Biochimica et Biophysica Acta, 1064 (1991) 103–110

ADONIS 000527369100176G

BBAMEM 75206

Monensin-mediated antiport of Na^+ and H^+ across liposome membrane

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(Received 10 August 1990)

Key words: Monensin; Liposome; Membrane potential; Sodium ion/proton antiport

The mechanism of monensin-mediated transport of Na^+ and H^+ across large unilamellar liposome membrane was investigated. The inside negative membrane potential ($\Delta\psi$) was generated by the addition of monensin to the liposomes with an outward Na^+ gradient. The effects of intravesicular H^+ bufferring power and medium pH on the initial rates of $\Delta\psi$ formation, Na^+ efflux and H^+ influx were examined. The results showed that (i) the initial Na^+ flux (J_{Na}) was larger than the initial H^+ flux (J_H) at any H^+ bufferring power, (ii) the J_H increased with increasing inner buffer concentration, but the effect of H^+ bufferring power on the J_{Na} was small, (iii) the initial rate of $\Delta\psi$ formation increased linearly with the increase in the value of (J_{Na}–J_H), and (iv) the J_{Na} increased with increasing H^+ concentration. The generation of $\Delta\psi$ was not due to H^+ leak from the liposome, since the $\Delta\psi$ was generated even when H^+ concentration gradient was inwardly directed. The monensin-mediated transport of Na^+ and H^+ in this system occurred at the ratio of $Na^+/H^+ > 1.0$ and the resultant net electric charge efflux is the cause of the inside negative membrane potential. Tetraphenylphosphonium retarded both the $\Delta\psi$ formation and the H^+ influx, but did not affect the Na^+ efflux, suggesting that the driving force of H^+ influx is the inside negative membrane potential generated by Na^+ efflux. This idea also well accounts for the observed H^+ bufferring power effects on the Na^+ efflux, H^+ influx and $\Delta\psi$ formation. It was suggested that Na^+ was transported in the form of 1:1 complex between protonated monensin and Na^+.

Introduction

Ionophore antibiotic, monensin, has been considered to be an electroneutral Na^+ and H^+ exchanger across biological membrane and model membranes [1–6]. Sandeaux et al. [4] had studied the monensin-mediated transmembrane Na^+ flux across planar bilayer lipid membrane, and reported that the electric charge flux was very small as compared to the measured Na^+ flux under the steady-flux conditions. However, the mechanism of monensin-mediated Na^+/H^+ exchange is not yet fully clarified. Obviously, the most direct approach to reveal the mechanism of monensin-mediated transport of Na^+ and H^+ is to measure the initial fluxes of both Na^+ and H^+. The use of unilamellar liposome is of advantage for obtaining further information regarding the mechanism of monensin's action, since initial rates of Na^+ and H^+ transport and those of membrane potential formation can easily and independently be determined.

In this report, we show that monensin causes an inside negative membrane potential ($\Delta\psi$) in the liposome with outward Na^+ gradient. In order to elucidate the mechanism of membrane potential generation, the initial rates of Na^+ efflux and H^+ influx, respectively, were measured by means of flow dialysis with $^{22}Na^+$ and the fluorescence change of pH indicator, pyranine, entrapped in the liposome. Further, the effects of proton bufferring power and pH of the medium on the Na^+ efflux, H^+ influx and $\Delta\psi$ formation were examined. The mechanism of $\Delta\psi$ generation in the system

Abbreviations: PC, phosphatidylcholine; pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid; $diSC_3(5)$, 3,3′-dipropyl-2,2′-thiodicarbocyanine iodide; $\Delta\psi$, electrical potential across the membrane; ΔpH, transmembrane pH difference; ΔpNa, sodium gradient across the membrane; $\Delta\tilde{\mu}_{H^+}$, electrochemical potential of proton; $\Delta\tilde{\mu}_{Na^+}$, electrochemical potential of sodium; TPP^+, tetraphenylphosphonium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Bes, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid.

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is discussed in terms of the net charge efflux as a consequence of the difference between the Na^+ efflux and the H^+ influx.

Materials and Methods

Large unilamellar liposomes consisting of egg yolk phosphatidylcholine (PC) and cholesterol (7:2, molar ratio) were prepared by the reverse phase evaporation method, as described previously [7]. Typically, liposomes were loaded with 200 mM NaCl, 50 μM pyranine, $^{22}Na^+$ (2 μCi) and appropriate concentration of Hepes at pH 8.0. In order to remove extravesicular pyranine and $^{22}Na^+$ and to form sodium concentration gradient ($\Delta pNa = 2.0$), the liposome suspension was passed through a Sephadex G-50 column (1.5 × 20 cm) pre-equilibrated with 198 mM choline chloride, 2.0 mM NaCl and appropriate concentration of Hepes at pH 8.0 ($[Hepes]_{in} = [Hepes]_{out}$). Encapsulation efficiency (%) of liposomes after the gel filtration was determined by the comparison of $^{22}Na^+$ radioactivities of the liposome suspension before and after the gel filtration. The liposome suspensions diluted to final encapsulation efficiency of 0.05% with the same buffer solution used for the gel filtration were used for the following measurements.

$^{22}Na^+$ efflux from liposomes was measured at 20 °C by the flow dialysis method, the apparatus of which was reported previously [8]. A liposome suspension (3.0 ml) was placed in the upper chamber and then an appropriate amount of monensin stock solution (10 μM in ethanol) was added. The flow rate of dialysis buffer passing through lower chamber was 1.63 ml/min, and the contents of both chambers were stirred with small magnetic stirring bars. Each 0.65 ml fraction was collected and its $^{22}Na^+$ radioactivity was measured with a liquid scintillation counter (Aroka LSC-700).

