ELSEVIER

Biochimica et Biophysica Acta 1558 (2002) 54–62

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Anacardic acid-mediated changes in membrane potential and pH gradient across liposomal membranes

Masaaki Toyomizu [a,*], Katsuyuki Okamoto [b], Yukio Akiba [a], Tetsuo Nakatsu [c], Tetsuya Konishi [d]

[a] *Science of Biological Function, Life Science, Graduate School of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Sendai 981-8555, Japan*

[b] *Animal Nutrition, Niigata University, 2-8050 Ikarashi, Niigata 950-8555, Japan*

[c] *Takasago Institute for Interdisciplinary Science, Inc., 4 Volvo Drive, Rockleigh, NJ 07647, USA*

[d] *Radiochemistry-Biophysics, Niigata College of Pharmacy, 5-13-2 Kamishin'ei, Niigata 950-2081, Japan*

Received 31 May 2001; received in revised form 13 September 2001; accepted 13 September 2001

Abstract

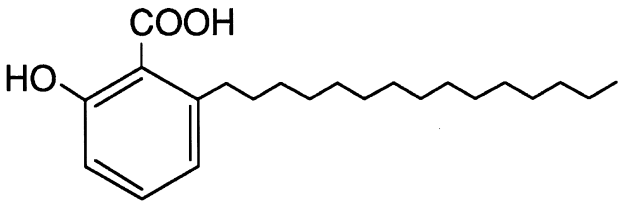
We have previously shown that anacardic acid has an uncoupling effect on oxidative phosphorylation in rat liver mitochondria using succinate as a substrate (Life Sci. 66 (2000) 229–234). In the present study, for clarification of the physicochemical characteristics of anacardic acid, we used a cyanine dye (DiS-C3(5)) and 9-aminoacridine (9-AA) to determine changes of membrane potential ($\Delta\Psi$) and pH difference (ΔpH), respectively, in a liposome suspension in response to the addition of anacardic acid to the suspension. The anacardic acid quenched DiS-C3(5) fluorescence at concentrations higher than 300 nM, with the degree of quenching being dependent on the log concentration of the acid. Furthermore, the K^+ diffusion potential generated by the addition of valinomycin to the suspension decreased for each increase in anacardic acid concentration used over 300 nM, but the sum of the anacardic acid- and valinomycin-mediated quenching was additively increasing. This indicates that the anacardic acid-mediated quenching was not due simply to increments in the K^+ permeability of the membrane. Addition of anacardic acid in the micromolar range to the liposomes with $\Delta\Psi$ formed by valinomycin-K^+ did not significantly alter 9-AA fluorescence, but unexpectedly dissipated $\Delta\Psi$. The $\Delta\Psi$ preformed by valinomycin-K^+ decreased gradually following the addition of increasing concentrations of anacardic acid. The $\Delta\Psi$ dissipation rate was dependent on the pre-existing magnitude of $\Delta\Psi$, and was correlated with the logarithmic concentration of anacardic acid. Furthermore, the initial rate of ΔpH dissipation increased with logarithmic increases in anacardic acid concentration. These results provide the evidence for a unique function of anacardic acid, dissimilar to carbonylcyanide *p*-trifluoromethoxyphenylhydrazone or valinomycin, in that anacardic acid behaves as both an electrogenic (negative) charge carrier driven by $\Delta\Psi$, and a 'proton carrier' that dissipates the transmembrane proton gradient formed.

Keywords: Anacardic acid; Ionophore; Protonophore; Membrane potential; Transmembrane pH difference; Liposome

Abbreviations: DiS-C3(5), 3,3′-dipropyl-2,2′-thiadicarbocyanine iodide; 9-AA, 9-aminoacridine; $\Delta\Psi$, change of membrane potential; ΔpH, transmembrane pH difference; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\mu$H, proton electrochemical potential; MES, 2-[*N*-morpholino]ethanesulfonic acid

* Corresponding author. Fax: +81-22-717-8691. *E-mail address:* toyomizu@bios.tohoku.ac.jp (M. Toyomizu).

0005-2736/02/$ – see front matter
PII: S0005-2736(01)00422-9



Anacardic acid

Scheme 1.

1. Introduction

Anacardic acid (2-hydroxy-6-pentadecylbenzoic acid; Scheme 1) is the product of hydrogenation of the naturally occurring unsaturated anacardic acids, namely, the 8′*Z*-monoene, the 8′*Z*,11′*Z*-diene, and the 8′*Z*,11′*Z*,14′*Z*-triene, which are the chief constituents (about 75%) of cashew nutshell liquid [1]. Many significant biological and pharmacological activities of these compounds have been described in recent years. The compounds exhibit antitumor [2] and antimicrobial [3,4] activities as well as having potent molluscicidal [5] effects. Recently, we reported that all four anacardic acids with C15:0, C15:1, C15:2 or C15:3 alkyl side chains exhibited uncoupling effects (similar to the classical uncoupler 2,4-dinitrophenol) on the ADP/oxygen (ADP/O) ratio, state 4 and respiratory control ratio in succinate-oxidizing rat liver mitochondria [6].

