Selective transport of potassium ions across a planar phospholipid bilayer by a calix[4]arene-crown-5 as a synthetic carrier

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The ion transport ability in a planar phospholipid bilayer, of two *p-tert*-butylcalix[4]arene-crown-5 derivatives (**1**, **2**), a *p-tert*-butylcalix[5]arene ester **3**, dibenzo-18-crown-6, and valinomycin have been investigated using a voltage clamp method. Membrane current measurements showed that the synthetic calixarene ionophores except for dibenzo-18-crown-6, show ion transport activities for K^+ in the bilayer membranes. The order of K^+ transport activities of the compounds was valinomycin **>1 > 3 > 2**. From the measurements of reversal potentials, the relative ion permeability across the bilayer was determined for **1**, **3** and valinomycin. Both **1** and **3** showed ion transport selectivity for K^+, while valinomycin showed ion transport selectivity for Rb^+. Among the calixarene ionophores, compound **1** showed the highest K^+ conductivity and K^+/Na^+ selectivity. Although the K^+/Na^+ selectivity of **1** is less than that of valinomycin by a factor of *ca.* 2, calix[4]arene-based ionophore **1** has the potential for use as a synthetic K^+ carrier in phospholipid bilayer membranes.

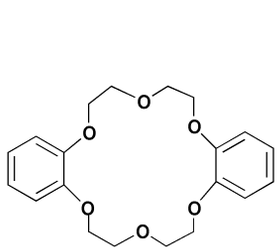
Introduction

Selective ion transport across biological membranes is of crucial importance for all living cells and organisms.[1] To understand the mechanism of ion transport in the living systems, artificial phospholipid bilayer membranes have been widely used as models of biological membranes.[1,2] Phospholipid bilayer membranes have a thickness of about 30 Å (the region of hydrocarbon chains), and they are highly impermeable to ions, because of the low relative permittivity of hydrocarbon chains of the lipid.[2] However, incorporation of ionophoric compounds into phospholipid bilayers mediates ion transport across the bilayer membrane. It is well known that naturally occurring antibiotic ionophores such as monensin,[3] valinomycin,[4] gramicidin,[5] and alamethicin[6] have the abilities for selective ion transport across phospholipid bilayer membranes. In the past decade, a variety of synthetic ionophores have been designed for mimicking selective ion transport in biological membrane systems.[7]

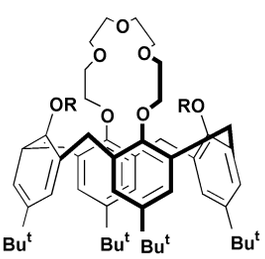
We have recently reported that calixarene-based ionophores (*p-tert*-butylcalix[*n*]arene ester derivatives) are easily incorporated into phospholipid bilayer membranes, and they can mediate ion transport across the bilayer membranes.[8] It has been shown that the ester derivatives of calix[4]arene and calix[5]arene have ion transport activities for Na^+ and K^+ in phospholipid bilayer systems.[8a,b,d,9] In the case of the calix[4]arene ester, the rates of Na^+ transport across the bilayer are comparable to the rates of monensin-mediated Na^+ transport.[8a] Although, the calix[5]arene esters show K^+ transport activity in phospholipid bilayers, they do not have a high enough K^+/Na^+ selectivity to be used as selective K^+ carriers in biological membranes.[8b] In spite of numerous new ionophores synthesized,[10] valinomycin is still used as the most effective K^+ ionophore in biological membrane systems.[1,11]

The calixarene-based K^+ ionophores (calix[4]arene-crown-5 derivatives),[12] first reported by Ungaro and co-workers are unique ionophores in which a calix[4]arene is combined with a crown ether bridge. Reinhoudt and co-workers[12d] have investigated their ion transport properties in supported liquid membranes, and found that calix[4]arene-crown-5 derivatives can act as selective K^+ carriers in the membrane. They also found that a 1,3-alternate calix[4]arene-crown-5 conformer has a high K^+/Na^+ selectivity and its selectivity is better than that of valinomycin in a supported liquid membrane.[12e] However, there has been no study of ion transport by calix[4]arene-crown-5 derivatives in lipid bilayer systems. The objective of the present study is to determine whether calix[4]arene-crown-5 derivatives have a similar capacity to valinomycin for selective K^+ transport across phospholipid bilayer membranes.

