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Biochimica et Biophysica Acta 1512 (2001) 98–110

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Influence of plant terpenoids on the permeability of mitochondria and lipid bilayers

Andrey Y. Abramov [a], Maria V. Zamaraeva [a], Albert I. Hagelgans [a], Rustam R. Azimov [b], Oleg V. Krasilnikov [c,*]

[a] *Department of Biophysics, Tashkent State University, 700095 Tashkent, Uzbekistan*
[b] *Institute of Physiology and Biophysics, Academy of Science of the Republic of Uzbekistan, 700095 Tashkent, Uzbekistan*
[c] *Laboratory of Membrane Biophysics, Department of Biophysics and Radiobiology, Federal University of Pernambuco, Av. prof. Moraes Rego, S/N, Cidade Universitaria, 50670-901 Recife, PE, Brazil*

Received 15 March 2000; received in revised form 27 February 2001; accepted 1 March 2001

Abstract

Five sesquiterpene alcohol esters of the carotane series, from plants of the genus *Ferula*, were investigated with regard to their capacity to modify the ion permeability of both planar lipid bilayers and mitochondria. These compounds are subdivided into two structural groups that differ in their effects on membrane permeability. Complex esters of sesquiterpene alcohols with aliphatic acids, which constituted the first group (lapidin and lapiferin), do not possess ionophoric properties. The second group comprised complex esters of sesquiterpene alcohols with aromatic acids (ferutinin, tenuferidin and ferutidin), all of which increase cation permeability of lipid bilayers and mitochondria in a dose-dependent manner. A pronounced selectivity of the terpenoid-modified membranes for divalent cations versus monovalent cations was found. Evidence of a carrier mechanism for terpenoid-induced ion transport is demonstrated. A tentative complex composed of a divalent cation with two molecules of membrane-active terpenoid is proposed.

Keywords: Terpenoid; Carrier; Ion transport; Lipid bilayer; Mitochondria; Divalent cation

1. Introduction

Plants of the genus *Ferula* (family Apiaceae) are long-lived plants that are widespread in Europe and Asia. They are often used as spices in cooking and in preparation of canned food and they are usually rich in terpenoids, an abundant class of biologically active compounds which occurs in all living organisms and can display high biological activity [1,2]. In a systematic investigation of 40 Central Asian species of *Ferula*, more than 100 new terpenoids have been isolated and structurally characterized in the laboratory of Dr. A.I. Saidkhodzjaev since 1970 (Institute of Chemistry of Plant Substances, Academy of Science of Republic of Uzbekistan).

Panoferol, a mixture of terpenoids from *Ferula*, has been previously shown [3] to accelerate pubescence in chickens. The mechanism of action is unknown, but it has been suggested that some components of panoferol may increase sex hormone levels and calcification rates in developing eggs [4], suggesting that panoferol acts on calcium homeostasis. This

* Corresponding author. Fax: +55-81-3271-8560;
E-mail: kras@npd.ufpe.br

0005-2736/01/$ – see front matter
PII: S0005-2736(01)00307-8

suggestion was confirmed by the discovery that one of the components of the panoferol mixture (ferutinin) possesses Ca^{2+}-ionophoric properties [5].

Intracellular calcium concentration plays a pivotal role in many physiological and pathological processes in all cell types. Because the regulation of calcium homeostasis is a key element of metabolic control, it is very important to find new molecular modulators.

Planar lipid membranes (BLM) and mitochondria provide convenient experimental systems for screening membrane-active compounds and for analyzing transport mechanisms. The present communication presents a study of the mechanism of terpenoid-induced ion transport and of the correlation between terpenoid structure and ionophoric properties.

2. Materials and methods

2.1. Chemicals

Sesquiterpene alcohol esters used in the present study were generously provided by Dr. Saidkhodzjaev. Ferutinin and tenuferidin were isolated from *Ferula tenuisecta* [6,7]. Ferutidin was isolated from *Ferula orina* [8], and lapidin [9] and lapiferin [10] were isolated from *Ferula lapidosa*. The terpenoids studied in the present communication are complex esters of ferutinol with *n*-oxybenzoic acid (ferutinin, tenuferidin), methoxybenzoic acid (ferutidin), angelic acid (lapidin) or angelic and acetic acids (lapiferin). Ethanol was used as a solvent to prepare terpenoid stock solutions (5 mM). EGTA, EDTA and rotenone were purchased from Sigma (St. Louis, MO, USA). Tris was from Fluka. All other reagents, of the highest purity available, were purchased from Reakhim (Mikhailovsk, Russia).

ChemWindowDB Version 4.0 software (SoftShell International) was used to draw the two-dimensional chemical structures of terpenoids presented in Fig. 1. Three-dimensional chemical structures were built with CS Chem3D Version 4.0 software (CambridgeSoft).

2.2. Membranes and electrophysiology

Lipid bilayers were formed at room temperature (25°C) by the technique of Montal and Mueller

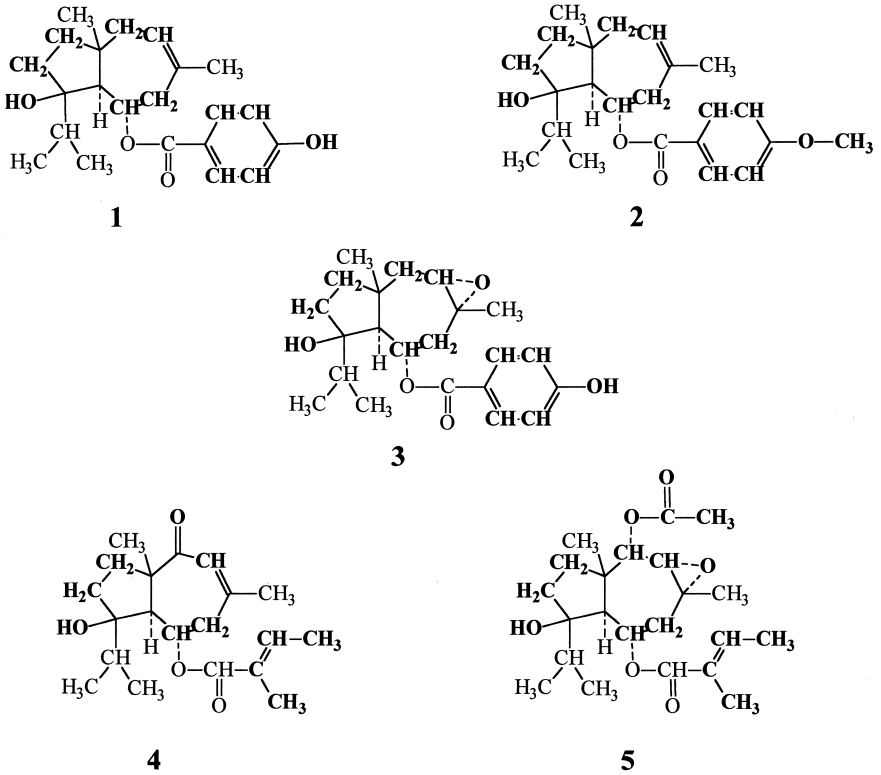

Fig. 1. Chemical structures of the terpenoids: 1, ferutinin; 2, ferutidin; 3, tenuferidin; 4, lapidin; 5, lapiferin.

