) from the slope of a linear least-squares fit to the pH versus time (sec). After the acidification rate measurement, the flow was restored for 110 s, during this time the pH was re-equilibrated to a value close to that of the perfusion medium. The entire flow on/off cycle time was 150 s. Acidification rate data (μV/s) were mathematically normalized to a 100% (metabolic rate) value at a time before any treatment. The perfusion/halt cycle and data processing was controlled by a Macintosh PC interface. This allowed a direct comparison of the acidification rate collected from separate chambers with different initial rates. Multiple determinations of the acidification rate were

obtained and the data expressed as the average of at least 3 determinations at a given time point and shown as metabolic rate in percent.

The time dependency of the peptide induced metabolic activity of the endothelial cells was determined by loading the cells into the chambers of the micro-physiometer and determining the steady basal rate. The cells were treated with a 14 μM peptide solution for 3 min and then the chambers were perfused again with normal bicarbonate-free MEM and the metabolic rate was monitored for an additional 60 min. To determine the dose dependency of the Alam-P14 induced increase in the metabolic rate the cells were exposed to different concentrations of Alam-P14. The maximal response was calculated as the percentage of the basal metabolic rate for each chamber.

2.5. Dye release experiments

Large unilamellar vesicles (LUVs) in Tris-buffer (10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, pH = 7.4) containing 70 mM calcein for dye release experiments were prepared from 1-palmitoyl-2-oleoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, USA) (POPC) by repeated filtration using a thermobarrel extruder (Lipex Biomembranes, Vancouver, Canada) as described in [29]. Untrapped calcein was removed from the vesicles by gel filtration on a Sephadex G75 column. 10 μl LUV suspensions were injected into a quartz cuvette containing magnetically stirred peptide solutions of different concentration to a final volume of 2.6 ml. Calcein release from the vesicles was monitored fluorometrically by measuring the decrease in self-quenching (excitation 490 nm, emission 520 nm) at room temperature on a Perkin/Elmer LS 50B spectrofluorimeter. The fluorescence intensity corresponding to 100% leakage was determined by the addition of 100 μl of 10% Triton X-100 solution usually after 5 min of fluorescence recording. Percent leakage, F (%) after 1 min, was calculated by the equation $100(\%) * (I - I_o) / (I_{100\%} - I_0)$ where I is the intensity observed in the peptide solution, and I_o and $I_{100\%}$ are the fluorescence intensities measured in the absence of peptide and in the presence of Triton X-100, respectively. Peptide concentrations causing 50% dye efflux (EC_{50}) were estimated from dose-response curves.

2.6. Conformational investigations

Small unilamellar vesicles (SUVs) for CD investigations were prepared by suspending, vortexing and sonicating POPC in 10 mM Tris buffer, pH 7.4 containing 154 mM NaF and 0.1 mM EDTA [29]. CD spectra at 20 μM peptide concentration in the presence of 10 mM lipid were recorded on a J 720 spectrometer (Jasco, Japan) between 185 and 260 nm at room temperature. Usually six scans were accumulated for each sample and at least two independent preparations for each type of sample were measured, smoothed and averaged. Circular dichroism and differential scattering of the SUVs were eliminated by subtracting the lipid spectra of the corresponding peptide-free suspensions. The helicity was determined from the mean residue elipticity $[\Theta]$ at 222 nm according to the relation $[\Theta]_{222} = -30300\ (\alpha) + 2340\ ((\alpha)$ amount of helix) [30]. The error was 5% helicity.

CD spectroscopically derived binding isotherms were determined from the changes of the CD of peptide solutions (three different concentrations between $5 \cdot 10^{-5}$ mol/l and $5 \cdot 10^{-6}$ mol/l) after adding different amounts of SUVs. Using for the CD of the peptide the relations: $F = \Theta_{222}(0) - \Theta_{222}$ (F – relative signal, $\Theta_{222}(0)$ – ellipticity at 222 nm in the absence of lipid, Θ_{222} – measured ellipticity in the presence of lipid) and $F = F_\infty\ (C_b/C_p) = F_\infty\ (C_l/C_p)r$, where F_∞ is F of the completely bound peptide, C_l the lipid concentration, C_p the total peptide concentration, C_b the lipid-associated peptide concentration and $r = C_b/C_l$. F can be plotted against C_l/C_p. From these equations and the mass conservation equation the binding isotherm can be evaluated [31]. As an approximation peptide binding at low r values can be described in terms of a partition equilibrium ($K_{app} = r/C_f$) (C_f – concentration of free peptide). We evaluated the apparent binding constants (K_{app}) from the initial slope of the isotherms.