Intra icular pH (pH_{in}) was determined from pyranine fluorescence as reported by Kano and Fendler [9]. The excitation wavelengths for the protonated and deprotonated form of pyranine were 400 nm and 450 nm, respectively, and the fluorescence intensity (I) was detected at 510 nm. An aliquot of monensin stock solution was added to the liposome suspension (3.0 ml), then the time course of change in fluorescence intensities I_{450} and I_{400} was measured. Here, I_{450} and I_{400} were the fluorescence intensities upon excitation of pyranine at 450 and 400 nm, respectively. The values of intravesicular pH before and after the addition of monensin were determined by the use of the correlation curve obtained by plotting log (I_{450}/I_{400}) against pH. The total number of protons accumulated in liposomes was calculated as the increment of protonated forms of Hepes and pyranine in the liposomes by using the measured pH_{in}, $pK_a = 7.55$ for Hepes and $pK_a = 7.22$ for pyranine.

The membrane potential ($\Delta\psi$, inside negative) in liposomes was determined from quenching of $diSC_3(5)$ fluorescence (exitation wavelength 620 nm, emission wavelength 670 nm). To a liposome suspension (3.0 ml), 5 μl of $diSC_3(5)$ stock solution (0.5 mg/ml in ethanol) was added. Subsequently an aliquot of monensin stock solution was added after the fluorescence intensity had reached steady level, then the time course of change in fluorescence intensity was recorded. All fluorescence measurements were carried out at 20 °C with Shimadzu fluorescence spectrophotometer RF-501.

The amounts of PC and cholesterol in each liposome suspension (encapsulation efficiency 0.05%, 3.0 ml) were determined with Phospholipid-test Wako kit and Cholesterol B-test Wako kit (Wako Pure Chemical Industries, Japan) and were $(2.52–3.30) \cdot 10^{-7}$ mol for PC and $(0.791–1.19) \cdot 10^{-7}$ mol for cholesterol. The PC/cholesterol (mol/mol) of liposomes ranged from 6.3:2 to 6.9:2.

Materials used were as follows: egg yolk PC from Nihon-seika (Japan); $diSC_3(5)$ from Japanese Research Institute for Photosensitizing Dyes; pyranine from Molecular Probes; choline base from Sigma; $^{22}NaCl$ (carrier-free) and $H^{36}Cl$ (0.1 mCi/g) from New England Nuclear; cholesterol, monensin (sodium salt), Hepes, tetraphenylphosphonium chloride, choline chloride and other chemicals were purchased from Wako, Japan.

Results

Generation of $\Delta\psi$ due to the monensin-mediated antiport of Na^+ and H^+ across liposome membrane

In this study, inside negative $\Delta\psi$ in the liposome was measured by the fluorescence quenching of $diSC_3(5)$. It was shown by us [8] that valinomycin-induced potassium diffusion potential (inside negative) in liposomes, prepared by the same method described here, was well represented by the Nernst equation,

$$\Delta\psi = -59 \log([K^+]_{in}/[K^+]_{out})$$

where $[K^+]_{in}/[K^+]_{out}$ is the imposed K^+ concentration ratio. As Fig. 1 shows, the plots of the decrement of fluorescence intensity of $diSC_3(5)$ (%) against $-59 \log [K^+]_{in}/[K^+]_{out}$ (mV) are linear in the range of -100 to 0 mV. The value of the inside negative $\Delta\psi$ due to the monensin-mediated Na^+/H^+ antiport was determined by using this correlation curve.

When monensin was added to the liposomes with outward Na^+ gradient ($[Na^+]_{in} = 200$ mM, $[Na^+]_{out} = 2$ mM, $pH_{in} = pH_{out} = 8.0$), the $diSC_3(5)$ fluorescence was quenched as shown in Fig. 2 (curve A), and at the same time the pH_{in} measured with fluorescent pH indicator, pyranine, was lowered (curve B in Fig. 2). The subsequent addition of NaCl (final 200 mM) to the external

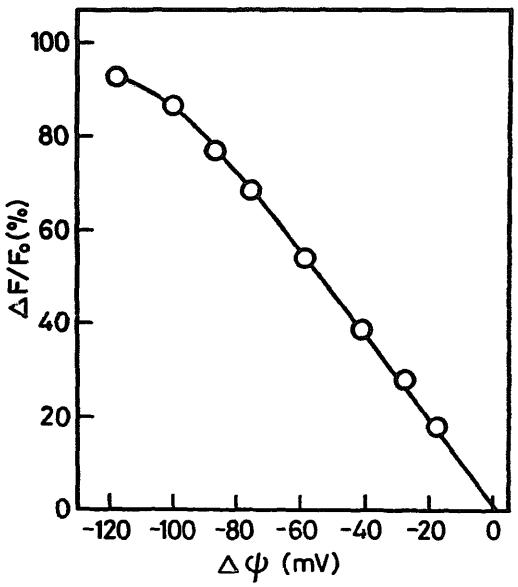

Fig. 1. Calibration of fluorescence quenching of $diSC_3(5)$ in terms of $\Delta\psi$. Liposomes loaded with 200 mM KCl and 2 mM Hepes (pH = 8.0) was suspended in isoosmotic external 2 mM Hepes buffer containing KCl and NaCl in desired proportions at pH 8.0. An aliquot (5 μl) of $diSC_3(5)$ solution (0.5 mg/ml in ethanol) was added to the liposome suspension (3.0 ml, encapsulation efficiency = 0.05%). $\Delta F = F_0 - F_q$, where F_0 and F_q are the fluorescence intensities of $diSC_3(5)$ before and after the addition of valinomycin ($7.0 \cdot 10^{-8}$ M), respectively. $\Delta\psi$ corresponds to the K^+-diffusion potential, $\Delta\psi = -(RT/F)$ ln $([K^+]_{in}/[K^+]_{out})$.