[11]. If not mentioned specially, a solution of bovine brain phospholipids (10 mg/ml in *n*-hexane) was used to spread monolayers on the surface of two buffered salt solutions (4 ml). These were separated by a Teflon partition 25 μm thick in a Teflon chamber. After solvent evaporation the membrane was formed by raising the monolayers above the level of the hole (0.2–0.4 mm in diameter) connecting the hemichambers through the partition. The hole was pretreated with a solution of 5% hexadecane in *n*-hexane.

Experiments were done under voltage-clamp conditions. Current that passed through the bilayers was measured with Ag/AgCl electrodes connected through salt bridges (3% agar with 3 M KCl) in series with a voltage source and a current amplifier (K284UD1A; Svetlana, Leningrad, USSR). The *trans* compartment of the experimental chamber was connected to the virtual ground. Voltage pulses were applied to the *cis* compartment of the chamber. The amplifier signal was monitored with a storage oscilloscope (C1-13; Box B-2970, Vilnus, USSR; where the bilayer formation was also continuously monitored via change in capacitive current) and recorded on a strip chart recorder (KCP-4; Box A-7859, Mahachkala, USSR). Current traces were read by hand. Capacity of the bilayers employed was equal to 0.75 ± 0.03 μF/cm^2. The conductance of BLMs (G) in symmetrical solutions was defined as $G = I/V$, where I is the transmembrane current flowing through the channels and V corresponds to the fixed potential. Basal conductance of BLMs was less than 5 pS.

Modified BLMs with steady-state conductance were used to determine the steady-state current-voltage relationship. Transmembrane voltage was switched from zero to different values of positive and negative potentials for approx. 1 min, and the corresponding currents were measured.

Cation-anion selectivity of modified BLMs was measured in the presence of 3-fold concentration differentials of either monovalent or divalent cation electrolytes (50 mM/150 mM and 5 mM/15 mM, *cis*/*trans*, respectively). Zero current potential (V^*) was defined as the potential that must be applied to the experimental cell in order to reach zero transmembrane current, equal to that of a symmetrical system with zero mV applied potential.

The cation-cation selectivity of modified BLMs was evaluated from values of zero current potential in bi-ionic systems when bilayers separated 10 mM solutions (in case of divalent cation electrolytes) or 20 mM solutions (in case of monovalent cation electrolytes). Assuming an ideal cation selective membrane, the Goldman equation [12] was used to estimate the ratio of BLM permeability coefficients for cations (P).

Comparison of BLM permeability for mono- and divalent cations was performed under conditions in which the BLM separated a 20 mM solution of monovalent cation electrolyte in the *cis* compartment from a 10 mM solution of divalent cation electrolyte in the *trans* compartment. In this case the ratio of the BLM permeability coefficients for mono- (P_m) and divalent cations (P_d) was calculated using an equation derived from equation A6 of Lewis [13]. The derived equation is as follows:

$$\frac{P_m}{P_d} = 4\frac{Me^{2+}}{Me^{+}} \frac{\exp(A)}{1+\exp(-A)} \quad (1)$$

where $A = V^*F/RT$; Me^{2+} and Me^{+} are activities of divalent and monovalent cations, respectively; V^* is the zero current potential; F is the Faraday constant, R is the gas constant, and T is the absolute temperature. Activity coefficients were taken from [14].

If the solutions in the two compartments of the experimental cell contained mixtures of monovalent and divalent electrolytes, respectively, the selectivity of modified BLMs was analyzed using the equation suggested by Lewis [13]:

$$C = \frac{Me_o^{+} + \dfrac{4P_d Me_o^{2+}}{P_m(1+C)}}{Me_i^{+} + \dfrac{4P_d Me_i^{2+} C}{P_m(1+C)}} \quad (2)$$

where $C = \exp(A)$.

With the bi-ionic solutions used the positive value of V^* indicates that the cations presented in the solution in the *trans* compartment of the chamber pass through terpenoid-modified BLM better than one presented in the *cis* compartment.

Thick phospholipid membranes (TLM) were formed at room temperature (25°C) on the hole (approx. 0.2 mm in diameter) connecting the two Teflon hemichambers (of 2 ml) filled with buffered salt solutions equal to those used for BLM experiments. A

2% solution of a phosphatidylcholine-cholesterol mixture (3:1, by mass) in *n*-decane was used. To form thick membranes approx. 10 μl of the lipid mixture was applied over an orifice. Experiments were done under voltage-clamp conditions. A binocular microscope and the monitoring of the capacitive current in reply to continue triangular voltage pulses were used to control the absence of the bilayer zones. At the chosen diameter of the hole and the quantity of the applied lipid solution, the bilayer zones did not appear for hours and the capacity of the thick membranes was always less than 0.001 μF/cm^2. The estimated thickness of such membranes was 25–30 μm which was several thousand times thicker than BLM (approx. 5 nm). All other electrical characteristics of TLM were defined as described above for BLM.

2.3. Estimation of the apparent stoichiometry of a terpenoid-Ca^{2+} complex

It is reasonable to assume that terpenoids interact with ions on the phase boundary (water solution/lipid membrane) and that the resulting complex diffuses through a membrane and determines the final BLM conductance. The general reaction can be given by the following equation:

$$n\mathrm{Ter} + m\mathrm{Ca}^{2+} \Leftrightarrow \mathrm{Ter}_n\mathrm{Ca}_m^{2+}$$

where n and m are numbers of terpenoid molecules (Ter) and calcium ions in a conductive complex ($\mathrm{Ter}_n\mathrm{Ca}_m^{2+}$).

In the presence of a constant concentration of Ca^{2+} the rates of the forward (V_1^{Ter}) and the reverse (V_2^{Ter}) reactions are the following:

$$V_1^{\mathrm{Ter}} \propto [\mathrm{Ter}]^n \qquad V_2^{\mathrm{Ter}} \propto [\mathrm{Ter}_n\mathrm{Ca}_m^{2+}]$$

where [Ter] is the terpenoid concentration in solution and $[\mathrm{Ter}_n\mathrm{Ca}_m^{2+}]$ is the concentration of conductive complex in membrane.

