Conductance Change in Phospholipid Bilayer Membrane by an Electroneutral Ionophore, Monensin†

Minoru Inabayashi,‡ Seiji Miyauchi,‡ Naoki Kamo,*,‡ and Takashi Jin§

Laboratory of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, and Section of Physiology, Institute of Electronics Science, Hokkaido University, Sapporo 060, Japan

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ABSTRACT: Monensin is a polyether antibiotic ionophore and is considered an electroneutral Na/H antiporter. Its addition, however, increased the conductance of phospholipid bilayer membrane, and this increase was observed only when the medium contained Na^+. Analysis of the current−voltage curve suggested that the increase was due to the formation and the translocation of an univalently charged species. The conductance at zero external voltage was proportional to the second power of monensin concentration and increased with the decrease in pH of the medium. Modified monensin whose terminal carboxyl was esterified showed much larger increase (*ca.* 100 times) in conductance than intact monensin. We concluded that the complex between the dimer of protonated monensin and Na^+ contributed to the electrogenic transport of monensin. This complex bears a +1 charge, which is consistent with the analysis of current−voltage curves. Contrary to the conductance, the Na^+ transfer rate of liposomal membrane measured with ^{23}Na-NMR was proportional to the monensin concentration, meaning that the electrogenic component contributes little to the total monensin-mediated Na^+ transport in the present system. It should be noted that this electrogenic component may change the membrane potential.

Monensin is classified as a polyether antibiotic ionophore, and its chemical structure is well-characterized: at one terminal, there is a carboxyl group and at the opposite end there is a tertiary hydroxyl. When monensin is present in the membrane, its carboxyl group becomes deprotonated at the interface with an alkaline solution and a hydrogen bond forms between the deprotonated carboxyl and the hydroxyl group, which leads to the characteristic ring conformation (Pressman, 1976; Westley, 1982; Riddell et al., 1988; Mollenhauer et al., 1990). Metal ions, especially sodium ions, are incorporated into the ring, and electroneutral M^- − Na^+ complexes diffuse to another interface, where deprotonated monensin (M^-)[1] associates with H^+ to release Na^+. The neutral protonated monensin, MH, generated there returns to the interface facing a more acidic solution. The cycle is, thus, completed within the membrane, which results in an exchange of Na^+ and H^+ (Na/H antiporter). It is of note that monensin's function as antiporter is electroneutral or electrically silent because the species transferring across the membrane are electrically neutral.

Sandeaux et al. (1982) measured transmembranous $^{22}Na^+$ fluxes across a planar bimolecular lipid membrane doped with monensin and observed very small short-circuit electrical currents in comparison with the measured Na^+ flux. They proposed the electroneutral scheme of Na^+ transport described above.

More recently, Nakazato and Hatano (1991) measured fluxes of $^{22}Na^+$ and H^+ directly using liposomes containing monensin and concluded that Na^+ is transported in the form of a 1:1 complex between MH and Na^+. This conclusion is greatly different from the widely-accepted transport mechanism of monensin in that the process is electrogenic. They found supporting evidence that the Nernstian membrane potential was generated when monensin was added into liposomes separating NaCl solution of different concentrations. In addition, Suzuki et al. (1988) reported an ion-selective electrode using monensin: if monensin were an electroneutral Na/H antiporter, it would be impossible to fabricate the electrode.

In order to resolve these contradictory observations, we attempted to measure the conductance change of a phospholipid bilayer membrane by addition of monensin and found that the increase in the membrane conductance was proportional to the second power of monensin concentration (>several micromolar) and was larger in acidic solutions. These findings suggest that the monensin transport is electrogenic and that dimers of protonated monensin are involved in the electrogenic transport. Na^+ exchange rate across liposomal membrane was measured with ^{23}Na-NMR. In contrast to the results of conductance measurements, the rate was proportional to the monensin concentration. This suggests that transfer of Na^+ by monensin is composed of two pathways: the major one is electroneutral and the smaller component is electrogenic. It follows that this electrogenic component may change the electrical

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* Corresponding author: fax +81-11-706-4984; tel +81-11-706-3923.