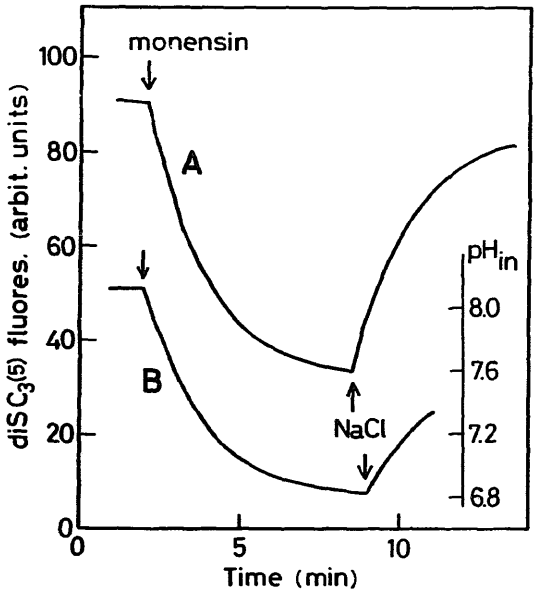

Fig. 2. Time-courses of the changes in fluorescence intensity of $diSC_3(5)$ and pH_{in} after the addition of monensin to liposomes with outward Na^+ gradient. Curve A: $diSC_3(5)$ fluorescence; curve B: pH_{in}. Initial conditions: $[Na^+]_{in} = 200$ mM, $[Na^+]_{out} = 2.0$ mM, $[choline^+]_{out} = 198$ mM, $[Hepes]_{in} = [Hepes]_{out} = 30$ mM, $pH_{in} = pH_{out} = 8.0$, [pyranine]$_{in} = 50$ μM. Allows show the time when 2 μl of monensin stock solution (10 μM in ethanol) and an aliquot of 3.0 M NaCl solution was added (final concentration $[Na^+]_{out} = 200$ mM). Further details are described in Materials and Methods.

medium restored the $diSC_3(5)$ fluorescence to initial level and increased pH_{in} up to 7.9 after 10 min. In the absence of Na^+ concentration gradient, the addition of monensin caused neither the fluorescence quenching of $diSC_3(5)$ nor the decrease in pH_{in}. This fact clearly shows that the inside negative $\Delta\psi$ generation is due to Na^+ efflux.

The effects of H^+ bufferring power in liposomes on Na^+ efflux, H^+ influx and $\Delta\psi$ formation

In order to elucidate the mechanism of $\Delta\psi$ generation, the initial outward Na^+ flux (J_{Na}, mol cm^{-2} min^{-1}) and the initial inward H^+ flux (J_H, mol cm^{-2} min^{-1}) were measured by using liposomes loaded with different concentration of Hepes (2, 30 and 100 mM) at pH 8.0. Here, the surface area of total liposomes required to calculate the fluxes was estimated to be 740–790 cm^2 from the half amount of PC and cholesterol in the liposome suspension. The surface area occupied by one molecule of PC and that of cholesterol in bilayer structure were taken to be 72 $Å^2$ and 38 $Å^2$ [10], respectively. The time course of Na^+ ($^{22}Na^+$) efflux from liposomes were followed by the flow dialysis method. The total number of protons accumulated in liposomes was calculated from the internal pH change, as described in Materials and Methods. Fig. 3 shows the typical time-course of Na^+ efflux and H^+ influx after the addition of monensin in the liposomes loaded with 30 mM Hepes. The pH_{in} and extravesicular Na^+ concentration leveled off within about 15 min. The Na^+ efflux was obviously more rapid than the H^+ influx. Similar results were obtained at other concentrations of

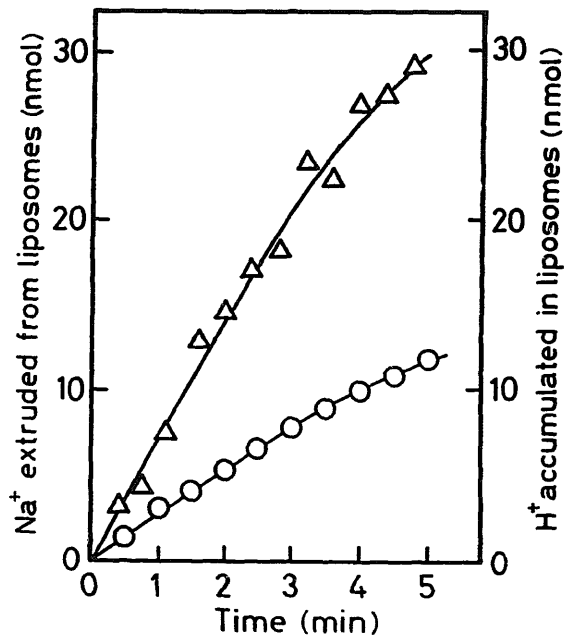

Fig. 3. Time-courses of Na^+ efflux and H^+ influx. △, Na^+ extruded from liposomes; ○, H^+ accumulated in liposomes. Initial conditions were the same as in the case of Fig. 2. [monensin] = 6.7 nM. See Materials and Methods for further details.