At equilibrium $V_1^{\mathrm{Ter}} = V_2^{\mathrm{Ter}}$ and $[\mathrm{Ter}_n\mathrm{Ca}^{2+}m] \propto [\mathrm{Ter}]^n$. Reasonably assuming that BLM conductance (G_{BLM}) is proportional to the concentration of the conductive complex one can obtain $G_{\mathrm{BLM}}^{\mathrm{Ter}} \propto [\mathrm{Ter}]^n$. If so,

$$\log G_{\mathrm{BLM}}^{\mathrm{Ter}} \propto n \log[\mathrm{Ter}]$$

where n is the slope of the $G_{\mathrm{BLM}}^{\mathrm{Ter}}$ against [Ter] dependence in a double log plot that is numerically equal to the number of terpenoid molecules in a conductive complex.

Applying the same reasoning to Ca^{2+} in the presence of a constant concentration of terpenoids the following equations can be obtained:

$$V_1^{\mathrm{Ca}} \propto [\mathrm{Ca}^{2+}]^m \qquad V_2^{\mathrm{Ca}} \propto [\mathrm{Ter}_n\mathrm{Ca}_m^{2+}]$$

$$V_1^{\mathrm{Ca}} = V_2^{\mathrm{Ca}} \qquad [\mathrm{Ter}_n\mathrm{Ca}_m^{2+}] \propto [\mathrm{Ca}^{2+}]^m$$

$$G_{\mathrm{BLM}}^{\mathrm{Ca}} \propto [\mathrm{Ca}^{2+}]^m \text{ and } \log G_{\mathrm{BLM}}^{\mathrm{Ca}} \propto m \log[\mathrm{Ca}^{2+}]$$

where m is the slope of $G_{\mathrm{BLM}}^{\mathrm{Ca}}$ against [Ca^{2+}] in a double log plot that is numerically equal to the number of Ca^{2+} in a conductive complex.

In both cases these theoretical slope values reflect the maximal number of components (terpenoid molecules and calcium ions) in the conducting complex when the complex formation is the limiting step in the whole sequence of the transport process. In the real situation the slope depends on the experimental condition and can vary from 1 to the maximum theoretically predicted. In all cases a slope value larger than 1 unequivocally points out that more than one molecule of carrier and/or ion participate in the conducting complex formation and the ratio of the slopes (n/m) gives an estimating stoichiometry of the complex and has been employed in our study to evaluate the complex.

A separate set of experiments were done to verify if there is a decrease in concentration of the terpenoids in aqueous phase caused by a possible adsorption onto the wall of the chamber and other reasons. In order to do this the spectroscopy method was employed. No marked difference in maximum of absorption (257 nm) was detected during 24 h experiments with ferutinin, ferutidin, or tenuferidin (data not shown).

The concentrations of free calcium ions were calculated using the program 'Bound and Determined' [15].

2.4. Isolation of mitochondria and determination of the permeability of their membranes

Mitochondria were isolated from rat liver using the routine differential centrifugation protocol [16], in a solution containing 250 mM sucrose, 1 mM

EDTA, and 19 mM Tris-HCl, pH 7.4. The pellet was resuspended and washed in a second solution containing 250 mM sucrose, 10 mM Tris-HCl, pH 7.4. The resultant pellet was resuspended in the second solution at a concentration of approx. 50 mg/ml of protein (measured by the biuret method), stored on ice and used within a few hours.

The passive permeability of mitochondrial membranes for ions was measured by following energy-independent swelling in isosmotic nitrate solutions as described by Brierley [17]. According to this method, the permeability of mitochondrial membranes can be determined quantitatively and rather simply, based on the kinetics of their energy-independent swelling in various saline solutions. In all investigations of charged particle transport through the inner membrane of mitochondria, the electrical phenomena accompanying these processes should be taken into account. As indicated previously [18], osmotic swelling in the presence of electrolytes occurs only when both an anion and a cation permeate into the matrix compartment of mitochondria, increasing the osmotic pressure inside the organelle without creating a significant diffusion potential. Application of ionophores with known properties and systematic variation of cationic and anionic constituents of the medium permit one to study the permeability of inner mitochondrial membranes for specific ions under normal and experimental conditions.

Nitrate salts of different cations were used to study the passive permeability of inner membranes of mitochondria to cations in the presence of terpenoids. Salt concentrations were 120 mM for Na^+ and K^+, 80 mM for Mg^{2+} and Ba^{2+}, and 40 mM for Ca^{2+} and Sr^{2+}. In addition, Ca^{2+} and Sr^{2+} solutions contained 120 mM sucrose. All solutions were buffered with Tris-NO_3 to pH 7.4. To exclude possible energy-dependent transport in these experiments, the incubation medium was always supplemented with rotenone (0.33 μg/ml). Measurements were performed at room temperature in 3 ml glass cuvettes. The final concentration of mitochondria, evaluated in terms of protein concentration, was about 1.0 mg/ml. The suspension was continuously agitated with a magnetic stir bar. Swelling was observed as the decrease in absorbance at 520 nm, using an LMF-2 photometer (LOMO, Leningrad, USSR).

The ratio between rates of swelling in the presence (S_i) and absence (S_o) of terpenoids ($R = S_i/S_o$) was used to quantify the change in permeability of mitochondrial membranes: $R > 1$ reflects increased permeability, while $R < 1$ indicates that modified membranes are less permeable than controls.

2.5. Statistics

Student's *t*-test was used to evaluate the significance of the difference between mean values. Data are presented as mean ± S.D.

3. Results

Addition of 1–3 μM ferutidin or tenuferidin in the experimental chamber was sufficient to considerably increase the bilayer conductance. On the other hand, when either of the other two terpenoids (lapidin or lapiferin) was added to the solution up to 200 μM we saw no increase at all in BLM conductance (data not shown). Hence the terpenoids studied by us can be divided into two groups which do or do not possess the ability to increase BLM conductance. Representatives of the membrane-active group of terpenoids became objects of our rapt study. Usually under influence of ferutidin or tenuferidin (Fig. 2), a rise in BLM conductance began soon after the addition of these terpenoids. A new quasi-steady-state level of BLM conductance was reached within 10–20 min. The kinetics with which a higher conductance level was attained under the influence of these two terpenoids resembles the ferutinin influence [5]. In all cases the final conductance level depends on both the concentration of the membrane-active terpenoids (ferutidin, ferutidin or tenuferidin) and on whether these terpenoids were added into one or both BLM bathing solutions. In the latter case, BLM conductance was at least 5-fold higher (Fig. 2B). Constant mixing of bathing solutions on both sides of the bilayer was important and was done consistently in all experiments. It appears that the membrane-active terpenoids do not require a specific lipid because they increase the conductance of lipid bilayers formed from bovine brain phospholipids as well as from pure phosphatidylcholine. To address the terpenoid-mediated transport mechanism and to rule out non-specific effects of the compounds studied,

