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Effect of K^+ on the membrane functions of an alkalophilic *Bacillus*

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We have examined the involvement of K^+ in the membrane functions of a facultatively alkalophilic *Bacillus* at neutral and alkaline pH. The effects of K^+ on membrane functions, such as maintenance of the membrane potential, leucine uptake and respiratory activity, were dependent on the external pH. K^+ uptake, which induced alkalinization of the cytoplasm, is suggested to be electrogenic at neutral pH and 'electroneutral' at alkaline pH, resulting in a similar level of net accumulation. We suggest that the bacterial membrane is highly permeable to K^+ at neutral pH, compared to alkaline pH, which results in a pH-dependent effect of K^+ on the above membrane functions.

Introduction

Monovalent cations, such as Na^+ and K^+, are involved in important membrane functions for bacterial growth. For instance, Na^+ and K^+ participate in the regulatory systems for internal pH in several species of bacteria [1–8]. In alkalophilic bacteria, most attention has been paid to the role of Na^+. It has been suggested that the alkalophilic bacteria require Na^+ for amino acid transport, motility and acidification of the cytoplasm at alkaline pH [2,8–14]. A facultatively alkalophilic *Bacillus*, which exhibits growth over a wide pH range from 7 to 10.5 [15], requires both Na^+ and K^+ to regulate the internal pH by acidification and alkalinization of the cytoplasm at alkaline and neutral pH, respectively [8]. Loss of either of the acidification or alkalinization systems by mutation resulted in the loss of ability to grow at alkaline or neutral pH, respectively [16]. K^+ seems to be essential for the growth, especially at neutral pH, in *Bacillus*.

We have studied how K^+ is involved in the membrane functions of the bacterium at neutral and alkaline pH. We propose that membrane functions, such as maintenance of membrane potential ($\Delta\psi$), leucine uptake and respiratory activity, were affected by K^+ depending on the external pH. This pH-dependent effect of K^+ may result from a pH-dependent change of the K^+ permeability of the membrane.

Materials and Methods

Culture. A facultatively alkalophilic *Bacillus* YN-2000 [8,15] was used. The bacteria were aerobically grown at pH 10 at 37°C in the culture medium as previously described [15,17]. The cells, grown to a late logarithmic phase, were collected, washed and suspended in 20 mM Tris-H_3PO_4

Abbreviations: ΔpH, transmembrane pH gradient; $\Delta\psi$, membrane potential; $\Delta\bar{\mu}_{H^+}$, proton electrochemical potential.

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buffer (pH 8.2) containing 150 mM NaCl and 0.5 mM $MgCl_2$. The bacterial suspension was stored at 0 °C until used.

Transmembrane pH gradient (ΔpH) and membrane potential ($\Delta\psi$). The ΔpH and $\Delta\psi$ of the bacteria were determined by measuring the distribution across membranes of [^{14}C]methylamine or 5,5-dimethyl[^{14}C]oxazolidine-2,4-dione for ΔpH and [^{14}C]tetraphenylphosphonium for $\Delta\psi$ as described previously [8]. The reaction was started by adding 40 μl of a cell suspension to 6 ml of the reaction medium (medium A) consisting of 20 mM Tris-H_3PO_4, 150 mM NaCl, 0.5 mM $MgCl_2$, 3 mM ascorbate and 0.07 mM tetramethylphenylene diamine in the presence or absence of 5 mM KCl at pH 7.5 or 9. After 5-min incubation at 25 °C, the bacteria were collected on to a membane filter (TM-100, Toyo) by filtration. The filters were transferred into toluene-Triton (X-100) scintillation liquid and assayed for radioactivity in an Aloka liquid-scintillation system LSC-700 [8]. To study the effects of various monovalent cations on ΔpH and $\Delta\psi$, NaCl in medium A was replaced by the chloride salts of other cations. When the bacteria were suspended in buffer containing no Na^+ and stored at 0 °C, the bacteria appear to become unstable and the results were not reproducible. Thus, the bacteria were suspended and stored in the buffer containing 150 mM NaCl as described above, and an application of the suspension introduced 1 mM NaCl to the reaction mixture. The proton electrochemical potential ($\Delta\bar{\mu}_{H^+}$) was calculated from the equation, $\Delta\bar{\mu}_{H^+} = \Delta\psi - Z\Delta pH$ ($Z = 2.3RT/F$, where F is the Faraday constant) using the experimental values determined for ΔpH and $\Delta\psi$.

