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Biochimica et Biophysica Acta 1240 (1995) 167–178

BBA Biochimica et Biophysica Acta

Identification of the structural elements of amphotericin B and other polyene macrolide antibiotics of the hepteane group influencing the ionic selectivity of the permeability pathways formed in the red cell membrane

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Received 26 June 1995; accepted 10 July 1995

Abstract

The selectivity of the transmembrane permeability induced by polyene antibiotics was studied in human erythrocytes and related to the hemolytic potency of the drugs. The selectivity induced was differently, dependent on the antibiotic structure in aromatic (vacidin A, gedamycin) and nonaromatic heptaenes (amphotericin B, candidin). Aromatic heptaenes were more effective than nonaromatic in inducing permeability to K^+. For both groups of antibiotics, permeability to K^+ was not affected by substitution at the carboxyl group but important differences in the induction of permeability to H^+, OH^- and Cl^- were found. The strongly hemolytic aromatic heptaenes vacidin A and gedamycin exhibited much higher protonophoric activity than the nonaromatic ones: amphotericin B, and candidin. The protonophoric properties of aromatic heptaenes were related to the presence of a free carboxyl group in the antibiotic molecule. Indeed the esterification or amidation of the carboxyl group of vacidin A or gedamycin eliminated the ability of the antibiotic to increase H^+ conductance and consequently diminished their hemolytic activity to an important extent. Both groups of antibiotics differed also in the efficiency of anion permeability induction. Only unsubstituted aromatic heptaenes, at high concentration, induced Cl^-/OH^- exchange and conductive flux of Cl^- in a concentration-dependent manner. Substitution at the carboxyl group of vacidin A or gedamycin eliminated this property. Amphotericin B as well as its carboxyl-substituted derivatives formed a pathway characterized by low K^+ over Cl^- selectivity, whatever the concentration. The hemolytic activity, related to K^+ permeability increased by heptaenes was dependent on simultaneous increase of the permeability to anions, and net KCl influx. Carboxyl-substituted derivatives of aromatic heptaenes presenting a remarkably high selectivity for K^+, had consequently a very poor hemolytic activity.

Keywords: Amphotericin B; Aromatic polyene antibiotic; Antibiotic; Vacidin A; Erythrocyte; Hemolysis; Ionic selectivity

1. Introduction

The lack of an effective and nontoxic drug for the treatment of a variety of systemic mycotic infections, especially common in patients with induced or acquired immunological deficiencies, is the major problem in antifungal chemotherapy [1]. The polyene macrolide antibiotics remain most promising as a source of potential antifungal drugs of clinical significance. This group of compounds, unlike other antifungal agents, combines all features essential for the effective antifungal drugs: high antifungal activity, broad antifungal spectrum, fungicidal action and absence of resistance development. Up to now only one member of this large group of antibiotics, amphotericin B (Fungizone) is being used in clinics for the treatment of systemic infections. However, polyene macrolides still should be considered as leading compounds in the chemical modification programme aimed at the removal or diminishment of their undesirable properties, which are insolubility in water and poor selective toxicity. In our studies on the improvement of the selective toxicity of these antibiotics via chemical modification we

Abbreviations: RBC, red blood cells; CCCP, carbonylcyanide-*m*-chlorophenyl hydrazone; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; Gr, gramicidin D; Val, valinomycin; diS-C_3-(5), 3,3′-dipropylthiadicarbocyanine; DMSO, dimethyl sulfoxide; Abbreviations of all polyene antibiotics used in this study are given in Table 1.

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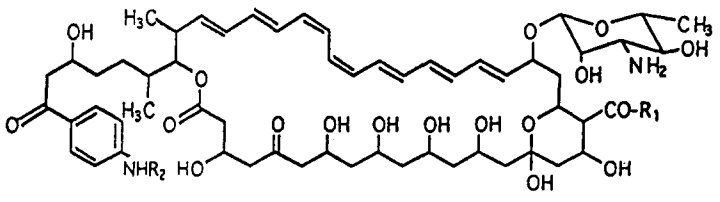
SSDI 0005-2736(95)00170-0

focused our interest on the large macrolide ring heptaenes, aromatic and nonaromatic, since they exhibit a high antifungal activity and also a high specificity of membrane effects related to channel formation.

Among animal cells, erythrocytes are very sensitive to the toxic effects of polyene macrolides. These cells provide a good model to study at the cellular level the selectivity of the permeability pathways induced by polyene antibiotics and its relation to the toxicity for animal cells, which is in this case, hemolysis. Lysis of the red cells induced by membrane active compounds is the final result of complex changes of membrane permeability and membrane structure. In the case of large ring polyenes it is due to a rather specific increase of permeability mainly to monovalent cations (a primary effect). The flux of ions through the channels formed in the cell membrane leads to a secondary effect – colloidosmotic hemolysis.

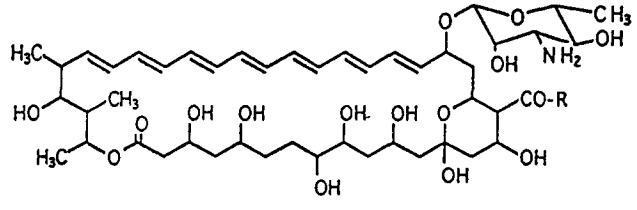
A comparative study of the action on red cells of a series of a natural or semisynthetic heptaenes revealed large differences in the hemolytic potency of closely related compounds. Moreover, for many of them, hemolytic activity did not parallel permeabilizing activity, pointing to different structural relations in both processes. For one of the most potent aromatic heptaenes, vacidin A, it had been

Aromatic heptaenes

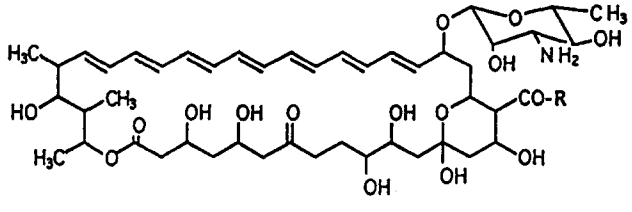


	R_1	R_2
Vacidin A (Vac)	OH	H
VacGlyMe	$NHCH_2COOCH_3$	H
VacGlyEt	$NHCH_2COOC_2H_5$	H
VacGlytBu	$NHCH_2COOC(CH_3)_3$	H
VacME	OCH_3	H
Gedamycin (Ged)	OH	CH_3
GedGlyMe	$NHCH_2COOCH_3$	CH_3

Nonaromatic heptaenes



	R
Amphotericin B (AmB)	OH
AmBGlyMe	$NHCH_2COOCH_3$
AME	OCH_3



	R
Candidin (CD)	OH
CDGlyOMe	$NHCH_2COOCH_3$
CDME	OCH_3

Fig. 1. Structures of the antibiotics tested.

established that significant reduction of the hemolytic activity may be attained by substitution at the carboxyl group resulting in the absence of carboxylate anion [2–5]. Further studies of the ionophoric and hemolytic properties of two aromatic heptaenes, vacidin A and perimycin, also pointed to the carboxyl group as the important element of aromatic heptaenes responsible for their high hemolytic activity. The results obtained suggested the relation between the presence of this group, protonophoric properties of the polyene antibiotics and hemolysis [2]. Data which would indicate similar relations for nonaromatic heptaenes were not available.

