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Electrogenic and nonelectrogenic ion fluxes across lipid and mitochondrial membranes mediated by monensin and monensin ethyl ester

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ABSTRACT

Monensin is a carrier of cations through lipid membranes capable of exchanging sodium (potassium) cations for protons by an electroneutral mechanism, whereas its ethyl ester derivative ethyl-monensin is supposed to transport sodium (potassium) cations in an electrogenic manner. To elucidate mechanistic details of the ionophoric activity, ion fluxes mediated by monensin and ethyl-monensin were measured on planar bilayer lipid membranes, liposomes, and mitochondria. In particular, generation of membrane potential on liposomes was studied via the measurements of rhodamine 6G uptake by fluorescence correlation spectroscopy. In mitochondria, swelling experiments were expounded by the additional measurements of respiration, membrane potential, and matrix pH. It can be concluded that both monensin and ethyl-monensin can perform nonelectrogenic exchange of potassium (sodium) ions for protons and serve as electrogenic potassium ion carriers similar to valinomycin. The results obtained are in line with the predictions based on the crystal structures of the monensin complexes with sodium ions and protons (Huczyński et al., Biochim. Biophys. Acta, 1818 (2012) pp. 2108–2119). The functional activity observed for artificial membranes and mitochondria can be applied to explain the activity of ionophores in living systems. It can also be important for studying the antitumor activity of monensin.

1. Introduction

Monensin (Fig. 1) is extensively used as an anticoccidial drug in poultry and as a growth promoter in ruminants. It belongs to a broad class of antibiotics termed polyether ionophores, i.e. compounds capable of transporting monovalent and divalent cations through cellular membranes [1]. It is widely used in cell biology to dissipate intracellular pH gradients and disturb the functioning of Golgi apparatus [2]. However, its toxicity on mammalian cells is associated with the action on their mitochondria, which exhibit substantial swelling [3]. As an antibiotic, monensin is predominantly effective against Gram-positive bacteria [4,5]. Moreover, it also exhibits antiviral, antifungal, antiparasitic, antimalarial, anti-inflammatory, and tumor cell cytotoxic activity [6–9]. Recently, the antitumor activity of polyether ionophores was substantiated by the finding that they have the ability to kill cancer stem cells and inhibit breast cancer growth and metastasis in mice [10,11]; effects that could be a result of the alteration of the intracellular pH by ionophores [12].

The major mechanism of monensin action as a cation carrier is the electrically silent exchange of sodium (or potassium) cations for protons across the membrane [1,7]. A scheme of this process is displayed in Fig. 2, showing that monensin carboxylate anion (I-COO^-) forms complexes with a monovalent cation M^+ (I-COOM) and a proton (I-COOH) and diffuses across the membrane. The coordination of a metal cation by I-COO^- anion is always accompanied by formation of a pseudo-cyclic structure of monensin anion, which is stabilized by the 'head-to-tail' intramolecular hydrogen bonds formed between the carboxylate anion and hydroxyl groups. In contrast to the other popular polyether ionophore such as nigericin, monensin has a substantial preference for sodium over potassium ions as shown in the binding experiments [13,14] or in the transport experiments [15–17]. However, the experiments with planar lipid bilayers (BLM) [18,19] and liposomes [20,21] showed that monensin was able to transport sodium and potassium cations in an electrogenic manner, that is, generating electrical current on BLM and electrical potential on liposomes. The structural basis for this type of transport can be the structure of the acidic form of monensin with a sodium cation (I-COOH-M^+) present in crystal and in solutions, as has been proved by Huczyński et al. [22]. Diffusion of I-COOH-M^+ complex in one direction and a backward diffusion of

Abbreviations: DPhPC, diphytanoylphosphatidylcholine; DPhytanylPC, diphytanylphosphatidylcholine; BLM, bilayer lipid membrane; FCCP, carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; TMRE, tetramethylrhodamine ethyl ester; Ethyl-monensin, monensin ethyl ester; FCS, fluorescence correlation spectrometry; TPP^+, triphenylphosphonium cation; BCECF, pH probe 2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein; TTFB, tetrachlorotrifluoromethylbenzimidazole

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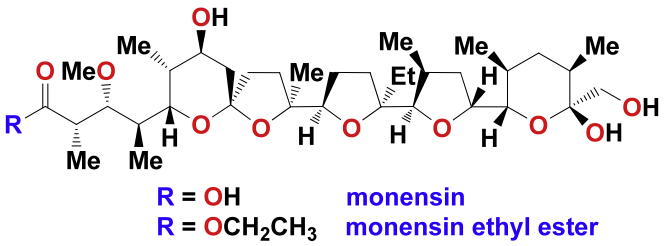

Fig. 1. The structures of monensin and monensin ethyl ester (ethyl-monensin).

I-COOH without the metal cation can be a mechanism of this type of electrogenic ion transport (Fig. 2B). This type of transport is characteristic of another popular ionophore, valinomycin.

The carboxylic group of monensin (COOH) can be chemically modified to obtain ester derivatives blocking COOR group (Fig. 2), which eliminates the possibility of protonation–deprotonation [5]. According to the scheme in Fig. 2A, modification of the carboxylic group of monensin must substantially change the cation transport properties of monensin. However, it has been proved that monensin derivatives show a certain activity against Gram-positive bacteria [5]. The complexation of monovalent and divalent metal cations by monensin esters and the structure of these complexes have been studied using spectroscopic methods and are discussed in detail [5,23–29]. These esters have been found to show especially high affinity to Na^+ and Ca^{2+} cations. The structures of the complexes formed between one of the monensin esters, i.e. monensin 1-naphthylmethyl ester, with sodium and lithium cation have been determined by crystallographic methods [23].

One of the most commonly studied esters of monensin is its methyl ester (methyl-monensin), which was shown to elicit electrogenic sodium transport through liposomes better than the parental monensin [21]. This type of valinomycin-like action can be described in the scheme in Fig. 2C displaying the formation of cationic I-COOR-M^+ complex [29,30], its transmembrane diffusion, metal ion dissociation, and a backward diffusion of the neutral I-COOR molecule. Methyl-monensin was used for the design of a sodium-selective electrode [31], although sodium electrodes based on the parental monensin have also been described [14].

In the present work, we compared the ion carrier properties of monensin and chemically synthesized monensin ethyl ester (ethyl-monensin) with an emphasis on the determination of the electrogenic or nonelectrogenic nature of the ion fluxes. We used artificial membranes (BLMs and liposomes), as well as natural membranes (mitochondria). Importantly, mitochondria are very useful systems to study electrogenic ion fluxes because, in these systems, the actions of electrogenic carrier (valinomycin) and the nonelectrogenic one (nigericin) are qualitatively different [7,32]. In particular, valinomycin decreases the membrane potential of mitochondria while nigericin increases it. This is a result of the dependence of the activity of the respiratory enzymes on the proton-motive force which consists of two components, electrical membrane potential and pH gradient. Nigericin decreases pH gradient and the proton pumps restore the proton-motive force by the increase in the membrane potential. It was shown in the present paper that monensin and ethyl-monensin exhibited both electrogenic and nonelectrogenic types of activity, i.e. monensin can transport potassium ions electrogenically, while ethyl-monensin can perform electrically silent exchange of potassium ions for protons.