Leucine uptake. After incubating the bacteria for 2 min at 25 °C in 20 mM Tris-H_3PO_4 buffer containing 150 mM NaCl or RbCl, 0.5 mM $MgCl_2$, 3 mM ascorbate and 0.07 mM tetramethylphenylenediamine in the presence or absence of 5 mM KCl at pH 7.5 or 9, leucine uptake was initiated by the addition of 10 μM [^{14}C]leucine (16 Ci/mol, Amersham International). Control experiments were carried out in the presence of 1 μM gramicidin, as this inhibits the uptake reaction. The bacteria were then collected on a membrane filter (TM-100, Toyo) by filtration and assayed for radioactivity.

K^+ uptake. K^+ uptake was measured at 25 °C as described previously [8].

Respiratory activity. Respiratory activity was measured at 25 °C in 20 mM Tris-H_3PO_4 buffer containing 150 mM NaCl, 0.5 mM $MgCl_2$, 3 mM ascorbate and 0.07 mM tetramethylphenylenediamine in the absence or presence of KCl at various pH values, using a Clark-type oxygen electrode (Model 5/6 H Oxygraph, Gilson).

Fluorescence. The change of $\Delta\psi$ was assayed at 25 °C by following the fluorescence intensity of Rhodamine 6G as described previously [8].

Protein determination. Protein concentration was determined by the method of Gornal et al. [18].

Results and Discussion

Effects of various monovalent cations on the proton electrochemical potential ($\Delta\bar{\mu}_{H^+}$) and its components

We have earlier suggested that the facultatively alkalophilic *Bacillus* acidifies and alkalinizes the cytoplasm through an exchange system of Na^+ and K^+ for H^+ at alkaline and neutral pH, respectively [8]. The internal pH, which was decreased in the presence of Na^+, was raised by the addition of K^+, and the resulting increase of ΔpH was compensated for by the decrease of $\Delta\psi$, so that $\Delta\bar{\mu}_{H^+}$ was maintained. Such a compensatory change of $\Delta\psi$ for the maintenance of $\Delta\bar{\mu}_{H^+}$ was studied only in the presence of Na^+ [8]. In order to examine how $\Delta\bar{\mu}_{H^+}$ is maintained in the absence and presence of K^+ without the addition of Na^+ at neutral and alkaline pH, ΔpH and $\Delta\psi$ were measured in the presence of various monovalent cations at pH 7.5 and 9.

In the presence of Na^+ alone, the internal pH of the bacteria became lower than the external pH of 7.5 and 9 (Table I). This may indicate acidification of the cytoplasm by Na^+ through operation of the Na^+/H^+ exchange system [8]. On addition of KCl, the internal pH increased at both pH values studied, 7.5 and 9, and the internal pH became higher than the external pH at pH 7.5. At pH 9, however, the internal pH was still lower than the external pH (Table I). In the presence of K^+ alone, the internal pH was only slightly higher than the external pH at both pH values studied, 7.5 and 9. Acidification of the cytoplasm was also observed with Li^+. These results suggest that only

TABLE I

EFFECTS OF VARIOUS MONOVALENT CATIONS ON $\Delta\bar{\mu}_{H^+}$ AND ITS COMPONENTS

Total concentrations of chloride salts of monovalent cations in the reaction mixture were maintained at 150 and 155 mM in the absence and presence of KCl, respectively. NaCl was always present at 1 mM (see Materials and Methods). pH_o and pH_i represent external and internal pH, respectively. Δ values are expressed as the $values_{internal} - values_{external}$.

pH_o	Cation	KCl (5 mM)	pH_i	$-Z\Delta pH$ (mV)	$\Delta\psi$ (mV)	$\Delta\bar{\mu}_{H^+}$ (mV)
7.5	Na^+	−	7.0	30	−145	−115
		+	8.0	−30	−80	−110
	Li^+	−	7.2	18	−141	−123
		+	7.8	−18	−87	−105
	K^+	−	7.8	−18	−114	−132
	Rb^+	−	7.8	−18	−98	−116
		+	8.0	−30	−71	−101
	Cs^+	−	7.6	−6	−96	−102
		+	7.9	−24	−83	−107
9.0	Na^+	−	8.1	53	−157	−104
		+	8.5	30	−131	−101
	Li^+	−	8.3	41	−152	−111
		+	8.7	18	−134	−116
	K^+	−	9.1	−6	−151	−157
	Rb^+	−	9.0	0	−139	−139
		+	9.1	−6	−132	−138
	Cs^+	−	9.0	0	−135	−135
		+	9.2	−12	−124	−136

