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A Comparison between Sodium and Potassium in the Inhibition of Stomatal Opening by Cyclic 'Crown' Polyethers

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Cyclic 'crown' polyethers are found to inhibit stomatal opening in abaxial epidermal peels of Commelina communis *when they are floated on bathing media containing 10 mM MES and 50 mM KCl or NaCl buffered to pH 6.1 and irradiated with light in CO_2-free air. Increasing potassium ion concentration in the bathing medium overcomes this inhibition to stomatal opening by reducing the leakage of potassium out of the guard cells. Evidence is presented for 2:1 ligand to sodium complexes in the guard cell membrane when sodium is used in the bathing medium.*

Introduction

In a series of studies, we have reported the effect of macrocyclic 'crown' polyethers on potassium (rubidium and caesium) fluxes into respiring rat liver mitochondria [1] and on stomatal opening in epidermal peels of *Commelina communis* [2, 3]. Potassium ions move into the guard cells of stomata as they open [5–10], thus lowering the guard cell water potential. Water then flows into the guard cells, increasing their turgor, and the construction of the guard cells is such that stomatal opening occurs, leading to transpiration and also carbon dioxide assimilation. The results from experiments with *C. communis* suggested [3] that the crown ethers partition into membranes and allow potassium ions to flow down their chemical gradient. In this paper, we report the results of increasing potassium ion concentration in the bathing medium of epidermal peels of *C. communis*, and discuss their implications on the role of synthetic ionophores. We also report the inhibitory effect of crowns to stomatal opening when the cation provided in the bathing medium is sodium instead of potassium. Although earlier work suggested that sodium could be taken up into the guard cells of stomata in place of potassium and thus provide the osmotic potential for stomatal opening [7, 11, 12], recent work has shown that stomatal responses to carbon dioxide, light and abscisic acid are reduced under these conditions [13].

Experimental

Seeds of *C. communis* were germinated and grown under conditions previously described [2, 3]. Following the method of Travis and Mansfield [4], 4–6 weeks old plants were used for experiments, and the plants kept in darkness prior to peeling. The youngest fully developed leaves were then excised and cut into strips. Peels of the lower epidermis were made and cut into 5 × 10 mm strips.

In the first series of experiments, the effect on stomatal opening of increasing potassium ion concentration in the bathing medium was determined. Previous work [3] had established the concentrations of both benzo-18-crown-6 (12 m*M*) and t-butylbenzo-15-crown-5 (0.2 m*M*) just to inhibit stomatal opening when epidermal peels prepared as above were incubated in CO_2-free air on a light box for 3 hr in plastic petri dishes containing 10 ml of an aqueous solution of 50 m*M* KCl and 10 m*M* MES [4-morpholine ethanesulphonic acid] buffer adjusted to pH 6.1 with potassium hydroxide. In the present work, epidermal peels were incubated in light and CO_2-free air in solutions containing either 12 m*M* benzo-18-crown-6 or 0.2 m*M* t-butylbenzo-15-crown-5 and concentrations of 50, 100 and 150 m*M* potassium chloride. The solutions also contained 10 m*M* MES buffered to pH 6.1 with potassium hydroxide. After three hours' incubation the stomatal apartures were measured on a Vickers M17 microscope as previously described (Table Ia).

Partition coefficients of the two crowns between n-octanol and 50, 100 and 150 m*M* aqueous KCl solutions were determined [3] by vigorously shaking 4 ml of 5 m*M* crown solution in n-octanol with 4 ml

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TABLE I.

a. Stomatal Width against Varying Concentration of Potassium Ions in the Presence of either 12 m*M* Benzo-18-crown-6 or 0.2 m*M* t-butylbenzo-15-crown-5, and Compared with Apertures of Controls.

Potassium Ion Concentration m*M*	Stomatal aperture (mμ)			
	t-butylbenzo-15-crown-5		benzo-18-crown-6	
	0	0.2 m*M*	0	12 m*M*
50	9.0	0.83	11.2	0
100	13.0	5.77	13.2	3.99
150	13.3	8.39	13.7	9.58

b. Partition Coefficients of Benzo-18-crown-6 and t-butylbenzo-15-crown-5 between n-octanol and Aqueous Potassium Chloride Solutions of Varying Concentrations.

Crown	Partition coefficient		
	50 m*M* KCl	100 m*M* KCl	150 m*M* KCl
Benzo-18-crown-6	1.44	0.88	0.65
t-Butylbenzo-15-crown-5	181	167	158

TABLE II. Variation of Stomatal Aperture with Varying Concentrations of Benzo-18-crown-6 after 3 hr Incubation in 50 m*M* NaCl and 10 m*M* MES at pH 6.1.

