Studies on the Chemical Modification of Monensin. II. Measurement of Sodium Ion Permeability of Monensylamino Acids Using Sodium-23 Nuclear Magnetic Resonance Spectroscopy

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A technique to assay Na^+ ions in cells is presented. Intracellular and extracellular Na^+ ions in a suspension of guinea pig erythrocytes were conveniently determined by using sodium-23 nuclear magnetic resonance (^{23}Na -NMR), in combination with two anionic shift reagents: $\text{Dy}(\text{TTHA})^{3-}$ and $\text{Dy}(\text{PPPi})_2^{7-}$. Monensin (1), monensylalanine (2b), monensylserine (2c), and monensylphenylalanine (2d) induced large increases of intracellular Na^+ ion concentration ($[\text{Na}]_{\text{in}}$), while monensylglycine (2a), monensyltyrosine (2e), monensylaspartic acid (2f), and monensylglutamic acid (2g) showed slight increases. The values of initial increasing rate (V_i) of 2a—g were much smaller than that of 1. This fact was probably due to the lower lipophilicity of 2a—g than 1, because a good correlation was observed between V_i and Rm_{50} values of 1 and 2a—g. This lower lipophilicity is a consequence of conformational differences between 1 and 2a—g.

Keywords monensin; monensylamino acid; sodium ion permeability; intracellular sodium ion concentration; ^{23}Na -NMR; guinea pig blood; shift reagent; external reference; Rm_{50} value; positive inotropic effect

The polyether antibiotic monensin (1, Fig. 1) is one of a large class of naturally occurring ionophores, and preferentially transports Na^+ ions across the cell membrane by forming a pseudocyclic complex. In a preceding paper,¹⁾ we reported chemical transformations of monensin (1) into monensylamino acids (2a—g) by condensing the carboxylic group with optically active amino acids, because amino acids have the carboxylic groups which were expected to stabilize the pseudocyclic conformations of 2a—g by forming head-to-tail hydrogen bonds to the terminal hydroxy group. X-Ray crystallographic analysis showed that the NaBr complexes of 2a—g were quite different from the NaBr complex of 1. This crystallographic difference prompted us to study the Na^+ permeability of 2a—g since the permeability of Na^+ ions into the cell is supposed to be deeply concerned with the biological activities of the polyether antibiotics.²⁾ In this paper, we wish to report our results on the Na^+ transport ability of 1 and 2a—g across the membrane of living erythrocytes of guinea pig using sodium-23 nuclear magnetic resonance (^{23}Na -NMR) spectroscopy.

Recently, measurement of intracellular concentration of Na^+ ions in living cells has become feasible by using ^{23}Na -NMR, in combination with anionic paramagnetic shift

Chart 1. Monensylamino Acid (2a—g) NaBr Complexes

reagents. These reagents do not enter intact cells and remain localized in the extracellular compartments, so that the NMR resonance of extracellular Na^+ ions is shifted away from the resonance of intracellular Na^+ ions.^{3,4)} Gupta *et al.* reported a method to determine intracellular Na^+ ions by using dysprosium triphosphate ($\text{Dy}(\text{PPPi})_2^{7-}$) as a shift reagent.³⁾ However, $\text{Dy}(\text{PPPi})_2^{7-}$ is toxic to living cells,⁴⁾ and their method requires determination of the extracellular Na^+ concentration prior to NMR measurement. We therefore employed $\text{Dy}(\text{PPPi})_2^{7-}$ as an external reference and nontoxic dysprosium triethylenetetramine hexacetate ($\text{Dy}(\text{TTHA})^{3-}$)⁴⁾ as a shift reagent for discrimination of the signals due to intracellular and extracellular Na^+ ions in an erythrocyte suspension.⁵⁾ The integral of the intracellular Na^+ resonance relative to the integral of an external reference was determined at various times and compared with the calibration line prepared previously, in order to obtain precisely the concentration of intracellular Na^+ ions.

In addition, we attempted to evaluate the Rm values of 1 and 2a—g as parameters of lipophilicity, because lipophilicity seemed to be closely related to the Na^+ ion-transporting capability through the cell membrane, which is constituted of lipid bilayers.

Fig. 1. Chemical Structure of Monensin (1) NaBr Complex

Experimental

Materials Monensin (1) was prepared from commercially available sodium monensin by the reported method⁶⁾ while monensylamino acids (2a–g) were prepared by the condensation of monensin (1) with the corresponding amino acid benzyl esters followed by debenzylation, as described in a preceding paper.¹⁾

Guinea pig blood was collected by cardiocentesis of several guinea pigs and treated with heparin to avoid coagulation.