2. Materials and methods

Palmitoyloleoylphosphatidylcholine (POPC), diphytanoylphosphatidylcholine (DPhPC), diphytanylphosphatidylcholine (DPhytanylPC),

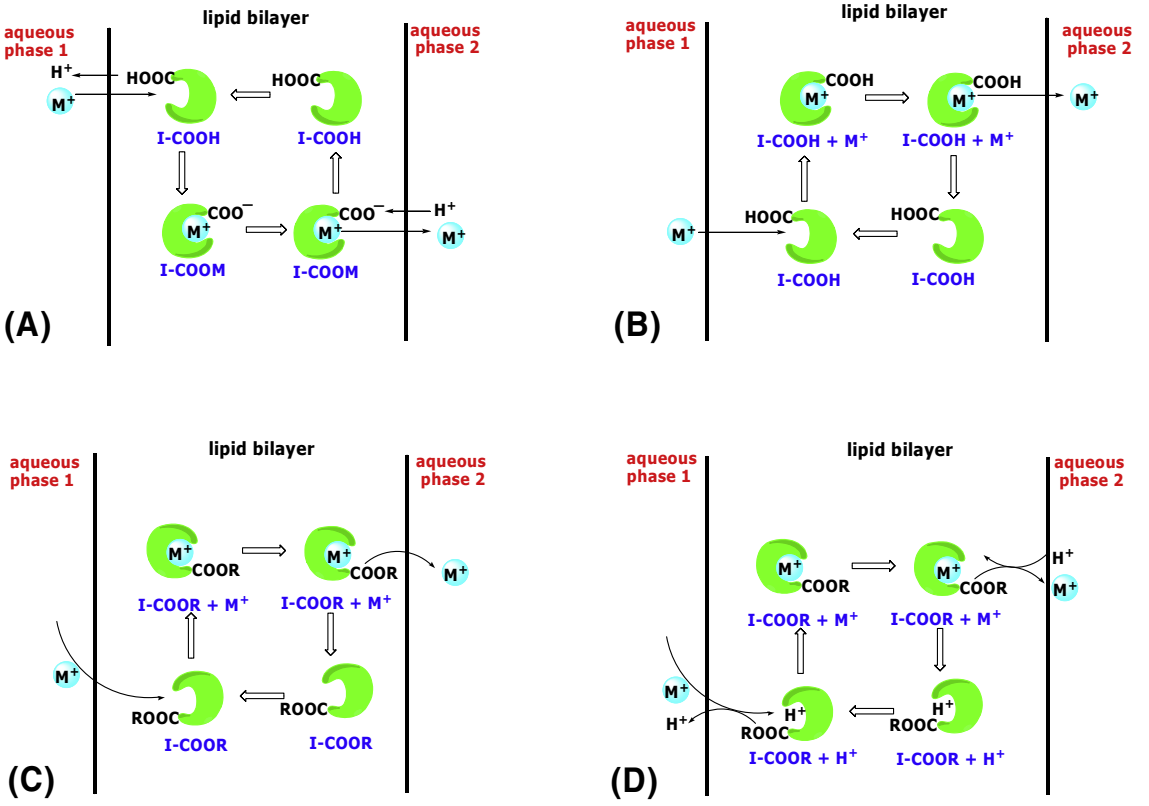

Fig. 2. Four possible ways of cation transport by monensin and ethyl-monensin. In the electroneutral transport (A), the metal cation is incorporated into the monensin skeleton of the pseudocyclic structure of ionophore, and electroneutral I-COOM complex diffuse to another interface, while the neutral ionophore acid molecule I-COOH returns. In electrogenic transport (B), the metal cation is bound and transported by ionophore acid molecule I-COOH forming I-COOH + M^+ complex. In the electrogenic (C) or nonelectrogenic transport (D), the cation fluxes is carried out by monensin with modified carboxylic group (I-COOR) such as ethyl-monenin.

egg yolk phosphatidylcholine (EggPC), and cholesterol (Chol) were purchased from the Avanti polar lipids (Alabaster, Alabama). 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS or Pyranine) and DiS-C3-(5) were purchased from Invitrogen. The protonophore tetrachlorotrifluoromethylbenzeimidazole (TTFB) was a gift from Prof. Lev Yaguzhinsky (Moscow State University). Other chemicals were purchased from Sigma.

Ethyl-monensin was obtained according to the method described previously [5]. The purity of this compound was controlled by elemental analysis, FT-IR, ^{1}H, and ^{13}C NMR spectroscopic methods.

The measurements of K^+/H^+ or Na^+/H^+ exchange on a planar bilayer lipid membrane (BLM) were carried out by a previously developed technique based on monitoring steady-state pH shifts in the unstirred layers (USLs) near a BLM [33–35]. Planar BLMs were formed on an 0.8-mm diameter hole in a Teflon partition separating two compartments of a cell containing aqueous solutions of 100 mM choline chloride, 1 mM MES, 1 mM Tris at pH 6.5. The membrane-forming solutions contained 20 mg DPhPC or 30 mg DPhytanylPC in 1 ml *n*-decane. Gradients of pH on the BLM were measured by recording the open-circuit potential in the presence of a protonophore (1 μM TTFB), which was added at both sides of the BLM. The open-circuit BLM potentials were recorded with the help of a Keithley 301 amplifier, digitized by a LabPC 1200 (National Instruments, Austin, TX) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). The electrical current was measured with a Keithley 428 amplifier. Ag-AgCl electrodes were connected to membrane-bathing solutions via agar-agar bridges filled with 100 mM KCl.

2.1. Detection of proton transport in pyranine-loaded liposomes

The lumenal pH of the liposomes was assayed with pyranine by a slightly modified procedure given in [36]. To prepare pyranine-loaded liposomes, lipid (5 mg DPhPC and 1 mg cholesterol) in a chloroform suspension was dried in a round-bottomed flask under a stream of nitrogen. The lipid was then resuspended in a buffer (100 mM KCl, 20 mM MES, 20 mM MOPS, 20 mM tricine titrated with KOH to pH 6.0) containing 0.5 mM pyranine. The suspension was vortexed and then freeze-thawed three times. Unilamellar liposomes were prepared by extrusion through 0.1-μM pore size nucleopore polycarbonate membranes using an Avanti Mini-Extruder. The unbound pyranine was then removed by passage through a Sephadex G-50 coarse column equilibrated with the same buffer solution. To measure the rate of pH dissipation in liposomes with lumenal pH 6.0, the liposomes were diluted in a solution buffered to pH 8 and supplemented with 2 mM *p*-xylene-bis-pyridinium bromide to suppress the fluorescence of leaked pyranine. The pH was estimated from the ratio F_{455}/F_{410} of the intensities of fluorescence measured at 505 nm upon excitation at 455 nm (F_{455}) and 410 nm (F_{410}), respectively [36], as monitored with a Panorama Fluorat 02 spectrofluorometer. At the end of each recording, 1 μM of nigericin was added to dissipate the remaining pH gradient. The measurements were carried out at $T = 15$ °C.