Oxidative phosphorylation is mediated by a proton electrochemical potential ($\Delta\mu$H), as described by the chemiosmotic hypothesis. Although general anesthetics, such as halothane and chloroform, uncouple oxidative phosphorylation without significant reduction of $\Delta\mu$H [7], the explanation of uncoupling in mitochondria, chloroplasts and bacterial cells has been verified in numerous cases for a large and diverse number of potent ionophores as well as uncouplers [8–11]. For example, proton translocators, i.e. weak acids, increase the membrane proton permeability and collapse the transmembrane proton gradient, ΔpH, by shuttling protons across the membrane [12], whereas electrogenic ionophores collapse the membrane potential ($\Delta\Psi$) and thereby $\Delta\mu$H [8,13]. Thus, it is of importance to elucidate whether the uncoupling effect of anacardic acids is due to their potency as protonophores or as ionophores.

It was previously noted that by selecting the proper fluorescent probes, a cyanine dye (3,3′-dipropyl-2,2′-thiadicarbocyanine iodide, DiS-C3(5)) for $\Delta\Psi$ and 9-aminoacridine (9-AA) for ΔpH, photodiode array spectrophotometry could be used to determine kinetic changes of $\Delta\Psi$ and ΔpH simultaneously in unilamellar liposomes [14]. In the present study, for identification of the potential physicochemical characteristics of anacardic acid underlying the increase in the uncoupling of oxidative phosphorylation in mitochondria [6], we examined the effect of anacardic acid on $\Delta\Psi$ and ΔpH in liposomes using DiS-C3(5) and 9-AA as $\Delta\Psi$ and ΔpH probes, respectively.

2. Materials and methods

2.1. Materials

The anacardic acids used for assay were isolated and identified through spectroscopic analysis as previously reported [15]. Cashew nut shell, which was crushed and extracted with MeOH, was obtained from a cashew nut producer in Indonesia. The final purification was performed using recycling HPLC (LC-908, Japan Analytical Industry, Tokyo, Japan) with a reverse phase chromatographic column (JAI-GEL-ODS A-343-10: i.d. 20×250 mm, 10 μm, Japan Analytical Industry) [16]. Sufficient amounts of pentadecyl derivatives for assay were obtained by hydrogenation.

2.2. Liposome preparation

Large unilamellar liposomes were prepared by the reverse phase evaporation method, as described previously [14]. Briefly, phosphatidylcholine and cholesterol (9:1, molar ratio) were dissolved in a diethyl ether/chloroform mixture (5:1, v/v), following which the organic solvent was removed under reduced pressure by a rotary evaporator to obtain a thin film of lipids. Aliquots of 2 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer containing 150 mM KCl (pH 7.0) were added as the loading buffer to the lipids resolved in diethyl ether. The resulting two phase system was sonicated in a bath-type sonicator for 3 min at 0°C. The homogeneous lipid emulsion obtained was treated under reduced pressure (about

100 mm Hg) until a stable gel was formed, after which residual ether was further evaporated off at about 700 mm Hg. The liposome dispersion obtained was centrifuged for 20 min at 12 000×*g* to remove aggregated materials. Finally a K^+ gradient was generated by passing the liposome suspension through a Sephadex G-50 column with 2 mM MES buffer (pH 7.0) containing 150 mM NaCl.

2.3. Simultaneous determinations of $\Delta\Psi$ and ΔpH in liposomes by photodiode array spectrometry

A photodiode array detector (MCPD-100, Otsuka Electronics) was used for determinations of $\Delta\Psi$ and ΔpH in liposomes essentially as described before [14]. A sample cuvette was illuminated with a 500 W Xe actinic light source through a silica fiber light guide without using any filter. 2 ml of K^+-loaded liposomes suspended in 2 mM MES buffer (pH 7.0) containing 150 mM NaCl was placed in the cuvette and was stirred with a magnetic stirrer. After taking a background spectrum, a 6 μl aliquot of 9-AA (10 mM aqueous solution) and a 4.5 μl aliquot of DiS-C3(5) (1 mM ethanol solution) were added successively, unless otherwise indicated.

For the preliminary kinetic studies that could be monitored by using the appropriate dyes as described above, a 2 μl aliquot of valinomycin (1 mM ethanol solution) was injected into the liposome suspension to generate a K^+ diffusion potential. A 2 μl aliquot of carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 10 μM ethanol solution) was added 1 min after the valinomycin addition in order to induce a $\Delta\Psi$-driven, FCCP-mediated ΔpH formation in the liposomes.

For evaluating the potency of anacardic acid to produce $\Delta\Psi$ in K^+-loaded liposomes, changes in the fluorescence of DiS-C3(5) were recorded 1 min after the addition of a known concentration of anacardic acid (10 nM–10 μM). This was followed by the addition of valinomycin (final concentration 1 μM) so that the potency of each concentration of anacardic acid could be analyzed. In further experiments to clarify the concentration dependence of anacardic acid on the initial rate of $\Delta\Psi$ dissipation, various amounts of anacardic acid were added to the liposome suspension 6 min before the valinomycin addition. Finally, the question of whether or not the $\Delta\Psi$ dissipation mediated by anacardic acid is dependent on the magnitude of $\Delta\Psi$ preformed by valinomycin-K^+ was also examined. Liposomes were loaded with 150 mM KCl and 2 mM MES (pH 7.0), and suspended in isoosmotic 2 mM MES buffer containing KCl and NaCl in desired proportions at pH 7.0.