‡ Faculty of Pharmaceutical Sciences.

§ Institute of Electronics Sciences.

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[1] Abbreviations: $G(0)$, membrane conductance at the limit of zero applied voltage; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; M^-, deprotonated monensin; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; methyl-monensin, compound derived from monensin whose terminal carboxyl is esterified with a methyl group; MH, protonated monensin; TPB^-, tetraphenylboron; Tris, tris(hydroxymethyl)aminomethane; μ, reduced potential difference ($=FV/RT$).

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properties of membranes such as conductance and membrane potential.

MATERIALS AND METHODS

Materials. Phospholipids used for formation of bilayer membrane and large unilamellar vesicles were partially purified phosphatidylcholine from soybean phospholipid (Nacalai Tesque, Kyoto, Japan) by the method of Kagawa and Racker (1971). The composition of the lipid used was described in our previous paper (Ono et al., 1994). Monensin was obtained from Wako Pure Chemicals (Osaka, Japan) and recrystallized from ethanol. The purity was checked by NMR (270 MHz, Jeol GX-270) spectra. Pure water was prepared with a Millipore Milli-Q apparatus (Tokyo, Japan), and its specific resistance was 18.3 MΩ·cm.

Methyl Esterification of Monensin. Methyl esterification of carboxyl group of monensin (hereafter called methyl-monensin) was carried out with diazomethane which was prepared as follows: nitrosomethylurea (100 mg) was dissolved in 5 mL of 50% KOH, followed by extraction with 5 mL of diethyl ether. Diazomethane in diethyl ether was dried with anhydrous sodium sulfonate. An acid form of monensin was extracted three times to 0.1 N HCl from Na-monensin (10 mg) in ethyl ether. The acid form of monensin and diazomethane, both of which were dissolved in diethyl ether, were mixed and stirred on ice for 5 min. After excess diazomethane was quenched by addition of small amounts of acetic acid, the solvent was evaporated. Methyl-monensin was purified by silica gel thin-layer chromatography (hexane: acetone = 2:1). Complete methyl esterification was confirmed with NMR spectra. The yield was 94%.

Formation of Planar Bilayer Membrane and Measurement of Current−Voltage Curve. The planar lipid bilayers were formed by the folding method of Takagi et al. (1965) and Montal and Mueller (1972). Details on the formation of the membrane and electrical measurements were described earlier (Ono et al., 1994; Miyauchi et al., 1993). Bilayer membranes whose electric resistance and membrane capacitance were respectively more than 200 GΩ and 0.5−0.7 μF/cm^2 were used.

Preparation of Large Unilamellar Vesicles for ^{23}Na-NMR Measurement. One hundred milligrams of the partially-purified phosphatidylcholine described above was suspended in a buffer solution containing 200 mM NaCl and 50 mM Hepes-Tris (at pH 7.0) by sonication (output power of 180 W, 30 min). Small unilamellar vesicles thus obtained were frozen in liquid N_2 and thawed. This freeze−thaw procedure was repeated three times, and the suspension was centrifuged using an RP55T rotor (Hitachi Koki Co., Ltd., Hitachi, Japan) at 38 000 rpm for 30 min. The pellet was suspended in 2 mL of 100 mM NaCl, 20 mM Na_5PPP_i (sodium tripolyphosphate), and 50 mM Hepes-Tris buffered at pH 7.0. Ten microliters of 1 M $DyCl_3$ solution as a shift reagent was added slowly with microsyringe, and the solution was mildly agitated with a vortex mixer.

^{23}Na-NMR Measurement. Measurements were carried out on a Bruker MSL 300 FT NMR spectrometer operating at 79.39 MHz, and temperature was 297 K. The spectrometer was field/frequency locked on the ^{2}H resonance of ^2H_2O in the inner compartment of a coaxial tube. Typically, 1000 free induction decays were collected into 8192 data points with a sweep width of 50 kHz. Monensin or methyl-monensin was added from methanol stock solution (20 mM). The rate constants for the exchange in the direction Na(in) → Na(out) were determined from line broadening according to a reference of Riddell and Hayer (1985). This line broadening method has been used by several authors (Xie et al., 1994; Tanaka et al., 1992; Riddell & Tompsett, 1990; Buster et al., 1988) as an established method for measuring the transport rate through membranes.