Hepes. As Fig. 4 shows, the J_H increased and the $\Delta\psi$ formation was retarded with increasing Hepes concentration, whereas the effect of H^+ bufferring power on the J_{Na} was small. Regardless of initial conditions, the values of $\Delta\psi$ at a stationary state were consistent with those calculated by the equation,

$$\Delta\psi = -59 \log([Na^+]_{in}/[Na^+]_{out}) = -59 \log([H^+]_{in}/[H^+]_{out})$$

where, each ion concentration is the value at the stationary state (data not shown). Thus, only the conversion of $\Delta\tilde{\mu}_{Na^+}$ into $\Delta\tilde{\mu}_{H^+}$ occurs in this system. As Fig. 5 shows, the initial rate of $\Delta\psi$ formation showed linear relation with $J_{Na} - J_H$, suggesting that the $\Delta\psi$ was generated by the net charge efflux, $J_{Na} - J_H$.

It is of interest to see if such inside negative membrane potential can be generated under the conditions of an inward H^+ gradient ($pH_{in} > pH_{out}$). As Fig. 6 shows, when monensin was added to the liposome suspension under such a condition, the fluorescence intensity of $diSC_3(5)$ decreased before pH_{in} became lower than pH_{out}. This finding clearly shows that inside negative $\Delta\psi$ can be generated without H^+ leak from liposomes.

The effect of medium pH on Na^+ efflux, H^+ influx and $\Delta\psi$ formation

As shown in Table I, the increase in proton concentration accelerated the initial Na^+ efflux, H^+ influx and $\Delta\psi$ formation. The J_{Na} was larger than J_H at any pH, though the ratio, J_{Na}/J_H, became close to 1.0 as the initial pH was lowered. The initial rate of $\Delta\psi$ formation increased with the increase in the value of $J_{Na} - J_H$, as in the case of the effect of H^+ bufferring power on the relation between $\Delta\psi$ and $J_{Na} - J_H$. This result shows that H^+ concentration is the important factor in determining not only the transport rates of both Na^+ and H^+ but also the ratio of J_{Na} to J_H. The $\Delta\psi$, however, was generated due to the net charge efflux, $J_{Na} - J_H$, at the pH of at least above 6.0 (see the data points in Fig. 5 (filled circles)). In Table I, the

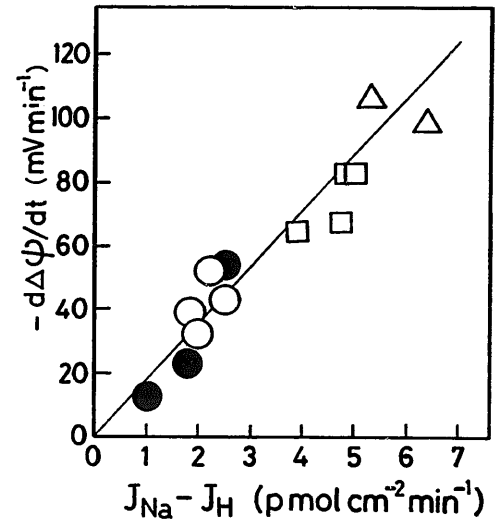

Fig. 5. Correlation between initial rate of $\Delta\psi$ formation and $J_{Na} - J_H$. Experimental conditions were the same as in the case of Fig. 4. $[Hepes]_{in} = [Hepes]_{out} = 2.0$ mM (△), 30 mM (□) and 100 mM (○). Data in Table I are also plotted (filled circles).

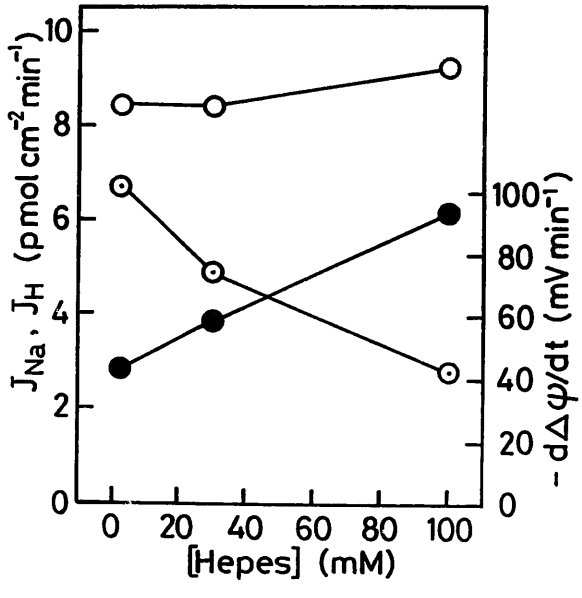

Fig. 4. Effects of intravesicular H^+ bufferring power on the initial rates of Na^+ efflux, H^+ influx and $\Delta\psi$ formation. ○, Na^+ efflux; ●, H^+ influx; ⊙, $\Delta\psi$ formation. Conditions: $[Na^+]_{in} = 200$ mM, $[Na^+]_{out} = 2.0$ mM, $[choline^+]_{out} = 198$ mM, $[Hepes]_{in} = [Hepes]_{out} = 2$, 30 and 100 mM, $pH_{in} = pH_{out} = 8.0$, $[pyranine]_{in} = 50$ μM, [monensin] = 6.7 nM. Experimental conditions for $\Delta\psi$ measurement with $diSC_3(5)$ were described in Materials and Methods.