Li^+ is able to substitute for Na^+ in the Na^+/H^+ exchange system. In contrast to Na^+ and Li^+, addition of Rb^+ or Cs^+ alone caused a slight rise in the internal pH at pH 7.5, but remained the same as the external pH at pH 9. Adding K^+, increased the internal pH only slightly in the presence of Rb^+ or Cs^+ at pH 7.5 and 9. Increase of internal pH by the addition of KCl was accompanied by a decrease of $\Delta\psi$, and $\Delta\bar{\mu}_{H^+}$ was maintained almost constant in the presence of Na^+, Li^+, Rb^+ or Cs^+ at pH 7.5 and 9 (Table I). In the presence of K^+ alone, $\Delta\bar{\mu}_{H^+}$ was higher than in the presence of other cations at pH 7.5 and 9. In the presence of Na^+, Li^+, Rb^+ or Cs^+, $\Delta\bar{\mu}_{H^+}$ was almost the same independent of the cations at pH 7.5, but lower by approx. 30 mV in the presence of Na^+ or Li^+ than in the presence of Rb^+ or Cs^+ at pH 9. The differences in the magnitudes of $\Delta\bar{\mu}_{H^+}$ at pH 9 can be mainly ascribed to the lower magnitudes of ΔpH in the presence of Na^+ or Li^+, which resulted from acidification of the cytoplasm. We proposed earlier that the exchange system of Na^+ for H^+ exhibits an optimum activity at alkaline pH [8]. In the presence of Na^+ or Li^+ at alkaline pH, ΔpH may be partially converted to sodium chemical potential (ΔpNa^+) or lithium chemical potential (ΔpLi^+) by the operation of the exchange system of Na^+ or Li^+ for H^+.

Effects of Na^+ and K^+ on the leucine uptake

In order to examine whether the change of components of $\Delta\bar{\mu}_{H^+}$ (ΔpH and $\Delta\psi$) by the addition of KCl affects the energy coupling system, such as amino acid transport, the leucine uptake of *Bacillus* was measured at pH 7.5 and 9. In the presence of Na^+ alone, the bacteria exhibited a similar uptake of leucine at pH 7.5 and 9 (Fig. 1). When Na^+ was replaced by Rb^+, leucine uptake was very low at pH 7.5 and 9. Since $\Delta\bar{\mu}_{H^+}$ was not lower in the presence of Rb^+ than in the presence of Na^+ at pH 7.5 and 9 (Table I), the very low uptake of leucine in the presence of Rb^+ suggests that leucine uptake of this *Bacillus* is not driven by $\Delta\bar{\mu}_{H^+}$ alone, but also depends on the presence of Na^+ as indicated in the amino acid transport of other alkalophiles

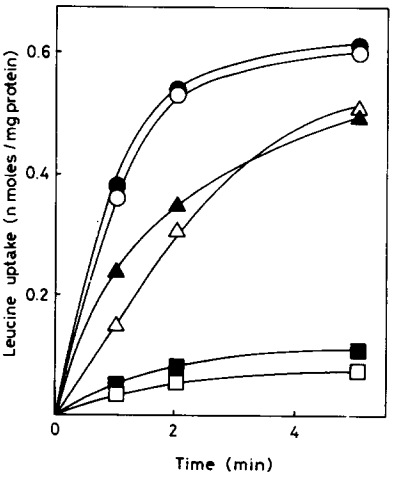

Fig. 1. Effects of Na^+, Rb^+ and K^+ on leucine uptake at pH 7.5 (○, △, □) and 9 (●, ▲, ■). The reactions were carried out in the medium containing 150 mM RbCl (□, ■), or 150 mM NaCl in the absence (○, ●) or presence (△, ▲) of 5 mM KCl, respectively.