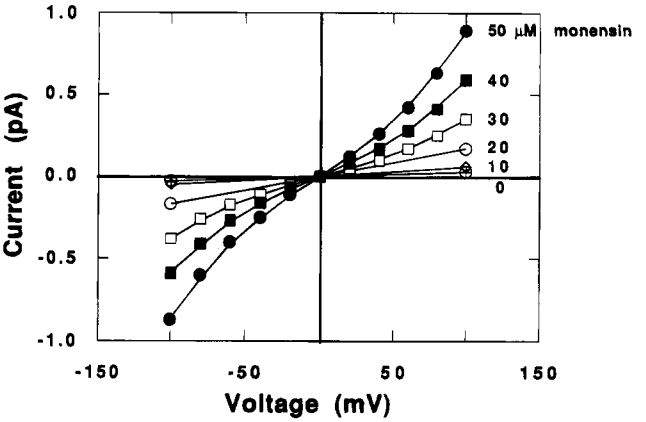

FIGURE 1: Current−voltage relationship under varying concentrations of monensin. Bilayer membrane was formed in 100 mM NaCl buffered at pH 7.0 with 50 mM Hepes-Tris. Temperature was 20 °C. Surface area of the membrane was 1.28 × 10^{-4} cm^2. Monensin concentrations were as follows: ●, 50 μM; ■, 40 μM; □, 30 μM; ○, 20 μM; ◇, 10 μM; ⊕, 0 μM.

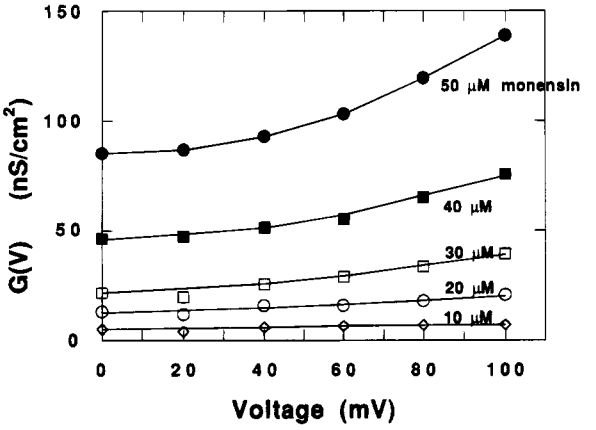

FIGURE 2: Dependence of conductance on externally-applied voltage. Experimental conditions and notations are the same as in Figure 1. The points on the ordinate axis are $G(0)$ values obtained by fitting with eq 1.

RESULTS AND DISCUSSION

Membrane Current Using Monensin. Figure 1 shows the current−voltage relationship under varying concentrations of monensin when the membrane was formed in 100 mM NaCl (pH, 7.0 buffered with 50 mM Hepes-Tris). This figure indicates that the magnitude of the electric current depends on the monensin concentration. When the same experiments were performed in 100 mM KCl, no electric current was observed. If monensin behaved as an electroneutral Na^+/H^+ antiporter, the current would not be observed.

In Figure 2, conductances were plotted against the externally-applied voltage, showing that the conductance was voltage-dependent. Ketterer et al. (1971) developed a theory describing the electric current due to the translocation of lipophilic ions through phospholipid bilayer membranes.

According to their theory, the whole transport process consists of three steps: the entry of ions to membrane from an aqueous solution, translocation within the membrane, and the desorption from the membrane. As to the translocation within the membrane, ions should pass above the top of an intramembrane energy barrier whose height depends on the applied voltage. This determines the voltage-dependent conductance. The ion energy profile can be calculated according to Flewelling and Hubbell (1986) model.

