Roles of K^+ and Na^+ in pH homeostasis and growth of the marine bacterium *Vibrio alginolyticus*

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The marine bacterium *Vibrio alginolyticus*, containing 470 mM- K^+ and 70 mM- Na^+ inside its cells, was able to regulate the cytoplasmic pH (pH_{in}) in the narrow range 7.6–7.8 over the external pH (pH_{out}) range 6.0–9.0 in the presence of 400 mM- Na^+ and 10 mM- K^+ . In the absence of external K^+ , however, pH_{in} was regulated only at alkaline pH_{out} values above 7.6. When the cells were incubated in the presence of unusually high K^+ (400 mM) and 4 mM Na^+ , the pH_{in} was regulated only at acidic pH_{out} values below 7.6. These results could be explained by postulating a K^+/H^+ antiporter as the regulator of pH_{in} over the pH_{out} range 6.0–9.0. When Na^+ -loaded/ K^+ -depleted cells were incubated in 400 mM- Na^+ in the absence of K^+ , an inside acidic ΔpH was generated at pH_{out} values above 7.0. After addition of diethanolamine the inside acidic ΔpH collapsed transiently and then returned to the original value concomitant with the extrusion of Na^+ , suggesting the participation of a Na^+/H^+ antiporter for the generation of an inside acidic ΔpH . In the presence of 400 mM- K^+ , at least 5 mM- Na^+ was required to support cell growth at pH_{out} below 7.5. An increase in Na^+ concentration allowed the cells to grow at a more alkaline pH_{out} . Furthermore, cells containing more Na^+ inside could more easily adapt to grow at alkaline pH_{out} . These results indicated the importance of Na^+ in acidification of the cell interior via a Na^+/H^+ antiporter in order to support cell growth at alkaline pH_{out} under conditions where the activity of a K^+/H^+ antiporter is marginal.

Introduction

Bacterial cells have the capacity to regulate cytoplasmic pH (pH_{in}) at around neutrality regardless of their external pH (pH_{out}) (Booth, 1985). For example, the pH_{in} of *Vibrio alginolyticus* (Nakamura *et al.*, 1984) and *Escherichia coli* (Slonczewski *et al.*, 1981) is regulated at about 7.6–7.8 over the pH_{out} range 6.0–9.0. At pH_{out} values below 7.6 the generation of an inside alkaline ΔpH is necessary for the regulation of pH_{in} . Since the pH_{in} under an acidic pH_{out} condition is regulated only in the presence of K^+ , the uptake of K^+ is considered to help in the generation of an alkaline pH_{in} (Tokuda *et al.*, 1981; Kroll & Booth, 1983). However, it is not clear how the magnitude of ΔpH is controlled depending on pH_{out} .

At pH_{out} values above 7.6, the generation of an inside acidic ΔpH is necessary and Na^+/H^+ and K^+/H^+ antiporters were implicated to function in achieving this (Brey *et al.*, 1980). In alkalophilic bacteria, it became apparent that a Na^+/H^+ antiporter acidifies pH_{in} , co-operating with the H^+ -extruding respiratory chain (Mandel *et al.*, 1980; Garcia *et al.*, 1983). Mutants of *E. coli* defective in the Na^+/H^+ antiporter grow poorly in alkaline media (Ishikawa *et al.*, 1987; Padan *et al.*, 1989) and the antiporter was shown to be required for growth of *E. coli* under alkaline pH_{out} (McMorrow *et al.*, 1989). Na^+/H^+ antiporter genes (*nhaA*) of *E. coli* (Niiya *et al.*, 1982; Goldberg *et al.*, 1987; Karpel *et al.*, 1988; Padan *et al.*, 1989) and *Bacillus firmus* (Ivey *et al.*, 1991) have been cloned and sequenced. The *E. coli* NhaA protein has been purified and reconstituted in proteoliposomes and shown to function as a Na^+/H^+ antiporter (Taglicht *et al.*, 1991). However, it is uncertain whether this antiporter functions as a regulator of pH_{in} .