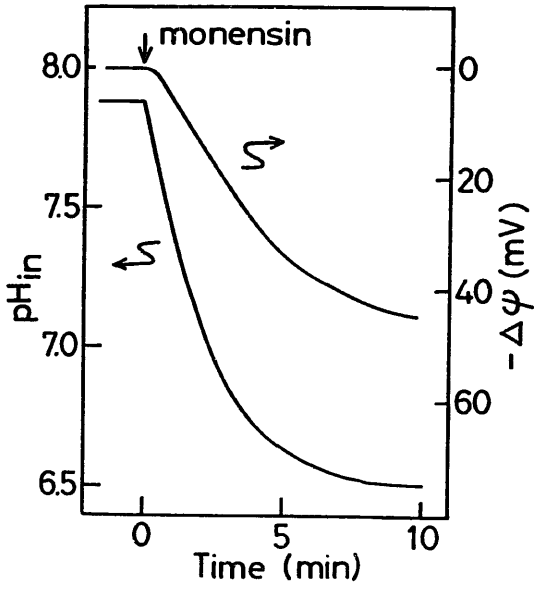

Fig. 6. Time-courses of the changes of $\Delta\psi$ and pH_{in} after the addition of monensin to the liposomes with inward H^+ gradient and outward Na^+ gradient. Initial conditions: $[Na]_{in} = 200$ mM, $[Na]_{out} = 20$ mM, $[cholineCl]_{out} = 180$ mM, $pH_{in} = 7.80$, $pH_{out} = 7.03$, encapsulation efficiency of liposomes 0.05%, total liposome suspension volume 3.0 ml, $[Hepes]_{in} = [Hepes]_{out} = 100$ mM. The concentration of monensin was $1.0 \cdot 10^{-8}$ M.

TABLE I

Effects of medium pH on the initial rates of Na^+ efflux, H^+ influx and $\Delta\psi$ formation

[monensin] = 1 nM, the encapsulation efficiency of liposome suspension (3 ml) = 0.05%, ΔpNa ($\log [Na^+]_{in}/[Na^+]_{out}$) = 2.0. The intra- and extravesicular H^+ buffers were 30 mM Hepes for pH 8.0, 30 mM Bes for pH 7.2 and 30 mM Mes for pH 6.2. All data were an average of the data taken from three experiments. The deviation from the average did not exceed ±5%.

pH	$[Na^+]_{in}$ (mM)	J_{Na} (mol cm^{-2} min^{-1})	J_H (mol cm^{-2} min^{-1})	J_{Na}/J_H	$J_{Na} - J_H$ (mol cm^{-2} min^{-1})	$d\Delta\psi/dt$ (mV min^{-1})
8.0	200	$2.79 \cdot 10^{-12}$	$1.75 \cdot 10^{-12}$	1.59	$1.04 \cdot 10^{-12}$	12
7.2	200	$7.67 \cdot 10^{-12}$	$5.84 \cdot 10^{-12}$	1.31	$1.83 \cdot 10^{-12}$	22
6.2 [a]	200	$1.83 \cdot 10^{-11}$	$1.57 \cdot 10^{-11}$	1.17	$2.60 \cdot 10^{-12}$	54
6.2 [b]	20		$7.00 \cdot 10^{-12}$			28
6.2 [b]	2		$1.53 \cdot 10^{-12}$			8

[a] When 1.0 nM monensin was used at pH 6.2 ($[Na^+]_{in}$ = 200 mM), the Na^+ efflux from the liposomes was too rapid to follow the initial Na^+ efflux accurately with the flow dialysis system used. Therefore 0.33 nM monensin was used in order to obtain the initial rate of Na^+ efflux at pH 6.2 and the datum in Table I was represented as the value/1 nM monensin.

[b] 2 mM Mes buffer was used.

values of initial rates of $\Delta\psi$ formation and J_H at different internal Na^+ concentration (ΔpNa = 2.0) at pH 6.2 were also listed.

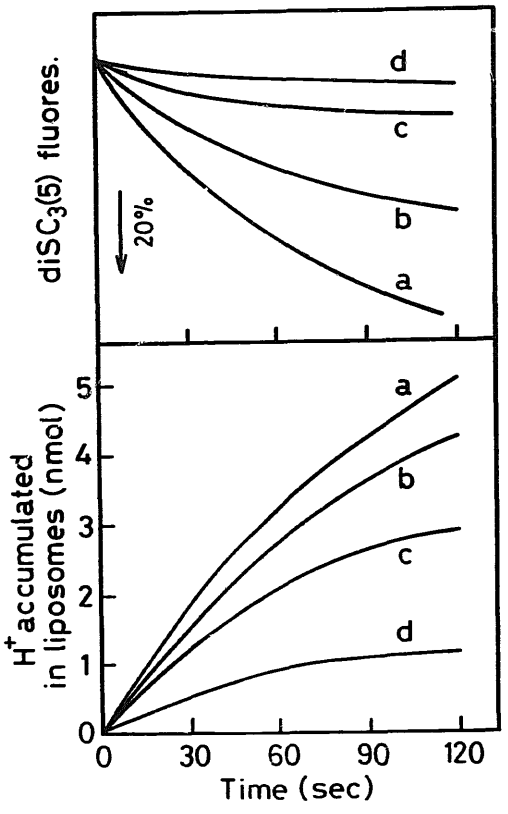

Fig. 7. The effect of TPP^+ on H^+ influx and $\Delta\psi$ formation. The experimental conditions were identical to those of Fig. 2, except that TPP^+ was present. After the incubation of the liposome with TPP^+ for 30 min, monensin (final concentration 6.7 nM) was added to the system. [TPP^+] = 0 mM (curve a), 2 mM (curve b), 6 mM (curve c), 18 mM (curve d). Other experimental conditions are described in Materials and Methods.