Recently, we (Ono et al., 1994) measured the electric current due to various lipophilic phosphonium cations in phospholipid bilayer membranes prepared by the same lipids and procedure as those in the present paper and found that the conductance followed this equation:

$$G(V)/G(0) = (2/z\mu)\exp(0.008\mu^2)\sinh(z\mu/2) \quad (1)$$

where $G(V)$ stands for the conductance at the externally applied voltage V, $G(0)$, the conductance at zero voltage, μ, the reduced potential difference defined as $\mu = FV/RT$, and z, the valence of mobile species. F, R, and T have their usual thermodynamic meaning. The conductance $G(0)$ is given by:

$$G(0) = (F^2/RT)\beta k_i C \quad (2)$$

where k_i stands for the rate constant of transmembrane ion transport at zero externally-applied voltage, β, the linear partition coefficient, i.e., a ratio of surface density of adsorbed ions in membrane to their volume in the aqueous solution, and C, the cation concentration in the aqueous solutions. The term of βC, hence, represents the surface density of the monensin−Na^+ complex in the ion potential energy well, which is proportional to the complex concentration within the membrane. In addition, $G(0)$ is similar to the permeability coefficient. It is generally accepted that the rate-determining step for the permeation of lipophilic cations is the translocation across the barrier and not that at the membrane/solution interfaces. Equation 1, then, has no parameters concerning the translocation through the interfaces.

The data shown in Figure 2 can be fitted with eq 1, and the solid curves in the figure indicate the fit. This good agreement may suggest that the rate-determining step is the translocation across the intramembrane barrier, and not the process at the interfaces such as association or dissociation of Na^+ with monensin. The best values of z in eq 1 ranged between 0.95 and 1.07, meaning that the monensin−Na complex has a univalent electric charge. Unfortunately, the sign cannot be determined from the present data. According to the well-known scheme for monensin acting as an electroneutral Na/H antiporter, a univalent species is M^-, but this species cannot be responsible for the electric current because in acidic solutions where the concentration of M^- may be decreased, the current increased (see later).

Figure 3 shows plots of $G(0)$ versus monensin concentration, revealing that the conductance $G(0)$ is not proportional to the monensin concentration. The solid curve in this figure indicates that $G(0) = 0.032 \times$ (monensin concentration)^2. This suggests that the electrogenic transport is a bimolecular process, if we assume β to be a constant.

Figure 4 shows the plot of $G(0)$ versus the NaCl concentration when the monensin concentration was kept

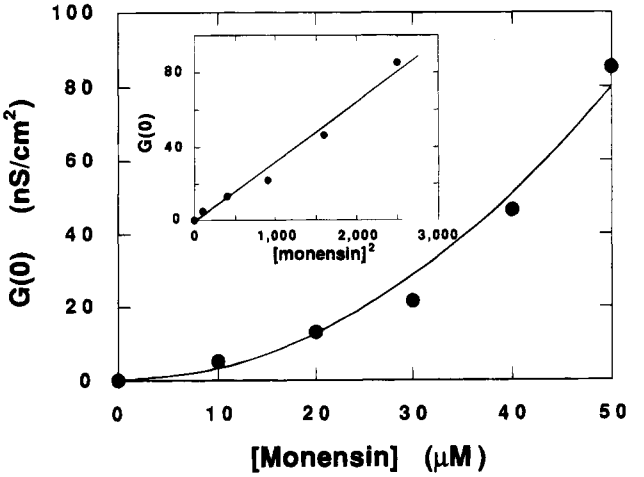

FIGURE 3: $G(0)$ values were plotted against monensin concentrations. Solutions separated by bilayer membrane contained 200 mM NaCl buffered at pH 7.0 with 50 mM Hepes-Tris. Temperature was 20 °C. The inset shows the plot of $G(0)$ versus [monensin]^2. The solid curve in the main figure represents $G(0) = 0.032 \times$ [monensin]^2.