Although the pH_{in} of *V. alginolyticus* was acidified by Na^+/H^+ antiporter activity at alkaline pH_{out} , the pH_{in} was not regulated at a constant value in the absence of K^+ (Nakamura *et al.*, 1984). Since the K^+/H^+ antiporter

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Abbreviations: ΔP , protonmotive force; ΔpH , chemical gradient of H^+ ; ΔpK , chemical gradient of K^+ ; ΔpNa , chemical gradient of Na^+ ; pH_{in} , internal pH; pH_{out} , external pH.

of this organism is strongly dependent on the pH_{in} and only active at pH_{in} values above 7.6, we proposed that the K^+/H^+ antiporter functions as a regulator of pH_{in} at alkaline and acidic pH_{out} (Nakamura *et al.*, 1984). To test this hypothesis, we examined the effects of K^+ and Na^+ on pH homeostasis and the role of Na^+ for growth of *V. alginolyticus* at alkaline pH_{out} .

Methods

Preparation of normal and Na^+ -loaded cells. *V. alginolyticus* strain 138-2 was grown aerobically at 37 °C in a synthetic medium containing 0.3 M-NaCl, 10 mM-KCl, 2 mM- K_2HPO_4 , 15 mM- $(NH_4)_2SO_4$, 10 μ M- $FeSO_4$, 5 mM- $MgSO_4$, 1% (w/v) glycerol, 50 mM-Tris/HCl (pH 7.5). The cells were harvested in the exponential phase of growth and then washed with 0.4 M-NaCl by centrifugation. These cells contained 470 mM- K^+ and 70 mM- Na^+ and are referred to as normal cells. Na^+ -loaded/ K^+ -depleted cells were prepared using diethanolamine treatment as previously described (Nakamura *et al.*, 1982). These cells contained about 400 mM- Na^+ and less than 1 mM- K^+ .

Growth conditions. Cells were grown aerobically in a Monod-tube at 37 °C and growth was monitored by measuring the optical density (OD) at 600 nm in a Perkin-Elmer model 35 spectrophotometer. The culture doubling time was calculated from the linear part of the exponential growth curve. For measurement of cell growth at alkaline pH_{out} in the presence of 0.5 M- K^+ (see Fig. 4), the cells were first cultured in a complex medium containing 0.5% (w/v) yeast extract and 0.5% (w/v) polypeptone, supplemented with 0.4% (w/v) K_2HPO_4 and 0.5 M-NaCl (pH 7.4). When the culture density reached an OD at 600 nm of 0.4, a 1/50 vol. of the culture was inoculated into fresh complex medium of specific pH, supplemented with 0.5 M-KCl and buffer. Complex medium of appropriate pH was prepared by mixing a volume of complex medium containing 50 mM-HEPES, 10 mM-KOH (pH 6.6) with a volume of complex medium containing 50 mM-Tris, 25 mM-CHES, 10 mM-KOH (pH 9.3). Since the complex medium contains about 5 mM- Na^+ , and 10 mM- Na^+ is carried over from the preculture, the concentration of Na^+ in the growth medium was about 15 mM. Taking this into consideration, further Na^+ was added as required. To adjust the Na^+ concentration to 5 mM in the experiments depicted in Fig. 4, the cells were first cultured in the synthetic medium described above except that the salts were replaced with 0.5 M-KCl and 5 mM-NaCl and the pH was adjusted to 7.0.

To prepare cells containing high or low internal Na^+ (see Fig. 5), the cells were first cultured in complex medium containing 56 mM-MES, 10 mM-Tris and 15 mM-KOH (pH 6.8) and supplemented with either 0.4 M-NaCl or 0.4 M-KCl. The former cells contained 70 mM- Na^+ and the latter less than 5 mM- Na^+ . The culture was then diluted 8-fold with complex medium containing 0.4 M-KCl, 25 mM-TAPS, 50 mM-Tris, 15 mM-KOH (pH 8.8). The final Na^+ concentration in the growth medium was adjusted to 55 mM.