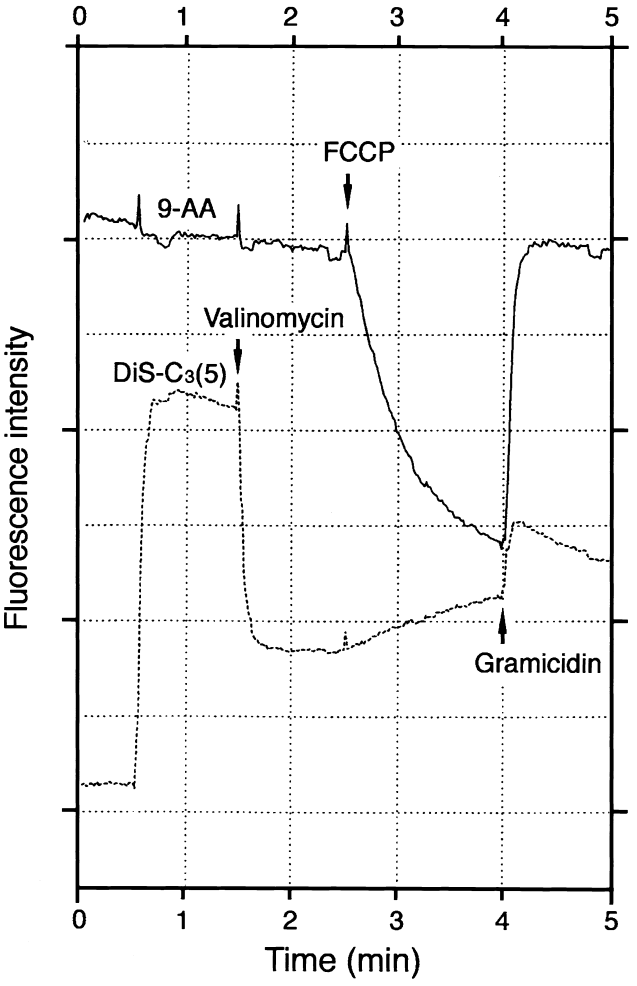


Fig. 1. Time course of the fluorescence changes due to $\Delta\Psi$ and ΔpH changes in K^+-loaded liposomes after additions of valinomycin and FCCP. Liposomes loaded with 150 mM KCl and 2 mM MES (pH 7.0) were suspended in isoosmotic external buffer containing 2 mM MES and 150 mM NaCl (pH 7.0), as described in Section 2. After recording background spectra from 9-AA (final concentration 30 μM) and DiS-C3(5) (final concentration 2.25 μM), valinomycin (final concentration 2 μM) was injected to the liposome suspension to generate a K^+ diffusion potential. FCCP (final concentration 10 nM) was then added 1 min after the valinomycin addition in order to induce a $\Delta\Psi$-driven, FCCP-mediated ΔpH formation in the liposomes. Final concentration of gramicidin used was 100 nM.

FCCP and 9-AA were purchased from Tokyo Kasei. DiS-C3(5) was from the Japanese Research Institute for Photosensitizing Dyes. Gramicidin was purchased from Sigma and phosphatidylcholine (99% pure) from Nippon Fine Chemicals. Cholesterol and all other reagents used were purchased from Wako.

3. Results and discussion

Fig. 1 clearly shows that the fluorescence from DiS-C3(5) sharply decreased after addition of valinomycin according to the formation of a K^+ diffusion potential. The subsequent addition of FCCP initiated the quenching of 9-AA fluorescence corresponding to the formation of an inside-acidic ΔpH due to the $\Delta\Psi$-driven, FCCP-mediated H^+ influx into the liposomes. Further addition of 100 nM gramicidin restored in full both fluorescence intensities to their original levels. It is also notable that no significant volume change of the liposomes occurred during the ion flux process since no drift of the baseline due to changes in light scattering was observed (results not shown). These results suggest that the experimental conditions employed for analyzing time-dependent changes in fluorescence using photodiode array spectrometry as well as in the preparation of the large unilamellar liposomes are sufficiently suitable for identification of the physicochemical characteristics of anacardic acid.

Fig. 2A shows that when anacardic acid (final concentration 500 nM) was added to the K^+-loaded liposomes, the DiS-C3(5) fluorescence was quenched, but the extent to which this occurred was far less than that seen above for valinomycin. Following the addition of FCCP, the $\Delta\Psi$ was slightly affected but on this occasion did not give rise to any detectable ΔpH change. It is known that the fluorescence of cyanine dyes is quenched by dinitrophenol and by carbonylcyanide *m*-chlorophenylhydrazone, both of which uncouple mitochondrial oxidative phosphorylation, but that the changes in the optical signals of probes are not related to the membrane potential