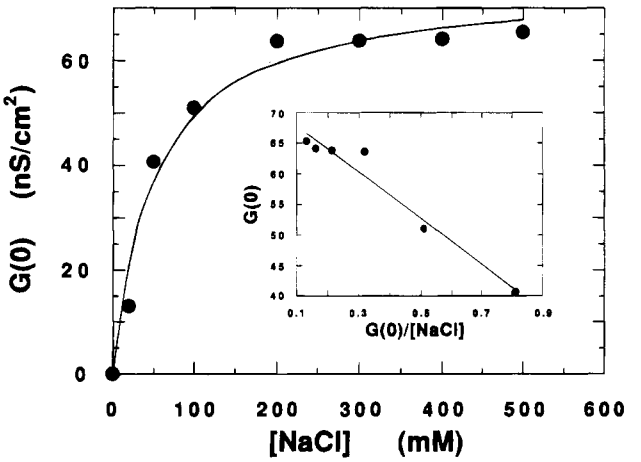

FIGURE 4: Zero voltage conductance, $G(0)$, as a function of NaCl (50 mM, pH = 7.0 with 50 mM Hepes-Tris). The concentration of monensin was 50 μM. Temperature was 20 °C. The inset shows the Eadie plot. Here, data for 20 mM were omitted due to the large deviation from the line. The solid curve in the main figure represents $G(0) = (74.76 \times [NaCl])/(51.4 + [NaCl])$.

constant (50 μM). $G(0)$ increased with an increase of NaCl concentration and reached saturation at high NaCl concentration. The conductance $G(0)$ is determined by two factors: one is the rate constant of the translocation over the barrier (k_i in eq 2), and the other is the surface density of the monensin−Na complex in the ion potential energy well (βC in eq 2). If we assume that the rate constant is independent of NaCl concentration, this curve may represent the surface density of the monensin−Na complex which determines the electrical conductivity of the membrane. Analysis by Eadie plot gave 51.4 mM of NaCl as the Na concentration at half the saturating current.

We measured the current−voltage relationship when the concentrations of NaCl solutions separated by the bilayer membrane were changed (data not shown). Values of the reversal potential were 62, 40, and 15 mV, respectively, when NaCl concentration gradients were 20/200, 50/200, and 100/200 (mM). These values are approximately equal to those calculated from the Nernst equation, meaning that the membrane is permeable only to Na^+. This conclusion is

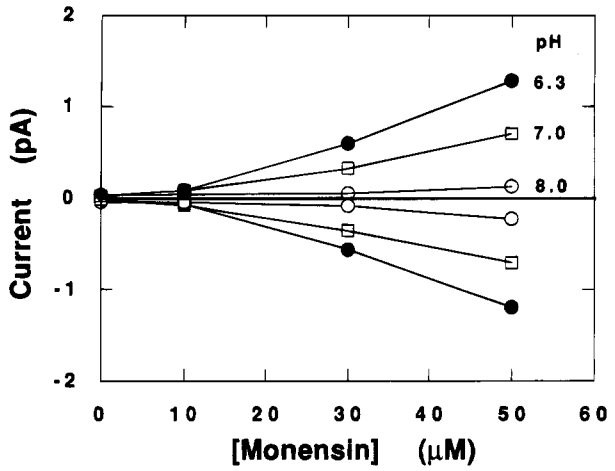

FIGURE 5: Dependence of the electric current on pH. The current passing through the bilayer membrane was measured in 100 mM NaCl solutions whose pH was 6.3 (●), 7.0 (□), and 8.0 (○). Positive values in the ordinate were obtained when voltage was applied in the forward direction; negative values, for reverse voltage. Monensin concentrations were 10, 30, and 50 μM. Hepes-Tris buffer (50 mM) was used for pH 7.0 and 8.0, and 50 mM Mes-Tris was used for pH 6.3. Temperature was 20 °C.