Measurement of internal pH, and internal Na^+ and K^+ concentrations. The inside alkaline or acidic ΔpH was determined from the distribution of either [^{14}C]acetylsalicylic acid (New England Nuclear) or di[^{14}C]methylamine (Amersham) as described previously (Nakamura *et al.*, 1984). Intracellular Na^+ and K^+ concentrations were determined by the filtration method and flame photometry, also as described previously (Nakamura *et al.*, 1982).

Protein-assay. Protein concentration was determined by the Lowry method with bovine serum albumin as standard.

Fig. 1. Effects of K^+ and Na^+ on regulation of pH_{in} at various pH_{out} values. Normal cells of *V. alginolyticus* containing 470 mM- K^+ and 70 mM- Na^+ were incubated in K^+ -medium (●) or Na^+ -medium (▲) for 6–8 min at 25 °C and then the pH_{in} at the respective pH_{out} was determined as described in Methods. The K^+ -medium contained 0.3 M-KCl, 0.1 M-potassium phosphate (pH 6.0–7.7) or 0.4 M-KCl, 50 mM Tris/HCl (pH 8.0–9.0). The Na^+ -medium was prepared by replacing all of the K^+ in the above medium with Na^+ .

Results

Effects of K^+ and Na^+ on the regulation of pH_{in}

When normal cells of *V. alginolyticus* were incubated in a medium containing 400 mM- Na^+ and 10 mM- K^+ , the pH_{in} was regulated within 7.6–7.8 over the pH_{out} range 6.0–9.0 (Nakamura *et al.*, 1984). Since an outwardly directed K^+ chemical gradient (ΔpK) was required for the regulation of pH_{in} at pH_{out} above 7.6, the normal cells were incubated in a medium containing 400 mM- K^+ to minimize the magnitude of ΔpK . As shown in Fig. 1, the inside acidic ΔpH was not generated at pH_{out} values above 7.6, but the pH_{in} was regulated at pH_{out} values below 7.6. The Na^+ -loaded/ K^+ -depleted cells gave the same results (data not shown).

Conversely, when normal cells were incubated in a medium containing 400 mM- Na^+ in the absence of K^+ , the pH_{in} was regulated only at alkaline pH_{out} and an inside alkaline ΔpH was not generated at acidic pH_{out} (Fig. 1). Although the cells retained about 470 mM- K^+ and a large ΔpK was maintained under these conditions, the pH_{in} was not regulated at acidic pH_{out} . The addition of more than 1 mM- K^+ was necessary for the regulation of pH_{in} . These results clearly indicated that for pH homeostasis the presence of ΔpK is essential at alkaline, but not acidic, pH_{out} values and that the presence of K^+ in the external medium is essential at acidic, but not alkaline, pH_{out} values. The requirement for external K^+ in regulation of pH_{in} at acidic pH_{out} suggested that

Fig. 2. Time course of changes in pH_{in} after addition of KCl at different pH_{out} values. Na^+ -loaded cells were suspended in 0.3 M-NaCl, 20 mM-glycerol and 0.1 M-sodium phosphate at pH 6.1 ($\triangle$), or 6.8 ($\circ$) or in 0.4 M-NaCl and 50 mM-Tricine/NaOH at pH 9.0 ($\square$). After 2 min incubation, 10 mM-KCl was added (filled symbols) and the pH_{in} was then determined.

alkalinization of the cytoplasm induced by the K^+ uptake is a prerequisite for pH homeostasis.

As shown previously (Nakamura *et al.*, 1984), Na^+ -loaded/ K^+ -depleted cells incubated in the absence of K^+ generate an inside acidic ΔpH at pH_{out} values above 7.0, but the addition of 10 mM- K^+ is necessary to maintain a constant pH_{in} . To examine the effect of K^+ uptake on regulation of pH_{in} , time courses of changes in pH_{in} after addition of K^+ were measured. The pH_{in} of the Na^+ -loaded cells suspended in medium of pH_{out} 6.1 and 6.8 was the same as the respective pH_{out} (Fig. 2). Within 2 min of the addition of 10 mM- K^+ the pH_{in} was alkalinized concomitant with K^+ uptake and was regulated at about pH 7.6 within 10 min. At a pH_{out} of 6.8, the pH_{in} was alkalinized to 7.8 within 3 min of addition of K^+ and then slightly acidified to a pH_{in} of 7.6. When Na^+ -loaded cells were suspended in medium of pH 9.0, the pH_{in} was maintained at about 7.8; upon addition of K^+ , the pH_{in} immediately changed to 8.3 and then gradually decreased to 7.8. These results indicated that the K^+ uptake induces alkalinization of the cell interior over the pH_{out} range 6.0–9.0, and that the pH_{in} is regulated by an acidification mechanism. Similar results have been reported for *E. coli* (Kroll & Booth, 1983).