3. Results

Alamethicin (Alam-P14) induced a dose-dependent release of hemoglobin from human red blood cells

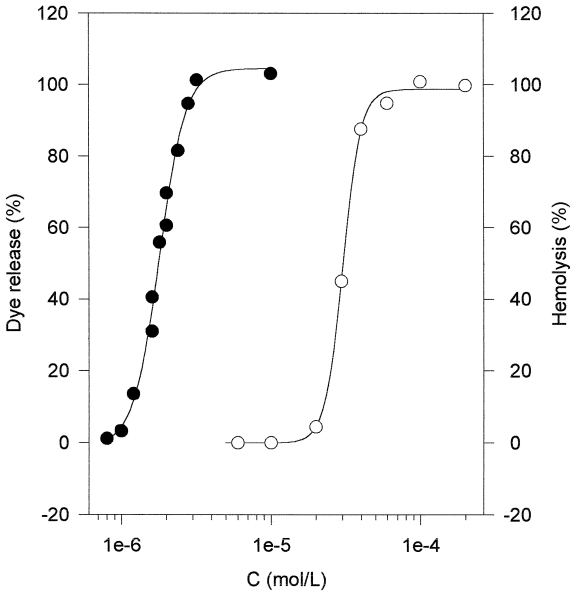

Fig. 1. Lytic activity of Alam-P14: Dye release from POPC LUVs (black circles). The lipid concentration was 12 μM. The EC_{50} was determined to be 1.8 μM. Hemoglobin release from human red blood cells (open circles) using 1.8×10^8 cells/ml. The EC_{50} was found to be 30 μM. Values determined in repeat experiments differed by less than 5%.

(Fig. 1). The concentration of the half maximal effect (EC_{50}) was determined to be 30 μM, corresponding to 10^{11} molecules per erythrocyte. Total hemolysis was reached at 100 μM peptide concentration. No hemolytic activity was found for the alamethicin analogs with proline in positions 11 to 13 and 15 to 17 at peptide concentrations up to 200 μM. The results indicated that alamethicin-induced hemoglobin release from erythrocytes depends on the location of proline at the native position 14.

Incubation of bovine adrenal chromaffin cells with Alam-P14 in Ca^{2+}-containing medium caused secretion of catecholamines in a peptide concentration-dependent mode (Fig. 2) while no effect was observed under Ca^{2+} free conditions. A mild increase in catecholamine secretion compared to the control was found in the absence of Ca^{2+} at 50 μM peptide concentration. Even at this highest investigated concentration, alamethicin analogs with proline in different positions showed no effect. Obviously, catecholamine secretion from chromaffin cells was initiated by the influx of Ca^{2+} into the cells and the ability of the peptides to promote this ion uptake was strongly dependent on proline at position 14.

Incubation of bovine aortic endothelial cells for 3 min with 14 μM Alam-P14 induced after the application of peptide a metabolic stimulation reaching about 210% of the activity of control cells after about 15 min (Fig. 3). About 50 min after peptide treatment the cells returned to the steady metabolic rate. The stimulation of the metabolic rate was concentration dependent up to about 20 μM alamethicin (Fig. 4). At higher concentrations cell metabolism was inhibited and the inhibition was not completely reversible after washout of the drug, suggesting a toxic effect. The six alamethicin analogs tested with proline at position 12, 13, 15 (Fig. 3) and 11, 16 and 17 did not induce any stimulation of cell metabolism up to a concentration of 100 μM (data not shown). These results indicated that the position 14 of proline in the native sequence is an essential prerequisite for the activity of alamethicin peptides with respect to metabolic stimulation of endothelial cells.

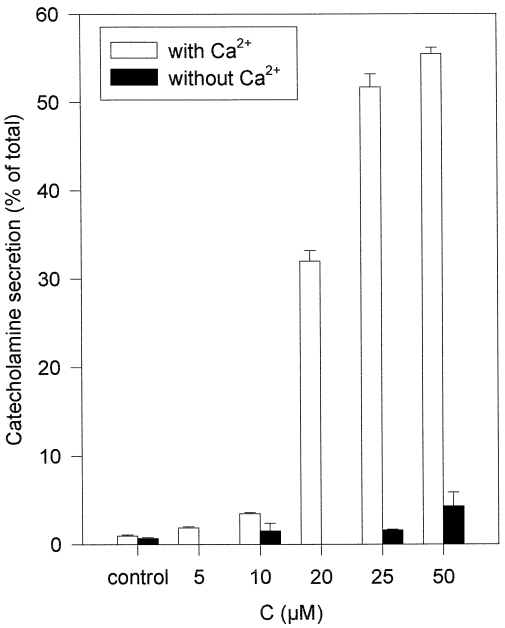

Fig. 2. Concentration dependence of Alam-P14 induced catecholamine secretion from bovine adrenal chromaffin cells. The effect was investigated in Ca^{2+} containing (white columns) and Ca^{2+} free (black columns) medium. The catecholamine secretion is shown as percentage of total content. Data are means ± S.D from three experiments.