The purpose of the present work was:

1. to identify the structural elements of aromatic and nonaromatic heptaenes determining the permeabilizing and hemolytic activities;
2. to analyse the relationship between the hemolytic activity, the ability of the channel formation and the ionic selectivity of the induced pathway. The studies were performed with natural aromatic and nonaromatic heptaenes and their carboxyl-substituted derivatives, esters and amides.

The structures of the investigated compounds are given in Fig. 1. Aromatic heptaenes are represented by vacidin A and gedamycin which differ only by the presence of the methyl group in aromatic residue of the latter antibiotic. Nonaromatic heptaenes are represented by amphotericin B and candidin which are different in the hydrophilic fragment of the macrolide ring. All four native antibiotics have a macrolide ring of the same size, the same amino sugar and they also have a carboxyl group. The major differences between vacidin A and amphotericin B are the following: at the carbon atom of the macrolide ring adjacent to the lactone bond there is a methyl group in amphotericin B but a substituted aliphatic chain bearing an aromatic residue in vacidin A; there is an all-*trans* heptaenic chromophore in amphotericin B but a *cis-trans* one in vacidin A [6]; the heptaenic chromophore in vacidin A is shifted towards the lactone bond as compared to amphotericin B. There are also some differences in the hydrophilic fragment of macrolide ring comprising the amount and distribution of the carbonyl and hydroxyl groups.

2. Materials and methods

2.1. Polyene antibiotics and their derivatives

Vacidin A and gedamycin were isolated by counter-current distribution from crude aureofacin, obtained from Pharmaceutical Works POLFA (Tarchomin, Poland); candidin was from the same source. Amphotericin B was a gift from Squibb France (Neuilly, France). Isolation of vacidin A and synthesis of all derivatives has been performed by one of us (A.C.). The method of synthesis of a series of carbonyl methyl amides of vacidin A and gedamycin has been already described [3]. The methyl esters were prepared following the method of Bruzzesse et al. with some modifications [7]. The compounds to be tested were dissolved in dimethyl sulfoxide (DMSO), usually at 1 mg/ml for aromatic heptaenes and 5 mg/ml for nonaromatic ones (stock solutions). Appropriate dilutions in DMSO were applied to the red cell suspension in microliter amounts. The concentration of DMSO did not exceed 1% and this concentration was harmless to red blood cells. The concentrations of the antibiotics were expressed as micromoles per liter and were calculated according to absorption for pure compounds in methanol. (For amphotericin B and candidin $\varepsilon = 150\,000$ at 409 nm; for vacidin A, $\varepsilon = 120\,000$ at 382 nm.) For the derivatives absorption was measured at the same wave length as native antibiotics with the extinction recalculated according to the changes of molecular weight.

2.2. Other compounds

CCCP (carbonylcyanide-*m*-chlorophenyl hydrazone), DIDS (4,4′-diisothiocyano-2,2′-disulfonic acid stilbene), valinomycin and gramicidin D were form Sigma (Poole, UK).; diS-C_3-(5) (3,3′-dipropylthiadicarbocyanine) was from Eastman Kodak (Rochester, NY, USA),

2.3. Media

Isotonic media of pH 7.4 were composed of 150 mM KCl or choline chloride and were buffered with 5 mM Tris-HCl to pH 7.4, or they were used unbuffered.

2.4. Erythrocytes

Human blood, citrate anticoagulated, was kept at 4°C and used during 3 weeks. Just before use, erythrocytes were separated from plasma and buffy coat by centrifugation for 15 min at $700 \times g$ and were washed three times by suspending in four volumes of buffered isotonic choline chloride followed by centrifugation. A suspension of washed cells was kept on ice and used within a few hours.

2.5. Hemolytic activity

[Hb-50] was determined graphically from the hemolysis dose–response curves as described previously [2]. Determination was performed in buffered isoosmotic potassium chloride, pH 7.4 at cell concentration $2 \cdot 10^7$ per ml; temperature 22 or 37°C; time of incubation with an antibiotic 60 min; CCCP (10^{-5} M) was added prior to polyene. Values are the mean of four different samples.

2.6. Potassium release

[EK-50] was determined from the dose response curves as described previously [2]. Determination was performed

in buffered, isoosmotic choline chloride, pH 7.4 at cell concentration $2 \cdot 10^7$ per ml; temperature 22 or 37°C; time of incubation with antibiotic 60 min. Values are the mean of three independent experiments.

2.7. Kinetic of potassium efflux

A potassium-selective electrode (F 2312 K, Radiometer Copenhagen) was introduced to cell suspension containing 10^8 cells/ml in unbuffered 150 mM choline chloride; pH was adjusted with choline hydroxide solution; temperature 22°C. When the recorder reading stabilized, antibiotic solution was added and potassium level in the medium was monitored continuously until new stable level had been reached. Calibration of the amount of potassium released has been done by titration with 0.1 M KCl of cell suspension untreated with antibiotic. Determination was repeated for each antibiotic concentration.

2.8. Kinetic of proton influx [8]

Cells at concentration 10^8 per ml were suspended in unbuffered 150 mM choline chloride pH 7.4 at 22°C. The pH of the suspensions (pH_e) was monitored continuously, using a combined glass electrode (GK 2401), connected with a pH-meter (Radiometer, Copenhagen). The tested compounds were added after stabilization of the recorded signal. Experiments were repeated for each concentration of a given antibiotic.

2.9. Treatment with DIDS [9]

A dense cell suspension (hematocrit 75–80%) in isotonic choline chloride containing 40 mM sucrose, pH 7.4, (choline-sucrose medium) and 0.1 mM DIDS was incubated for 60 min at 37°C and then kept on ice until use. DIDS was dissolved in 20 mM NaOH just before use. For pH measurements, 100 microliters of DIDS-treated cell suspension has been added to 5 ml of 150 mM unbuffered choline-sucrose medium. Final cell concentration was 10^8 per ml.