2.2. Measurements of electrical potential on membranes of liposomes with DiS-C3-(5).

Liposomes were prepared by evaporation under a stream of nitrogen of a solution of egg yolk phosphatidylcholine in chloroform followed by hydration with a buffer solution containing 100 mM KCl, 10 mM Tris, pH 7.4. The mixture was vortexed, passed through a cycle of freezing and thawing, and extruded through 0.1-μM pore size Nucleopore polycarbonate membranes using an Avanti Mini-Extruder. The membrane potentials of liposomes were generated and measured via fluorescence of DiS-C3-(5) of a suspension of liposomes after the addition of valinomycin, monensinin, or ethyl-monensin in a buffer containing 100 mM choline chloride, 10 mM Tris, pH 7.4 as described previously [37]. The fluorescence at 690 nm (excitation at 650 nm) was monitored with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia). Experiments were carried out using 0.03 mg/ml liposomes and 1 μM DiS-C3-(5).

2.3. Rhodamine 6G uptake by liposomes measured by fluorescence correlation spectrometry (FCS)

As shown in our previous works, FCS can be used to measure the uptake of cationic rhodamines to mitochondria upon the formation of electrical potential on membranes of mitochondria [38]. In the present work, we used the same approach for measurements of the uptake of cationic rhodamine 6G by liposomes having potential on their membranes. The setup of our own construction was described previously [38]. Briefly, fluorescence excitation and detection were provided by a Nd:YAG solid state laser with a 532-nm beam attached to an Olympus IMT-2 epifluorescence inverted microscope equipped with a 40× NA 1.2 water immersion objective (Carl Zeiss, Jena, Germany). The fluorescence passed through an appropriate dichroic beam splitter and a long-pass filter and was imaged onto a 50-μM core fibre coupled to an avalanche photodiode (SPCM-AQR-13-FC, Perkin Elmer Optoelectronics, Vaudreuil, Quebec, Canada). The output signal $F(t)$ was sent to a personal computer using a fast interface card (Flex02-01D/C, Correlator.com, Bridgewater, NJ). The signal was measured as the number of photons per second and expressed in Hz. The data acquisition time T was 30 s. The card generated the autocorrelation function of the signal, $G(\tau)$, defined as

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t+\tau) \rangle}{\langle F(t) \rangle^2}, \quad (1)$$

where $< F(t) >$ is the mean fluorescence intensity and $\delta F(t) = F(t) - < F(t) >$ is the deviation from the mean. Autocorrelation function measurements are usually employed to determine diffusion coefficients by $G(\tau)$ curve fitting to the theoretical equation of three-dimensional diffusion [39]. The experimental data were obtained upon stirring, which increased the number of events by about three orders of magnitude, thus substantially enhancing the resolution of the method. Under these conditions, the dependence of $G(\tau)$ on τ is defined by the rate of liquid flow generated by the stirrer. The $G(\tau)$ value in the limit of low τ is used for quantitative estimation of the average number of fluorescent particles, namely, for suspension of identical particles [39]:

$$G(\tau \to 0) = \frac{1}{N}, \quad (2)$$

where N is the average number of fluorescent particles in the confocal volume. A decrease in the parameter N in our system can be attributed to the uptake of free dye molecules by liposomes leading to a decrease in the total number of fluorescence particles due to multiple binding of the dye to liposomes.

2.4. Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by differential centrifugation [40] in a medium containing 250 mM sucrose, 10 mM MOPS, and 1 mM EGTA, pH 7.4. The final washing was performed in the medium that additionally contained bovine serum albumin (0.1 mg/ml). Protein concentration was determined using the Biuret method. Handling of animals and experimental procedures were conducted in accordance with the international guidelines for animal care and use and were approved by the Institutional Ethics Committee of A.N. Belozersky Institute of Physico-Chemical Biology at the Moscow State University.

2.5. Mitochondria respiration and mitochondrial membrane potential measurements

Membrane potential of isolated mitochondria was measured with the help of a tetraphenylphosphonium (TPP^+)-sensitive electrode (NIKO-ANALIT, Moscow, Russia) in the medium of 250 mM sucrose, 5 mM MOPS, 5 mM KH_2PO_4, 1 mM EGTA, pH 7.4 at $T = 25$ °C. TPP^+ measurements were accompanied by measurements of respiration by a Clark-type electrode in a homemade two-electrode setup similar to that described in [41].

2.6. Swelling of mitochondria

The ionophoric ability of monensin and ethyl-monensin was tested by induction of swelling of non-respiring rat liver mitochondria incubated in buffered isotonic potassium acetate in the absence or in the presence of a protonophore FCCP [32,42]. Without nigericin, the mitochondria do not swell because isotonic potassium acetate can cross the membrane only as undissociated acetic acid. Nigericin enables potassium ions to influx into the matrix and equilibrate the matrix pH by efflux of protons, thus allowing the mitochondria to swell. In the presence of FCCP, intramitochondrial accumulation of potassium acetate becomes possible if K^+ can be imported by an electrogenic potassium carrier such as valinomycin. The swelling of mitochondria was recorded as a decrease in the absorbance of the mitochondrial suspension at 550 nm. In short, an aliquot of mitochondria (0.2 mg mitochondrial protein) was added to 1 ml of the 'swelling medium' containing 145 mM potassium acetate, 5 mM Tris, 0.5 mM EDTA, and 1 μM rotenone at pH 7.4.

2.7. Measurements of intramitochondrial pH with BCECF

The matrix pH of isolated rat liver mitochondria was assayed with a pH-sensitive probe 2′,7′-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF), which was subjected to hydrolysis by the intramitochodrial esterases according to Jung, Davis, and Brierley [43]. The resulting charged forms of the probe were retained in the mitochondrial matrix and appear well-suited for the estimation of pH in this compartment. A slightly modified procedure was used: 10 μl of BCECF/AM solution in DMSO (500 μg/ml) was added to a 5 mg/ml suspension of rat liver mitochondria in sucrose (0.25 M) containing MOPS (20 mM, pH 7.4) and EGTA (1 mM). The suspension was incubated for 10 min at 25 °C and the mitochondria were removed by centrifugation, resuspended at 50 mg/ml in sucrose-MOPS-EGTA, and kept in ice during the experiments. Intramitochondrial pH was estimated by recording the ratio of fluorescence of matrix BCECF at its excitation maximum of 510 nm to that at 440 nm using a Panorama Fluorat 02 spectrofluorimeter (emission, 530 nm). The incubation medium was 250 mM sucrose, 5 mM MOPS, 1 mM EGTA, pH 7.4. The concentration of mitochondrial protein was 0.25 mg/ml.

3. Results

As shown earlier, nonelectrogenic cation/H^+ exchange can be measured on a planar BLM via recording the open-circuit electrical potential in the presence of a protonophore, which corresponds to a pH gradient on the BLM [33–35]. The pH gradient was a result of pH shifts upon the formation of a gradient of the metal cation (for example, potassium cations), which proceeds predominantly in the unstirred layers adjacent to the membrane requiring low buffer capacity of the buffer solution [44,45]. In the presence of a protonophore, pH gradient of one unit leads to a potential of about 60 mV according to the Nernst equation $\Delta\psi = (RT/F)(\log([H^+]_{cis}/[H^+]_{trans}))$.