Concentration of benzo-18-crown-6 (m*M*)[a]	No. of stomata measured	Stomatal opening μm	σ μm
0	80	12	1.4
3.0	40	8.0	1.1
4.0	40	3.6	1.0
4.8	40	3.1	1.3
5.6	40	1.6	0.7
6.0	40	0	0
8.0	40	0	0

[a]Concentration required just to inhibit stomatal opening is 6.04 m*M* for benzo-18-crown-6.

of the respective aqueous potassium chloride solution, separating the layers, and subsequently determining the crown concentration in each layer using the absorption of the crown at 273 nm as measured on a Cecil CE 5095 UV/visible spectrometer (Table Ib).

The second series of experiments was designed to observe the effect of changing potassium for sodium in the bathing medium in the presence of cyclic polyethers. Epidermal peels were floated cuticle side up in petri dishes containing 10 ml of 50 m*M* NaCl and10 m*M* MES buffered at pH 6.15 with sodium hydroxide, and containing a series of concentrations of the cyclic polyether being tested. At this stage, all stomata were closed. The dishes were illuminated over a light box (21,000 lux), whilst carbon dioxide-free air was bubbled through the solutions *via* syringe needles at a rate of 0.16 l min^{-1} per dish. After three hours of such incubation, the stomatal apertures were measured as described above [3]. In this way, stomatal apertures for various crown concentrations were determined (Table II) and the values plotted to give the concentration of crown required just to inhibit stomatal opening. Some crowns were not sufficiently soluble in the buffer solution alone, and therefore were initially dissolved in the minimum amount of absolute alcohol and then diluted with buffer solution. The same quantity of alcohol was tested both on control epidermal peels and with water soluble crowns, and shown to have no effect on stomatal aperture.

The partition coefficients of crowns between n-octanol and 50 m*M* aqueous sodium chloride solution were determined by vigorously shaking 5 m*M* crown solution in octanol with 50 m*M* sodium chloride solution and subsequently measuring the crown concentration in each layer as before.

The cyclic polyethers were mostly prepared by literature methods [14], and the syntheses of acetylbenzo-18-crown-6, ethylbenzo-18-crown-6 and carboxybenzo-18-crown-6 will be described elsewhere [15]. All polyethers were either sublimed or crystallised to literature melting points before use.

Results and Discussion

In previous papers [2, 3] we have suggested that the action of crowns on stomata is governed by their

TABLE III. The Partition Coefficients of Crowns between n-Octanol and 50 m*M* NaCl Solution, and also the Minimum Concentrations of Crowns Just to Inhibit Stomatal Opening in 50 m*M* NaCl and 10 m*M* MES Buffered at pH 6.1.

Crown	Partition[a] Coefficient	$\log_{10}$ P	Minimum Concentration	$\log_{10}$ 1/C
Benzo-18-crown-6	2.67	0.42	6.04×10^{-3}	2.22
Acetylbenzo-18-crown-6	1.30	0.11	9.97×10^{-3}	2.00
Ethylbenzo-18-crown-6	22.9	1.36	1.05×10^{-3}	2.98
t-Butylbenzo-18-crown-6	108	2.03	4.5×10^{-4}	3.35
Benzo-15-crown-5	4.8	0.68	8.19×10^{-3}	2.09
			(4.1×10^{-3})[b]	(2.39)
t-Butylbenzo-15-crown-5	167	2.22	7.4×10^{-4}	3.13
			(3.7×10^{-4})[b]	(3.43)
Dibenzo-30-crown-10	62	1.79	4×10^{-4}	3.4

[a]Averages of several determinations. (Greatest error in log P of 0.1). [b]The effective concentration of crown for sandwich formation.

partition into the membranes of the guard cells. Once there, the ionophores make the membranes leaky in both directions to potassium ions which are therefore able to flow down their chemical concentration gradient. To test this model further, we have varied the concentration of potassium ions in the bathing medium in the presence of a fixed concentration of crown. This crown concentration is that which just inhibits stomatal opening when the bathing medium is at 50 m*M* KCl concentration. Increasing potassium ion concentration in the bathing medium in this way should reduce potassium leakage from the guard cells. The results are shown in Table III for two crowns, benzo-18-crown-6 and t-butylbenzo-15-crown-5, and indicate that, as the potassium concentration is increased, then so does the average stomatal opening observed under the incubation conditions employed. An alternative explanation is however possible, namely that the partition coefficient of the crown between the guard cell membrane and the aqueous salt solution may alter significantly as the potassium ion concentration in the aqueous phase is increased. We have therefore determined the partition coefficients of benzo-18-crown-6 and t-butylbenzo-15-crown-5 between n-octanol and aqueous KCl solutions in the concentration range 50–150 m*M*. The results show that, whilst the partition coefficient for benzo-18-crown-6 is halved, that for t-butylbenzo-15-crown-5 is only reduced by ~13% (Table Ib). However, the change in stomatal apertures for both crowns on going from 50 to 150 m*M* KCl are comparable, and hence a reduced rate of potassium ion leakage from the guard cells is linked with the increased external K^+ concentration and not simply due to a reduction in the concentration of crown in the guard cell membranes.