Studying the permeabilizing activity of the alamethicin peptides on model membranes, the native alamethicin was found to be most active in inducing dye release from neutral, large unilamellar vesicles. The EC_{50} at 12 μM POPC concentration was 1.8 μM (Fig. 1) while all the analogs were completely inactive at this concentration. Slightly enhanced dye release was observed with the analogs bearing proline at more C-terminal positions at 80 μM concentration (data not shown) leading to the suggestion that the proline shift towards the N-terminus is more effective in reducing activity than the shift to more C-terminal positions.

The helicities of the peptides in the presence of POPC vesicles varied significantly (Table 2). Comparison of the peptide affinities at POPC vesicles revealed a reduction of the apparent binding constant from about 4000 M^{-1} for the native peptide, Alam-P14 to about 800 M^{-1} for Alam-P12, a representative inactive analog (Fig. 5). The hydrophobicity of the alamethicin peptides per residue, $< H > = 0.364$ and the hydrophobic moments $< \mu >$ assuming an ideal helix were calculated using the Eisenberg consensus scale of hydrophobicity [32] (Table 2).

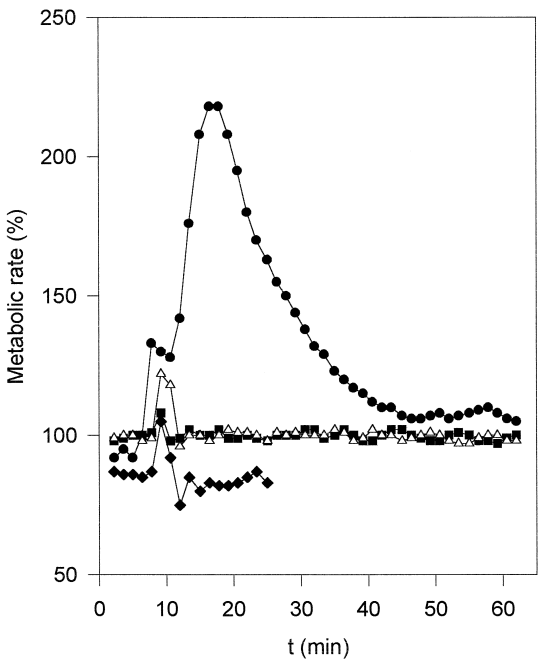

Fig. 3. Time dependence of metabolic rate changes of bovine aortic endothelial cells induced by alamethicin peptides. Cultivated endothelial cells were loaded into a micro-physiometer and treated with 14 μM peptide solutions for 3 min. Data shown are representative of three separate experiments: Alam-P 14 (●); Alam-P12 (♦); Alam-P13 (Δ); Alam-P15 (■).

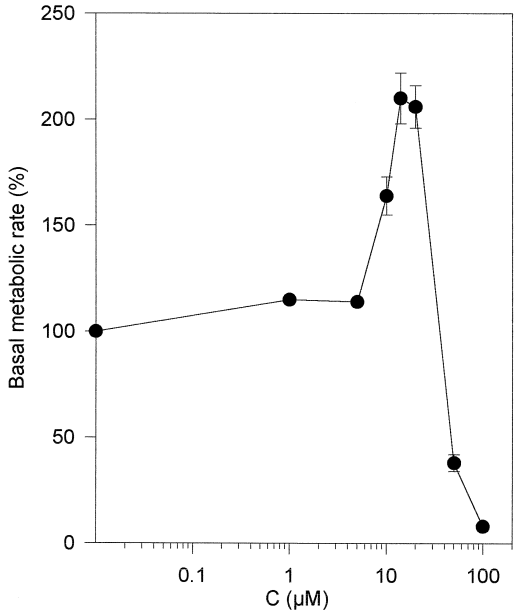

Fig. 4. Dose dependence of Alam-P14 induced increase in metabolic rate of bovine aortic endothelial cells. The endothelial cells were exposed to different concentrations of alamethicin for 3 min, and the metabolic rate was monitored for an additional 60 min. The maximal responses were calculated as the percentage of the basal metabolic rate. Data shown are the average of 3 similar experiments.