Fig. 3 shows a typical recording of $\Delta\psi$ on a BLM illustrating the measurements of K^+/H^+ exchange mediated by monensin at the KCl gradient of 60 mM (*cis* side) versus 30 mM (black curve in Fig. 3). The addition of 10 mM sodium ions at the *trans* side led to reversal of the potential because monensin is more effective Na^+/H^+ exchanger than K^+/H^+ one [34]. The red curve in Fig. 3 shows the results of a similar experiment with ethyl-monensin. This compound is also able to facilitate the K^+/H^+ exchange, although at higher concentrations. Besides, ethyl-monensin was less selective for sodium ions because the addition of 10 mM NaCl at the *trans* side did not reverse the potential. Importantly, the potentials resulted from the pH gradients on the membrane and were not related to electrogenic fluxes of potassium induced by monensin and ethyl-monensin, as concluded from the measurements of the BLM electrical current in the absence of a protonophore TTFB (tetrachlorotrifluoromethylbenzimidazole). Neither monensin nor ethyl-monensin induced detectable electrical current under our experimental conditions, while the addition of 1 μM TTFB increased the current by over three orders of magnitude.

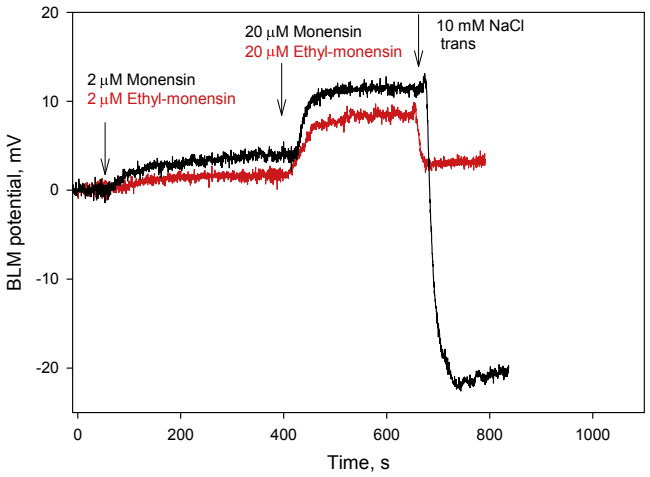

Fig. 3. Measurements of K^+/H^+ exchange on the lipid membrane via the generation of the electrical potential on the BLM with KC1 concentration gradient (60 mM KC1 against 30 mM KCl) in the presence of a protonophore TTFB (1 μM) upon the addition of monensin (black curve) or ethyl-monensin (red curve). The solution was: 1 mM Tris, 1 mM MES, 30 mM KCl, pH 7.0. The BLM was formed from a solution of DPhPC in *n*-decane. The potential had plus on the *trans* side of the BLM where the concentration of KCl was lower.

Fig. 4A shows the results of measurements of Na^+/H^+ exchange mediated by monensin and ethyl-monensin. Similar to K^+/H^+ exchange, discussed above, the data show that the acting concentrations of ethyl-monensin were substantially higher than those of monensin. The reliable effect (about 4 mV) was recorded at 20 μM ethyl-monensin. Fig. 4, panels A and B permit a comparison of sodium and potassium fluxes induced by monenin and ethyl-monensin measured under the same conditions. The ratio of the monensin-mediated Na^+/K^+ fluxes estimated from the corresponding potentials was about 15, while the ratio of the ethyl-monensin-mediated fluxes was only 2.

A question arises about the nature of cation complexes of ethyl-monensin, which accounted for this electrically silent K^+/H^+ and Na^+/H^+ exchange, namely, are these complexes neutral or are they positively charged (for example, I-COOR-Na^+). To address this question, one can use lipids having a lower dipole potential, which determines the rate of diffusion of charged complexes of carriers through the membrane [46]. In our experiments, we used diphytanylphysphatidylcholine (DPhytanylPC) carrying alkyls linked through ether bonds (not ester bonds as in conventional lipids) and having a dipole potential of about 100 mV lower when compared to diphytanoylphosphatidylcholine (DPhPC) leading to an about 30-fold cation transport increase [47,48]. As seen from a comparison of Fig. 4C (DPhytanylPC) and Fig. 4A (DPhPC), the activity of monensin remained the same while the acting concentrations of ethyl-monensin were reduced substantially. The potential induced by 2 μM of ethyl-monensin in a DPhPC membrane was undetectable, while it reached about 4 mV (i.e. $\Delta pH = 4/60$) in