Preparation of Shift Reagents $\text{Dy}(\text{TTHA})^{3-}$ was purchased from Dojin Co., Ltd. The sodium salt of $\text{Dy}(\text{PPPi})_2^{7-}$ was prepared *in situ*³⁾ by treating DyCl_3 with Na_5PPPi .

^{23}Na -NMR Measurement NMR spectra were recorded using a JEOL GSX-400 spectrometer at 105.60 MHz and 37 °C. A concentric NMR tube combination (3-mm-o.d. inside a 10-mm-o.d. NMR tube) was used in the experiments (Fig. 2). The inner tube containing 20 mM $\text{Na}_7\text{Dy}(\text{PPPi})_2 \cdot 3\text{NaCl}$ solution was used as an external reference while the annular space between the inner and outer tubes contained 1.8 ml of guinea pig blood, 0.2 ml of 100 mM $\text{Dy}(\text{TTHA})^{3-}$ solution, and 20 μl of 10 mM test compound in dimethylsulfoxide (DMSO). A calibration line was constructed by addition of NaCl solutions (2 ml; 1, 10, 50, and 100 mM) to the annular space in the concentric NMR tube.

Concentrations of the intracellular and extracellular Na^+ ions ($[\text{Na}_{\text{in}}]$ and $[\text{Na}_{\text{out}}]$) were calculated from the corresponding integrals (INT_{in} and INT_{out}) using the following Eqs. 1 and 2.

$$[\text{Na}_{\text{in}}] = (\text{INT}_{\text{in}}/A) \times (V_{\text{a}}/V_{\text{b}} \times \text{Hem}) \quad (1)$$

$$[\text{Na}_{\text{out}}] = (\text{INT}_{\text{out}}/A) \times (V_{\text{a}}/V_{\text{b}} \times (1 - \text{Hem})) \quad (2)$$

where V_{a} and V_{b} represent the total volume of the sample included in the annular space and the volume of the blood used, respectively. Hem is the hematocrit and A is the slope of the calibration line.

R_m Values⁷⁾ The stationary phase was precoated thin layer chromatography (TLC) plates of Silica gel 60 F254 silanized (layer thickness 0.25 mm, Merck no. 5747), and the mobile phase was aqueous MeOH solution of various concentrations, e.g., 40, 50, 60, and 70% (w/v). The test compounds were dissolved in MeOH and spotted in randomized locations in order to avoid any systematic error. The plates were developed about to 15 cm from the spotted line at room temperature, dried, sprayed with $\text{Ce}(\text{III})_2(\text{SO}_4)_3$ solution, and heated in an oven at 85 °C. The R_m values were calculated from R_f values by means of the following equation.^{7a)}

$$R_m = \log(1/R_f - 1) \quad (3)$$

Results

^{23}Na -NMR The relation between the integrals of 1, 10, 50, and 100 mM NaCl solutions and integrals of the corresponding external reference is shown in Fig. 3. The regression line was obtained by the least-squares method

Fig. 2. The Concentric NMR Tube Combination Used for the ^{23}Na -NMR Measurement (3-mm-o.d. Inside a 10-mm-o.d. NMR Tube)

A) The inner tube contained the external reference, 20 mM $\text{Na}_7\text{Dy}(\text{PPPi})_2 \cdot 3\text{NaCl}$. B) The annular space contained guinea pig blood (1.8 ml), 100 mM $\text{Dy}(\text{TTHA})^{3-}$ (0.2 ml), and 10 mM test compound DMSO solution (20 μl).

and is expressed by the following equation:

$$\text{INT} = 0.0471 \times [\text{Na}] + 0.00 \quad (\gamma = 1.00; \gamma: \text{correlation coefficient})$$

where 0.0471 corresponds to the value of A in Eqs. 1 and 2.

Figure 4 shows representative ^{23}Na -NMR spectra acquired before (a; 0 time, several minutes after addition of $\text{Dy}(\text{TTHA})^{3-}$) and 90 min after (b) addition of 2d. The resonances of intracellular and extracellular Na^+ ions were clearly separated into two well-resolved signals. The resonance indicated as (B) corresponds to intracellular Na^+ ions while the downfield resonance (C) due to extracellular Na^+ ions which interacted with $\text{Dy}(\text{TTHA})^{3-}$.