2.10. Membrane potential determination

2.10.1. By pH method [8]

Cells (10^8 per ml), treated or untreated with DIDS, were suspended in unbuffered choline-sucrose medium or in isoosmotic choline-sucrose containing KCl at different concentrations; pH_e of the suspension was continuously recorded. If needed, pH was adjusted with 0.1 M KOH. CCCP (10^{-5} M) was added prior to the tested antibiotic. For testing and calibrating the system, DIDS-treated cells were suspended in isotonic choline-sucrose media containing potassium chloride at different concentrations and valinomycin ($2 \cdot 10^{-6}$ M) or gramicidin (10^{-7} M) was added just after CCCP; temp. 23°C

2.10.2. By fluorescence with DiS-C_3-(5) [10]

2 ml of the tested medium was placed in the cuvette of a spectrofluorimeter (Jobin Yvon spectrofluorimeter JY3D), solution of DiS-C_3-(5) ($2 \cdot 10^{-7}$ M) was added and fluorescence intensity was continuously recorded. After addition of $4 \cdot 10^8$ cells a stable level of fluorescence, corresponding to a distribution of the probe between cells and medium was reached after a few minutes. Then the tested antibiotic was added and changes of fluorescence intensity were recorded until a new stable level of fluorescence was obtained. Excitation and emission wavelengths were 620 and 670 nm, respectively; slits 10 mm. Determination of the membrane potential as a function of KCl concentration was done on DIDS-treated cells in isoosmotic buffered choline chloride containing KCl at different concentrations. Valinomycin, used as a reference potassium selective ionophore was tested under the same conditions.

3. Results

3.1. Hemolytic potency and potassium release

Potassium release and hemolytic activity of the tested compounds expressed as the concentration of antibiotic needed for releasing 50% of intracellular potassium [EK-50] or of hemoglobin [Hb-50], determined graphically from the dose-response curves are shown in Table 1.

Table 1
Potassium release and hemolytic activity

Antibiotic	EK-50	Hb-50		Hb-50/EK-50	
		−CCCP	+CCCP	−CCCP	+CCCP
Vacidin A (Vac)	0.025	0.24	0.04	10	1.6
VacGlyMe	0.03	11.0	0.07	367	2.3
VacGlyEt	0.03	7.0	0.10	233	3.3
VacGlyBu	0.07	4.0	0.15	57	2.1
VacME	0.10	20.0	0.30	200	3.0
Gedamycin (Ged)	0.01	0.35	0.025	40	3.0
GedGlyMe	0.02	9.0	0.10	400	6.0
Amphotericin B (AmB)	0.5	2.0	2.0	4.0	4.0
AmBGlyMe	1.5	3.0	3.0	2.0	2.0
AME	2.0	7.0	7.0	3.5	3.5
Candidin (CD)	0.28	3.7	1.6	13.2	5.7
CDGlyMe	0.52	3.7	2.4	7.1	4.6
CDME	1.65	4.1	3.6	2.5	2.1

EK-50 is the concentration of antibiotic (μM) causing 50% of intracellular potassium release from $2 \cdot 10^7$ cells/ml in isotonic buffered solution (pH 7.4) of choline chloride after 1 h incubation at 37°C.
Hb-50 is the concentration of antibiotic (μM) causing 50% hemolysis of $2 \cdot 10^7$ cells/ml in isotonic buffered solution of potassium chloride (pH 7.4) after 1 h incubation at 37°C; final concentration of CCCP 10^{-5} M. These values were determined graphically from the dose-response curves. The error of EK-50 and Hb-50 was ±5%.

3.1.1. [EK-50]

The aromatic heptaenes, vacidin A and gedamycin, were more than one order of magnitude more efficient in potassium permeability as well as hemolysis induction than the nonaromatic ones: amphotericin B and candidin. In both groups of heptaenes esterification or amidation of the carboxyl group, resulting in elimination of the negative charge in the molecule, diminished only to a small extent their permeabilizing activity, measured by potassium release; the highest decrease of [EK-50] (4-fold) was observed for methyl esters.

3.1.2. [Hb-50]

In contrast, substitution at the carboxyl group has essentially different influence on hemolytic activity in both groups of antibiotics studied. Such a modification strongly reduced the hemolytic activity of aromatic heptaenes. Depending on the derivative, this decrease ranged from 15- to 80-fold. On the other hand, similar substitution at the carboxyl group of nonaromatic heptaenes only slightly decreased their hemolytic activity. It should be noted that the derivatives of aromatic heptaenes were one order of magnitude less hemolytic than amphotericin B.

Hemolytic activity of aromatic heptaenes was stimulated by a protonophore, CCCP; a 6-, 65- and 160-fold increase of hemolysis was observed for vacidin A, VacGlyMe and VacME, respectively. In fact, CCCP shifted the hemolytic dose-response curves of aromatic heptaenes close to a concentration range inducing permeability to potassium, but had no effect on [EK-50] values (not shown).

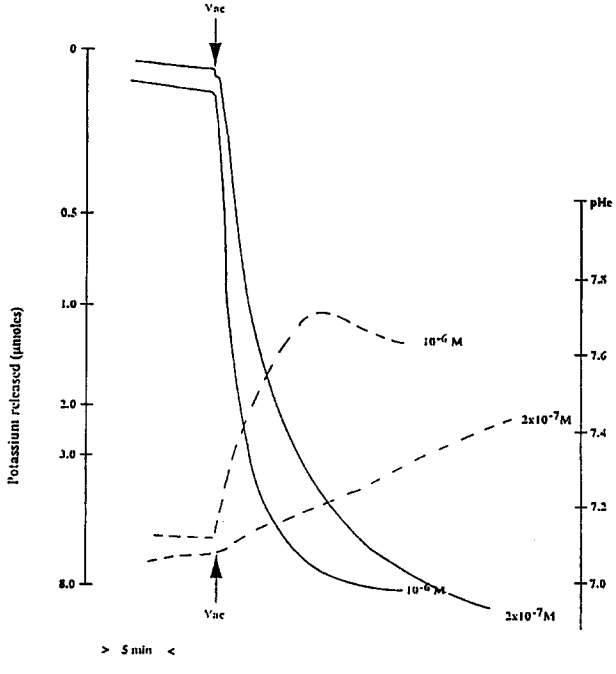


Fig. 2. K^+/H^+ exchange induced by vacidin A at concentrations: $2 \cdot 10^{-7}$ M and 10^{-6} M. K^+ efflux (—); H^+ uptake (- - -). Cell concentration: 10^8 cells/ml in unbuffered isotonic choline chloride; temperature 22°C.