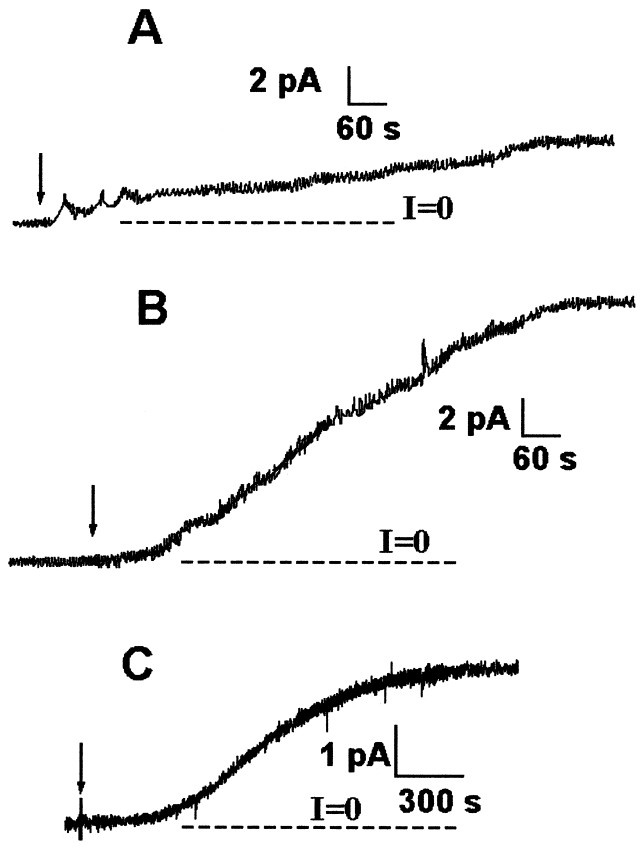

Fig. 2. Time course of the current in response to the addition of 10 μM of tenuferidin. In these experiments the bathing solutions contained 5 mM $CaCl_2$, 1 mM Tris-HCl, pH 7.5. Bilayer (A,B) and thick (C) membranes were clamped at −50 mV. Arrows indicate the moment of addition of tenuferidin to the *cis* side (A) or to both sides of the membrane (B,C). Time and current scales are given in the figure.

experiments with TLM (whose thickness (25–30 μm) is several thousand times larger than that of BLM (approx. 5 nm) and the size of terpenoids (approx. 1 nm)) were done. It was found that all three membrane-active terpenoids are able to increase TLM conductance, although TLM conductance increased slower and its final value was lower than the parameters established on bilayer membranes. An example of such records is shown in Fig. 2C. The ability of the membrane-active terpenoids to increase the conductance of TLM is consistent with the carrier mechanism in their action, because it is very difficult to imagine the formation of a channel-like structure from approx. 1 nm elements which runs through the 25–30 μm thick hydrophobic zone. In a symmetrical electrolyte system the one-side addition of terpenoids leads to the appearance of a negative potential on the side of their addition. We found the same results in BLM as in the TLM systems. Hence, the terpenoid-induced transport is electrogenic. It means that the complex of terpenoids with a cation possesses a free charge.

Steady-state current-voltage characteristics of bilayers modified by any of the 'membrane-active' terpenoids were linear and symmetrical in the range of potentials from −150 to +150 mV (Fig. 3). The shape was somehow different from the cyclic current-voltage relationships reported earlier for ferutinin-modified bilayers [5], due to the difference in the method employed. The difference in shape of the cyclic (relatively almost instant, obtained at a continuous triangular voltage pulse) and the steady-state current-voltage relationships reflects the time-consuming reorganization of the terpenoid-build ion-transporting units (presumably in bilayer) with voltage, which takes more time at lower voltages.

In the presence of a 3-fold transmembrane NaCl gradient (50 mM/150 mM; *cis*/*trans*), a zero current membrane potential for ferutidin-modified BLM was found reliably ($P < 0.05$) larger (13.9 ± 0.1 mV; $n = 5$) than for tenuferidin (12.8 ± 0.2 mV; $n = 5$). Examples of typical I-V curves of BLMs modified by these two terpenoids are presented in Fig. 4A. The positive value of V^* indicates that cations pass through terpenoid-modified BLM better than anions. These val-

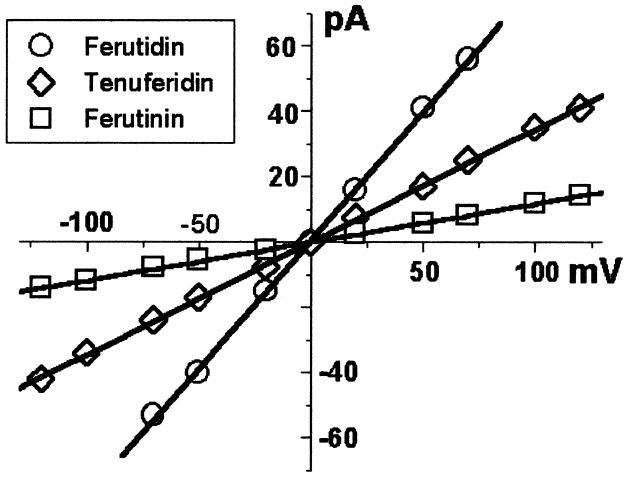

Fig. 3. Steady-state current-voltage relationships of terpenoid-modified bilayers. ○, ferutidin; ◇, tenuferidin; □, ferutinin. Concentration of terpenoids was 10 μM. All other experimental conditions are described in the legend to Fig. 2 and in Section 2. Results of a typical experiment are presented.