The time course of $\Delta[\text{Na}_{\text{in}}]$ is shown in Table I. DMSO, used as a solvent for the test compounds, caused a slight decrease of $[\text{Na}_{\text{in}}]$. So the corrected time course of $[\text{Na}_{\text{in}}]$, $\Delta C (= \Delta[\text{Na}_{\text{in}}]_t - \Delta[\text{Na}_{\text{in}}]_{\text{DMSO}})$, is shown in Fig. 5 and Table I. Monensin (1) and 2b–d caused remarkable increases of $[\text{Na}_{\text{in}}]$ (31.3–46.7 mM) whereas the other compounds showed slight increases (5.4–7.8 mM). The characteristic features of $[\text{Na}_{\text{in}}]$ increase with monensin (1) were a rapid increase within a short period, followed by a moderate decrease. Compounds 2e and 2g showed a similar $[\text{Na}_{\text{in}}]$ tendency to monensin (1), though values of $[\text{Na}_{\text{in}}]$ with 2e and 2g were extremely small.

R_m Values A linear relationship was observed between

Fig. 3. Relation between the Concentration of NaCl Solutions in the Annular Space and Integral of Those Solutions Relative to That of the External Reference

Fig. 4. ^{23}Na -NMR Spectra Acquired a) Before and b) 90 min After Addition of 2d

A, B, and C indicate the signals of the external reference, and intracellular and extracellular Na^+ ions, respectively.

TABLE I. Time Courses of Intracellular Na⁺ Ion Concentration

Time (min)	DMSO, <i>n</i> =4 $\Delta[\text{Na}_{\text{in}}]_i$ ($\pm$ S.E.)	1, <i>n</i> =5 $\Delta[\text{Na}_{\text{in}}]_i$ ($\pm$ S.E.)	ΔC	2a, <i>n</i> =5 $\Delta[\text{Na}_{\text{in}}]_i$ ($\pm$ S.E.)	ΔC	2b, <i>n</i> =6 $\Delta[\text{Na}_{\text{in}}]_i$ ($\pm$ S.E.)	ΔC	2c, <i>n</i> =3 $\Delta[\text{Na}_{\text{in}}]_i$ ($\pm$ S.E.)	ΔC
5	-0.3 ($\pm$ 0.7)	46.4 ($\pm$ 2.1)	46.7	—	—	—	—	—	—
10	-1.2 ($\pm$ 0.7)	44.3 ($\pm$ 3.4)	45.5	—	—	—	—	—	—
20	-2.7 ($\pm$ 0.7)	37.6 ($\pm$ 1.9)	40.3	-0.1 ($\pm$ 0.5)	2.6	5.9 ($\pm$ 1.0)	8.6	10.3 ($\pm$ 1.6)	13.0
40	-3.1 ($\pm$ 1.0)	35.0 ($\pm$ 1.7)	38.1	0.4 ($\pm$ 0.6)	3.5	10.8 ($\pm$ 1.8)	13.9	18.6 ($\pm$ 2.6)	21.7
60	-3.1 ($\pm$ 0.8)	35.8 ($\pm$ 0.9)	38.9	0.5 ($\pm$ 1.2)	3.6	14.9 ($\pm$ 1.0)	18.0	23.0 ($\pm$ 2.8)	26.1
90	-3.4 ($\pm$ 0.9)	37.5 ($\pm$ 2.5)	40.9	—	—	19.0 ($\pm$ 1.3)	22.4	31.2 ($\pm$ 3.6)	34.6
120	-3.2 ($\pm$ 1.0)	38.6 ($\pm$ 3.2)	41.8	1.9 ($\pm$ 1.8)	5.1	21.9 ($\pm$ 1.1)	25.1	32.3 ($\pm$ 2.6)	35.5
180	-4.7 ($\pm$ 0.5)	—	—	3.5 ($\pm$ 2.4)	8.2	24.9 ($\pm$ 1.8)	29.6	38.9 ($\pm$ 2.8)	43.6
240	-3.3 ($\pm$ 0.8)	—	—	4.5 ($\pm$ 2.9)	7.8	28.0 ($\pm$ 3.2)	31.3	42.9 ($\pm$ 1.9)	46.2