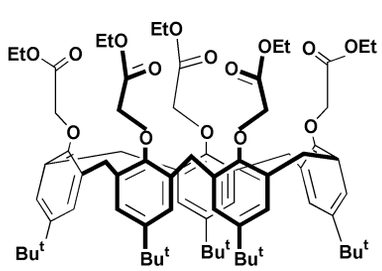
In this work, we have investigated alkali-metal ion transport across a planar phospholipid bilayer by *p-tert*-butylcalix[4]-arene-crown-5 derivatives (**1**,**2**; Scheme 1), *p-tert*-butylcalix[5]-arene ester **3**, dibenzo-18-crown-6 and valinomycin. The ion transport activities and ion permeabilities of the compounds in the bilayer were determined using a voltage clamp method.[2]



Dibenzo-18-crown-6



1 (R=OMe), **2** (R=H)



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Scheme 1 Synthetic ionophores tested for K^+ transport activity in a planar phospholipid bilayer system.

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Here we report that, among the synthetic calixarene ionophores studied, *p-tert*-butylcalix[4]arene-crown-5 (**1**) is a most effective K^+ carrier in a phospholipid bilayer system.

Results and discussion

Planar bilayer experiments using a patch clamp amplifier constitute a highly sensitive method for determining the ion transport activity of electrogenic ionophores, because the ion fluxes across the bilayer can be detected at pA level of membrane current.[2] Planar bilayers were formed across an aperture in a Teflon film between two aqueous salt solutions in the *cis* and *trans* chamber. Fig. 1 shows the generation of

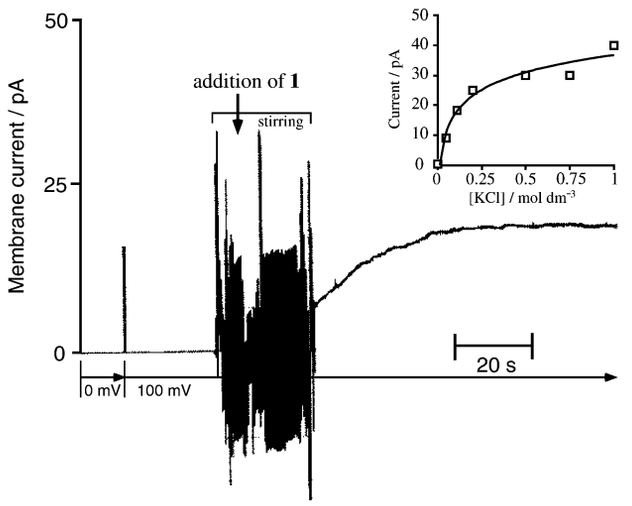

Fig. 1 Increase in membrane conductance by the addition of 20 μl of **1** (100 μM DMSO solution). The ionophore was added to the *cis* chamber with stirring, where two chambers were filled with KCl solution (100 mM) adjusted to pH = 7.2 by a HEPES–Tris buffer. The insert shows the dependence of the membrane current on KCl concentration.

membrane currents when an aliquot of a DMSO solution of **1** was added to the *cis* chamber. Upon addition of **1**, membrane conductance significantly increased and gave a stable level about 1 min after the addition. A control experiment was performed where only neat DMSO was added: a control level (0.5 pA at 100 mV) of the membrane current did not change upon addition of 200 μl of DMSO. The insert in Fig. 1 shows the dependence of the membrane current on KCl concentration. The plot of current *versus* the concentration of KCl gave a saturation curve, suggesting that the ion transport by **1** takes place by a carrier mechanism.[11] Similar saturation behavior of K^+ flux has been reported in a system in which **1** is incorporated into a supported-liquid membrane.[12d]