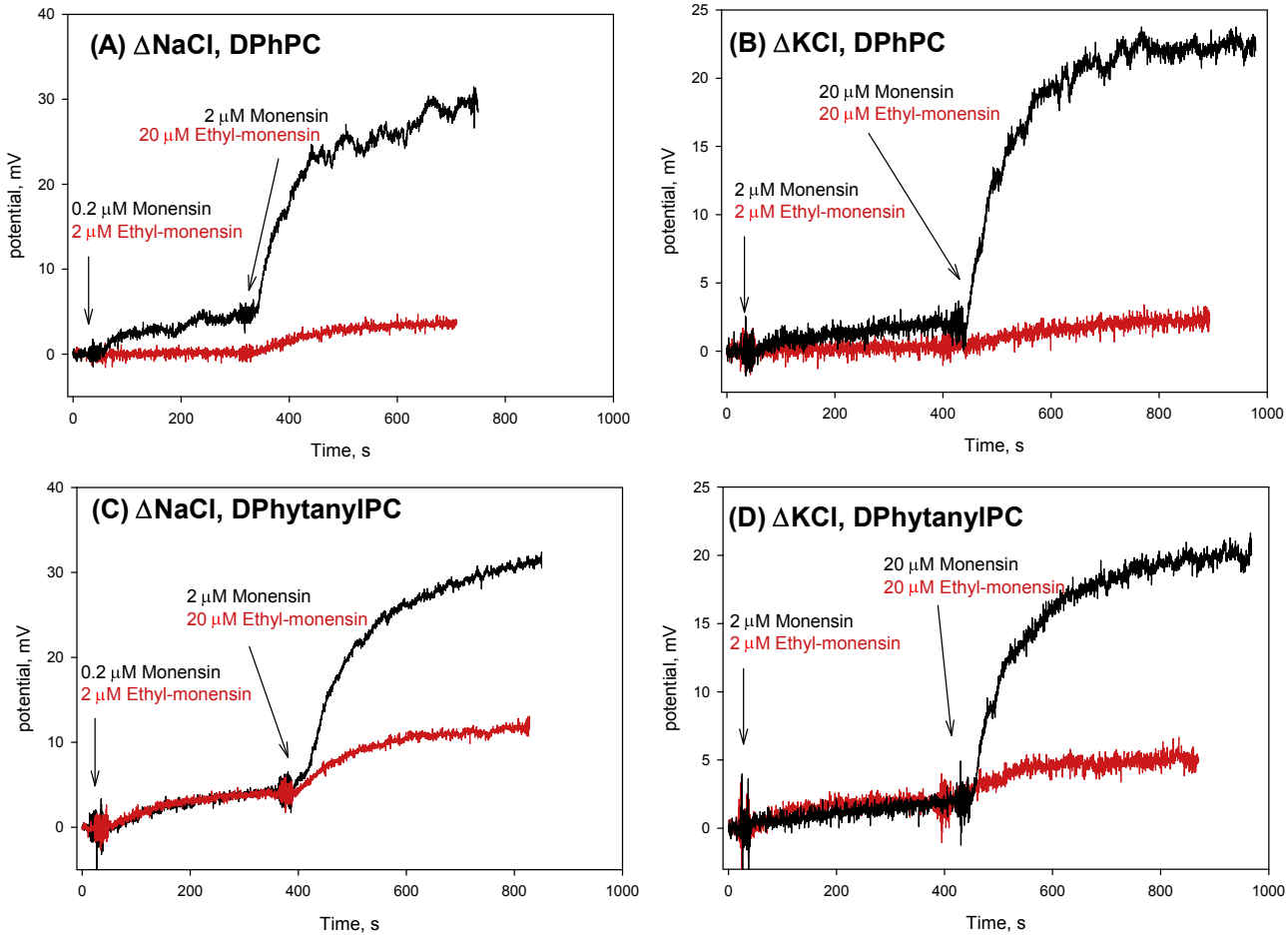

Fig. 4. Measurements of Na^+/H^+ exchange (panels A and C) and K^+/H^+ exchange (panels B and D) on the lipid membrane via the generation of electrical potential on the BLM with sodium or potassium ion concentration gradients (10 mM *cis* against 0 mM *trans*) in the presence of a protonophore TTFB (1 μM) upon the addition of monensin (black curve) or ethyl-monensin (red curve). The solution was: 1 mM Tris, 1 mM MES, 100 mM choline chloride, pH 6.5. The BLM was formed from a solution of DPhPC in *n*-decane (panels A and B) or diphytanylphosphatidylcholine in *n*-decane (panels C and D). The potential had plus on the *trans* side of the BLM where the concentration of sodium or potassium ions was lower.

DPhytanylPC membranes. A similar result was observed in the case of K^+/H^+ exchange (Fig. 4B and D). These experiments suggested that ethyl-monensin-mediated Na^+/H^+ exchange proceeding via the formation of cationic complexes with the Na^+ cations and possibly H^+, while monensin induced Na^+/H^+ exchange proceeding via neutral complexes. An alternative proof for the effect of dipole potential on the Na^+/H^+ exchange mediated by monensin and ethyl-monensin could be the result of experiments with known dipole potential modifier phloretin [49,50]. However, phloretin is a permeable weak acid, which can affect the formation of pH gradients on BLM [51].

An alternative system suitable for the study of H^+ fluxes through artificial membranes is a system of liposomes loaded with a pH sensitive probe pyranine and with a pH gradient on the membrane. Importantly, the protonophore by itself without valinomycin is not able to induce hydrogen ion flux in this system because this flux generates electrical potential blocking the flux. In contrast, nigericin is effective in this system without valinomycin. Fig. 5A shows a dose dependence of the action of monensin in this system without valinomycin in the range of 10–100 nM. The addition of valinomycin leads to a slight stimulation of the H^+ flux (red and black curves in Fig. 5A). Furthermore, Fig. 5B shows the effect 100 nM ethyl-monensin by itself (black curve) and in the presence of valinomycin (red curve). Similar to monensin, valinomycin only slightly stimulated the H^+ flux. It can be concluded that both monensin and ethyl-monensin induced predominantly electrically silent H^+ fluxes, similar to the case of planar BLM. However, the acting concentrations of monensin and ethyl-monensin were very close in the case of liposomes, monensin being somewhat more active compared to ethyl-monensin.

It has been shown previously with the help of a fluorescent potential dependent probe DiS-C3-(5), that monensin is capable of transporting K^+ cations through membranes of liposomes in an electrogenic manner similar to valinomycin [20,52]. To confirm this conclusion and to compare the actions of monensin and ethyl-monensin, experiments were performed to check the potential dependent uptake of a cationic dye rhodamine 6G by the method of fluorescent correlation spectroscopy (FCS). According to [39,53], the amplitude of $G(\tau)$ in the limit $\tau \rightarrow 0$ is determined by the reciprocal of the mean number (N) of fluorescent particles in the observation volume. In our experiments, N comprises dye-loaded liposomes and free dye molecules outside liposomes. To measure $G(\tau \rightarrow 0)$ more precisely, we performed FCS experiments upon stirring [38]. The addition of liposomes substantially increased the amplitude of $G(\tau)$ (from 0.03 to 0.26) suggesting that rhodamine 6G has high affinity to the lipid of liposomes (Fig. 6A, grey and black curves). The addition of valinomycin under the conditions of potassium ion gradient on the membrane (100 mM KCl inside liposomes and 100 mM choline chloride outside them) increased the amplitude of $G(\tau)$ by about twice (from 0.26 to 0.60, black and red curves in Fig. 6A). Subsequent addition of 100 mM KCl decreased the amplitude to 0.17, i.e. to a value lower than before the addition of valinomycin. This effect can be related to the generation of some electrical potential on the membrane of liposomes even in the absence of valinomycin due to potassium ion leak of the membrane.

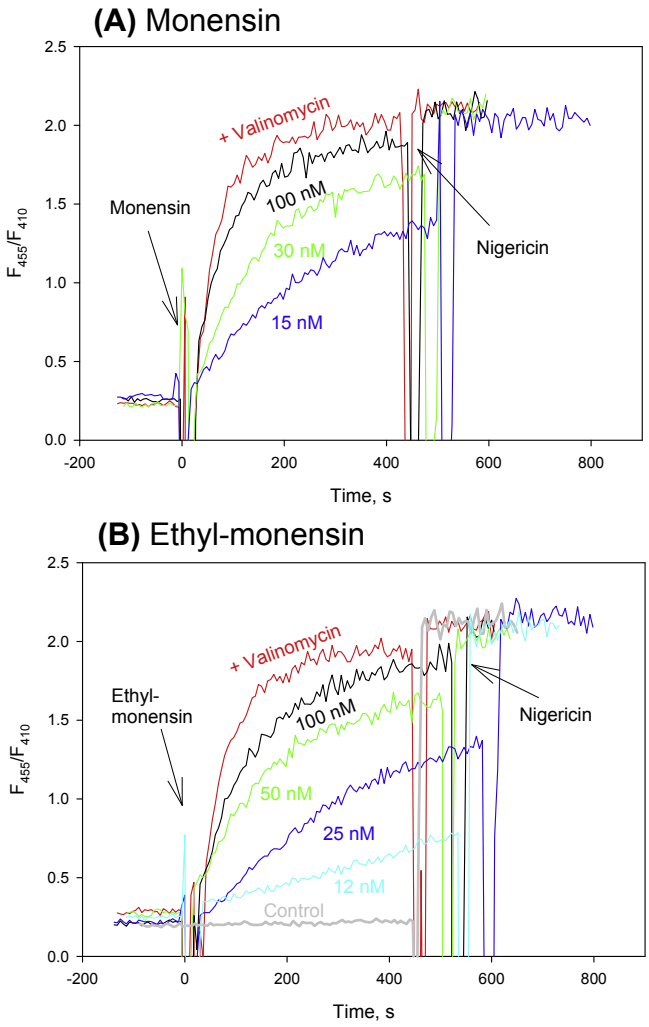

Fig. 5. Dissipation of pH gradient on membranes of pyranine-loaded liposomes by monensin (100 nM, panel A) or ethyl-monensin (100 nM, panel B) in the absence (black curves) and in the presence of 10 nM valinomycin (red curves). Colored curves represent different concentrations of the carriers (without valinomycin). Inner liposome pH was estimated from the ratio (F_{455}/F_{410}) of pyranine fluorescence intensities measured at 505 nm upon excitation at 455 nm and 410 nm, respectively. Nigericin (1 μM) was added at the end of each record to equilibrate the pH. Other conditions: see Materials and Methods. Lipid concentration was 20 μg/ml.