Time (min)	2d, <i>n</i> =7 $\Delta[\text{Na}_{\text{in}}]_i$ ($\pm$ S.E.)	ΔC	2e, <i>n</i> =3 $\Delta[\text{Na}_{\text{in}}]_i$ ($\pm$ S.E.)	ΔC	2f, <i>n</i> =4 $\Delta[\text{Na}_{\text{in}}]_i$ ($\pm$ S.E.)	ΔC	2g, <i>n</i> =5 $\Delta[\text{Na}_{\text{in}}]_i$ ($\pm$ S.E.)	ΔC
5	7.5 ($\pm$ 2.7)	7.8	—	—	—	—	—	—
10	17.0 ($\pm$ 3.5)	18.4	—	—	—	—	—	—
20	27.3 ($\pm$ 1.3)	30.0	1.2 ($\pm$ 0.5)	3.9	0.2 ($\pm$ 0.9)	2.9	0.6 ($\pm$ 1.6)	3.3
40	37.2 ($\pm$ 1.0)	40.1	-0.5 ($\pm$ 0.7)	2.6	-0.4 ($\pm$ 1.0)	2.7	-1.3 ($\pm$ 1.0)	1.8
60	39.8 ($\pm$ 2.0)	42.9	1.3 ($\pm$ 0.4)	4.4	-0.8 ($\pm$ 0.5)	2.3	0.7 ($\pm$ 1.0)	3.8
90	39.9 ($\pm$ 2.7)	43.3	1.2 ($\pm$ 0.9)	4.6	—	—	—	—
120	39.7 ($\pm$ 2.6)	42.9	1.6 ($\pm$ 0.8)	4.8	1.0 ($\pm$ 1.1)	4.5	0.8 ($\pm$ 1.7)	4.0
180	—	—	3.7 ($\pm$ 0.5)	8.4	1.9 ($\pm$ 1.7)	6.6	0.1 ($\pm$ 2.6)	4.8
240	—	—	3.7 ($\pm$ 0.6)	7.0	3.1 ($\pm$ 2.7)	6.4	2.1 ($\pm$ 1.2)	5.4

$\Delta[\text{Na}_{\text{in}}]_i = [\text{Na}_{\text{in}}]_i - [\text{Na}_{\text{in}}]_0$, S.E., and $\Delta C = \Delta[\text{Na}_{\text{in}}]_i - \Delta[\text{Na}_{\text{in}}]_i^{\text{DMSO}}$.

Fig. 5. Time Courses of ΔC Induced by 1 and 2a—g
●, 1; ○, 2a; ▲, 2b; △, 2c; ▼, 2d; ▽, 2e; ■, 2f; □, 2g.

Rm values of 1, 2a—g and concentration of MeOH in the mobile phase in the range from 40 to 60%. By means of the least-squares method, linear regressions were calculated from the *Rm* values, and the equation of each straight line was used in order to calculate an *Rm*₅₀ value corresponding to 50% MeOH in the mobile phase. The *Rm*₅₀ values are listed in Table II. There was no compound more lipophilic than monensin (1).

Discussion

We have developed a method to determine the concentrations of intracellular and extracellular Na⁺ ions using ²³Na-NMR spectroscopy. The hematocrit value of the guinea pig blood used in this study remained unchanged during the NMR measurement, which routinely took about 4 h. The initial extracellular Na⁺ concentration of blood

TABLE II. Parameter of Lipophilicity (*Rm*₅₀), Logarithmic Increasing Rates at 0—20 min (log *V*_i), and Ratio of Surface Area of Lipophilic and Hydrophilic Site at Amino Acid Moieties (*A*_i/*A*_h)

Compd.	<i>Rm</i> ₅₀	log <i>V</i> _i	<i>A</i> _i / <i>A</i> _h
1	1.85	0.97 ^{a)}	—
2a	0.65	-0.78	0.65
2b	0.73	-0.36	1.18
2c	0.63	-0.18	0.63
2d	1.17	0.18	2.72
2e	0.83	-0.65	1.75
2f	-0.04	-0.83	0.43
2g	-0.08	-0.78	0.65

a) log *V*_i at 0—5 min.

cells in the absence of the test compound was determined by measuring the ²³Na-NMR spectrum several minutes after addition of Dy(TTHA)³⁻. The resulting concentration was 123.2 mM (S.E. $\pm$ 1.1 mM) which was in good agreement with the standard value (125.0 mM).⁸⁾ The blood of guinea pig therefore appears to be a convenient experimental preparation to determine precisely the influx of Na⁺ ions across the erythrocyte membrane. Our NMR technique has the advantage of separating directly the resonances from intracellular and extracellular Na⁺ ions, and allowing us to determine quantitatively the influx and efflux of Na⁺ ions. The increase of integrals for intracellular Na⁺ ions was always parallel to the decrease of the corresponding integrals for extracellular Na⁺ ions. Since Dy(TTHA)³⁻ introduced in this study was nontoxic to the blood cells and caused effective resonance separation, this reagent should be widely useful in ²³Na-NMR studies of intact cells and tissues.