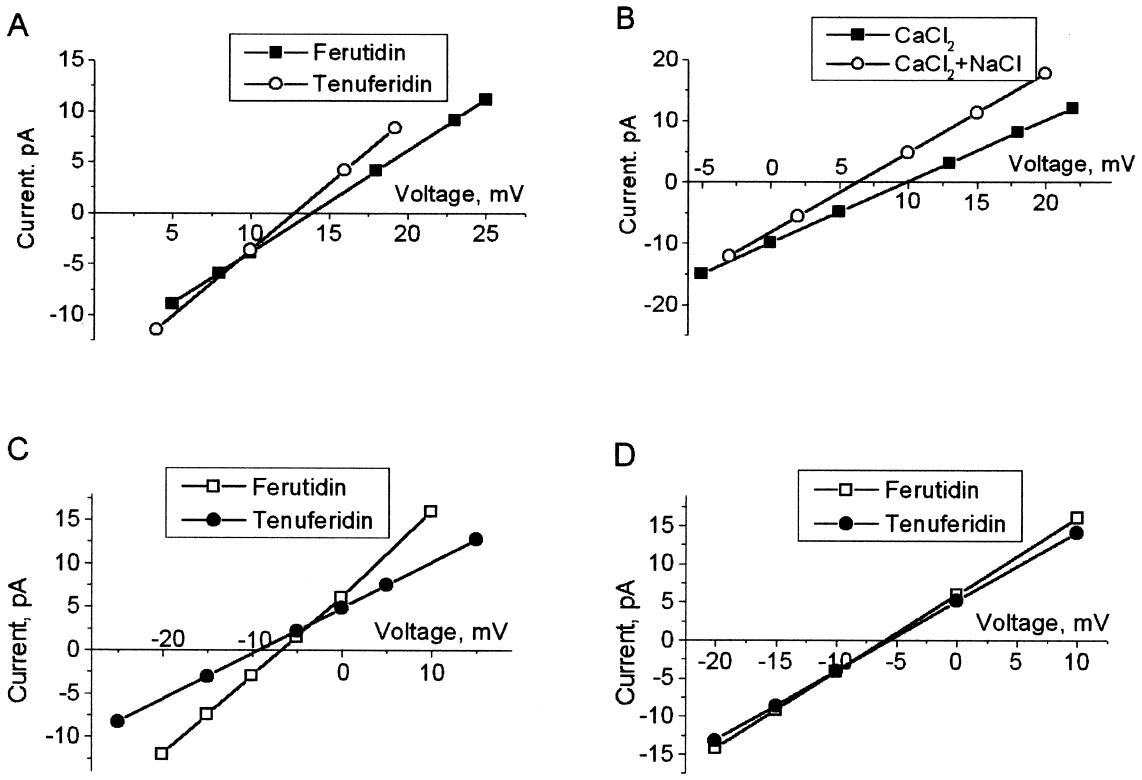

Fig. 4. Representative I-V curves of modified BLMs at asymmetrical ionic conditions. (A) A 3-fold transmembrane NaCl gradient (50 mM/150 mM; *cis*/*trans*) was used. BLMs were modified by ferutidin or tenuferidin as shown in the figure. (B) A gradient of $CaCl_2$ (5 mM/15 mM, *cis*/*trans*) in the absence or in the presence of 150 mM NaCl at both sides on bilayer membranes modified by ferutidin was used. (C) BLM modified by terpenoids separates dissimilar solutions: 10 mM $CaCl_2$ (*cis* compartment) and 20 mM NaCl (*trans*). (D) BLM modified by terpenoids separates dissimilar solutions: 10 mM $CaCl_2$ (*cis* compartment) and 20 mM KCl (*trans*). The presented data are of typical experiments. The concentration of terpenoids on both sides of BLM was 40 μM. Other conditions were as described in Section 2 and in the text.

ues of V^* were about half the potential expected for ideal cation-selective membranes (approx. 26 mV). Zero current membrane potentials were also far from ideal when a KCl gradient (50 mM/150 mM; *cis*/*trans*) was used as well. Therefore, in monovalent cation electrolyte solutions, terpenoid-modified bilayers are permeable for monovalent cations and, to a lesser extent, for chloride.

Cation-anion selectivity was considerably greater when modified membranes were bathed with divalent cation electrolyte solutions. V^*, measured in the presence of a gradient of $CaCl_2$ (5 mM/15 mM, *cis*/*trans*), on bilayer membranes modified by ferutidin and tenuferidin was found to be almost the same ($P > 0.1$): 9.9 ± 0.5 mV ($n = 5$) and 9.7 ± 0.7 mV ($n = 5$), respectively (Fig. 4B). Under these conditions, the Nernst potential for a $CaCl_2$ gradient is approx. 12.5 mV. Hence, tenuferidin- and ferutidin-modified bilayers demonstrate pronounced selectivity for calcium ions, which, however, is slightly less than that established earlier for the ferutinin-treated BLM under analogous conditions (12.5 ± 0.5 mV [5]).

If, in addition to a *cis*/*trans* $CaCl_2$ gradient (5 mM/15 mM), NaCl is added simultaneously to both compartments at a concentration of 150 mM, V^* is reduced to new statistically indistinguishable ($P > 0.1$) levels: 6.2 ± 0.2 mV ($n = 6$) and 6.3 ± 0.2 mV ($n = 6$) for ferutidin- and tenuferidin-modified membranes, respectively. Results of a typical experiment are presented in Fig. 4B. The observed reduction of V^* could be explained by a shunting of Ca^{2+} current by sodium and chloride ions. Moreover, it indicates that in the presence of calcium ions on both sides of BLM, the permeability ratio P_{Na}/P_{Ca} is lower (the application of Eq. 2 gives 0.30 and 0.29 for ferutidin- and tenuferidin-modified membranes, respectively)

than one would expect from the P_{Na}/P_{Cl} and P_{Ca}/P_{Cl} values obtained in simpler systems. It is even less than the P_{Na}/P_{Ca} ratio obtained in a bi-ionic system (0.52 and 0.56, respectively). These facts confirm that divalent cations are preferentially transported through terpenoid-modified lipid bilayers.

Bi-ionic systems are fruitful in comparative analyses of the selectivity of modified membranes for different cations. Experiments were performed in two stages. In the first, the relative permeability of BLM for Ca^{2+} and monovalent cations (such as K^+ and Na^+) was examined. During the second stage, the bilayer permeability for Ca^{2+} was compared with that for other divalent cations.

It was established that the V^* values for tenuferidin- and ferutidin-modified BLM were statistically ($P<0.05$) different (-9.2 ± 0.4 mV ($n=5$) and -6.7 ± 0.2 mV ($n=5$), respectively) when the BLM separated dissimilar solutions: 10 mM $CaCl_2$ (*cis* compartment) and 20 mM NaCl (*trans*). When KCl solutions were used instead of NaCl, the values of V^* obtained in the presence of these two terpenoids were smaller (-5.5 ± 0.5 mV ($n=5$) and -5.9 ± 0.3 mV ($n=5$)) and not significantly different ($P>0.1$). The results of the typical experiments are presented in Fig. 4C,D. The negative value of V^* indicates that the cation placed on the *cis* side passes through terpenoid-modified BLM better than the cation presented on the *trans* side. Using Eq. 1 and experimental values of V^*, as indicated above, the cations were ranked according to their permeability: $P_{Ca}>P_K>P_{Na}$. Quantitatively, the relative permeability of modified BLM was 1.0:0.57:0.52 (for tenuferidin) and 1.0:0.57:0.56 (for ferutidin), respectively. These data indicate that terpenoid-modified BLM discriminates well between mono- and divalent cations.

Table 1
Zero current potentials (mV) of modified bilayers for pairs 10 mM $CaCl_2$/10 mM $MeCl_2$ (*cis*/*trans*)

Terpenoids	Me^{2+}			
	Zn^{2+}	Mg^{2+} #	Ba^{2+} #	Sr^{2+}
Ferutinin	2.8 ± 0.2	-4.0 ± 0.5	-5.4 ± 0.5	-9.2 ± 0.7
Tenuferidin	4.0 ± 0.2	-2.8 ± 0.2	-3.8 ± 0.3	-18.1 ± 0.9*
Ferutidin	13.8 ± 0.3*	-3.1 ± 0.1	-4.0 ± 0.2	-8.9 ± 0.7

All experimental conditions are described in Section 2 and in the text. Values are given as means ± S.D. from at least five experiments.
$P\geq0.05$ between zero current potentials obtained for Mg^{2+} and Ba^{2+}. $P<0.05$ for all other neighboring ion pairs in rows.
* $P<0.05$ in columns.