The effect of TPP^+ on the H^+ influx, Na^+ efflux and $\Delta\psi$ formation

Under the initial conditions used here ($pH_{in} = pH_{out}$ = 8.0, $[Na^+]_{in}$ = 200 mM, $[Na^+]_{out}$ = 2.0 mM), only an outward Na^+ motive force is present. Thus the initial event occurs after the addition of monensin is the Na^+ efflux, and this generates an inside negative $\Delta\psi$ which should become a driving force for H^+ influx. In this case, the H^+ influx mediated by monensin is considered to be secondary transport. In this respect, the effect of tetraphenylphosphonium (TPP^+) on the H^+ influx was examined. As is well known, a large amount of TPP^+ diminishes the inside negative membrane potential. As Fig. 7 shows, TPP^+ (2–18 mM) retarded H^+ influx as well as $\Delta\psi$ formation depending on its concentration, but did not affect the initial Na^+ efflux even in the presence of 18 mM (data not shown). This result suggests that the $\Delta\psi$ generated by Na^+ efflux is the proton motive force in this system. This mechanism is quite reasonable for the explanation of the observed effect of the intravesicular proton bufferring power on the H^+ influx rate (see Discussion).

Discussion

The carboxylic polyether ionophore, monensin, is widely used for the analysis of the roles of transmembrane cation gradients on the function of cells and their organelles. The action of monensin on membrane has been considered to be the electroneutral exchange of Na^+ and H^+ [1–6]. In this report, we showed that the addition of monensin to the liposomes with outward Na^+ gradient causes an inside negative $\Delta\psi$, and that the J_{Na} was obviously larger than the J_H.

If the monensin-mediated exchange of Na^+ and H^+ across membrane is an electroneutral process, the fol-

lowing three factors have to be the causes of $J_{Na} > J_H$: (1) H^+ leak, (2) H^+ leak as the form of HCl, (3) H^+ bufferring power of inner surface of the liposome membrane.

(1) In the first case, a H^+ leak is expected from the liposomes after the establishment of inside acidic ΔpH, and this is possible cause of inside negative $\Delta\psi$. However, the inside negative membrane potential was generated even under an inward H^+ gradient which prevents H^+ leak (see Fig. 6). This result clearly shows the cause of the inside negative $\Delta\psi$ generation due to $J_{Na} > J_H$ is not the H^+ leak. The H^+ bufferring power in the liposome slightly affected the initial rate of $\Delta\psi$ formation but largely influenced the intial H^+ gradient formation. The initial rates of $\Delta\psi$ and H^+ gradient formation in the liposomes loaded with 100 mM Hepes were -44 mV/min and $2.5 \cdot 10^{-9}$ M/min, respectively, and those in the liposomes loaded with 2 mM Hepes were -100 mV/min and $6.2 \cdot 10^{-7}$ M/min, respectively. Thus, in spite of the significant difference in the rates of H^+ gradient formation (about 250 times), the initial rate of $\Delta\psi$ formation in the liposomes loaded with 2 mM Hepes was only twofold larger than that in the liposome loaded with 100 mM Hepes. These facts also support the above conclusion that H^+ leak is not the cause of $\Delta\psi$ formation and $J_{Na} > J_H$.

(2) The second factor lowering the observed J_H is the H^+ leak as an electroneutral form HCl. Gutknecht and Walter [11] reported a rather high permeability for molecular HCl across lipid bilayer membrane. However, no H^+ leak as the form of HCl was confirmed by the measurement of $^{36}Cl^-$ efflux during monensin-mediated transport of Na^+ and H^+. Moreover, the efflux of electroneutral HCl can not generate membrane potential.

(3) The third factor of underestimation for the J_H is the H^+ bufferring power of liposome inner surface itself. Grzesiek and Dencher [12] reported a very large contribution of lipid headgroups to the internal bufferring power of small soybean phospholipid vesicles. If such a high bufferring power would be present in the large unilamellar liposome used here, it must be taken into consideration for the determination of J_H. Assuming the H^+ bufferring power of inner surface of the liposomes is over 3 mM Hepes equivalent, the J_H would be larger than J_{Na} for the liposome loaded with 2 mM Hepes. This means an inside positive $\Delta\psi$ generation. However, the fact that an inside negative $\Delta\psi$ is generated rules out the possibility that the H^+ bufferring power of liposome surface is larger than 3 mM Hepes equivalent. Hence, the effect of the lipid headgroup had to be negligibly small, when the liposomes roaded with 30 or 100 mM Hepes was used.

From the above considerations, it is concluded that the net electric charge efflux ($J_{Na} - J_H$) is the cause of monensin-mediated $\Delta\psi$ generation. Although the transport rates of Na^+ and H^+ depend on both the intravesicular H^+ bufferring power and the initial pH, the monensin-mediated transport of Na^+ and H^+ in this system occurred at the ratio of $Na^+/H^+ > 1.0$ and the resultant net electric charge efflux caused the inside negative membrane potential.

Since primary ion motive force present is the Na^+ concentration gradient (ΔpNa) in this system, the initial event takes place after the addition of monensin must be the Na^+ efflux and the resultant generation of inside negative membrane potential. The influx of H^+ occurs immediately after the generation of $\Delta\psi$, forming ΔpH. If there is no H^+ pathway, $\Delta\psi$ thus generated rapidly equilibrates to ΔpNa and the net Na^+ efflux ceases just like the case of valinomycin-K^+ system. In this system, however, monensin facilitates H^+ influx driven by the $\Delta\psi$ generated. Thus, H^+ influx couples to Na^+ efflux via the $\Delta\psi$. In this situation, the Na^+ efflux and H^+ influx will be sustained and the difference of J_{Na} and J_H will be observed as a $\Delta\psi$ term until $\Delta\psi$ balances ΔpH as well as ΔpNa. The fact that H^+ influx is retarded by TPP^+, which diminish inside negative $\Delta\psi$, strongly supports the idea that $\Delta\psi$ is the driving force of H^+ influx.