Monensin (1) and 2b—d showed remarkable maximal ΔC . The positive inotropic effect (PIE) was then examined on isolated guinea pig papillary muscle since monensin (1)

Fig. 6. Relation between Rm_{50} and $\log V_i$ of **1** and **2a–g**.
●: **1**; ○: **2a**; ▲: **2b**; △: **2c**; ▼: **2d**; ▽: **2e**; ■: **2f**; □: **2g**.

exhibits the anticoccidial activity and PIE, which is known to be induced by influx of Na^+ ions into the cells. The PIE of **2a–g** was then examined. However, none of these compounds exhibited PIE although the maximal ΔC values for **2c** and **2d** were comparable to that of **1**. From these observations, it could be suggested that appearance of PIE might depend not on the maximal increase but on the initial increasing rate of $[\text{Na}_{\text{in}}] (V_i)$. Actually the V_i of **1** within 0–5 min is significantly faster than those of **2a–g** within 0–20 min. Therefore, those differences in V_i values are considered to be due to the differences in lipophilicity, which plays an important role in penetration of the compounds into the cell membrane consisting of lipid bilayers. Consequently, we have investigated the correlation between the logarithmic V_i and Rm_{50} . As shown in Fig. 6, a good correlation was observed except for **2f** and **2g**, thus suggesting that high lipophilicity could result in a more rapid Na^+ influx into the cells. The smaller Rm_{50} values of **2f** and **2g** are considered to be due to hydration of the two carboxyl groups in aqueous MeOH solution, used as the developing solvent for Rm value calculation, while the V_i values for **2f** and **2g** were comparable to that of **2a** because **2f** and **2g** may penetrate the lipid bilayers of erythrocyte membrane with the formation of intramolecular or intermolecular association between the two carboxylic groups in the molecule.

It is noteworthy that low lipophilicity of **2a–g** relative to that of **1** can be explained in terms of the molecular conformations coordinating to the Na^+ ion, as illustrated in Chart 1. The NaBr complex of monensin (**1**) possesses head-to-tail intramolecular hydrogen bonds leaving no hydrophilic substituent on the molecular surface, whereas the NaBr complexes of **2a–g** have hydrophilic amino acid substituents such as carboxyl and hydroxyl groups occupying positions toward the outside of the pseudocyclic molecule. A further study of the lipophilicity of **2a–g** was carried out to clarify the relationship between Rm_{50} and relative ratio of Van der Waals surface areas of the amino acid substituents, which could be calculated from the parameters of lipophilic groups (alkyl and aryl groups; A_l) and hydrophilic ones (hydroxy and carboxyl groups; A_h) according to the method reported by Yamakawa and co-workers.⁹⁾ As is clear from Fig. 7, a good correlation was observed between Rm_{50} and A_l/A_h values, except for **2f** and

Fig. 7. Relation between Rm_{50} and A_l/A_h .
○: **2a**; ▲: **2b**; △: **2c**; ▼: **2d**; ▽: **2e**; ■: **2f**; □: **2g**.

2g, which seemed to be more hydrophilic than other compounds on account of the presence of the two carboxylic groups. This correlation reveals that lower lipophilicity of **2a–g** is attributable to the hydrophilicity of the amino acid residues.

In conclusion, we have established a convenient method to determine precisely the concentrations of intracellular and extracellular Na^+ ions in a suspension of guinea pig erythrocytes using ^{23}Na -NMR spectroscopy, in combination with two anionic shift reagents; $\text{Dy}(\text{PPPi})_2^{7-}$ and $\text{Dy}(\text{TTHA})^{3-}$. This method could also be applicable for determination of K^+ and Ca^{2+} concentrations if suitable nontoxic shift reagents are available.

It is most likely that the initial increasing rate (V_i) of the compound is an important factor to predict the biological activity, and this may be closely related to the lipophilicity of the compounds. Similarly, lack of PIE and anticoccidial activity is also probably due to the less lipophilic character of the test compounds. Further investigation of chemical modifications of the monensin molecule with much more lipophilic functional groups is in progress.

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