To confirm the transport of K^+ ions by **1**, we measured resting membrane potentials when different concentrations KCl were placed on opposite sides of the planar bilayer membrane. If the planar bilayer membrane containing **1** is permeable only to K^+ ions, the resting membrane potentials should be expressed by the Nernst equation (1).[2]

$$\varphi_m = \frac{RT}{F} \ln \frac{[K^+]_{cis}}{[K^+]_{trans}} \quad (1)$$

When the concentration ratios of $[K^+]_{cis}/[K^+]_{trans}$ were increased to >1, the resting membrane potentials were positive on the side of the lower concentration, and the plot of the membrane potentials *versus* $\ln[K^+]_{cis}/[K^+]_{trans}$ showed a linear relationship (Fig. 2). It can be seen that the slope of the membrane potential is almost the same as the slope (59 mV decade^{-1}) of the theoretical Nerstian plot. Thus the selectivity, *i.e.* the permeability ratio for K^+/Cl^- is very large, suggesting that Cl^- anions cannot be transported by **1** across the bilayer.

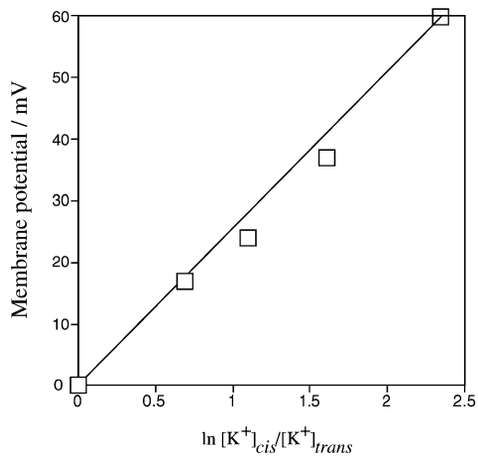

Fig. 2 Membrane potentials as a function of the ratio of KCl concentrations in the *cis* and the *trans* chamber separated by a planar bilayer containing **1**. Conditions: $[K^+]_{trans}$ = 100 mM, pH = 7.2, lipid/**1** = 100 : 1 (w/w). The solid line shows a theoretical Nernstian slope.

From the view of biological applications, it is of importance to compare the ion transport activity of the synthetic K^+ ionophores with that of valinomycin. Fig. 3 shows the depend-

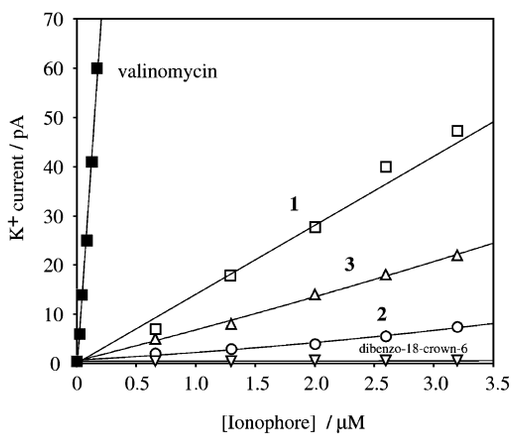

Fig. 3 Dependences of K^+ current on ionophore concentration: (■) valinomycin; (□) **1**; (△) **3**; (○) **2**; and (▽) dibenzo-18-crown-6. Ionophores were added as DMSO solutions (100 μM) to the *cis* chamber.

ence of the K^+ current at 100 mV on the ionophore concentrations (in the *cis* chamber). The K^+ current decreased in the order: valinomycin >**1** > **3** > **2** > dibenzo-18-crown-6. The ion transport activity may be evaluated by the value of the membrane current with respect to the ionophore concentration in the *cis* chamber: 300 and 14.3 pA μM^{-1} for valinomycin and **1**, respectively. The K^+ transport activity of **1** was less than that of valinomycin by a factor of *ca.* 20. Among the calixarene ionophores, compound **1** showed the highest K^+ transport activity in the bilayer membrane. It should be noted that the dependences of the K^+ current on the concentration of the calixarene ionophores (**1**–**3**) showed linear relationships. This suggests that the calixarene ionophores form a 1 : 1 complexes with K^+ ions in the bilayer, in a similar manner to the case of valinomycin. Interestingly, dibenzo-18-crown-6 did not show the K^+ transport activity in the bilayer. The poor activity of the dibenzo-18-crown-6 can be explained by lower lipophilicity in comparison to the other synthetic ionophores. Indeed, leaching of dibenzo-18-crown-6 into the aqueous phase has been observed in a supported liquid membrane system.[12d] In the case of **1** and valinomycin, these compounds are very lipophilic, which prevents leaching into the aqueous phase: the partition coefficient (log *P*) in octanol–water is reported as 15 and 8.6 for **1** and valinomycin, respectively.[12d] Thus, the lower K^+ transport activity of **1** *versus* valinomycin may be attributed to the lower