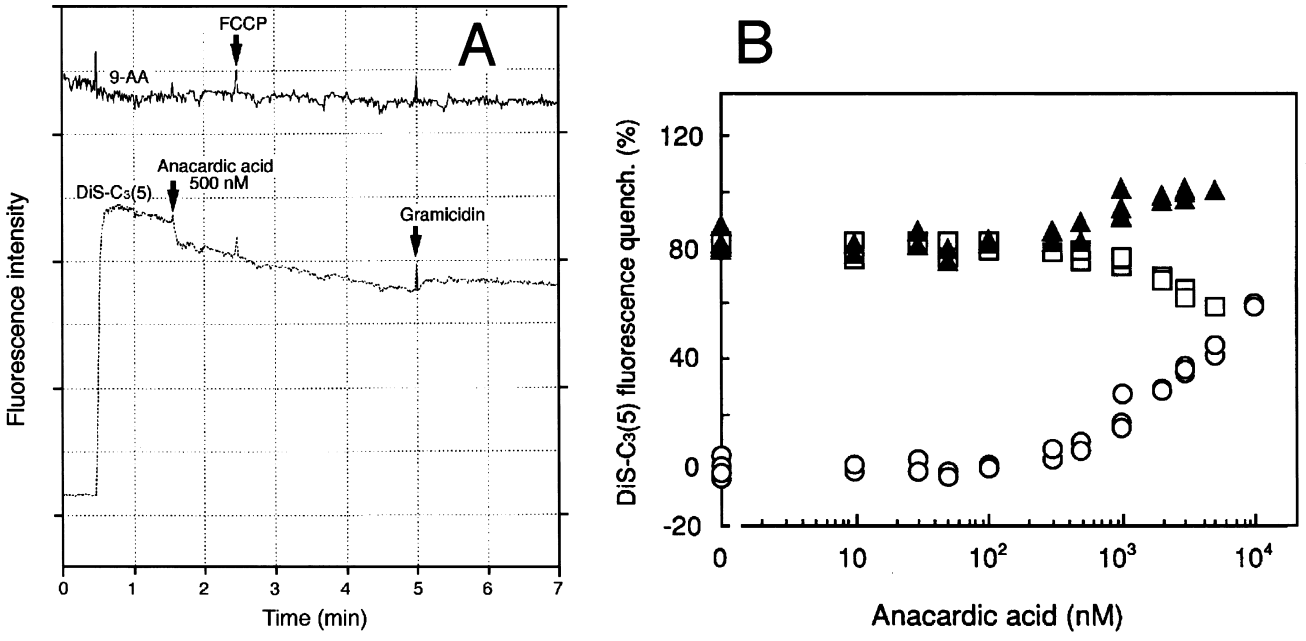


Fig. 2. Effect of anacardic acid on DiS-C3(5) fluorescence in the K^+-loaded liposomes. The liposomes and fluorescence probes were the same as in the legend to Fig. 1. (A) As an illustration, 500 nM anacardic acid (final concentration) was injected to the liposome suspension, instead of valinomycin at the time indicated in Fig. 1. (B) Anacardic acid-dependent DiS-C3(5) quenching in K^+-loaded liposomes. The fluorescence change of DiS-C3(5) was recorded 1 min after injection of a known concentration of anacardic acid (○). Valinomycin was then added to evaluate the quenching due to the valinomycin-mediated K^+ diffusion (□). Total change (▲) is the sum of anacardic acid- and valinomycin-dependent changes.

[17]. In this experiment, the addition of 100 nM gramicidin restored the DiS-C3(5) fluorescence intensity to its original level, supporting the idea that the quenching of the dye was still dependent on the membrane potential. As shown in Fig. 2B, it was also found that anacardic acid quenched DiS-C3(5) fluorescence at concentrations higher than 300 nM, with the degree of quenching being dependent on the log concentration of the acid. For each concentration of anacardic acid used, the subsequent addition of valinomycin generated membrane potential changes whose magnitude was almost the same as that predicted by the loaded K^+ concentration in the liposome: the anacardic acid- and subsequent valinomycin-mediated quenching of DiS-C3(5) fluorescence was almost additive, indicating that the anacardic acid-produced quenching of DiS-C3(5) fluorescence was not due to K^+ diffusion.

It has been shown that protonated fatty acids can flip-flop very rapidly in a lipid bilayer membrane, though the flip-flop or diffusion of the dissociated, negatively charged fatty acid was several orders of magnitude slower [18,19]. More recently, various fatty acids, such as 2-hydroxy fatty acids or dodecyloxybenzoic acid, were shown to acidify the liposome interior, whereas bipolar fatty acids, such as 12-hydroxylauric acid, dicarboxylic acids or phenylhexanoic acid, did not [20]. As anacardic acids are a group of fatty acids with a hydroxybenzoic acid as the polar head group [21], one may expect an interior acidification of the liposome to occur with the simple addition of anacardic acid. Successive experiments showed that, unlike FCCP, anacardic acid was unable to properly establish an inside-acidic ΔpH, but was found to dissipate the $\Delta\Psi$ when it was added to liposomes in which $\Delta\Psi$ was preformed by valinomycin-K^+ (Fig. 3A). The $\Delta\Psi$ changing profile after anacardic acid addition was rather complex such that $\Delta\Psi$ oscillated. It should also be noted that anacardic acid addition in these experiments induced a weak inside-acidic ΔpH (Fig. 3A). Moreover, to see the dependence of the anacardic acid-mediated $\Delta\Psi$ dissipation on the concentration, liposomes were preincubated for 6 min in known concentrations of anacardic acid. The anacardic acid-mediated $\Delta\Psi$ dissipation was remarkably enhanced at concentrations greater than 1000 nM (Fig. 3B). These findings can be explained as follows: addition of elevated