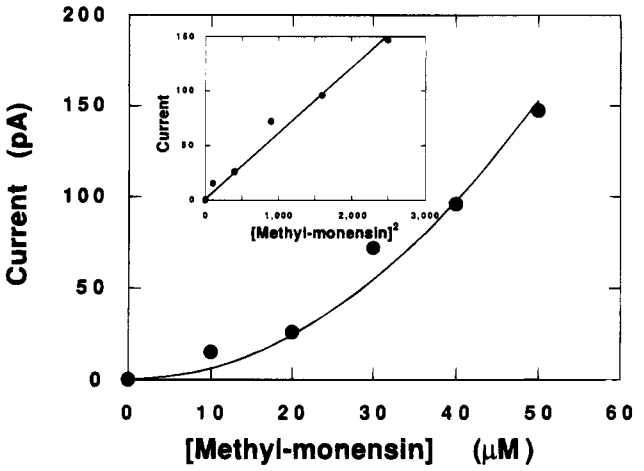

FIGURE 6: Electric currents observed at 100 mV of the externally-applied voltage plotted versus methyl-monensin concentration. Solutions separated by bilayer were 200 mM NaCl, buffered with 50 mM Hepes-Tris (pH 7.0). Temperature was 20 °C. The inset shows the current data plotted versus [methyl-monensin]². The curve in the main figure represents current = 0.061 × [methyl-monensin]².

consistent with observations by Suzuki et al. (1988) that a Na-selective electrode is possible and the finding by Nakazato and Hatano (1991) that membrane potential is generated by the addition of monensin to liposomes.

The membrane electric current in the presence of monensin was measured for different pH, and the results are shown in Figure 5. We found that the electric current increased with the decrease in pH. This fact, as described above, implies that the electric current is not due to the translocation of M^-. In addition, this indicates that, in acidic solutions, the formation of complex which contributes to the electric current is preferable. The protonation of carboxyl group of monensin therefore is necessary for the formation of the monensin−Na complex which carries charges within membranes.

Considering these properties together with the involvement of two monensin molecules, there are two possible compositions of the complex that affect the electrogenic transport; in one the complex is composed of two MH and Na^+, and in the other a complex is formed between M^-, MH, and Na^+. The latter case, however, can be ruled out because the complex is neutral. The overall charge of the former complex is +1, which is consistent with our result that $z = 1$.

Membrane Current by Monensin Methylester. The fact that the neutral form of monensin (protonated form) is involved in the electrogenic process led us to synthesize monensin methyl ester (methyl-monensin). We measured $G(0)$ in the presence of varying concentrations of methyl-monensin, and the results obtained are plotted in Figure 6. Here, $G(0)$ values were estimated from fitting of the current−voltage data with eq 1; the estimated value of z will be described later. We found that $G(0)$ values for methyl-monensin were about 100 times greater than that obtained by monensin. Because the partition coefficient of methyl-monensin to organic solvents is reported to be almost equal to that of monensin (Tohda et al., 1990), and because transmembrane rate constants of methyl-monensin and ordinary monensin seem not to be greatly different, the 100-fold increase in $G(0)$ can be interpreted as the change in the concentration of the univalent ionic form of monensin which contributes to the electrogenic transport.

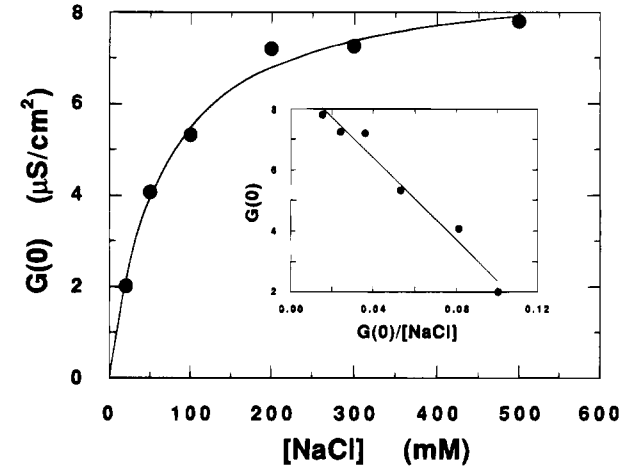

FIGURE 7: NaCl dependence of $G(0)$ using 50 μM methyl-monensin. $G(0)$ values were obtained under varying concentrations of NaCl whose pH was kept constant at 7.0 with 50 mM Hepes-Tris. Temperature was 20 °C. The inset shows the Eadie plot to obtain parameters. The solid curve in the main figure represents $G(0) = (8.91 \times [NaCl])/(64.2 + [NaCl])$.

In addition, we could not find any current when KCl was used instead of NaCl. This means that the selectivity of Na^+ against K^+ remains the same even after the structural modification.