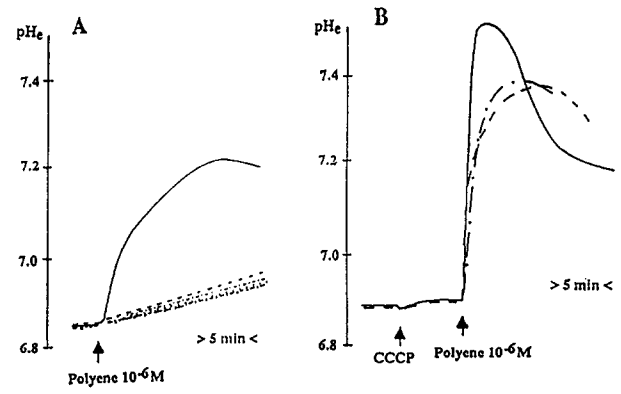


Fig. 3. pH changes in erythrocyte suspensions induced by vacidin A and its derivatives substituted at the carboxyl group. (A) antibiotics alone; (B) antibiotics in the presence of protonophore CCCP, 10^{-5} M. Vacidin A (—); VacME (– · –); VacGlyMe (- - -); Experimental conditions as in legend to Fig. 2.

In the case of nonaromatic heptaenes, such stimulation did not occur.

3.1.3. [Hb-50 / EK-50]

The degree of hemolysis determined in isoosmotic potassium chloride is dependent on the increase of KCl concentration inside the cells. The ratio [Hb-50/EK-50] reflects a difference between potassium influx and efflux. It can be seen that in the case of aromatic heptaenes, the hemolytic data did not parallel the permeabilizing activities. Similar values of [EK-50] and large differences in [Hb-50] permit to assume that the substituent at the carboxyl group of aromatic heptaene did not influence significantly channel-forming ability but had marked effect on ionic selectivity of the pathway induced. The same substitution at the carboxyl group of amphotericin B or candidin was meaningless for both channel formation and its selectivity.

3.2. Potassium and proton permeability

Kinetics of potassium and proton fluxes induced by vacidin A in red blood cells are shown in Fig. 2. The addition of this antibiotic at concentration $2 \cdot 10^{-7}$ or 10^{-6} M causes immediate potassium release and simultaneous uptake of protons. Effect on proton flux is essentially more dependent on antibiotic concentration than is the potassium efflux. The comparison of the protonophoric properties of vacidin A and its carboxyl group derivatives (Fig. 3) shows that at a concentration (10^{-6} M), inducing similar potassium efflux, only vacidin A was able to induce proton influx. None of the carboxyl-substituted derivatives tested is able to promote a significant proton uptake (Fig. 3A). For the derivatives, alkalinization of the medium occurred only in the presence of a protonophore. The pattern of pH changes in the presence of CCCP was similar for all derivatives. The results for VacGlyMe and VacME are shown in Fig. 3B.

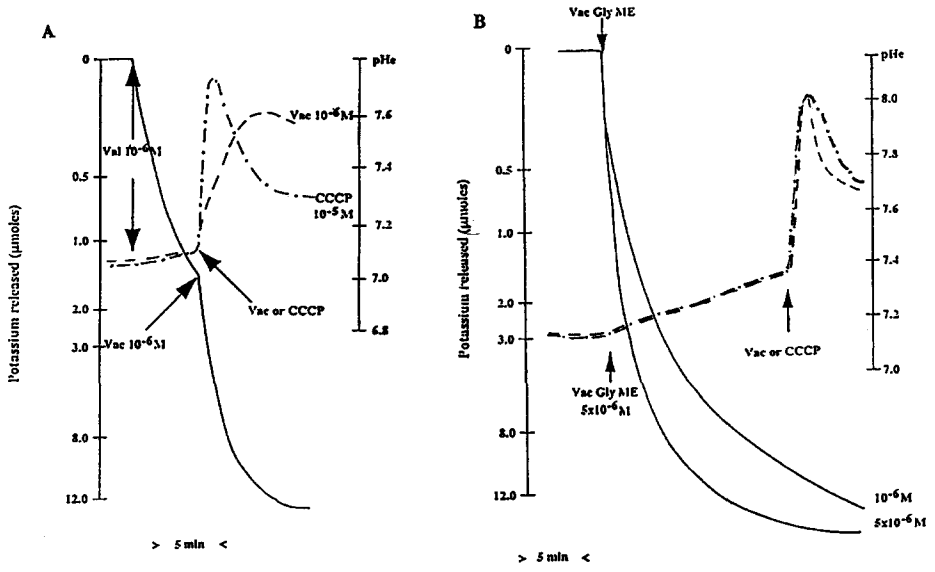


Fig. 4. Protonophoric properties of vacidin A. (A) Potassium efflux (—) induced by valinomycin 10^{-6} M and proton uptake induced by vacidin A, 10^{-6} M (- - -) or CCCP, 10^{-5} M (–·–). (B) Potassium efflux (—) induced by VacGlyMe $5 \cdot 10^{-6}$ M and proton uptake induced by vacidin A (- - -) or CCCP, 10^{-5} M (–·–). Experimental conditions as in legend to Fig. 2.

A similar diminishment of the protonophoric activity by substitution at the carboxyl group was observed also for gedamycin (data not shown).

The presented results provide additional evidence for our earlier suggestion, that although vacidin A and its carboxyl-substituted derivatives have similar ability of potassium permeability induction, the properties of the pathways formed are significantly different. Ionic selectivity of the channel formed by vacidin A is concentration dependent and its proton permeability increases with antibiotic concentration. In contrast, VacGlyMe or VacME form highly cation selective pathway independently on the antibiotic concentration.

The ability of vacidin A to increase permeability to proton was directly demonstrated in experiments with valinomycin (Fig. 4A) and VacGlyMe (Fig. 4B) In cells