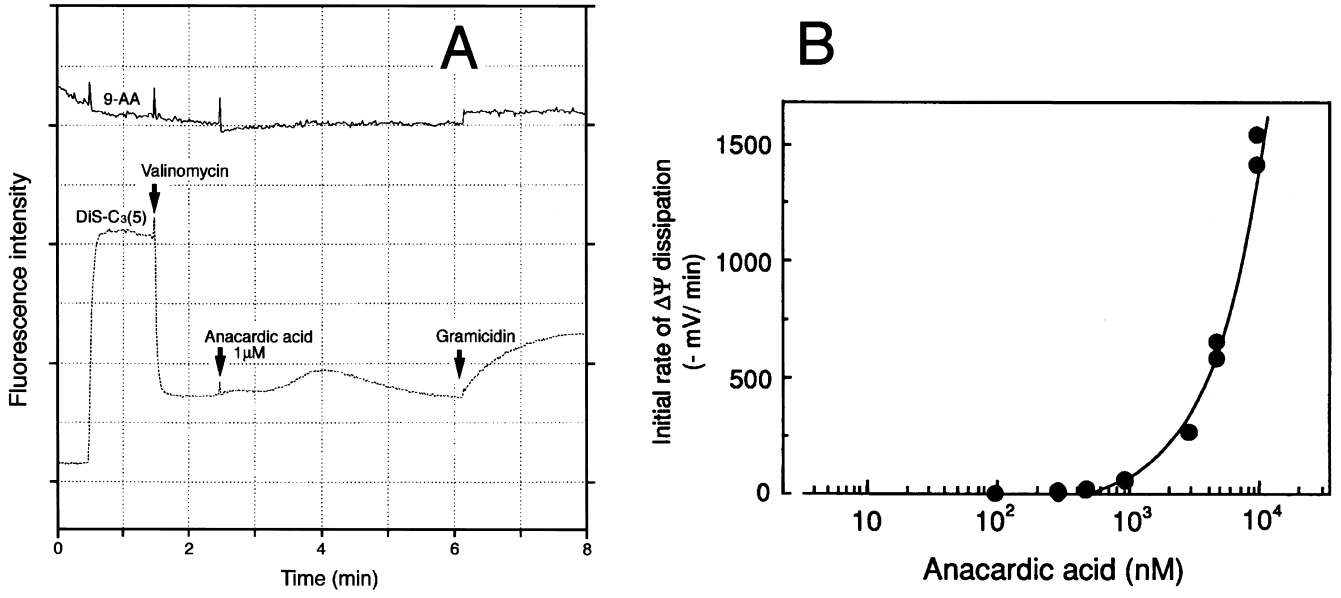


Fig. 3. Effect of anacardic acid on the dissipation of $\Delta\Psi$ formed by valinomycin-K^+ in the liposomes. The liposomes and fluorescence probes were the same as in the legend to Fig. 1. (A) As an illustration, 1 μM anacardic acid (final concentration) was added to the liposome suspension, instead of FCCP at the time indicated in Fig. 1. (B) Relationship between initial rate of anacardate-mediated $\Delta\Psi$ dissipation and anacardic acid concentration for experiments in which different amounts of anacardic acid were added to the liposome suspension 6 min before valinomycin addition. The $\Delta\Psi$ formed by valinomycin-K^+ without anacardic acid addition was calculated to be −118 mV from the Nernst equation given a $[K^+]_{in}/[K^+]_{out}$ of 100.