As is seen in Figure 6, $G(0)$ was not linear with the concentration of methyl-monensin. The inset shows that $G(0)$ is also proportional to [methyl-monensin]², as shown in the inset of Figure 3 for monensin. Fitting of the current−voltage curves with eq 1 gave $z = 1$, which is the same as found for monensin data (Figure 2). The complex is, therefore, composed of two neutral methyl-monensins and one Na^+ ion and has one unit of positive charge.

Values of $G(0)$ were estimated for various concentrations of NaCl and for a constant concentration of methyl-monensin equal to 50 μM (Figure 7). It should be pointed out that the curves in this figure are very similar to those in Figure 4, with the exception of the absolute values in the ordinate.

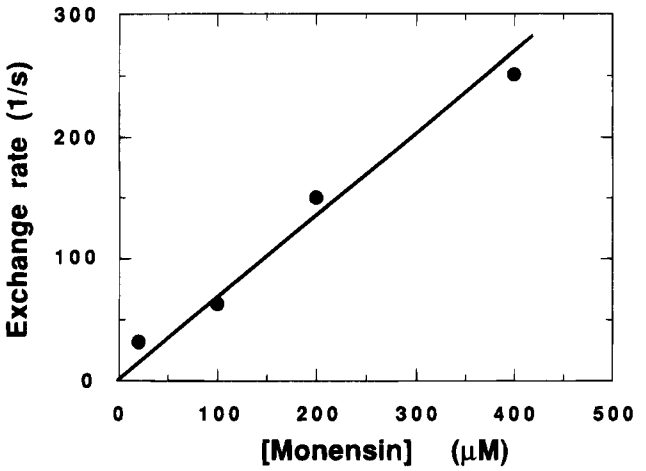

FIGURE 8: Na^+ exchange rate across liposomal membranes is proportional to monensin concentration, in contrast to data shown in Figure 3. The rate was obtained from line broadening of the ^{23}Na-NMR signal. Temperature was 20 °C. For details of experimental conditions, see text.

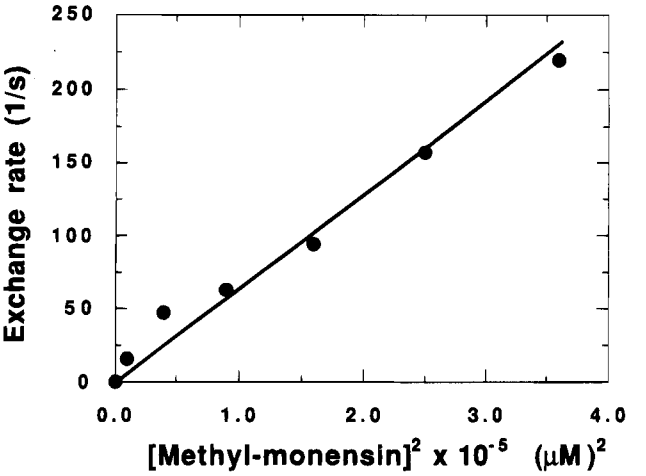

FIGURE 9: Na^+ exchange rate across liposomal membranes is proportional to the square of methyl-monensin concentration, which is in accord with data shown in Figure 6.

Actually, the Na^+ concentration for half of the saturated $G(0)$ was 62.4 mM, which is approximately equal to that of intact monensin (51.4 mM).

Exchange Rate of Na^+ through Liposomal Membrane. The exchange rate of Na^+ through liposomal membranes was determined from NMR studies. This rate was measured under conditions in which no membrane potential was generated. Therefore, the conductance at zero externally-applied voltage $G(0)$ and the rates determined by NMR can be compared. Figure 8 shows that the exchange rate by monensin was proportional to the monensin concentration, whereas $G(0)$ was proportional to (monensin concentration)^2 as shown in Figure 3. The difference of the monensin concentration dependency between $G(0)$ and the exchange rate was also observed for 300 mM NaCl solution. This difference can be interpreted as follows: the current passing through bilayer membranes is caused only by the translocation of the positively charged univalent complex, $[(MH)_2Na]^{1+}$, while NMR measurements give the total Na^+ transport rate, including the rate by electroneutral complex formed with M^- and Na^+. In other words, we found that the contribution of the electrogenic Na^+ transport to the total Na^+ transport by monensin was small.