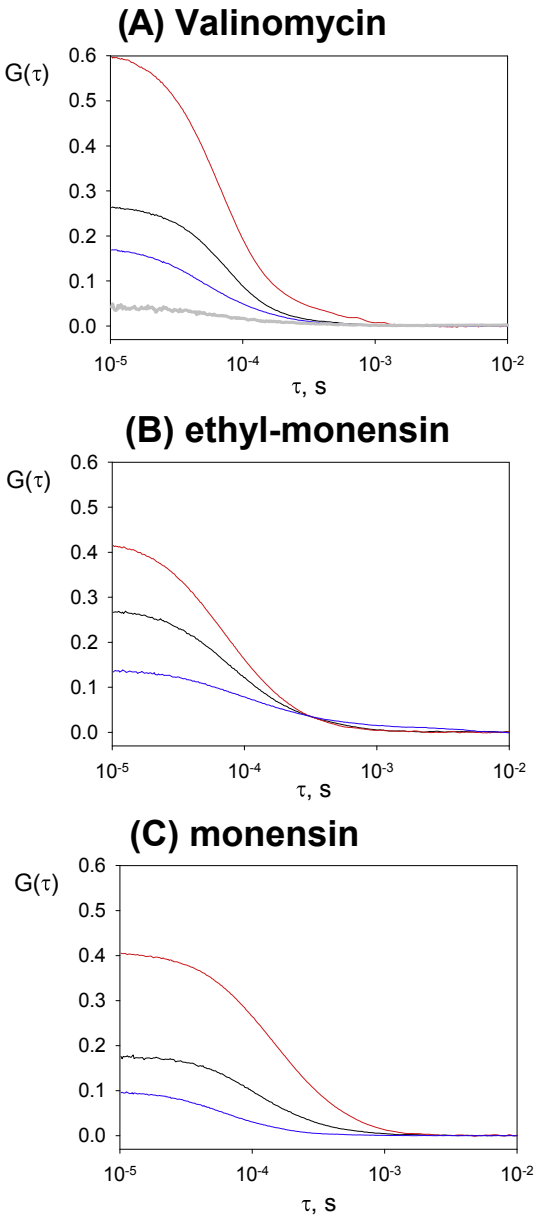

Fig. 6. FCS measurements of rhodamine 6G-uptake by liposomes with a potassium ion gradient on the membrane upon the addition of valinomycin (10 nM, panel A), ethyl-monensin (100 nM, panel B), and monensin (100 nM, panel C). Black curves: autocorrelation functions $G(\tau)$ of rhodamine 6G (concentration 10 nM) in a suspension of DPhPC liposomes (10 μg/ml) in 100 mM choline chloride, 10 mM TRIS, pH 7.4. Red curves: $G(\tau)$ of rhodamine 6G after the addition of the appropriate carrier. Blue curves: $G(\tau)$ of rhodamine 6G after subsequent addition of 100 mM KCl. Grey curve in panel A is a control $G(\tau)$ of rhodamine 6G without other additions. All measurements were carried out under stirring conditions.

Control experiments showed that in the absence of potassium ion gradient (in a 100 mM KCl buffer) the addition of valinomycin did not change the amplitude of $G(\tau)$ (data not shown). Fig. 6B and C show the effect of ethyl-monensin and monensin, respectively. Both monensin and ethyl-monensin increased the amplitude of $G(\tau)$. The acting concentrations of monensin and ethyl-monensin were similar in this system. The experiments confirmed the ability of monensin to induce electrogenic potassium ion fluxes through the membranes of liposomes [20] and proved that ethyl-monensin had the same ability. It can be concluded from the experiments on planar BLM and liposome membrane that both monensin and ethyl-monensin can perform nonelectrogenic exchange of potassium (sodium) ions for protons and serve as electrogenic potassium ion ionophores. To compare electrogenic K^+ transport mediated by different ionophores, we measured fluorescence of the potential-sensitive dye DiS-C3-(5) under the conditions of a transmembrane K^+ gradient. The activity of ethyl-monensin was similar to that of monensin. In particular, substantially higher concentrations (by about two orders of magnitude, data not shown) of both ionophores than those of valinomycin were required to induce comparable potentials on liposomes.

It is well known that isolated mitochondria can serve as useful models for the study of ionophores on natural membranes, especially for a comparison between electrogenic and nonelectrogenic ionophores [7,32]. A swelling of non-respiring mitochondria in a medium of potassium acetate indicates the functioning of nonelectrogenic K^+/H^+ exchanger because the protonated form of acetate readily permeates the mitochondrial membrane, while its anionic form is not permeable. Acidification of the matrix due to acetic acid accumulation prevents swelling while the addition of nigericin reduces matrix acidification and enables the influx of potassium ions thus promoting swelling. Valinomycin by itself is not active in this system but it induces swelling when in combination with a protonophore [32]. Fig. 7A shows the effect of monensin and ethyl-monensin on the swelling of rat liver mitochondria in a

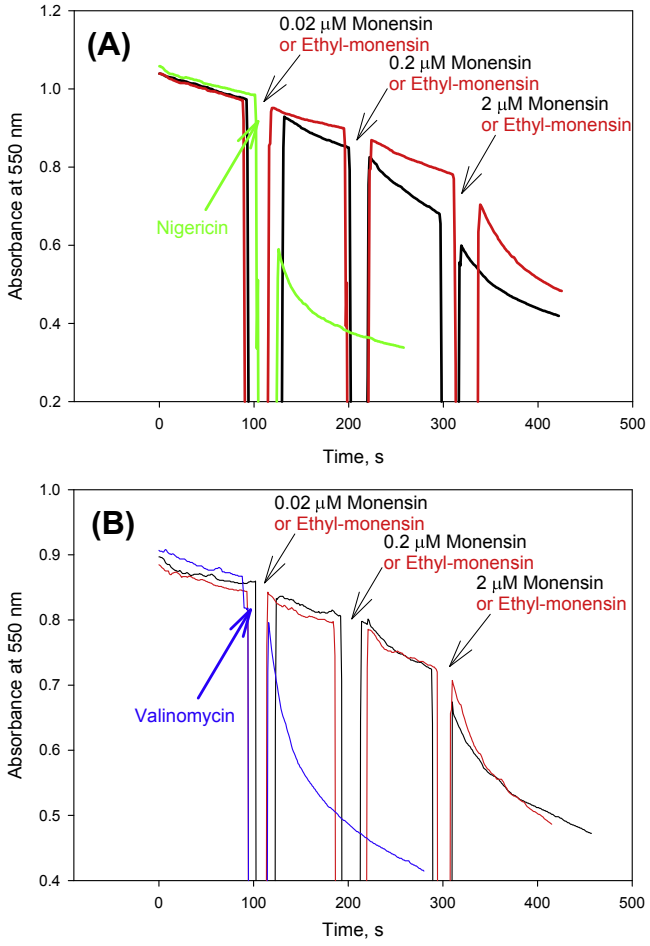

Fig. 7. Effect of monensin (black curves) and ethyl-monensin (red curves) on the swelling of rat liver mitochondria in potassium acetate medium in the absence (panel A) and in the presence of FCCP (1 μM, panel B). Incubation mixture: 145 mM potassium acetate, 5 mM Tris, 0.5 mM EDTA, pH 7.4, and 1 μM rotenone. Mitochondrial protein, 0.2 mg/ml. Green and blue curves are positive controls of the addition of 1 μM nigericin and 1 μM valinomycin, respectively.