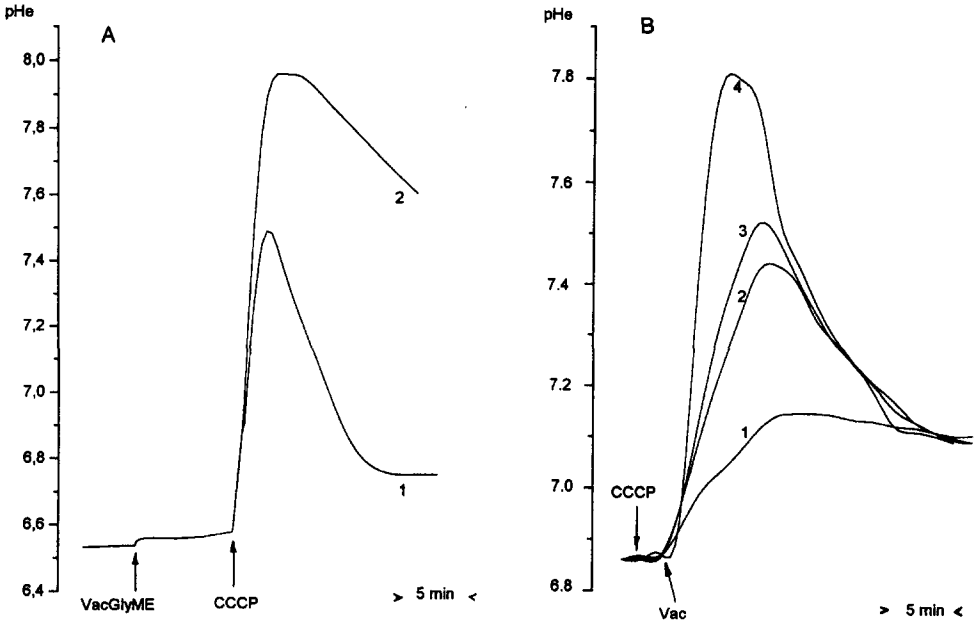


Fig. 5. (A) pH changes induced in suspension of: (1) untreated and (2) DIDS-treated red cells, by VacGlyMe, $5 \cdot 10^{-6}$ M. (B) pH changes induced in suspension of: (1) untreated, (2) DIDS-treated, (3) CCCP-treated and (4) DIDS- and CCCP-treated red cells, by vacidin A, 10^{-6} M. Experimental conditions as in legend to Fig. 2.

permeabilized by this polyene as well as by highly potassium-selective ionophore vacidin A worked in the same way as typical protonophore, CCCP.

3.3. Permeability to anions

The analogy with the reference cation-selective ionophore for which the mechanism of membrane permeability induction and selectivity of pathways induced are very well known [8] suggests the same explanation for the biphasic time-course of proton movement observed for the examined aromatic polyene macrolides. A treatment of red cells with VacGlyMe or VacME allows rapid potassium efflux down the concentration gradient and causes hyperpolarization of the membrane; the negative membrane potential produced by potassium leak is a sufficient driving force for proton uptake via CCCP, that is reflected by the increase of pH_e. The subsequent decline of pH_e indicates anion redistribution, due to the OH^-/Cl^- exchange governed by the natural, very efficient, anion transport system. A final result is KCl efflux. The experiments done on red cells in which the natural transport system was inhibited with DIDS [8,9] support such interpretation for VacGlyME (Fig. 5A). In cells with inhibited anion transport system negligible proton uptake induced by this polyene was abruptly increased by addition of CCCP whereas the subsequent decline of pH, being dependent on anion transport system, was strongly inhibited. Similar time-course of pH changes was observed only for low concentration of vacidin A (not shown); at concentration inducing permeability to protons, anion transport was not inhibited by DIDS (Fig. 5B). It suggested that the pathway formed at higher concentration of vacidin A has to be permeable not only to potassium and protons but also to anions.

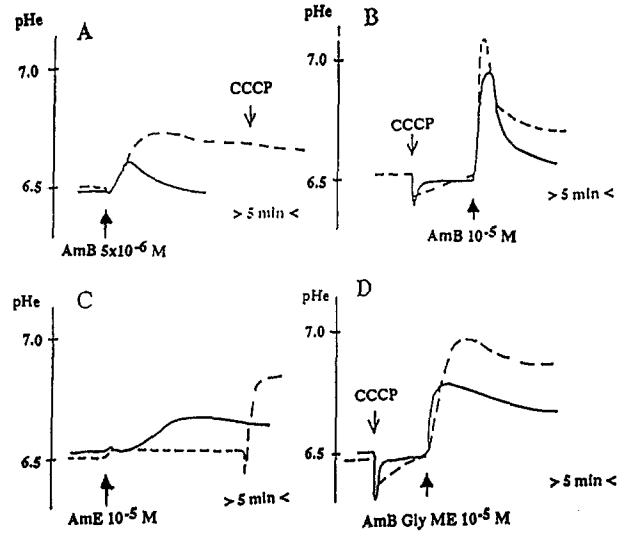


Fig. 7. pH changes induced in suspensions of normal (—) and DIDS-treated (- - -) red cells by: (A) AmB, $5 \cdot 10^{-6}$ M; (B) AmB, 10^{-5} M; (C) AME, 10^{-5} M; (D) AmBGlyMe, 10^{-5} M. Experimental conditions as in legend to Fig. 2.

The differences in the ability of anion permeability induction by polyene antibiotics are demonstrated in Fig. 6. In DIDS-treated cells, in the presence of a protonophore and potassium-selective ionophore, like valinomycin, pH_e is a reliable measure of membrane potential. Large pH_e increase and its stability indicated that membrane was

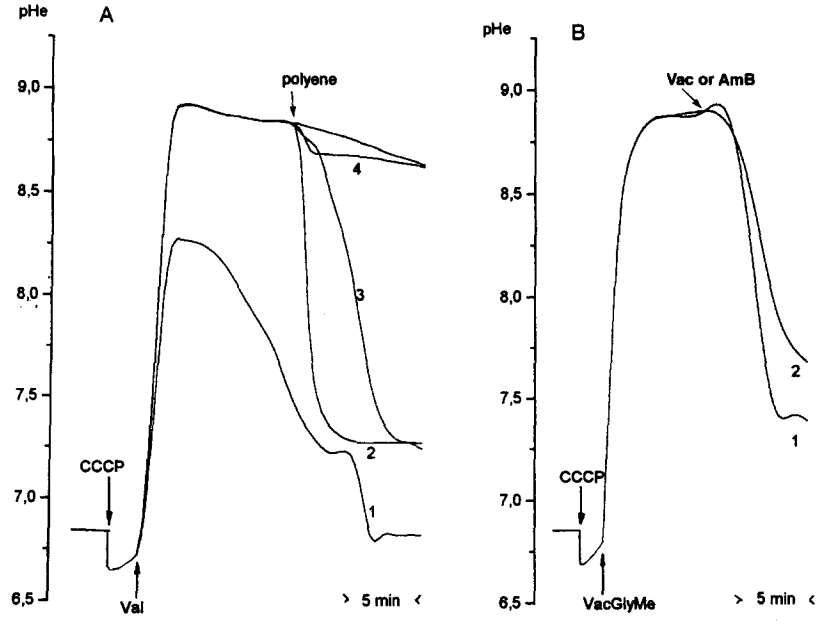


Fig. 6. OH^-/Cl^- exchange induced by vacidin A and amphotericin B in DIDS-treated cells. (A) Cells hyperpolarized with valinomycin, $2 \cdot 10^{-6}$ M and CCCP, 10^{-5} M and then treated with: (2) AmB, 10^{-5} M; (3) Vac, 10^{-6} M; (4) VacGlyMe, $5 \cdot 10^{-6}$ M; (1) time-course of pH changes in DIDS-untreated cells hyperpolarized with valinomycin and CCCP. (B) Cells hyperpolarized with VacGlyMe, $5 \cdot 10^{-6}$ M and then treated with: (1) AmB, 10^{-5} M and (2) Vac, 10^{-6} M.