Cyclic polyethers are also able to coordinate other alkali metals apart from potassium. In particular, sodium ions also form ion–dipole complexes with the ether oxygens of the heterocyclic ring, but may exhibit different stoichiometries from the corresponding potassium complexes. For example, the 15-crown-5 ring series yield 2:1 ligand to metal sandwich complexes with potassium salts from polar solvents [16, 17], whereas sodium generally (but not always [18]) gives 1:1 complexes in which either solvent molecules or anions complete the coordination of the cation. We were therefore interested in studying the effect of a series of crown ether molecules on stomatal opening when the cation used in the bathing medium was sodium. Original studies [11, 12] had shown that sodium could take the place of potassium as the ion which is taken up into the guard cells on stomatal opening, although this view has been modified in the light of more recent work [13] which shows reduced responses to light, carbon dioxide and abscisic acid. Table II shows the response of stomatal opening of abaxial epidermal peels of *C. communis* incubated in CO_2-free air on a lightbox in 50 m*M* NaCl, 10 m*M* MES buffer when treated with various concentrations of benzo-18-crown-6. In this way it is possible to determine graphically the minimum crown concentration required just to inhibit stomatal opening, and the results obtained for a series of crowns are listed in Table III. The partition coefficient of each crown between n-octanol and 50 m*M* NaCl was also determined and are listed in the Table, together with $\log_{10}$ P (P = partition coefficient) and $\log_{10}$ 1/C (C = concentration of crown in moles just to inhibit stomatal opening). The Hansch approach was then applied by testing an empirical fit of the data to eqn. 1 [19].

TABLE IV. Least Squares Regression of Minimum Concentrations of Crown for Stomatal Inhibition against Partition Coefficients Using Sodium Ions.[a]

	1	*2*
a	0.64	0.7
b	1.94	1.97
R square	0.86	0.97
Res. error	0.04	0.01
Max. abs. res.	0.33	0.21

[a] *1*) Using concentration of crown in the bathing medium. *2*) Correcting the concentration of 15-crown-5 ligands to take account of sandwich formation. $C_{eff} = C/2$. For the equation $Y = aX + b$ ($Y = \log 1/C$, $X = \log P$). R square is $= -\Sigma(Y_i - g(X_i))^2/\Sigma(Y_i - Y)^2$ where $Y = \Sigma(Y_i/N)$. Residual error is $\Sigma(Y_i - g(X_i))^2/(N - 2)$. Maximum absolute error is $\max(|Y_i - g(X_i)|)$.

$$\log 1/C = a \log P + b \quad (1)$$

where a and b are constants. A reasonable straight line resulted [see Table IV]. However, our earlier potassium results [3] showed a significant improvement in the fit when the possibility of sandwich formation by 15-crown-5 rings in the membrane was considered. The effective crown concentration in the membrane is then halved, since two crown molecules are required for each complex. When these values are plotted in eqn. 1, a considerably better straight line fit is obtained (Table IV). Although most complexes of sodium ions with 15-crown-5 rings which have been isolated have been 1:1 stoichiometry, it has been possible by using bulky non-coordinating anions or a very concentrated solution of excess benzo-15-crown-5 to isolate 2:1 sandwich complexes [18]. The latter method, in particular, where there are few solvent molecules, would involve a lipophilic-like solution, similar to the environment likely to be encountered in a membrane, and hence sandwich formation for sodium ions in a membrane is reasonable. A comparison between the partition coefficients of sodium and potassium between n-octanol and water show up the differences between sodium and potassium coordination [Table V]. Thus, although sodium is capable of sandwich formation, the fit of the ion between the two ligands is not so good, and similarly with dibenzo-30-crown-10, sodium is not so well coordinated in a wrap-around complex as is potassium. There is also a greater tendency for sodium to form a 1:2 ligand to metal complex with larger rings such as dibenzo-24-crown-8 and dibenzo-30-crown-10. The complexes of sodium with benzo-15-crown-5 and dibenzo-30-crown-10 are therefore likely to be more polar than those of potassium with the same ligands. This may lead to a lower partition coefficient of the crown into octanol from aqueous sodium chloride than from aqueous potassium chloride. Complexes of both potassium and sodium with 18-crown-6 ring systems are likely to be more similar in their polarity, and the partition coefficients in this case may depend more upon their respective stability constants, which are greater for potassium.

TABLE V. Comparison of Sodium and Potassium Partition Coefficients between n-Octanol and Water.

Crown	Partition coefficient	
	Sodium	Potassium
Benzo-18-crown-6	2.67	1.44
Acetylbenzo-18-crown-6	1.30	0.91
Ethylbenzo-18-crown-6	22.9	9.5
Carboxybenzo-18-crown-6	1.5	0.53
t-Butylbenzo-18-crown-6	108	51
Benzo-15-crown-5	4.8	5.04
Dibenzo-15-crown-5	91	167
t-Butylbenzo-15-crown-5	167	181
Dibenzo-30-crown-10	62	70

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