[9–12]. In the presence of Na^+ at pH 7.5, the leucine uptake rate was decreased dramatically by the addition of KCl. This retardation of leucine uptake rate was also observed at pH 9, although to a lesser extent compared to that at pH 7.5. Since the addition of KCl decreased $\Delta\psi$ and increased ΔpH, this retardation suggests that leucine uptake is not driven by ΔpH (alkaline, inside). The decrease of $\Delta\psi$ by the addition of KCl was larger at pH 7.5 than at pH 9 (Table I). These results suggest that the retardation of Na^+-dependent uptake of leucine by KCl is due to a decrease of $\Delta\psi$. Thus, together with the result that $\Delta\bar{\mu}_{H^+}$ was kept constant irrespective of the addition of KCl (Table I), the driving force is considered to be $\Delta\psi$ or the sodium electrochemical potential ($\Delta\bar{\mu}_{Na^+}$), and not $\Delta\bar{\mu}_{H^+}$.

pH-dependent effect of K^+ on $\Delta\psi$ and respiratory activity

As shown in Table I, the decrease of $\Delta\psi$ by the addition of KCl seems to be dependent on the external pH. Fig. 2 shows the effect of external pH on the decrease of $\Delta\psi$ by the addition of KCl. The depolarization of $\Delta\psi$ decreased considerably with increasing pH. The addition of KCl also accelerated the respiratory activity (Fig. 2). The acceleration of respiratory activity by the addition of KCl was observed at neutral pH and reduced with the increasing pH, in parallel with the decrease in depolarization of $\Delta\psi$. The decrease of $\Delta\psi$ on addition of KCl probably accelerates the respiratory activity so that $\Delta\bar{\mu}_{H^+}$ may be maintained constant.

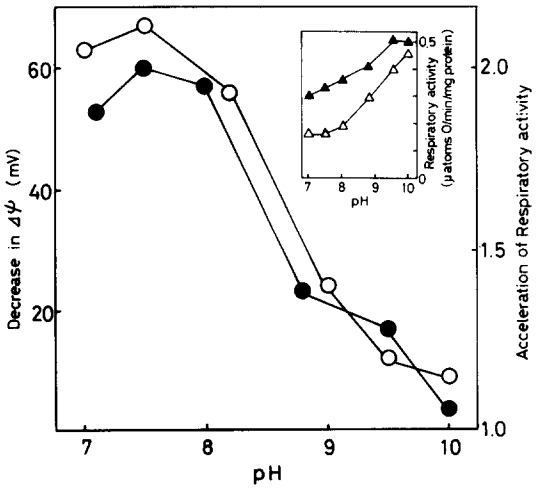

Fig. 2. Effect of pH on the decrease of $\Delta\psi$ (○) and acceleration of the respiratory activity (●) by the addition of KCl. Acceleration of the respiratory activity was expressed by the ratio of the respiratory rate in the presence of 5 mM KCl to that in the absence of 5 mM KCl at the respective pH. The inset shows the absolute rates of respiration in the absence (△) and presence (▲) of KCl.

Electrogenicity of K^+ uptake

We have earlier suggested that proton efflux for alkalinization of the cytoplasm by addition of KCl at neutral pH could be due to a decrease of $\Delta\psi$ through the electrogenic uptake of K^+, which was completed within 2 min after the addition of KCl [8]. Therefore, the larger decrease of $\Delta\psi$ by the addition of KCl at neutral pH than at alkaline pH may suggest that the net accumulation of K^+ through electrogenic uptake is higher at neutral pH than at alkaline pH. In order to examine this possibility, the net accumulation of K^+ was determined at various pH values, and was shown to be almost constant at pH 7.5–10 (0.4–0.5 μmol K^+/mg protein). This result rules out the possibility mentioned above and suggests that the apparent mechanism of K^+ transport in *Bacillus* is different at neutral and alkaline pH: the bacteria seem to take up K^+ by an electrogenic mechanism at neutral pH and rather 'electroneutrally' at alkaline pH, resulting in the similar level of net accumulation of K^+.

K^+ permeability of the membrane

The pH-dependent difference in the electrogenicity of K^+ transport might be due to a pH-dependent change of K^+ permeability of the membrane. This possibility was examined by the change of $\Delta\psi$, followed by fluorescence change of Rhodamine 6G on addition of KCl at pH 7.5 and 10 [8,19]. When the cells were added to medium containing Rhodamine 6G at pH 7.5, the fluorescence was quickly quenched (Fig. 3A). This quenching depended on the magnitude of $\Delta\psi$; the depolarization of $\Delta\psi$ resulted in the recovery of the fluorescence intensity [8]. When KCl was added at a steady level of quenching, the fluorescence intensity instantaneously increased to a new level, and then decreased gradually as a result of the