strongly hyperpolarized and that OH^-/Cl^- exchange via band 3 protein was inhibited. When membrane was hyperpolarized by valinomycin (Fig. 6A), subsequent addition of VacGlyMe practically did not change this situation. In contrast, upon addition of vacidin A (10^{-6} M), pH gradient collapsed. This new steady-state pH_e was a compromise between tendency of H^+ to distribute according to the membrane potential (via CCCP and polyene channel) and the tendency of OH^- to equilibrate according to Cl^- distribution, (via polyene channel). An extent and stability of membrane hyperpolarization upon addition of VacGlyMe (Fig. 6B) is comparable with that obtained with valinomycin. It confirms the high cationic selectivity of the pathway formed by this compound. Subsequent decrease of pH_e induced by Vac indicating OH^-/Cl^- exchange demonstrates the different ionic selectivity of the channels formed in the membrane by native antibiotic and its derivative in which negative charge was eliminated. This

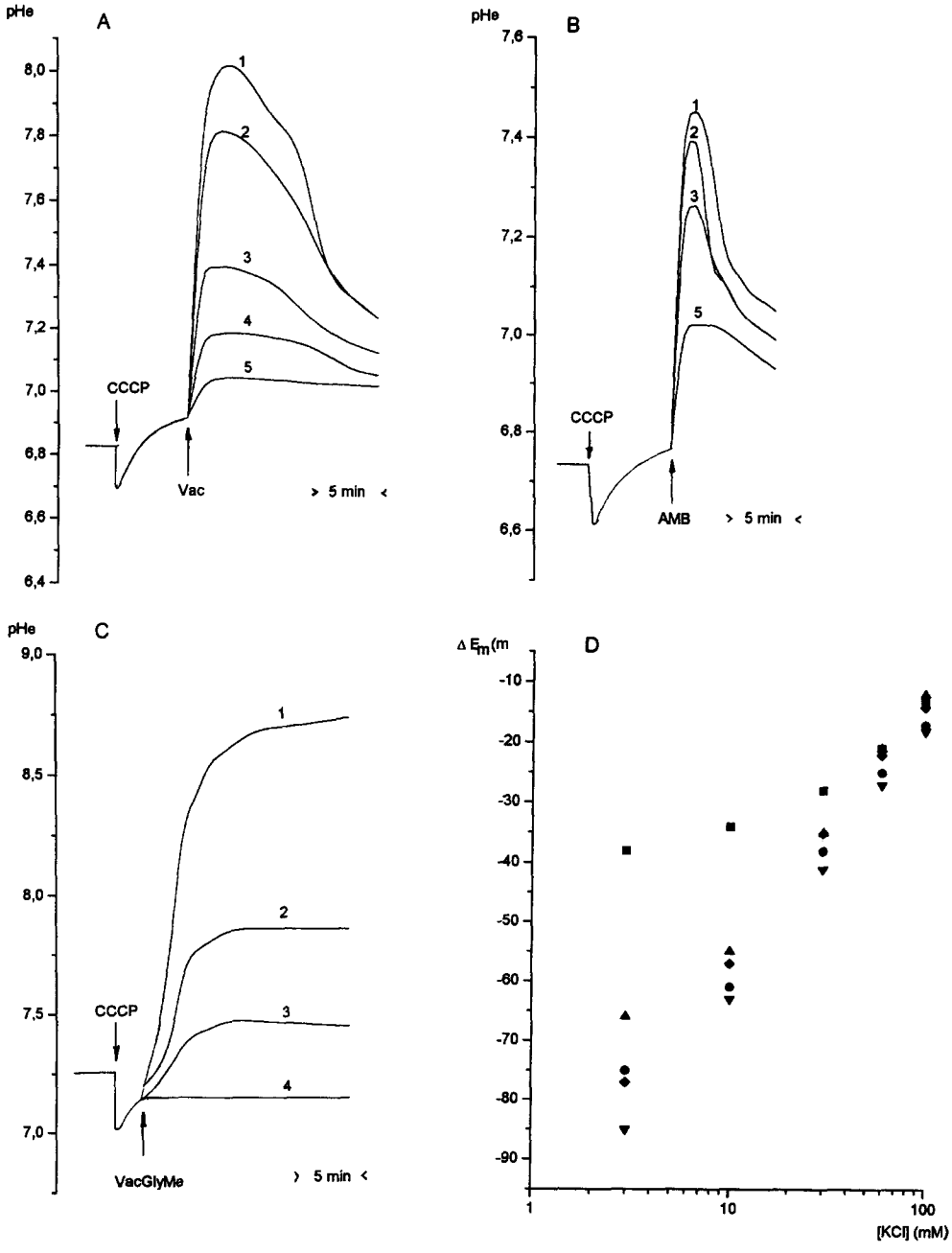


Fig. 8. Relationship between KCl concentration and membrane potential (E_m) measured by pH method in DIDS-treated cells. Time-course of pH changes in the presence of: (A) Vac, 10^{-6} M; (B) AmB, $5 \cdot 10^{-6}$ M; (C) VacGlyMe, $5 \cdot 10^{-6}$ M. Concentrations of KCl were as follows: 3 mM (1); 10 mM (2); 30 mM (3); 60 mM (4); 100 mM (5). (D) Relationship between changes in membrane potential (ΔE_m) and KCl concentration for: (▼) gramicidin 10^{-7} M; (◆) valinomycin $2 \cdot 10^{-6}$ M; (●) VacGlyMe $5 \cdot 10^{-6}$ M; (▲) Vac 10^{-6} M; (■) AmB $5 \cdot 10^{-6}$ M. Cell concentration: 10^8 cells/ml; temperature 24°C.

electrosilent exchange of anions has to be taken into account when the membrane potential is evaluated by the pH method.

Amphotericin B as well as it carboxyl group derivative induced OH^-/Cl^- exchange similar to that observed for vacidin A.

The patterns of pH changes obtained for amphotericin B, and its derivatives, AME and AmBGlyMe (Fig. 7A–D) was essentially different from those registered for aromatic heptaenes. Amphotericin B induced very small pH increase in both normal and DIDS-treated cells (Fig. 7A). Addition of protonophore 15 min after the antibiotic did not cause any pH changes, indicating that potassium gradient had been already lost. When CCCP was added prior to the antibiotic, biphasic curves was obtained but difference between untreated and DIDS-treated cells were very small (Fig. 7B); also the extent of pH changes was much smaller than that induced by vacidin A under the same conditions. Such results indicate the poor K^+/Cl^- selectivity of the channel formed. Both derivatives seem to have very similar properties to the native antibiotic (Fig. 7C,D).