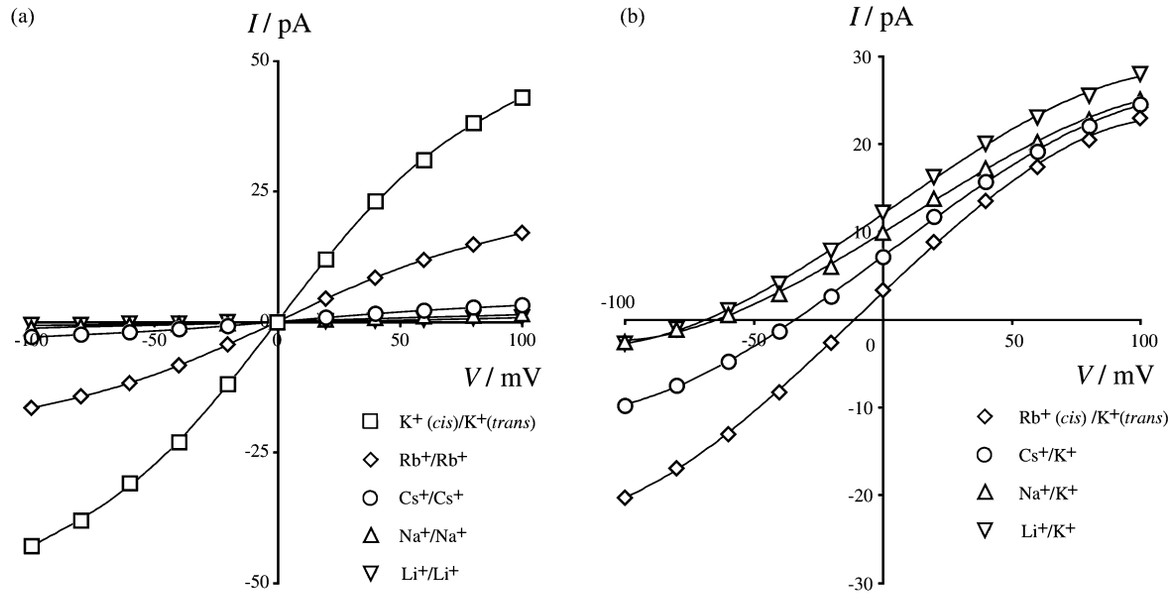

Fig. 4 Current–voltage relationships for a planar bilayer containing **1**: (*a*) symmetrical ionic conditions; $[MCl]_{cis} = [MCl]_{trans} = 100$ mM, pH = 7.2, lipid/**1** = 100 : 1 (w/w). (*b*) unsymmetrical ionic conditions; $[MCl]_{cis} = [KCl]_{trans} = 100$ mM, pH = 7.2, lipid/**1** = 200 : 1 (w/w).

binding constant of the K^+ complex ($K_{K^+} = 3.8 \times 10^8$ for **1** [12c] and $K_{K^+} = 2.2 \times 10^9$ for valinomycin [12e] in $CDCl_3$ saturated with H_2O).