potassium acetate medium without a protonophore (K^+/H^+ exchange assay) while Fig. 7B shows the swelling in the presence of a protonophore CCCP (electrogenic K^+ flux assay). Monensin induced nonelectrogenic fluxes at lower concentrations (starting from 200 nM) although micromolar concentrations of ethyl-monensin induced the swelling as well (Fig. 7A). The addition of CCCP had little effect on the activity of ethyl-monensin and monensin in this system (Fig. 7B), although in this case the actions of monensin and ethyl-monensin were similar even at low concentrations. The swelling data suggested that, in mitochondria, monensin and ethyl-monensin operated predominantly as K^+/H^+ exchangers.

These results refer to a system of nonenergized mitochondria lacking electrical potential and pH gradient on their membranes. To study the effect of monensin and ethyl-monensin on the proton fluxes of energized mitochondria, we used a fluorescent pH probe BCECF, according to [43]. It is known that nigericin decreases the pH gradient on the inner mitochondrial membrane, while valinomycin increases it, at least immediately after the addition [43]. The mitochondrial membrane potential is about 180 mV (negative inside) while the pH gradient is about 1 unit (matrix is alkaline). Owing to high electrical potential, the addition of valinomycin leads to an influx of potassium ions, temporarily depolarizing mitochondria, and to the efflux of protons by proton pumps. After the equilibration of potassium ion concentrations, the membrane potential and pH gradient are restored. In contrast, the addition of nigericin induces the efflux of potassium ions in exchange for protons, leading to a decrease in pH gradient and a subsequent increase in the membrane potential, because the enzymes of the electron transport chain (ETC.) pump protons against the total electrochemical potential of protons, which is a sum of the electrical potential and a pH gradient.

Fig. 8 shows the effects of monensin (panel A) and ethyl-monensin (panel B) on the matrix pH of mitochondria energized with succinate in the presence of rotenone. Both ion carriers decreased the pH gradient at concentrations of tenths of a nanomole, exhibiting the action of K^+/H^+ exchangers.

Fig. 9 shows the action of monensin and ethyl-monensin on the membrane potential of mitochondria as estimated by the distribution of tetraphenylphosphonium cation (TPP^+) with the help of TPP^+-selective electrode. The measurement setup was supplemented with a Clark-type electrode, enabling us to simultaneously measure respiration marked by numbers on the TPP^+ records. The content of 10 nM of the carriers did not affect the membrane potential of mitochondria in the presence of phosphate in the medium, which is known to decrease pH gradient and increase the membrane potential. However, 2 μM monensin increased the membrane potential (i.e. worked like nigericin;Fig. 9A), while 2 μM ethyl-monensin decreased the potential (Fig. 9B). Importantly, a decrease in the membrane potential of ethyl-monensin was not accompanied by an increase in respiration but led to a significant decrease in respiration (Fig. 9B), suggesting that ethyl-monensin can be an inhibitor of the succinate-dehydrogenase of

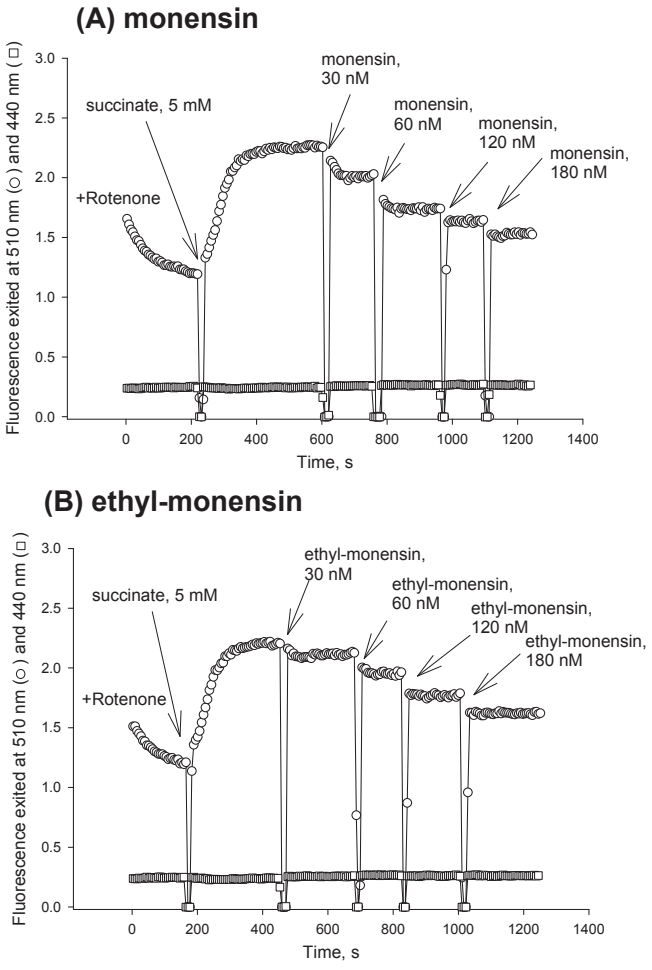

Fig. 8. Effect of monensin (panel A) and ethyl-monensin (panel B) on the matrix pH assayed with a pH probe BCECF loaded in rat liver mitochondria. Fluorescent signal at 530 nm exited at 510 nm (circles) is sensitive to pH decreasing upon acidification while the signal exited at 440 nm (squares) is pH independent. For experimental conditions see Materials and Methods.