3.4. Ionic selectivity indicated by the membrane potential

3.4.1. pH method

The influence of polyene antibiotics on relationship between KCl concentration and pH_e was measured in DIDS-treated cells in the presence of a protonophore. Assuming that in cells permeabilized to potassium and suspended in the medium containing impermeable cation, CCCP allows protons to equilibrate with E_m, the resulting changes in pH_e are related to membrane potential [E_m] as follows [11]:

$\Delta E_m = -(2.303RT/F) \times \Delta pH_e$, where $2.303RT/F = 58.7$ mV at 23°C and ΔpH_e is the difference between maximal pH_e obtained after addition of the antibiotic and protonophore and initial pH_e of the cell suspension.

For VacGlyMe (Fig. 8C) the response to KCl concentration was practically the same as for valinomycin and gramicidin. Resultant plot of ΔE_m vs. log KCl concentration was linear, and in the limit of error, the same for VacGlyMe, valinomycin and gramicidin, indicating a similar high cationic selectivity of VacGlyMe as that of reference ionophores (Fig. 8D).

In the case of vacidin A (Fig. 8A), if ΔE_m was calculated according to the maximal pH_e value, the plot had similar slope to that obtained for cations selective compounds; pH_e at the steady-state practically was independent on KCl concentration. It indicated that in DIDS-treated cells, in the presence of a protonophore K^+/H^+ exchange exceeded OH^-/Cl^- exchange. However, these two processes occurred simultaneously in cells untreated with DIDS and without protonophore (Fig. 5B); the net result of vacidin action was efflux of KCl.

Time-course and low value of maximal pH_e observed for amphotericin B (Fig. 8B) indicated low K^+/Cl^- selectivity of the channel formed by this antibiotic; cotransport of K^+ and Cl^- occurred also in DIDS-treated cells and seemed to surpass K^+/H^+ exchange even in the presence of protonophore.

3.4.2. Fluorescence with DiS-C_3-(5)

Differences in the selectivity of the pathway formed by vacidin A and VacGlyMe were also evidenced by changes

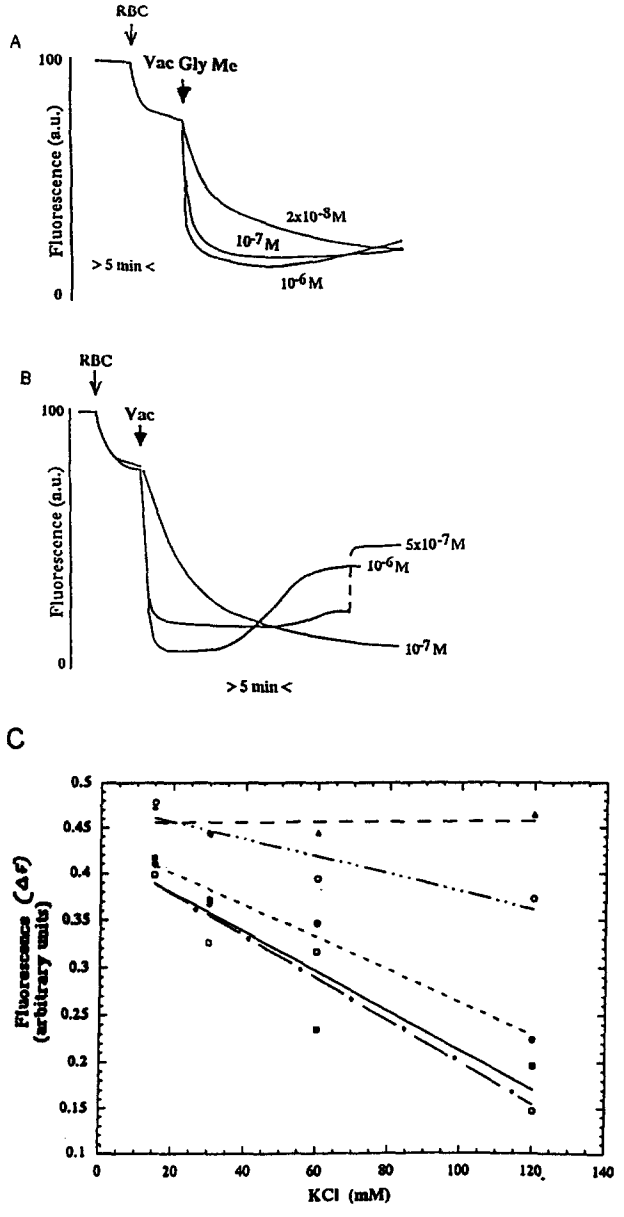


Fig. 9. Membrane potential measured by fluorescence of DiS-(C_3)-5. Effect of polyene concentration on time-course of changes in fluorescence determined for: (A) VacGlyMe; (B) Vac; decrease of fluorescence means membrane hyperpolarization. (C) Relationship between KCl concentration and fluorimetrically measured membrane potential changes (ΔF) induced by Vac at concentration: 10^{-7} M (–·–); $3 \cdot 10^{-7}$ M (– – –); $6 \cdot 10^{-7}$ M (–···–) and 10^{-6} M (– – –). Valinomycin (—) was used as a reference ionophore. ΔF is a difference between the fluorescence level at equilibrium after addition of antibiotic and the initial level (after addition of RBC). Cell concentration $2 \cdot 10^7$ cells/ml; temperature 23°C.

in the membrane potential measured by fluorescence, using DiS-C_3-(5) as a probe [10]. A decrease of fluorescence reflects membrane hyperpolarization. VacGlyMe (Fig. 9A) as well as VacME (not shown) induced membrane hyperpolarization to a steady level, independently of concentration. The first consequence of the addition of vacidin A (Fig. 9B) was also hyperpolarization but stability of the maximal level was dependent on antibiotic concentration. At 10^{-6} M hyperpolarization was followed by depolarization, insensitive to DIDS; these results and those obtained by pH measurements suggest that the primary event in the action of vacidin A is the formation of cation selective channels which subsequently transform to less selective ones. Determination of the membrane potential as a function of KCl concentration for various vacidin A concentration confirms the transformation of channel selectivity with antibiotic concentration (Fig. 9C). As expected, results obtained for VacGlyMe and VacME were similar to those obtained for valinomycin (not shown). However, due to concentration-dependent quenching of fluorescence, different for each aromatic heptaene, a quantitative estimation of K^+/Cl^- selectivity cannot be done by this method.

4. Discussion

It is well documented that large ring polyene macrolides, comprising aromatic and nonaromatic heptaenes, are channel – forming antibiotics (for review see [12]). However, the permeability characteristics of the channels, and the relation between selectivity and toxicity to animal cells, are not very well known. The present studies permitted: (1) to estimate the role of some structural factors of heptaene macrolides in their primary (membrane permeabilization to potassium) and secondary effects (hemolysis) on human red cells and (2) to explain large differences in the hemolytic activity in this group of antibiotics.