To evaluate the ion transport selectivities for **1**, we measured current–voltage curves under symmetrical ionic conditions [Fig. 4(a)]. The membrane conductivity at 100 mV decreased in the order: $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$. This result shows that **1**-mediated ion transport is selective toward K^+ ion. To obtain the values of the ion permeability, we examined the current–voltage relationships under unsymmetrical ionic conditions [Fig. 4(b)]. The reversal potentials (which correspond to zero current voltages) in the MCl(*cis*)/KCl(*trans*) system is related to the ion concentrations on the two sides of the membrane according to the Goldman–Hodgkin–Katz equation (2),[2,11,13]

$$\phi_{rev} = \frac{RT}{F} \ln \frac{P_M[M^+]_{cis} + P_{Cl}[Cl^-]_{trans}}{P_K[K^+]_{trans} + P_{Cl}[Cl^-]_{cis}} \quad (2)$$

where P_M, P_K, and P_{Cl} are the ion permeabilities of M^+ (M = Li, Na, Rb, and Cs), K^+, and Cl^- ions. Since the planar membrane containing **1** is impermeable to Cl^- ions, the ratio (P_M/P_K) of the cation permeabilities (under the condition $[MCl]_{cis} = [KCl]_{trans}$) can be easily calculated from the values of reversal potentials, eqn. (3).

$$\frac{P_M}{P_K} = \exp\left(\frac{\phi_{rev} F}{RT}\right) \quad (3)$$

The values of the reversal potentials are summarized in Table 1. Fig. 5 shows the relative ion permeability for **1**, together with the results for **3** and valinomycin. Both **1** and **3** show ion transport selectivity for K^+, while valinomycin shows ion transport selectivity for Rb^+. The K^+/Na^+ selectivities of these compounds in the bilayer system are 23, 12, and 2

Table 1 Values of reversal potentials (φ_{rev})[a]

	$-\varphi_{rev}$/mV			
Ionophore	Li^+/K^+	Na^+/K^+	Rb^+/K^+	Cs^+/K^+
Valinomycin	108	80	−27	22
1	67	63	11	33
3	77	18	3	18

[a] Ionic conditions: $[MCl]_{cis} = [KCl]_{trans} = 100$ mM, pH = 7.2.

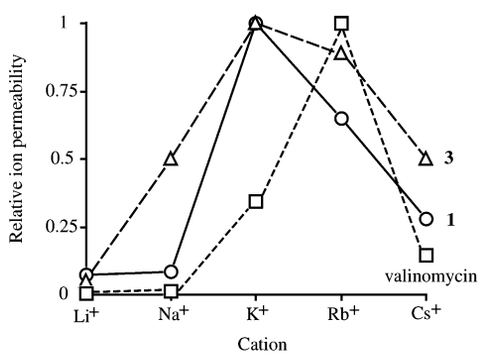

Fig. 5 Ion permeability across a planar lipid bilayer containing calixarene ionophores (**1**,**3**) and valinomycin. The values of the ion permeability are normalized by the largest value of the ion permeability observed.

for valinomycin, **1**, and **3**, respectively. Although the K^+/Na^+ selectivitiy of **1** is less than that of valinomycin by a factor of *ca.* 2, compound **1** has the highest K^+ selectivity among the calixarene-based synthetic ionophores.

Mechanisms for ionophore-mediated ion transport across bilayer membranes are divided into two categories: carrier and channel mechanisms.[11] The calix[4]arene-crown-5 (**1**) has a cylindrical structure based on a calix[4]arene moiety and its molecular length along the cylindrical structure is *ca.* 12 Å (estimated from CPK models). Thus there is a possibility that two molecules of **1** aligning across a bilayer form a channel-like structure. If such a channel exists, a single-channel current fluctuation[2,13] should be observed under conditions of very low ionophore concentration. Unfortunately, we could not observe such a single-channel current fluctuation for **1**. The carrier mechanism for the **1**-mediated transport of K^+ across phopholipid bilayer membranes is supported by the following findings: (1) the K^+ transport activity of **1** is less than that of the natural antibiotic carrier, valinomycin, by a factor of *ca.* 20; (2) the K^+ current (flux) of **1** shows saturation behavior at high concentrations of KCl solution; (3) the K^+ current of **1** increases linearly with the concentration of **1** added to the *cis* chamber, suggesting that **1** forms a 1 : 1 complex with K^+ ions in a bilayer.