A similar situation will occur in the effects of intravesicular H^+ bufferring power on the Na^+ efflux and the H^+ influx. Since, the primary ion gradient is only ΔpNa, it is likely that the initial Na^+ efflux depends only on the size of ΔpNa and is not affected by the intravesicular H^+ bufferring power. However, the subsequent H^+ influx must be affected by the H^+ bufferring power in the liposome, since the H^+ influx establishes an outward H^+ gradient which can retard H^+ influx. The high H^+ bufferring power in the liposome prevents the outward H^+ gradient formation and consequently increases the J_H and decreases the initial rate of $\Delta\psi$ formation.

The initial rate of $\Delta\psi$ formation was analyzed in terms of net electric charge flux as the result of the difference between the initial Na^+ efflux and H^+ influx. Considering a liposome of radius R, the initial rate of $\Delta\psi$ formation ($d\Delta\psi/dt$) is proportional to the initial transmembrane net current, I, inversely proportional to the electric membrane capacitance, C_m, and can be represented as follows:

$$d\Delta\psi/dt = I/C_m \quad (1)$$

$$I = e \cdot N \cdot (J_{Na} - J_H) 4\pi R^2 \quad (2)$$

where, e, N and ($J_{Na} - J_H$) are the elementary electric charge, the Avogadro's number and the net ion flux (mol s^{-1} (surface area)$^{-1}$), respectively. By the application of spherical condenser model to the liposome, C_m can be approximately represented as follows:

$$C_m = 4\pi\varepsilon\varepsilon_r R^2/D \quad (3)$$

where, D is the membrane thickness, ε_r is the relative dielectric constant of membrane and ε is the dielectric constant of vacuum. On the assumption of uniform size distribution for liposomes, the total membrane capacitance and the total electric charge flux, respectively, are the product of the number of liposomes and corresponding values for one liposome (C_m and I). Then, the $d\Delta\psi/dt$ can be rewritten for the total liposome suspension, independent of the number and size of liposomes.

$$d\Delta\psi/dt = (D/\varepsilon\varepsilon_r)\cdot e\cdot N\cdot(J_{Na} - J_H) \quad (4)$$

As Fig. 8 shows, the plots of $d\Delta\psi/dt$ vs. $eN(J_{Na} - J_H)$ gives a linear line with a slope of $1.84\cdot10^5$ (J cm^2 C^{-2}). From this slope value, the membrane capacitance per unit area and the value of ε_r of the membrane used here were calculated to be 5.3 μF cm^{-2} and 24, respectively, when the value of membrane thickness was taken to be $4.0\cdot10^{-7}$ cm. These values are considerably larger than those obtained with black lipid membrane (about 1 μF cm^{-2}, $\varepsilon_r < 10$ [13]). However, the permeability coefficient of H^+/OH^- for large liposome membrane (10^{-4}–10^{-3} cm s^{-1} [12,14–16]) is at least one order of magnitude greater than that of planar lipid bilayer (10^{-6}–10^{-5} cm s^{-1} [17]). This difference may be due to relatively high content of water and strands of water molecules in liposome membrane. The presence of water and its water bridges should increase the values of ε_r and membrane capacitance per unit area. Moreover, the value of ε_r of planar bilayer lipid membrane depends on the organic solvent used, indicating that the organic solvent remains in the membrane. Organic solvent such as decane, frequently used for the preparation of planar lipid membrane, decreases the content of water in lipid bilayer and lowers ε_r. This may explain the large value for ε_r and C_m of liposome membrane. Indeed, the range of ε_r from the center of lipid bilayer (liposome) to the point nearest to the bilayer interface has been estimated to be 10–25 for fluid-phase dipalmitoylphosphatidylcholine bilayers from the polarity profile obtained by the measurement of the isotropic splitting factor for different positional isomers of the phosphatidylcholine spin label [18].

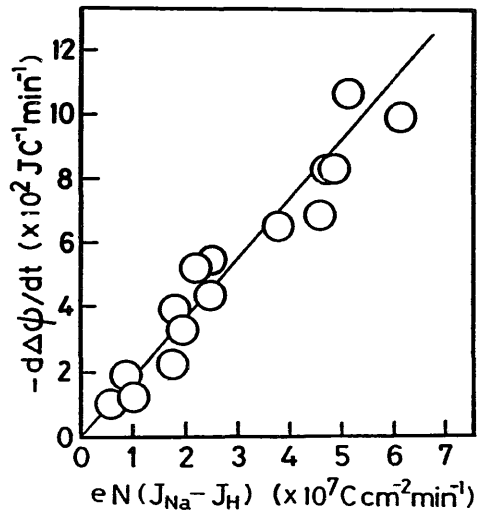

Fig. 8. The plots of $d\Delta\psi/dt$ against $e\cdot N\cdot(J_{Na} - J_H)$. $d\Delta\psi/dt$: initial rate of membrane potential formation; $e\cdot N\cdot(J_{Na} - J_H)$: initial net electric charge flux. e and N are the elementary electric charge ($1.6\cdot10^{-19}$ C) and Avogadro's number ($6.02\cdot10^{23}$ mol^{-1}), respectively.