Generation of inside acidic ΔpH by the Na^+/H^+ antiporter

To examine the generation of inside acidic ΔpH by Na^+ -loaded cells at pH_{out} above 7.0, the ΔpH was disrupted by addition of diethanolamine and changes in cellular Na^+ and pH_{in} were then measured. As shown in Fig. 3, cellular Na^+ was extruded against its chemical gradient

Fig. 3. Effects of diethanolamine on the extrusion of Na^+ at different pH_{out} values. The Na^+ -loaded cells were suspended in a reaction mixture containing 0.4 M-NaCl, 20 mM-glycerol and 4 mM-HEPES/NaOH (pH 6.6) and pre-incubated for 5 min at 25 °C ($\circ$). After 5 min, 40 mM-diethanolamine was added so as to adjust the pH of the medium to 7.0 ($\square$), 7.5 ($\triangle$), 8.0 ($\blacktriangle$), 8.5 ($\blacksquare$) or 9.0 ($\bullet$). At various times, cellular Na^+ was determined by a filtration method (—). For inhibitor experiments, the cells were pre-incubated for 5 min in the presence of 10 mM-KCN ($\bullet$ --- $\bullet$) or 50 μ M-HQNO ($\blacksquare$ --- $\blacksquare$); diethanolamine was then added to adjust the pH to 9.0 and the cellular Na^+ determined (---).

at pH_{out} values of 7.5–9.0 after addition of 40 mM-diethanolamine, and the extent of Na^+ efflux varied with pH_{out} . For example, at pH_{out} values of 7.5, 8.0, 8.5 and 9.0, the amounts of Na^+ extruded 7 min after addition of diethanolamine corresponded to 96, 182, 274 and 222 mM, respectively, and the pH_{in} was determined to be 7.1, 7.1, 7.5 and 7.8, respectively. These pH_{in} values were identical to those before addition of diethanolamine. Thus, the inside acidic ΔpH , disrupted by addition of diethanolamine, was regenerated by the extrusion of Na^+ and the pH_{in} returned to the respective original value within 7 min. Since diethanolamine distributes between the inside and outside of cells according to the magnitude of the inside acidic ΔpH , the amount of diethanolamine within the cells was calculated at the respective pH_{out} . This value coincided with the amount of Na^+ extruded at the respective pH_{out} . Thus, diethanolamine acted as a counter-cation for the Na^+ extruded. At a pH_{out} of 7.0, Na^+ was not extruded from the cells by addition of diethanolamine due to the absence of an inside acidic ΔpH . The Na^+ extrusion observed here was inhibited by 10 mM-KCN, but 50 μ M-HQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide) which is a specific inhibitor of the Na^+ pump (Tokuda & Unemoto, 1984), only delayed Na^+ extrusion (Fig. 3). These results indicated that Na^+ extrusion and the generation of an inside acidic ΔpH are apparently mediated by the functioning of the Na^+/H^+ antiporter, which is driven by a protonmotive force, ΔP . However, it should be emphasized that the pH_{in} maintained by the Na^+/H^+ antiporter at alkaline

Fig. 4. Effects of Na^+ and pH_{out} on the growth rate of cells in complex medium containing 0.5 M-KCl and 5 mM- (●), 15 mM- (▲) and 50 mM- (■) Na^+ at various pH_{out} values. Medium pH and Na^+ concentration were adjusted as described in Methods. Growth rate was calculated from the exponential phase of the growth curve and is expressed as the number of doublings h^{-1} .

pH_{out} varies with pH_{out} and the addition of K^+ is indispensable to the maintenance of a constant pH_{in} .