The structural features studied, essential for the activity against red blood cells, appeared to be: the aromatic residue and the carboxyl group (free or substituted).

The difference of one order of magnitude in the effective concentrations of native aromatic and nonaromatic heptaenes producing the same effects on red cells (Table 1) suggests the importance of the aromatic residue in the primary and secondary effects.

The role of the carboxyl group was different in aromatic and nonaromatic heptaenes. The ability of potassium channel formation was well preserved in the carboxyl-substituted derivatives in both aromatic and nonaromatic heptaenes. Substitution was insignificant for hemolytic activity of nonaromatic heptaenes. In contrast, the same substitution at the carboxyl group of aromatic heptaenes drastically reduced their hemolytic activity.

The mechanism of hemolysis caused by large macrolide ring polyene antibiotics is not yet completely understood. A colloidosmotic mechanism and lipid peroxidation have been proposed [2,5,13]. Whatever the exact mechanism, the induction of membrane permeability to monovalent cations and ion flux is an essential step preceding hemolysis. In fact, lipid peroxidation detected for amphotericin B seems to be a secondary event in the hemolytic process related to ion flux and swelling, because hemolysis can be prevented by addition of impermeants, balancing the osmotic pressure of hemoglobin and other large solutes inside the cells, to the external medium [5,14]. The complementarity between lipid peroxidation and ion fluxes in the mechanism of hemolysis induction needs to be analysed better.

Our previous studies [2,4,5] and results presented in this paper point to the importance of the permeability characteristics of the pathway formed for hemolytic efficiency of polyene antibiotics. All macrolides with a heptaenic chromophore confer a very large permeability of the red cell membrane to monovalent alkaline cations. For hemolysis according to coloidosmotic mechanism, a net increase of salt concentration inside the cells leading to swelling and lysis is required. For all antibiotics studied hemolysis occurred at higher concentration than potassium efflux. Therefore, hemolysis in isoosmotic KCl is limited by conductive Cl^- influx, which in contrast to electroneutral anion exchange is naturally slow. However, if membrane permeability was increased selectively to potassium, the rate of cell swelling and lysis was limited by Cl^- conductive influx. This process could be increased by induction of permeability to protons or by increasing the basic membrane permeability to anions. Different hemolytic activity was related to different mechanisms of Cl^- flux accompanying potassium influx, depending on antibiotic studied. Hemolysis was determined by the selectivity of the channels, formed by a given heptaene.

The data presented indicate that the relations between structure and the permeability characteristics were different in aromatic and nonaromatic heptaenes.

The selectivity of the channel formed by native vacidin A was strongly dependent on concentration; a channel formed at low antibiotic concentration was highly selective to potassium and within this concentration range hemolysis could be induced only in the presence of protonophore. Hemolysis occurred at a much higher concentration, and was correlated with the appearance of the permeability to H^+, OH^- and Cl^-. It is permitted to assume that vacidin A, depending on concentration, formed channels of different structure and selectivity. Formation of the low selectivity channel was determined by the presence of free carboxyl group in the molecule. Modification of this group by amidation or esterification, eliminated the ability of formation of low selectivity channel. The hemolytic activity of the carboxyl substituted-aromatic heptaenes was weak because the pathway they induced was highly selective to potassium and increase of the permeability to protons and anions practically was not observed. Similar changes in selectivity of the permeability induced with increasing

antibiotic concentration were reported for nystatin [8]. However, the three order of magnitude difference in the effective concentrations of vacidin A and nystatin exerting similar effects on the red cell membrane is worth noticing.

The permeability pathway formed by amphotericin B was of low selectivity, independent of concentration. The properties of the pathway were not changed by esterification or amidation of carboxyl group. Low selectivity of the channels formed in the human red cells was previously reported for amphotericin B ($PK^+/PCl^- = 6$) [9] and also for nystatin ($PK^+/PCl^- = 2$) [7].

Differences in the ionic selectivity of the permeability pathway formed by nonaromatic heptaene amphotericin B (and it derivatives) and aromatic heptaene, vacidin A have been lately demonstrated in large unilamellar vesicles [15]. Especially the selectivity between monovalent cations vs chloride varied with antibiotics. It was very strong with vacidin, weak with amphotericin B and none with amphotericin B derivatives. The selectivities observed were antibiotic, concentration and time dependent, which confirmed the existence of different type of channels. Various types of channels with different selectivity are induced also in *Leishmania sp.* upon the action of amphotericin B [16].

The modifications at the carboxyl group change the charge of the molecule from zwitterionic to positive one as well as the amphipatic properties of the antibiotic. Both properties are important for water solubility [17] and self-association [17,18]. The relation between the state of the antibiotic in solution and its effect on cell and model membranes was demonstrated lately [18,19].

It should be noted, that the same substituent at the carboxyl group differently change properties of aromatic and nonaromatic heptaenes. It has been demonstrated in the present study as well as at the previous one on lipidic vesicles [20–23]. It was especially evident in modification of the permeabilizing effectivity, affinity to ergosterol and cholesterol containing membranes [20] and in the structure of the permeabilizing species, reflected in circular dichroism (CD) spectra of polyenes bound to cholesterol-containing lipidic vesicles. Spectra of vacidin A and VacME differed qualitatively [4,21,22], whereas in the case of amphotericin B and AME the differences were only quantitative [23].

The mechanism of action of aromatic and nonaromatic heptaenes on the animal cells seems to be different.

5. Conclusions

The hemolysis induced by aromatic hepaenes is a secondary effect of the permeability induction to monovalent cations, protons and anions. The conductive flux of chloride is stimulated by the induction of the permeability to protons and increase of the basic permeability to anions. Only for this group of antibiotics the participation of proton conductance can explain the high hemolytic activity. For nonaromatic heptaenes permeability to protons seemed to be unimportant.

Structure–hemolytic activity relationships are different in aromatic and nonaromatic groups of heptaenes studied. In the group of aromatic heptaenes a qualitative changes in the selectivity of the pathway formed, leading to an essential diminishment of the hemolytic activity, can be obtained by elimination of the carboxylate anion. In the group of nonaromatic heptaenes this type of modification is not sufficient to decrease the hemolytic activity. Our results emphasize the interesting properties of the carboxyl-substituted derivatives of aromatic polyene antibiotics, the more as they have been shown to keep their antifungal activity [2].

Acknowledgements

We gratefully acknowledge the financial support (to B.C. and E.B.) of The Chemical Faculty, Techn. University of Gdansk and (to J.B. and O.S.) of CEFIPRA (grant 304-1) and CEE (grant CI 1-0517-F).

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