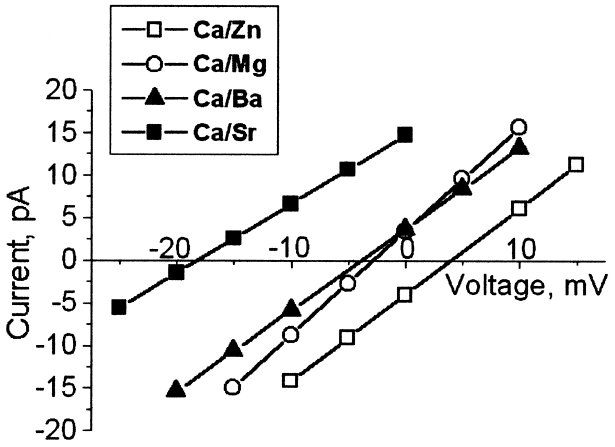

Fig. 5. I-V curves for BLM modified by tenuferidin in bi-ionic systems. The presented data are of typical experiments. BLM separated dissimilar solutions: 10 mM $CaCl_2$ (*cis* compartment) and 10 mM of one of the other bivalent metal chlorides (*trans*). The concentration of tenuferidin on both sides of BLM was 40 μM. Other conditions were as described in Section 2 and in the text.

The zero current potentials in bi-ionic systems, which reflected the relative permeability among divalent cations, are shown in Table 1. Representative I-V curves for tenuferidin are shown in Fig. 5. As indicated, zinc ions pass through terpenoid-modified BLM better than other cations and zero current was achieved by fixing positive potentials in the $CaCl_2$ compartment. With other divalent cations in the *trans* compartment, zero current was achieved by fixing negative potentials in the $CaCl_2$ compartment. The values of the permeability coefficient were calculated using a reduced Goldman equation adapted for this experimental model. As expected, zinc ions had the highest permeability coefficient and Sr^{2+} had the lowest. All tested divalent cations ranked as follows in order of decreasing permeability: $Zn^{2+}>Ca^{2+}>Mg^{2+}>Ba^{2+}>Sr^{2+}$. Qualitatively the same order was observed for BLM modified by any of the membrane-active terpenoids. The quantitative values of the relative permeability coefficient (for the examined ions) weakly depended on the type of terpenoids, and were 1.11:1.0:0.86:0.81:0.70, 1.17:1.0:0.88:0.86:0.49 and 1.7:1.0:0.89:0.85:0.69 for ferutinin, tenuferidin and ferutidin, respectively.

These data indicate that bilayers modified by terpenoids are able to discriminate between divalent cations although their Ca^{2+}/Mg^{2+} permeability ratio (1.12–1.16) appears smaller than the analogous ratio of A23187- (approx. 14) and X537A-modified (approx. 16) bilayers [19,20]. It needs to be noted, however, that the foregoing data for two well-known Ca^{2+} ionophores were obtained with different methods.

To estimate the number of terpenoid molecules forming a minimal structure to provide ion transport through BLMs, we studied the dependence of steady-state membrane conductance on concentration of ferutinin, ferutidin and tenuferidin, which were added to both BLM bathing solutions. It was established that such dependences were linear in double logarithmic plots (Fig. 6A). The values of the slopes were close to 3 for tenuferidin (2.9 ± 0.5) and considerably less (1.3 ± 0.2) for ferutidin. Comparison of these values with that reported for ferutinin (2.5 ± 0.3 [5]) gives the possibility to propose that three is the maximal number of terpenoid molecules forming the ion-transporting structure.

To establish a possible stoichiometry of the complex, it is also necessary to know the number of ions in the transporting structure (unit). This number can be estimated from the dependence of steady-state BLM conductance on cation concentration. Results obtained for $CaCl_2$ concentrations ranging from 50 μM to 5 mM are presented in Fig. 6B. Dependences were linear only when $CaCl_2$ concentrations ranged from 50 μM to 5 mM. Further increases in $CaCl_2$ concentration demonstrate a clear saturation effect (data not shown). The slope values on the linear part of the dependence were close to 1.5 for ferutinin and tenuferidin and almost one (approx. 1.2) for ferutidin. These data suggest that (with the exception of ferutidin) 2–3 molecules of terpenoids participate in the transport of 1–2 cations. We assume that these complexes may have the 'sandwich' type of structure suggested for the calcium ionophores A23187 and X537A [19,20].

Mitochondria were employed to examine the ability of terpenoids to affect cell membrane permeability. It has been shown that complex esters of sesquiterpene alcohols with aliphatic acids (lapidin and lapiferin) did not change the permeability of mitochondrial membranes to cations: mitochondria did not swell in nitrate solutions of several different mono- and divalent cations. The results obtained for a $Ca(NO_3)_2$ solution in the presence of different concentrations of sesquiterpenes are presented in Fig. 7.

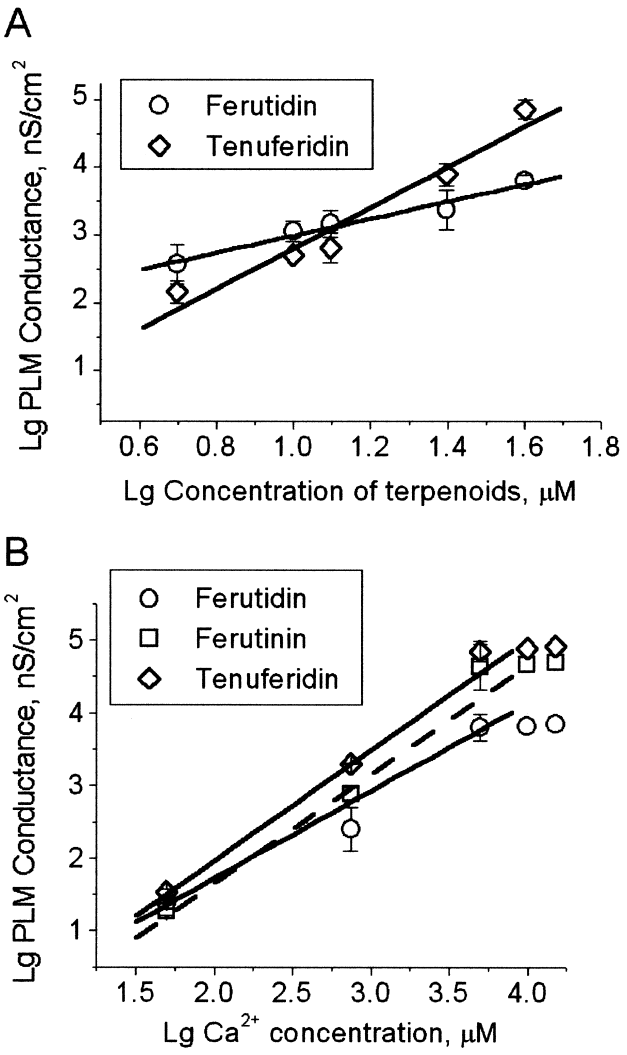

Fig. 6. Dependence of BLM conductance on concentration of terpenoids (A) and calcium ions (B). BLM were clamped at −50 mV. (A) The bathing solutions contained 5 mM $CaCl_2$ and 1 mM Tris-HCl, pH 7.5. Terpenoids were added on both sides of BLM in the concentrations shown on the abscissa. (B) The bathing solutions contained 1 mM EGTA, 1 mM Tris-HCl, pH 7.5 and suitable concentrations of $CaCl_2$ to maintain the concentrations of free calcium ions represented on the abscissa. The concentration of terpenoids on both sides of BLM was 40 μM. The BLM area was approx. 0.0007 cm^2. Each point represents the mean from 3–5 separate experiments ± S.D.