The results showed that any change in the position of proline 14 drastically reduced the bilayer permeabilizing activity of alamethicin. The effects did not

Table 2
Helicity and hydrophobic moment of alamethicin peptides

Peptide	α[%]	⟨μ⟩
Alam-Pro11	52	0.122
Alam-Pro12	85	0.108
Alam-Pro13	27	0.091
Alam-Pro14	**44**	**0.165**
Alam-Pro15	24	0.160
Alam-Pro 16	42	0.088
Alam-Pro 17	38	0.132

Helicity (α) of alamethicin peptides in the presence of POPC vesicles was determined at a peptide concentration of 25 μM and lipid concentration of 10.4 mM; the hydrophobic moment $< \mu >$ was calculated for an ideal helix according to Eisenberg [32].

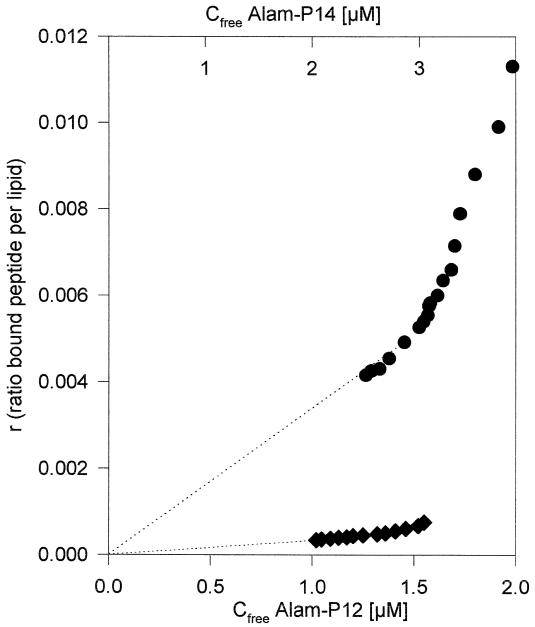

Fig. 5. Binding isotherms of Alam-P14 (●) and Alam-P12 (♦) to POPC vesicles. The apparent binding constants derived from the initial slope of the curves were $4000\,M^{-1}$ and $800\,M^{-1}$, respectively.

correlate with peptide helicity but appeared dependent on the peptide accumulation at the lipid matrix.

4. Discussion

This study shows that alamethicin F30 (Alam-P14) exhibits activities on different mammalian cells in the micromolar concentration range. The peptide induced lysis of human red blood cells, stimulated catecholamine secretion from bovine adrenal chromaffin cells and increased the metabolic activity of bovine aortic endothelial cells. Furthermore, it was found that these activities strongly depend on the location of proline at position 14 of the alamethicin chain.

The investigations of the action of Alam-P14 on erythrocytes confirmed previous reports of the hemolytic activity of alamethicin F50 bearing a glutamine at position 18 [5,8]. Interestingly, the peptide-induced damage of the red blood cells disappeared with any change in the position of proline 14. The loss of activity correlated with modifications in the permeability of model membranes exposed to alamethicin-peptides.

The lytic effect of alamethicin has been associated with its unique amphipathic bent-rod structure [5] which is mainly determined by the proline residue at position 14. The lipophilic N-terminal alamethicin helix has been suggested to be the most important determinant for the membrane disturbing effect. It may insert into the hydrophobic core of the membrane and disturb hydrophobic lipid–lipid interactions finally resulting in a release of membrane fragments [8]. However, the peptides investigated exhibited quite different helicities in the membrane bound state and there was no correlation between the amount of helix and membrane activity. Therefore, we associate the changes in the lytic activity of the alamethicin analogs first of all with changes in membrane affinity and consequently a modification of the amount of membrane associated peptides.

We have recently demonstrated, that the hemolytic activity of amphipathic peptides and their permeabilizing effect on neutral model membranes decreased substantially on the reduction of peptide hydrophobicity and hydrophobic moment [25,29,33]. Reduced binding was identified to be a decisive reason for the effect. A correlation with peptide hydrophobicity was also found comparing the lytic effects of alamethicin, suzukacillin and trichotoxine towards erythrocytes, leukocytes, mast cells and lymphocytes [7,8]. The hydrophobicities of the alamethicin peptides with proline at positions 11 to 17 are identical; however, as shown for Alam-P12 compared to Alam-P14, the apparent binding constant to neutral membranes was substantially reduced. The low affinity of the analogs might be related to changes in their hydrophobic moment (Table 2). Changes in the orientation of the N-terminal and C-terminal peptide regions caused by the proline shift with respect to the lipid acyl chains and polar membrane interface may additionally contribute to the low membrane disturbing activity of the proline substituted alamethicin analogs.