Conclusion

Synthetic ion carriers active in phospholipid bilayers must have

high lipophilicities as well as high ion selectivities. We have demonstrated that the calix[4]arene-crown-5 (**1**) can act as a synthetic K^+ carrier in a phosholipid bilayer membrane. Because of the high lipophilicity of **1**, this compound can be easily incorporated into the phospholipid bilayer membrane, and gives a stable K^+ flux across the bilayer. Among the synthetic ionophores studied, compound **1** shows K^+ transport ability with the highest K^+/Na^+ selectivity, while its selectivity is less than that of valinomycin by a factor of *ca.* 2. In the case of dibenzo-18-crown-6, no K^+ transport activitiy was detected. Although the ion transport ability of **1** is not as good as that of valinomycin, compound **1** can be regarded as a first example of a synthetic K^+ carrier active in lipid bilayer membranes. Reinhoudt *et al.* have found that several dialkoxycalix[4]arene crowns in the 1,3-alternative conformation have better K^+/Na^+ selectivity than **1** and valinomycin using supported liquid membranes.[12e] The systematic investigation of the relationships between the conformations of calix[4]arene crowns and their ion transport properties in bilayer systems will be the subject of future papers.

Experimental

Chemicals

Valinomycin and dibenzo-18-crown-6 were purchased from Aldrich Chemicals. The calix[4]arene-crown-5 derivatives (**1**,**2**) and calix[5]arene ester **3** were prepared according to the literature methods.[12,14] All compounds were identified by their ^1H NMR spectra and mass spectroscopy. Soybean phospholipids were purchased from Nakarai Tasque (Kyoto, Japan) and purified by the literature method.[15] Analytical reagent grade LiCl, NaCl, KCl, RbCl, and CsCl were purchased from Wako and used without further purification.

Planar bilayer formation

Planar bilayer membranes (soybean phospholipids) were prepared by the folding method.[2,16] Bilayers were formed at an aperture (diameter, 0.2 mm) in a Teflon film (thickness, 12.5 μm) which separated two Teflon chambers (internal volume of each chamber is 1.7 ml with surface area of 1 cm^2). The side to which compounds were added was defined as the "*cis*" chamber and the opposite side was the "*trans*" chamber. The aqueous salt solution (alkali metal chlorides) containing HEPES [*N*′-(2-hydroxyethyl)piperazine-*N*-(ethane-2-sulfonic acid]–Tris [tris(hydroxymethyl)aminomethane] buffer (25 mM, pH 7.2) (1.5 ml) was injected into both chambers with two syringes so that the water surface was just below the aperture. Then a 15 μl aliquot of phospholipid or phospholipid–calixarene mixtures dissolved in hexane (10 mg ml^{-1}) was placed on the surface of the solution in both chambers. After (*ca.* 10 min), the hexane was evaporated off leaving a phospholipid monolayer at the air–water interface in both chambers. The bilayer membrane was then formed by raising the water level sequentially in both chambers above the aperture.

Incorporation of the ionophore into planar bilayer membranes

Two methods were used to incorporate the ionophores into the planar bilayer membranes. In the measurements of concentration dependences of ionophores on membrane currents, microliter aliquots of ionophores dissolved in DMSO were added to the *cis* chamber after the formation of the bilayers. In the measurements of current–voltage curves, planar bilayer membranes were directly prepared from the mixture of lipids and ionophores.

Electrical recording

A patch clamp amplifier (CEZ-2300; Nihon Kohden, Ltd., Tokyo, Japan) was used in voltage clamp mode to amplify the currents and to control the voltages across the bilayer membranes. The command voltage was fed to the *trans* chamber *via* an Ag/AgCl electrode through an agar bridge and the *cis* chamber was earthed *via* an Ag/AgCl electrode through an agar bridge. The voltage was referenced to the *cis* side with respect to the *trans* side. The output signal from the amplifier was filtered at 1kHz and recorded using a Digidata 1200A A/D converter (Axon Instruments, Inc, USA). All measurements were performed at 25 °C.

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