uptake of K^+ into the cells, reaching a stationary level (Fig. 3A; see also Ref. 8). The ratio (increase of fluorescence intensity (ΔI) immediately after addition of KCl to fluorescence intensity (I) before addition of KCl) were considerably higher at pH 7.5 than those at pH 10 (Fig. 3B). The ratio of the increase of fluorescence at the stationary level after addition of KCl to the fluorescence (I) before addition of KCl was also higher to the similar extent at pH 7.5 than at pH 10 (data not shown), suggesting that the depolarization at the steady state after addition of KCl was higher at pH 7.5. The fact is consistent with the result shown by the determination of the stationary level of distribution of [^{14}C]tetraphenylphosphonium across the membrane, in which the degree of depolarization caused by the addition of 5 mM KCl was suggested to be 76 and 11 mV at pH 7.5 and 10, respectively [8]. The values of ratio ($\Delta I/I$) increased linearly with the logarithm of KCl concentration in the range from 1 to 10 mM at pH 7.5 and 10 (Fig. 3B). This result suggests that the ratio ($\Delta I/I$) corresponds relatively to the change of $\Delta\psi$ [19,20], and together with the result of the stationary level of fluorescence as described above, supports that the change of fluorescence of Rhodamine 6G reflects that of $\Delta\psi$ in the bacterium.

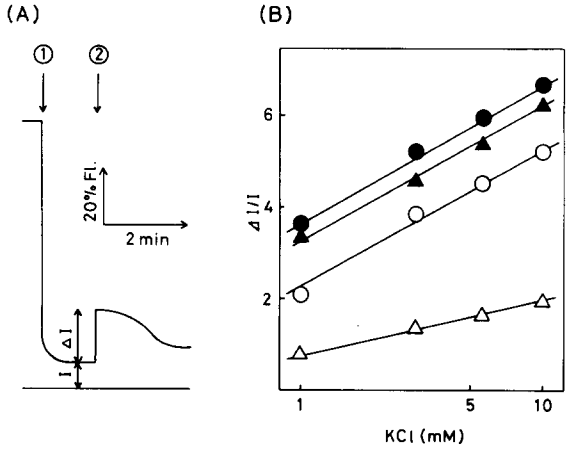

Fig. 3. Change in the fluorescence intensity of Rhodamine 6G by the addition of KCl (A) and as a function of KCl concentration at pH 7.5 and 10 (B). (A) At the times indicated by arrows 1 and 2, the cells and 1 mM KCl were added to the medium containing 1 μM Rhodamine 6G at pH 7.5, respectively. (B) The measurements were carried out in the absence (○, △) and presence (●, ▲) of 0.1 μM valinomycin at pH 7.5 (○, ●) and 10 (△, ▲), respectively.

The instantaneous increase after addition of KCl shown in Fig. 3A reflects the decrease of $\Delta\psi$ depending on K^+ permeability of the membrane. When the membrane became completely permeable to K^+ by the addition of valinomycin, the ratios ($\Delta I/I$) were almost the same at pH 7.5 and 10, and higher than those without addition of valinomycin (Fig. 3B). The higher value of ratio ($\Delta I/I$) at pH 7.5 in the absence of valinomycin suggests that the membrane was more permeable to K^+ at pH 7.5 as compared with that at pH 10. When the membrane is permeable to K^+, the $\Delta\psi$ (negative, inside) drives K^+ into the cells accompanied by depolarization of $\Delta\psi$. We, therefore, suggest that the higher depolarization of $\Delta\psi$ by the addition of KCl at neutral pH than at alkaline pH was due to the higher K^+ permeability of the membrane at neutral pH than at alkaline pH. The depolarization of $\Delta\psi$ allows the cells to expel more protons, which results in an increase of the internal pH [1,21]. The high K^+ permeability of the membrane at neutral pH contributes to the alkalinization of the cytoplasm on addition of K^+ at neutral pH.

In the present work, we suggest that the pH dependent decrease of $\Delta\psi$ on addition of K^+ was due to the pH-dependent difference in the electrogenicity of K^+ transport which depends on K^+ permeability of the membrane. On addition of K^+, the pH-dependent change of K^+ permeability of the membrane is likely to affect the degree of the depolarization of $\Delta\psi$ resulting from the uptake of K^+, and consequently affect the leucine uptake, respiratory activity and regulation of the internal pH. It was suggested that the decrease of $\Delta\psi$ and alkalinization of the cytoplasm at neutral pH was a specific effect of K^+, and other monovalent cations were not able to substitute for K^+ [8]. The pH-dependent change of K^+ permeability of the membrane may depend on the opening and closing of the specific channel for K^+. The further studies will be needed for the elucidation of the mechanism.

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