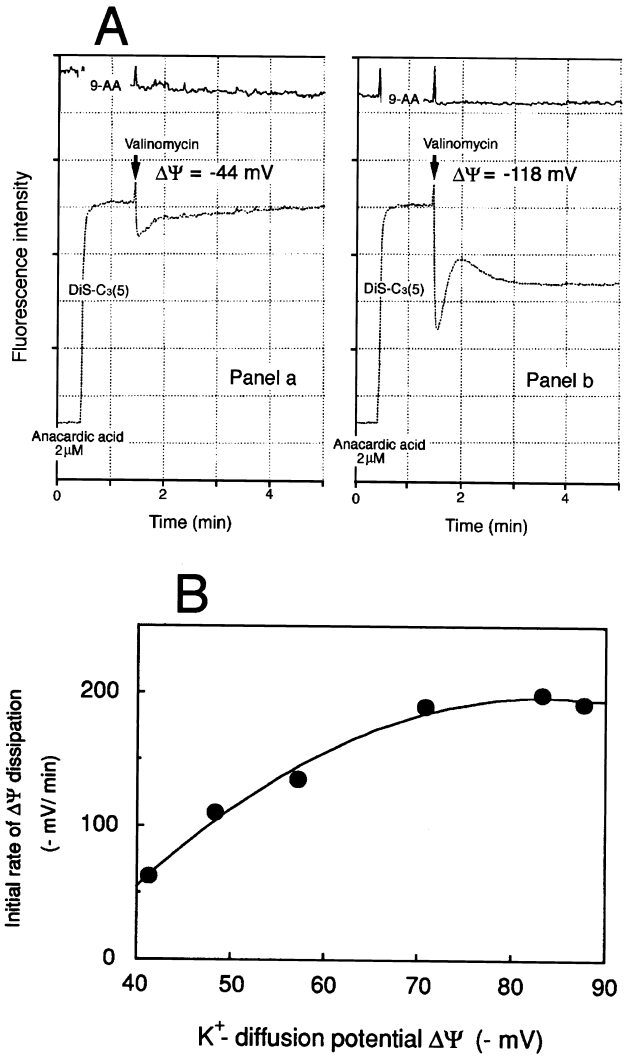


Fig. 4. ΔΨ-dependent efflux of anacardic acid anion. The liposomes and fluorescence probes were the same as in the legend to Fig. 1, but external 2 mM MES buffer was prepared with defined proportions of KCl and NaCl at pH 7.0. (A) As an illustration, 2 μM anacardic acid (final concentration) was added to liposome suspensions under gradient $[K^+]_{in}/[K^+]_{out} = 5.62$ ($\Delta\Psi = -44$ mV) and $[K^+]_{in}/[K^+]_{out} = 100$ ($\Delta\Psi = -118$ mV). (B) Relationship between initial rate of anacardic acid-mediated ΔΨ dissipation and K^+ diffusion potential ΔΨ. 2 μM anacardic acid (final concentration) was added to liposome suspensions 6 min before valinomycin addition. The diverse K^+ diffusion potentials ΔΨ in the range of −41 to −88 mV were formed in the liposome suspended in isoosmotic external buffer containing KCl and NaCl in proportions of 5.01, 6.62, 6.35, 15.8, 25.9 and 30.7 at pH 7.0, respectively, by the addition of valinomycin. The ΔΨ value formed by valinomycin-K^+ was calculated as in the legend to Fig. 3B.

concentrations of anacardic acid facilitates a concentration-dependent influx of anacardic acid. Uptake of the protonated form of anacardic acid at this stage is predominant because the presence of inside-negative ΔΨ restricts inflow of the dissociated form. The dissociated form of anacardic acid (anacardate) produced in the liposome is expelled from the liposome by the ΔΨ present, thereby generating ΔpH and a change in ΔΨ. Consequently, anacardic acid in the micromolar range was found to dissipate ΔΨ preformed by valinomycin-K^+.

The question should be raised as to whether or not the anacardate-mediated ΔΨ dissipation is sensitive to the extent of ΔΨ initially formed by valinomycin-K^+. In this way, the magnitude of the valinomycin-K^+-induced ΔΨ formation was determined in the presence of identical concentrations of anacardic acid in the liposome suspended in the different concentrations of K^+ (Fig. 4). The ΔΨ formed by valinomycin addition was immediately followed by its dissipation: at low initial ΔΨ (i.e. under a gradient of $[K^+]_{in}/[K^+]_{out} = 5.62$ (−44 mV: ΔΨ value calculated from the Nernst equation)), a monophasic, slow dissipation of ΔΨ was observed, but at higher initial ΔΨ (i.e. under a gradient of $[K^+]_{in}/[K^+]_{out} = 100$ (−118 mV)), the ΔΨ was rapidly dissipated and an overshoot was observed. These results again indicate that the efflux of anacardate was electrogenic and was accelerated by the ΔΨ applied. The ΔΨ dependence of the anacardate-mediated ΔΨ dissipation was further studied and the results are summarized in Fig. 4B. The initial rate of the anacardate-mediated ΔΨ dissipation, as a function of the K^+ diffusion potential in the range of −41 to −88 mV increased to a plateau at −71 mV, indicating that the anacardate-mediated ΔΨ dissipation was dependent on the magnitude of ΔΨ formed by valinomycin-K^+. Thus, the transport of the anionic form of anacardic acid is driven by ΔΨ.

The question also arises as to whether or not anacardic acid dissipates only ΔΨ but not ΔpH formed previously. We examined the effect of anacardic acid on ΔpH formed previously by the ΔΨ-driven, FCCP-mediated H^+ influx in liposomes. It was found that the anacardic acid-mediated dissipation of ΔpH was dependent on the log concentration of the acid (Fig. 5), indicating that the dissipation is due to the weak acid character of anacardic acid. It is reasonable to

consider that anacardic acid (whose pK_a is approx. 4.8) is almost fully dissociated at the experimental pH conditions (pH 7.0) used here, although it is known that long-chain fatty acids like anacardic acid tend to locate in the membrane with the head group at the interface, and in the circumstances the pK_a of the acid increases considerably [18,19]. Therefore, if one supposes that the dissociated form of anacardic acid is permeable to the lipid bilayer membrane, the observed dissipation of $\Delta\Psi$ and ΔpH will be accounted for by the transmembrane movement of anacardate because the K^+ diffusion potential was still maintained after anacardic acid addition (Fig. 2B). As mentioned above, dissociated fatty acids may not diffuse freely across the bilayer membrane and the flip-flop is also highly restricted. In order to explain the present data showing that anacardic acid dissipated the membrane potential, the structural characteristics of anacardic acid have to be considered, such that intramolecular hydrogen bonding between the phenolic and carboxylate oxygens is