On the other hand, esters of sesquiterpene alcohols

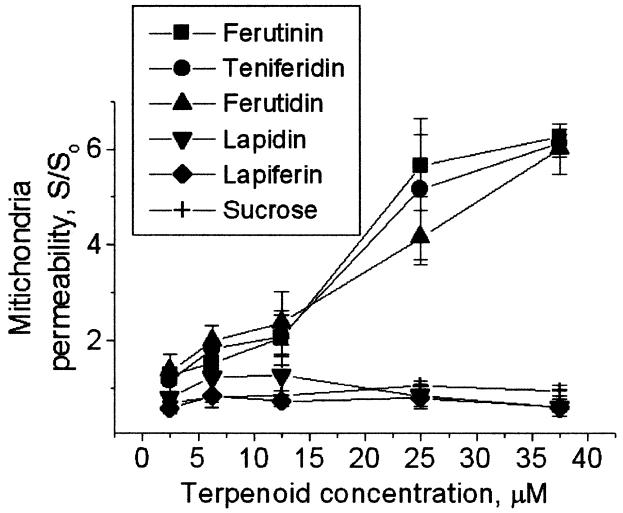

Fig. 7. Relative rate of energy-independent passive swelling of mitochondria (S_i/S_o) in the presence of different concentrations of terpenoids. The solution contains 40 mM $Ca(NO_3)_2$, 120 mM sucrose and 0.33 μg/ml rotenone with pH adjusted to 7.4 with Tris-NO_3. In the test of non-specific alteration of mitochondrial permeability the solution contains 250 mM sucrose and 0.33 μg/ml rotenone with pH adjusted to 7.4 with Tris-NO_3. Membrane-active (ferutidin, tenuferidin and ferutinin) and inactive (lapidin and lapiferin) terpenoids were examined. Other conditions were as described in Section 2. Each point represents the mean from five separate experiments ± S.D.

with aromatic moieties enhanced the passive permeability of mitochondrial membranes. The rate of mitochondrial swelling in solutions of $Ca(NO_3)_2$ grows quasi-linearly in the presence of membrane-active terpenoids (ferutidin, tenuferidin and ferutinin) in the concentration range 1–37.5 μM (Fig. 7). It seems that all of these terpenoids have essentially the same capacity to increase mitochondrial membrane permeability. The differences in the parameter S_i/S_o at each specific concentration of the three membrane-active terpenoids were not significant ($P > 0.05$).

Qualitatively the parameter S_i/S_o, which reflects the passive permeability of mitochondrial membranes, changes with terpenoid concentration similarly for all mono- and divalent cations examined (data not shown). Four to five experiments were done for each type of cation and each concentration of examined terpenoids. In all cases only membrane-active terpenoids were able to increase the passive permeability of mitochondrial membranes in a dose-dependent manner. Monovalent cations were found less permeable than divalent ones. The values of mitochondrila permeability (S_i/S_o) obtained at 25 μM concentration of membrane-active terpenoids were utilized to build the selectivity series of cations for modified mitochondrial membranes. For all membrane-active terpenoids the selectivity series were qualitatively and quantitatively similar to each other ($P \geq 0.057$ at any concentration). Because of this similarity, the data obtained for all membrane-active terpenoids were joined and the integral results are presented in Table 2 where the analogous integral results obtained in the presence of ineffective terpenoids (lapidin and lapiferin) are also presented for comparison. From these data the selectivity series of mitochondrial membranes modified with ferutidin, tenuferidin and ferutinin can be build as follows: $Ca^{2+}:Mg^{2+}:Na^{+}:K^{+}:Ba^{2+}:Sr^{2+} = 1.0:0.58:0.3:0.2:0.19:0.16$. The small difference between our data and those published earlier for ferutinin [5] is not significant. The induced permeability of mitochondrial membranes to Ca^{2+} was designated as equal to 1. Calcium ions appear to pass through mitochondrial membranes more readily than any other cation tested in the presence of membrane-active terpenoids. In other words, terpenoids made mitochondrial membranes selective for calcium ions.

It appears that swelling effects are caused by specific ionophoric properties of the membrane-active terpenoids because no terpenoid-induced mitochondrial swelling was observed under conditions (Fig. 7 and Table 2) (isosmotic sucrose solution) usually

Table 2
Relative mitochondrial swelling in different salt solutions in the presence of membrane-active and inactive terpenoids (25 μM)

Cation or substance	S_i/S_o	
	Active terpenoids	Inactive terpenoids
Ca^{2+}	5 ± 0.79*	0.83 ± 0.26
Mg^{2+}	2.9 ± 0.42*	0.75 ± 0.15
Na^{+}	1.5 ± 0.24*	0.80 ± 0.14
K^{+}	1 ± 0.16	0.88 ± 0.32
Ba^{2+}	0.95 ± 0.15	0.71 ± 0.30
Sr^{2+}	0.82 ± 0.14	0.82 ± 0.21
Sucrose	0.83 ± 0.11	0.83 ± 0.11

All experimental conditions are described in Section 2 and in the text. Results of at least 12 and eight experiments were integrated to get the presented values for membrane-active (ferutinin, ferutidin or tenuferidin) and inactive terpenoids (lapidin or lapiferin), respectively. Values are given as means ± S.D.
*$P \leq 0.05$ compared with data obtained in sucrose solution.

used to reveal the presence of non-specific, detergent-like properties of membrane-active substances. In these cases the relative swelling was slightly less than 1, due to the influence of a small amount of alcohol introduced to prepare terpenoid solutions.

4. Discussion

Our findings for BLM conductance in the presence of membrane-active terpenoids suggest a carrier mechanism, for the following main reason: all three membrane-active terpenoids are able to increase the conductance of TLM. The established electrogenic feature of this transport is in accordance with the chemical structure of membrane-active terpenoids.