Exchange rates of Na^+ mediated by methyl-monensin through liposomal membrane are shown in Figure 9, revealing that the rate was proportional to the square of the total concentration of methyl-monensin. This quadratic concentration dependence was also obtained for 300 mM NaCl. This is consistent with the finding of the quadratic concentration dependence of methyl-monensin conductance, meaning that all Na^+ transport mediated by methyl-monensin is carried by the electrogenic process in which $[(\text{methyl-monensin})_2Na]^+$ complex is involved.

Effect of Lipophilic Anion. It is well-known that a small amount of lipophilic anion such as tetraphenylboron (TPB^-) accelerates the transport of lipophilic cation through bilayer or biological membranes (Demura et al., 1985; Andersen et al., 1978). We found that addition of TPB^- (10 μM) actually increased the exchange rate by about 35%, for a monensin concentration of 200 μM (data not shown). A similar increase was observed with methyl-monensin. This is consistent with the notion that the electrogenic Na^+ transport mediated by positively charged $[(MH)_2Na]^+$ or $[(\text{methyl-monensin})_2Na]^+$ exists. In addition, we consider the possibility that lipophilic anion facilitates the formation of the positively charged $[(MH)_2Na]^+$ or $[(\text{methyl-monensin})_2Na]^+$ or increases their concentration in the membrane, which in turn yields an increase in the exchange rate.

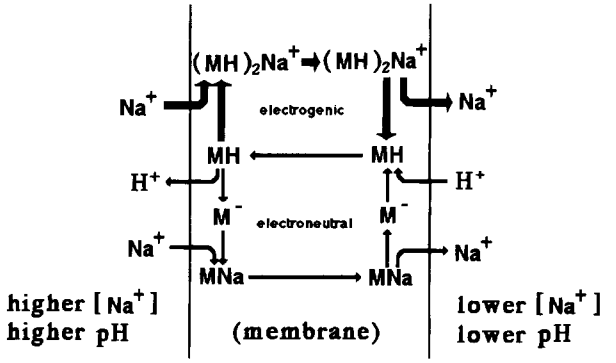

FIGURE 10: Proposed kinetic scheme for Na transport mediated by monensin. The lower cycle represents the well-known electroneutral Na/H antiporter, and the upper cycle, the new electrogenic cycle in accordance with the findings in the present paper.

Conclusion. We have shown that monensin is not only an electroneutral Na^+/H^+ antiporter but an electrogenic transporter: the latter is mediated by $[(MH)_2Na]^+$. Figure 10 illustrates the kinetic scheme of the transport of Na^+ by monensin. Although the amounts of Na transported by the electrogenic process were small compared to those by the electroneutral process in the present membrane system, the result of TPB^- suggests that the ratio of electrogenic to electroneutral transport may change depending on the composition of membrane. We should, therefore, be aware of the possibility that addition of monensin to cells or vesicles *does* change the membrane potential in addition to the intracellular Na^+ concentration. The change in membrane potential may alter the activities of ionic channels and/or transporters. This possibility should be considered especially when a high concentration of monensin is used and when Na^+ concentration in the medium is also relatively high (note that the K_m value is 51.4 mM). This explains why monensin decreased the photoinduced membrane potential of envelope

vesicles derived from *Halobacterium halobium* (Kamo et al., 1982) in 4 M NaCl.

It was reported that nigericin (Toro et al., 1976), grisorixin (Sandeaux et al., 1978), alborixin (Sandeaux et al., 1978), and X-537A (Kafka & Holz, 1976; Célis et al., 1974) increased the bilayer conductance and that conductance increase was approximately quadratic with the antibiotic concentration, as in the present work. This suggests that the formation of dimers of these antibiotics is not limited to monensin. Detailed studies of the translocation mechanism associated with these polyether antibiotics are now in progress in this laboratory.

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Registry Number Supplied by Author. Monensin, 17090-79-8.

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