In bovine adrenal chromaffin cells, influx of Ca^{2+} into the cell is essential for triggering the secretion of catecholamines. Our results demonstrate that alamethicin induces catecholamine release from chromaffin cells in Ca^{2+} containing media in a concentration-dependent mode thus suggesting a peptide mediated Ca^{2+} entry into the cells. Furthermore, we confirmed in a recent study that the alamethicin induces voltage-dependent ion conductivity by channel for-

mation in lipid bilayers [22]. Comparable properties have been described for trichosporin-Bs [9,10,34]. It has been concluded that both trichosporin-Bs induced macroscopic ion conductivity in lipid bilayers and stimulation of catecholamine secretion from chromaffin cells by the influx of external Ca^{2+} are caused by the formation of voltage-activated Ca^{2+}-permeable ion channels [9,10]. Although alamethicin is less active than trichosporin-Bs, it may be expected that all these peptaibols induce catecholamine secretion by a comparable mode of action. The barrel stave model is widely accepted as explanation of the formation of alamethicin ion channels by the parallel transmembrane arrangement of peptide helices. Taking into consideration the diameters of the water filled pores (between 0.2 and 2 nm) [22], Ca^{2+} exchange seems to be highly probable. Besides Ca^{2+} influx, alamethicin channel formation has also been suggested to evoke Mn^{2+} and Ni^{2+} entry into chromaffin cells [35]. Although our studies did not show a convincing correlation between catecholamine secretion and macroscopic conductance or single channel forming properties of the proline substituted alamethicin analogs, the reduced size of channels and drastically reduced life times [22] could at least partly explain the loss of catecholamine release stimulating activity. Changes in the channel properties, such as the reduced number of conductance levels and reduced life times, have also been suggested as being responsible for a decrease of catecholamine secretion induced by a trichosporin analog containing Aib instead of Pro at position 14 [9,20,34] and by des-Pro^{14} trichosporin [19].

Similar to trichosporin-B-VIa [20], Alam-P14 at high concentrations induced a Ca^{2+}-independent efflux of catecholamines, suggesting that the peptide may damage the membrane of chromaffin cells. The effect was not detectable for peptides with proline shifted towards the N- and C-terminus thus correlating with the observations on erythrocytes and POPC model membranes. The change in activity might be interpreted in terms of low membrane binding caused at least in part by changes in the hydrophobic moment as discussed above. The fact that the concentration of alamethicin necessary to induce membrane damage was higher than the trichosporin B-VIa concentration [9] implicated the role of peptide hydrophobicity which is much lower in Alam-P14 ($H = 0.364$) than trichosporin B-VIa ($H = 0.409$). Finally, channel formation at lower and membrane disruption at higher alamethicin concentrations are in accordance with the recently suggested principles for the peptide-induced catecholamine secretion from chromaffin cells [10].

Furthermore, using a new technique in measuring the extracellular acidification rate, it was shown that alamethicin transiently stimulated the metabolic rate of bovine aortic endothelial cells. The reversibility of alamethicin induced metabolic activation is interpreted as a transient change in cell membrane integrity followed by a disturbance of the balance between intracellular and extracellular ionic composition. To overcome this, ion pumps within the cell are activated. These pumps are ATP dependent, resulting in an enhanced cellular metabolism, and extrusion of protons into the extracellular space thus causing an increased acidification rate [28]. Changes in the position of proline 14 eliminated the stimulating activity on endothelial cells. The findings correlate strongly with the peptide induced permeabilization of the neutral membrane of phosphatidylcholine vesicles and largely with the macroscopic and single channel experiments which imply that the native peptide induced highest number, best resolved and most stable conductance levels [22]. A reliable mechanistic interpretation of the action of alamethicin on endothelial cell membranes cannot be derived on the basis of these results. Nevertheless, it seems clear that the effect depends on proline at position 14 and on the membrane-modifying properties of native alamethicin, which appear responsible for the exchange of ions through the membrane.

In conclusion, the activity of Alam-P14 on different mammalian cell systems was shown to depend strongly on the peptide structure. The activities are strongly related to proline at position 14 of the native sequence. Ion exchange as a result of channel formation and/or damage of the cell membrane may be responsible for the effects. However, it is difficult to arrive at an exact molecular interpretation of the mechanism. Conformational changes induced by proline substitution were found to change peptide affinity and probably influence peptide flexibility and orientation in the membrane. In addition, the architecture of the different cell membranes and the different transmembrane potentials of erythrocytes (about

−9 mV) [36], endothelial cells (about −10 mV) [37] and chromaffin cells (in the range of −60 to −80 mV) [38] may modulate peptide membrane interactions and are expected to influence the mode of peptide induced membrane permeabilization.

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