It has been considered that Na^+ is carried in the form of 1:1 complex of deprotonated monensin and Na^+ [2,4]. The equilibrium equations among protonated monensin (MH), deprotonated monensin (M^-), its Na^+ complex (MNa), Na^+ and H^+ at membrane interface are expressed as follows.

$$M^- + H^+ \rightleftharpoons MH \quad (5)$$

$$M^- + Na^+ \rightleftharpoons MNa \quad (6)$$

The concentration of MNa is given as

$$[MNa] = K_2[M]_0[Na^+]/(1 + K_1[H^+] + K_2[Na^+]) \quad (7)$$

$$[M]_0 = [M^-] + [MH] + [MNa]$$

Here K_1 and K_2 are the equilibrium constants of Eqns. 5 and 6, respectively. On the assumption that Na^+ is carried in the form of 1:1 Na^+-deprotonated monensin complex and that protonated monensin is not involved in the Na^+ transport process, J_{Na} is expected to increase with the increase in [MNa]. However, the fact that J_{Na} decreases with the increase in pH_{in} (Table I) indicates that J_{Na} depends on other forms of monensin Na^+ complex which decreases with the decrease in H^+ concentration. Thus, we assumed that the carrier species of Na^+ is 1:1 Na^+-protonated monensin complex $MHNa^+$. As the interfacial equilibrium equations, following two equations are considered.

$$M^- + H^+ = MH \quad (8)$$

$$MH + Na^+ = MHNa^+ \quad (9)$$

The concentration of $MHNa^+$ is given as follows.

$$[MHNa^+] = K_1K_2[M]_0[H^+][Na^+] / \{1 + K_1(K_2[Na^+] + 1)[H^+]\} \quad (10)$$

$$[M]_0 = [M^-] + [MH] + [MHNa^+]$$

Here K_1 and K_2 are the equilibrium constants of Eqns. 8 and 9, respectively. When K_1 and K_2 are taken to be $10^{5.6}$ and 10^2, respectively, J_{Na} linearly increases with $[MHNa^+]$, as shown in Fig. 9. The value of log K_1 is close to pK_a of monensin, 6.6, [19] (when titrated in 66% dimethylformamide). The value of K_2 used here is also close to the value of the apparent stability constant for the monensin-Na^+ complex in the membrane surface of egg PC liposomes, 32.6 [20]. Then the dependency of

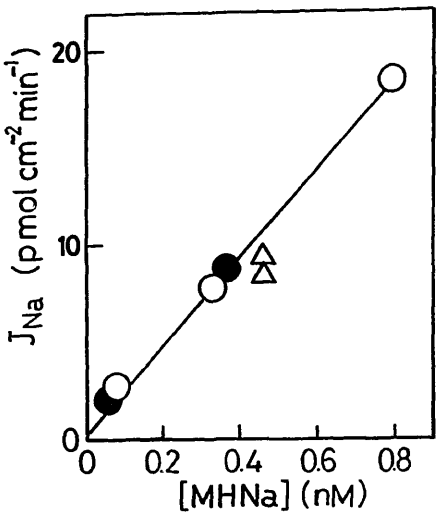

Fig. 9. Correlation between J_{Na} and [MNNa$^+$]. MHNa$^+$ represents the 1:1 Na$^+$-protonated monensin complex. The concentration was calculated using Eqn. 10. ○, values of J_{Na} in Table I; ●, values of J_{Na} calculated from the empirical Eqn. 4 using the values of J_H and $d\Delta\psi/dt$ in Table I; △, values of J_{Na} in Fig. 4.

J_{Na} for Na$^+$ concentration was examined at constant ΔpNa at pH 6.2. The J_H and initial rate of $\Delta\psi$ formation increased with the increase in $[Na^+]_{in}$ at pH 6.2, as shown in Table I. From the values of J_H and the initial rate of $\Delta\psi$ formation, the values of J_{Na} were calculated by using the empirical Eqn. 4 described above, and were plotted against calculated [MHNa$^+$] from Eqn. 10 in Fig. 8 (filled circles). The plots of J_{Na} against the calculated [MHNa$^+$] give almost the same line in both cases (the dependency of J_{Na} on $[Na^+]_{in}$ and $[H^+]_{in}$). These results suggest that Na$^+$ is transported in the form of MHNa$^+$.

Sandeaux et al. [4] studied the monensin-mediated transmembrane Na$^+$ flux across planar bilayer lipid membrane, and reported that the electric charge flux was very small as compared to the measured Na$^+$ flux. In our study, the difference between J_{Na} and J_H was clearly shown. However, the fact that net electrical charge flux across liposome membrane ($(2-12)\cdot 10^{-14}$ mol cm^{-2} s^{-1}) is only about 4–20-times larger than that across planar lipid membrane ($6\cdot 10^{-15}$ mol cm^{-2} s^{-1}) suggests that membrane potential was generated even in the black lipid membrane system. We measured the initial fluxes of both Na$^+$ and H$^+$ in liposome system while they measured only the steady flux of Na$^+$ in planar lipid membrane system. Thus our results can not be directly compared with their results, since the relations between $\Delta\psi$ and J_{Na} (J_H) are unknown from their results.

In conclusion, monensin-mediated initial exchange of Na$^+$ and H$^+$ across liposome membrane is not electroneutral process. The flux of Na$^+$ is always larger than that of H$^+$ and the resultant net electric charge efflux increases the inside negative $\Delta\psi$ until the $\Delta\psi$ balances both H$^+$ and Na$^+$ concentration gradients. The H$^+$ influx is considered to be the monensin-mediated secondary transport driven by the inside negative $\Delta\psi$ generated by initial Na$^+$ efflux, in which Na$^+$ is suggested to be transported in the form of MHNa$^+$. Present our results give the general aspect for the action of so-called cation carrier. Some cation carriers, such as valinomycin having a high selectivity for a specific cation behave as electrogenic carrier. On the other hand, other carrier such as monensin having low cation selectivity act as so-called electroneutral carrier. In the cation exchange process mediated by latter carrier in membrane system, the $\Delta\psi$ generated by transport of cation from the cis side to the trans side becomes the force to brake cation transport of this direction and may become a driving force for reversely directed cation transport. Studies for the role of $\Delta\psi$ in cation-cation exchange reaction across liposome membrane by carriers other than monensin are now in progress.

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