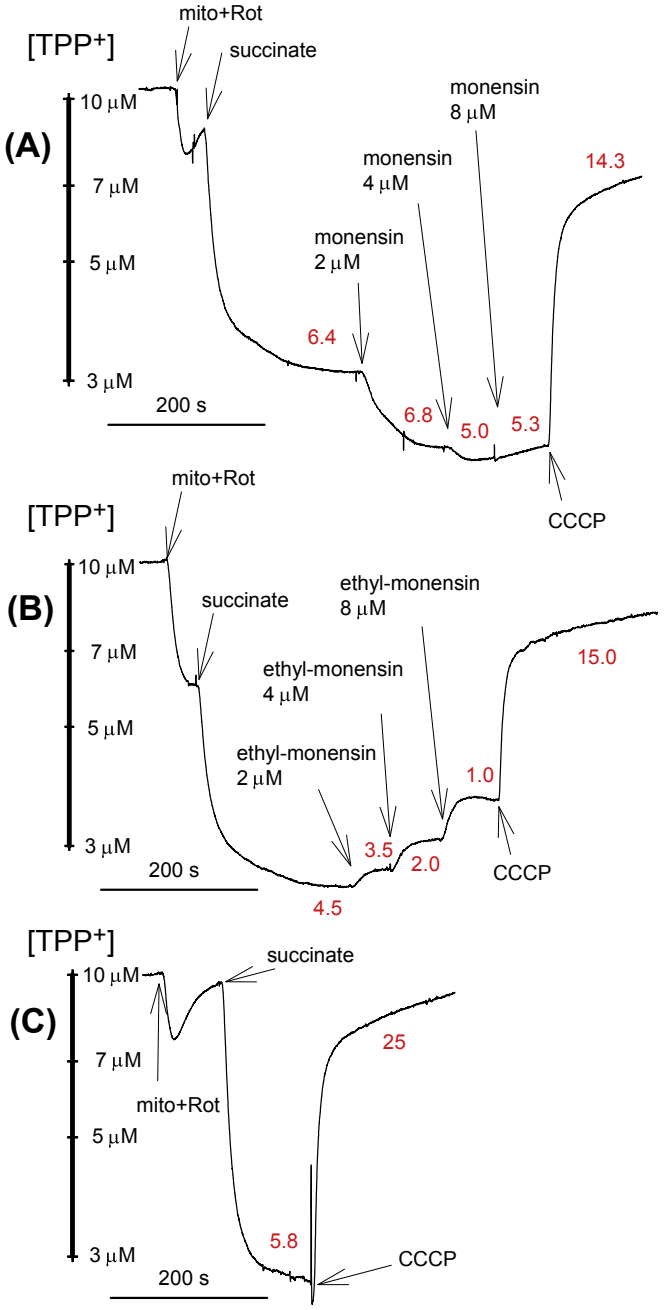

Fig. 9. Effect of monensin (panel A), ethyl-monensin (panel B), and a protonophore CCCP (panel C) on the membrane potential of rat liver mitochondria and their respiration. Shown are traces of TPP^+ accumulation in the medium described in "Materials and Methods" and the rates of respiration marked in red on the traces (in arbitrary units). Mitochondrial protein 1 mg/ml. Additions: rotenone, 2 μM; succinate, 5 mM; CCCP, 0.5 μM.

mitochondria. The inhibition of the respiratory enzymes by nigericin was observed previously [54]. Subsequent addition of an uncoupler led to the collapse of the membrane potential and a partial stimulation of respiration of mitochondria (from 1 to 15, Fig. 9B), the maximal rate of respiration was 25 (Fig. 9C).

4. Discussion

It was shown in the present work that monensin and ethyl-monensin can transport potassium ions both electrogenically as well as by a process of nonelectrogenic exchange for protons. The extent of these two types of activities can be substantially different depending on the conditions and such parameters as phospholipid composition of the membrane, cation gradients in the membrane (metal cation and proton), and the concentration of the carrier. The question of the stoichiometry of metal cation/proton transport as well as the stoichiometry of metal complexes with different polyether ionophores was discussed in the literature in reference to nigericin [55,56], calcium ionophore A23187 [57], and others [58]. Measurements of monensin-mediated hydrogen ion transport indicated that the cationic complexes of protonated monensin with sodium (potassium) ions contributed substantially to the ion carrier bound to the liposomal membranes [17].

As mentioned in the Introduction, the acting mechanism of monensin and ethyl-monensin can be directly related to the mechanism of K^+/H^+ exchange by nigericin and K^+ transport by valinomycin (panels A and C in Fig. 2). The two other types of activity observed (K^+/H^+ exchange by ethyl-monensin and K^+ transport by monensin) are obviously more complicated. The structural basis for this type of transport can be the crystal structure of a complex of acidic form of monensin with a sodium ion (I-COOH-M^+, Fig. 2B) postulated by Huczyński et al. [22,30]. A diffusion of the complex in one direction and a backward diffusion of I-COOH without the metal cation can be a mechanism of this type of electrogenic ion transport (Fig. 2B). It has been proposed [22] that electrogenic K^+-transport by monensin could proceed via potassium complexation with acidic forms of monensin leading to the formation of a positively charged form of I-COOH-M^+ (a scheme in Fig. 2B). This mechanism implies that monensin has high affinity to protons and the anionic form I-COO^- is in a minority even at neutral pH. This is in agreement with the pKa of monensin measured in methanol (10.2; [13]). This mechanism can be accounted for by the uptake of rhodamine 6G in liposomes having a gradient of potassium ions (Fig. 6) and possibly for the swelling of mitochondria in potassium acetate buffer in the presence of a protonophore (Fig. 7). The scheme of K^+/H^+ exchange by ethyl-monensin (Fig. 1D) is also based on the high affinity of neutral I-COOR species to protons implying that, under our experimental conditions, the I-COOR form is virtually absent. In this case, the unidirectional transport of electrical charges with potassium must be forbidden while the exchange of K^+ for H^+ (by I-COOR-K^+ form and I-COOR-H^+ form) can be permitted. This mechanism can explain the absence of the BLM electrical current in the presence of ethyl-monensin related to potassium ion flux. It can be suggested that the BLM experiments could reveal the current related to the translocation of the cationic forms of ethyl-monensin itself. However, it is well known that in contrast to the current of penetrating anions (such as tetraphenylborate), the current of penetrating cations (such as tetraphenylphosphonium) is hard to observe in a BLM system [59]. This is a result of the presence of a layer of oriented water molecules having high dipole moments on the surface of the membrane, which generate a high dipole potential, positive inside the membrane [46]. As mentioned above, the molecules of phospholipids also contribute to this dipole potential (Fig. 4).

The described mechanism of ethyl-monensin-mediated K^+/H^+ exchange can explain the dependence of its action on the membrane potential of mitochondria (i.e. on mitochondrial energization). In fact, ethyl-monensin can induce the swelling of de-energized mitochondria in potassium acetate suggesting the process of K^+/H^+ exchange (Fig. 7). On the other hand, under the conditions of polarized mitochondria with negative potential inside, the efflux of positively charged potassium complex I-COOR-K^+ is hindered and the K^+/H^+ exchange does not proceed. This is the reason for the inability of ethyl-monensin to increase the membrane potential of mitochondria in contrast to monensin (Fig. 9). Interestingly, ethyl-monensin was able to decrease the pH gradient on the mitochondrial membrane in a way similar to monensin (Fig. 8). However, this process obviously proceeded without the exchange for potassium ions but via the translocation of a protonated form of ethyl-monensin (I-COOR-H^+) into the matrix and the back-translocation of a neutral form of the carrier I-COOR. The process is similar to the work of a conventional uncoupler.

Summarizing, it can be concluded that we confirmed the previously observed ability of monensin to transport potassium ions in an electrogenic manner, thus in a way similar to valinomycin, as well as in exchange for protons in a nonelectrogenic manner. These results are in line with the predictions based on the crystal structures of the monensin complexes with sodium ions and protons [30]. Besides, it has been shown that ethyl-monensin is able to exchange potassium ions for protons across membranes. The functional activity observed on artificial membranes and mitochondria can be applied to explain the activity of monensin and its derivatives in living systems related to the ionophorous properties and the action on the oxidative phosphorylation. The work can be important in particular for the study of the antitumor activity of monensin and similar polyether ionophores because apoptotic cell death is frequently associated with the perturbation of mitochondria via an induction of oxidative stress, activation of pro-apoptotic proteins localized in mitochondria and a release of cytochrome C. It could be assumed that the swelling of mitochondria can trigger the process of mitochondrial perturbation.

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