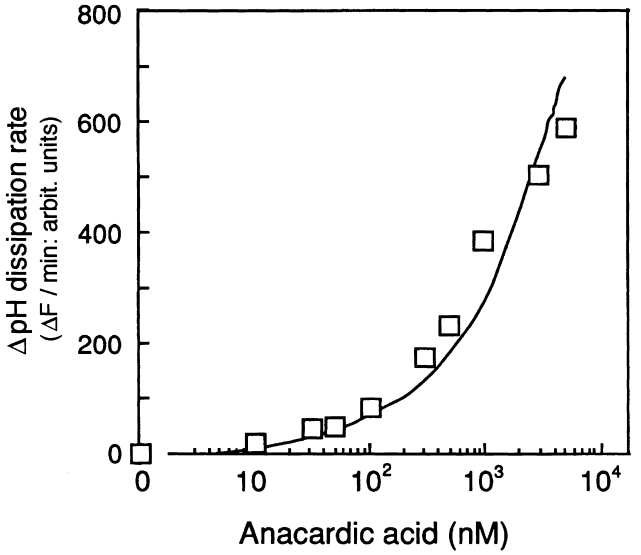
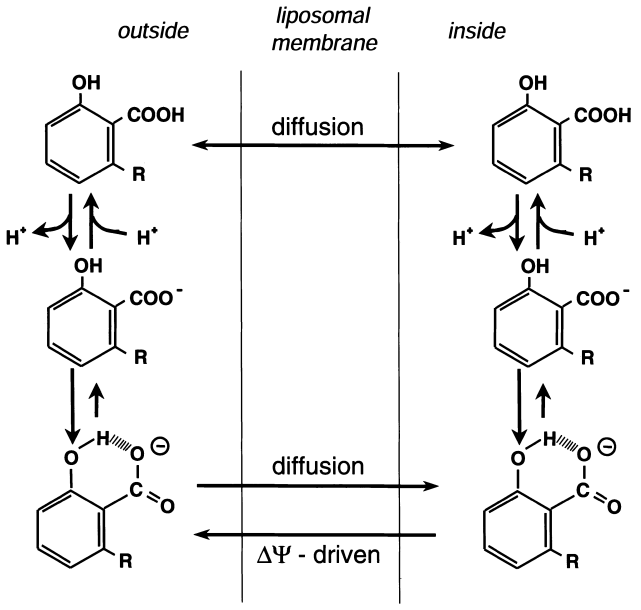


Fig. 5. Correlation between initial rate of the anacardic acid-mediated ΔpH dissipation and anacardic acid concentration. The liposomes, fluorescence probes and artificial uncouplers, FCCP and valinomycin were the same as in the legend to Fig. 1, but FCCP was added before valinomycin addition. Various amounts of anacardic acid were added 3 min after the valinomycin addition, and the fluorescence change of 9-AA recorded thereafter to estimate the initial dissipation rate of ΔpH formed previously. The initial rate of ΔpH dissipation was calculated by dividing the magnitude of restoration of 9-AA quenching by the time unit.



Scheme 2.

formed in anacardate, the dissociated form of anacardic acid, resulting in a stable six-membered ring structure. Such a ring structure will be effectively diffused across the lipid membrane even if it is negatively charged, as shown in Scheme 2 describing the proposed mechanism.

We have previously demonstrated that anacardic acids in the micromolar range exhibited uncoupling effects on the ADP/O ratio, state 4 and respiratory control ratio in succinate-oxidizing rat liver mitochondria, but cardanols, in which the carboxyl group in anacardic acids is lost, did the only weak effects on state 4 and control respiratory ratio [6], suggesting that not only the C15 alkyl side chain but also the carboxyl group may play an important role in assisting the uncoupling activity of anacardic acids in mitochondria isolated from the rat liver. However, it is known that the ionized form of fatty acid is not usually fully permeable across the bilayer membrane. Therefore, we suggest the mechanism through which the head group can form an internal hydrogen bond when the carboxyl in anacardic acid is ionized, and this shields the charge to allow the fatty acid to diffuse through the bilayer. Accepting the Mitchellian chemiosmotic hypothesis of energy coupling [22], it can be expected that anacardic acid behaves either as an FCCP-like protonophore or as a valinomycin-like

ionophore. Protonophores increase the membrane proton permeability and collapse the proton electrochemical gradient, $\Delta\mu H$, by shuttling protons across the membrane, whereas ionophores collapse the membrane potential, and thereby the proton electrochemical potential gradient, through the modulation of charge transfer across the membrane. However, the present data are hardly compatible with the classical scheme. It is more probable that the uncoupling effect of anacardic acid on oxidative phosphorylation may be assisted by the dissipation of both $\Delta\Psi$ and ΔpH, which might be mediated by the membrane-permeable weak acid as the H^+ and (negative) charge carrier. This is also the basis of a different mechanism by which general anesthetics, such as halothane and chloroform, uncouple oxidative phosphorylation without significant reduction of $\Delta\mu H$ [7].

Similar orders of magnitude of anacardic acid concentrations were effective both in uncoupling oxidative phosphorylation in the mitochondrial experiments described above [6] and in the $\Delta\Psi$ and ΔpH dissipation efficiency observed in this study. It must, however, be kept in mind that the similarity does not necessarily indicate that the concentrations required for the uncoupling effect on oxidative phosphorylation by anacardic acid are reflected in the uncoupling effect on mitochondria. A planar bilayer phospholipid membrane occupies such a small part of the experimental system that even a low concentration of uncoupler is sufficient to saturate this membrane. This may give a significantly higher uncoupler concentration in the water phase than in the case of mitochondria [23]. Thus, the bilayer phospholipid membrane should have been around several orders of magnitude more sensitive to anacardic acid than the mitochondria. It can be assumed, therefore, that mitochondrial membrane protein(s), which facilitate transmembrane diffusion of anacardic acid anion, may partly assist in some way the circulation of both neutral and anionic forms via the phospholipid regions of biological membranes, as shown even in the case of low DNP concentration [24].