The results of this study indicate that the ionophoric properties of plant terpenoids depend on their chemical structures. Complex esters of sesquiterpene alcohol with aromatic acids, ferutinin, tenuferidin and ferutidin, promoted dose-dependent increases in BLM conductance principally for cations. On the other hand, complex esters of sesquiterpene alcohol with aliphatic acids (lapidin and lapiferin) did not possess any ionophoric property. This difference in membrane activity may be caused by differences in their affinity for membranes. This assumption is supported by recent experiments with the surface-localized fluorescent probe 1-anilinonaphthalene-8-sulfonic acid (ANS). It has been shown [21] that esters of sesquiterpene alcohols of the carotane series with aromatic, but not with aliphatic moieties displace part of ANS from the membrane, indicating their high membrane tropic activity. Hence, aromatic moieties probably provide the additional lipophilicity necessary for their incorporation into membranes.

As shown in Section 2, the slope value of the dependence of BLM conductance on the concentration of terpenoids or Ca^{2+} (in double log plot) has to reflect the maximal number of the components (terpenoid molecules and calcium ions) in the conducting complex. This is absolutely true if complex formation is the limiting step in the whole sequence of the transport process. The real situation is more intricate and leads to a deflection in the derived stoichiometry from whole numbers. Hence, this ap-

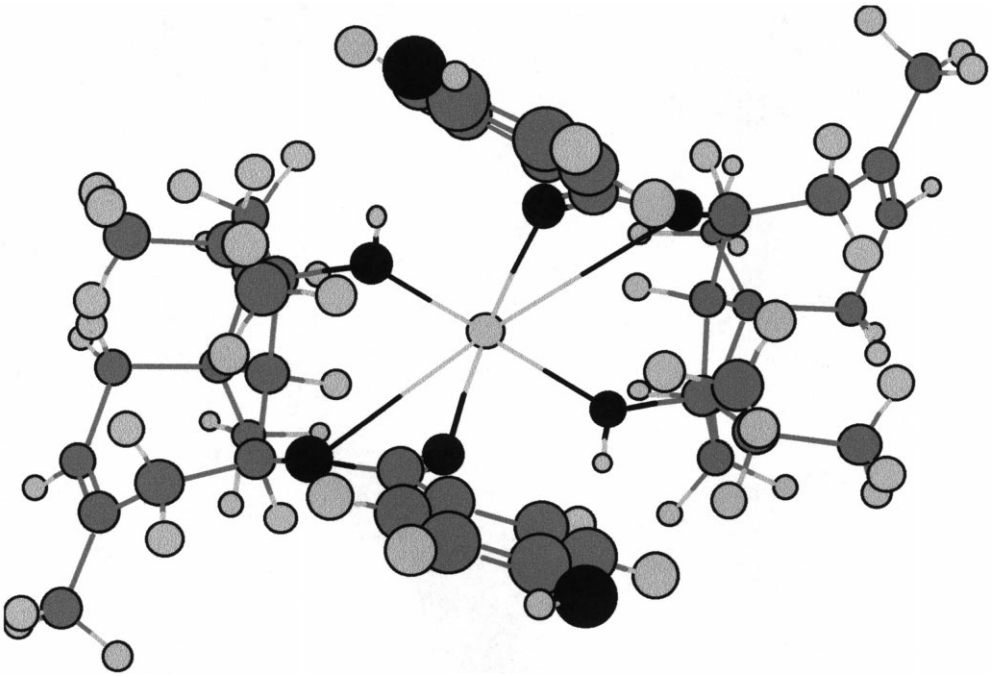

Fig. 8. Ball-and-stick tentative model for the complex of Ca^{2+} with two molecules of ferutinin. The top view on the complex is presented. The atoms are represented with the following color scheme: O in black, C in gray, H in light gray and Ca^{2+} in white. The distance between Ca^{2+} and the hydroxyl oxygens of ferutinol and between Ca^{2+} and the carbonyl oxygen of *n*-oxybenzoic acid is between 0.221 nm and 0.226 nm. The distance between carboxyl oxygen of *n*-oxybenzoic acid and Ca^{2+} is almost twice as large (approx. 0.383 nm). The proposed electrostatic bonds are indicated by lines for clarity.

proach can give us only an estimating value for complex stoichiometry. However, the process of complex formation is the main limiting step in our case, because the observed slopes for double log dose-effect dependences are much larger than the value (1.0) that is typical for the diffusion processes. It justifies the use of the double log dose-effect plot to estimate the apparent stoichiometry of the terpenoid-Ca^{2+} complex.

Some apparent differences were discovered in the stoichiometry of the different terpenoid-Ca^{2+} complexes. It appears to be 3:2 or, more probably, 2:1 for ferutinin and tenuferidin and about 1:1 in the case of ferutidin. The reason of a smaller stoichiometry of the ferutidin-Ca^{2+} complex is not clear yet. The assumption of a decrease in the effective terpenoid concentration during the experiments due to adsorption on the chamber wall (which can lower the slope of the dose-effect dependence, and, as a result, lead to underestimation of the stoichiometry for the ferutidin-Ca^{2+} complex) was checked in a separate set of experiments and not confirmed.

Various possible structures could be formed between calcium ions and terpenoids. The structure of a tentative complex between Ca^{2+} and two molecules of ferutinin that appears to have the lowest value of structural energy is presented in Fig. 8. In this model, Ca^{2+} interacts electrostatically and tightly with the hydroxyl oxygen of ferutinol and the carbonyl oxygen of *n*-oxybenzoic acid, forming four strong and two weak electrostatic bonds with two closely placed molecules of ferutinin. In this way the calcium ion is hidden from its environment and can freely pass through the hydrophobic zone of membranes. It appears that other membrane-active terpenoids can build analogous 'sandwich' complexes with divalent cations where the benzoic acid ring plays an important role in hiding a divalent cation inside the complex.

In the case of the lapidin-Ca^{2+} complex, the angelic acid residue is too small to shield Ca^{2+} effectively from its environment. This would not permit the complex to pass through the hydrophobic zone of membranes. The lapiferin-Ca^{2+} complex suffers the same restriction as the lapidin complex, but because of the presence of acetic acid and an epoxy group in the structure, lapiferin may form a second cation-binding center with the oxygens of these additional groups. We hypothesize that lapiferin, the most hydrophilic of all tested terpenoids, has an increased ability to form chelates in water solutions.

The origin of the cation selectivity of terpenoid-modified membranes is not yet clear. To better understand the problem, more detailed analyses of three-dimensional structures of membrane-active terpenoids are needed. The most important remaining question concerns possible conformations of terpenoids in solutions with different dielectric constants that were not examined in the present study. We plan to explore this matter using a computer simulation approach. However, already at this stage it is quite clear that membrane-active terpenoids constitute a new group of natural, divalent cation-selective ionophores.

Acknowledgements

We are grateful to Dr. Steven D. Aird (Mestrado em Ciências Fisiológicas, Universidade Estadual do Ceará) for corrections that improved the clarity of the manuscript. This research was partially supported by a CNPq fellowship (O.V.K.).

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