4. Conclusions

In the present experiments, anacardic acid did not behave as a 'typical' weak acid. Moreover, it behaved neither as an FCCP-type H^+ carrier nor as a valinomycin-type K^+ transporter. However, it was able to dissipate both ΔpH and $\Delta\Psi$ in liposomes. The ΔpH and $\Delta\Psi$ dissipation activities increased as a function of the membrane potential present. These characteristics are adequately explained if one supposes that the anacardic acid anion is freely permeable, in the same way as the protonated form, across the lipid bilayer to act as a (negative) charge carrier. This is highly likely given that the dissociated carboxylic group of anacardic acid is able to form intramolecular hydrogen bonding with a neighboring hydroxyl group to delocalize the negative charge. In addition, almost all anacardic acid is present in the dissociated form under the present experimental conditions (neutral pH), so that the charge carrier property of anacardic acid might be predominant.

Acknowledgements

We thank Dr. T. Wharton, University of Alberta, for his careful proofreading of the manuscript.

References

[1] S.V. Shobha, B. Ravindranath, Supercritical carbon dioxide and solvent extraction of the phenolic lipids of cashew nut (*Anacardium occidentale*) shells, J. Agric. Food Chem. 39 (1991) 2214–2217.
[2] I. Kubo, M. Ochi, P.C. Vieira, S. Komatsu, Antitumor agents from the cashew (*Anacardium occidentale*) apple juice, J. Agric. Food Chem. 41 (1993) 1012–1015.
[3] M. Himejima, I. Kubo, Antibacterial agents from the cashew *Anacardium occidentale* (Anacardiaceae) nut shell oil, J. Agric. Food Chem. 39 (1991) 418–421.
[4] H. Muroi, I. Kubo, Bactericidal activity of anacardic acids against *Streptococcus mutans* and their potentiation, J. Agric. Food Chem. 41 (1993) 1780–1783.
[5] I. Kubo, S. Komatsu, M. Ochi, Molluscicides from the cashew *Anacardium occidentale* and their large-scale isolation, J. Agric. Food Chem. 34 (1986) 970–973.
[6] M. Toyomizu, K. Okamoto, T. Ishibashi, Z. Chen, T. Nakatsu, Uncoupling effect of anacardic acids from cashew nut shell oil on oxidative phosphorylation of rat liver mitochondria, Life Sci. 66 (2000) 229–234.
[7] H. Rottenberg, Uncoupling of oxidative phosphorylation in rat liver mitochondria by general anesthetics, Proc. Natl. Acad. Sci. USA 80 (1983) 3313–3317.
[8] H. Rottenberg, ATP synthesis and electrical membrane potential in mitochondria, Eur. J. Biochem. 15 (1970) 22–28.

[9] P. Mitchell, Keilin's respiratory chain concept and its chemiosmotic consequences, Science 206 (1979) 1148–1159.
[10] S.G.A. McLaughlin, J.P. Dilger, Transport of protons across membranes by weak acids, Physiol. Rev. 60 (1980) 825–836.
[11] D.G. Nichols, Bioenergetics, Academic Press, London.
[12] E.A. Liberman, V.P. Skulachev, Conversion of biomembrane-produced energy into electric form: IV. General discussion, Biochim. Biophys. Acta 216 (1970) 30–42.
[13] P. Mitchell, J. Moyle, Estimation of membrane potential and pH difference across the cristae membrane of rat liver mitochondria, Eur. J. Biochem. 7 (1969) 471–484.
[14] T. Konishi, M. Murakami, Y. Hatano, K. Nakazato, Simultaneous determination of membrane potential and pH gradient by photodiode array spectroscopy, Biochim. Biophys. Acta 862 (1986) 278–284.
[15] M. Toyomizu, S. Sugiyama, R.L. Jin, T. Nakatsu, α-Glucosidase and aldose reductase inhibitors constituents of cashew, *Anacardium occidentale*, nut shell liquids, Phytother. Res. 7 (1993) 252–254.
[16] I. Kubo, T. Nakatsu, Recent examples of preparative-scale recycling high performance liquid chromatography in natural products chemistry, LC-GC 8 (1990) 933–939.
[17] C.L. Bashford, J.C. Smith, The use of optical probes to monitor membrane potential, Methods Enzymol. 55 (1979) 569–586.
[18] F. Kamp, J.A. Hamilton, pH gradients across phospholipid membranes caused by fast flip-flop of un-ionized fatty acids, Proc. Natl. Acad. Sci. USA 89 (1992) 11367–11370.
[19] F. Kamp, J.A. Hamilton, Movement of fatty acids, fatty acid analogues, and bile acids across phospholipid bilayers, Biochemistry 32 (1993) 11074–11086.
[20] P. Jezek, M. Modriansky, K.D. Garlid, Inactive fatty acids are unable to flip-flop across the lipid bilayer, FEBS Lett. 408 (1997) 161–165.
[21] J.H.P. Tyman, Non-isoprenoid long chain phenols, Chem. Soc. Rev. 8 (1979) 499–537.
[22] P. Mitchell, Biol. Rev. 41 (1966) 445–502.
[23] V.P. Skulachev, Uncoupling: new approaches to an old problem of bioenergetics, Biochim. Biophys. Acta 1363 (1998) 100–124.
[24] A.Yu. Andreyev, T.O. Bondareva, V.I. Dedukhova, E.N. Mokhova, V.P. Skulachev, N.I. Volkov, Carboxyatractylate inhibits the uncoupling effect of free fatty acids, FEBS Lett. 226 (1988) 265–269.