Effect of Na^+ on cell growth at alkaline pH_{out} in the presence of a high concentration of K^+

Since the presence of high concentrations of K^+ disrupts pH homeostasis at alkaline pH_{out} (Fig. 1) and since the Na^+/H^+ antiporter acidifies the cell interior, the effect of Na^+ on cell growth in the presence of 0.5 M- K^+ was examined. The marine *V. alginolyticus* is unable to grow in the absence of Na^+ . As shown in Fig. 4, growth was supported in the presence of 5 mM- Na^+ at pH_{out} values below 7.5. The inability of the cells to grow at a pH_{out} above 7.5 may be related to the difficulty in regulating pH_{in} under these conditions (see Fig. 1). Interestingly, an increase in Na^+ concentration allowed the cells to grow at more alkaline pH_{out} values. For example, in the presence of 15 and 50 mM- Na^+ , cells grew at pH_{out} values up to 8.1 and 8.8, respectively (Fig. 4). Precultured cells inoculated into fresh medium containing a high K^+ concentration were unable to grow immediately after the transfer, especially at alkaline pH_{out} , and the length of the lag phase increased with the increase in medium pH. The lag phases were shortened by increasing the concentration of Na^+ in the medium.

For acidification of the cell interior by the Na^+/H^+ antiporter, the externally added Na^+ must previously be transferred into the cells. Fig. 5 shows the effect of internal Na^+ on cell growth in the complex medium

Fig. 5. Effects of cellular Na^+ on adaptation to complex medium containing 0.4 M-KCl and 55 mM- Na^+ at pH 8.8. The cells were precultured in complex medium containing either 0.4 M- NaCl (▲) or 0.4 M-KCl (●) at pH 6.8. Each preculture was diluted 8-fold with fresh complex medium containing 0.4 M-KCl at pH 8.8. The final Na^+ concentration was adjusted to 55 mM. In one experiment (■), the cells were precultured in complex medium containing 0.4 M-KCl and 55 mM- Na^+ at pH 8.8, and then diluted 8-fold with fresh complex medium containing 0.4 M-KCl at pH 6.8.

containing 0.4 M-KCl and 55 mM- NaCl at pH 8.8. As was expected, cells containing 70 mM- Na^+ were able to grow after a short lag phase, whereas cells containing less than 5 mM- Na^+ required a long lag phase before growth. In the latter case, a significant decrease in optical density of the culture was observed during the lag phase, which was confirmed to be partly caused by cell death. Since the transfer of cells growing in medium of high K^+ concentration from pH_{out} 8.8 to pH_{out} 6.8 resulted in no lag phase (Fig. 5), the lag phase observed here was apparently related to the regulation of pH_{in} . Thus, cells seem to be fragile in medium of high K^+ concentration at alkaline pH under conditions where the cell interior was not acidified.

The pH_{in} of exponentially growing cells in the presence of 0.4 M-KCl and 55 mM- NaCl at pH 8.8 was maintained at about 7.5–7.7 (data not shown). Thus, the inside acidic ΔpH was maintained in the growing cells indicating that the pH_{in} was not strictly regulated.

Discussion

The present results clearly indicate that the K^+ requirement for pH homeostasis is not identical at acidic and

alkaline pH_{out} . At alkaline pH_{out} the presence of an outwardly directed ΔpK is indispensable, but the addition of K^+ to the external medium is not necessary for regulation of pH_{in} (Fig. 1). This means that the ΔpK is required for the generation of an inside acidic ΔpH . Since the electroneutral K^+/H^+ antiporter converts ΔpK to ΔpH until the pH_{in} becomes about 7.8 and then ceases to function at pH_{in} below 7.6 (Nakamura *et al.*, 1984), the ΔpK is apparently utilized as the driving force for the K^+/H^+ antiporter to maintain pH_{in} at about 7.6–7.8. Indeed, ΔpK is the only effective driving force, especially at alkaline pH_{out} .

Conversely, at acidic pH_{out} the addition of K^+ to the external medium is indispensable, even in the presence of a large magnitude of ΔpK (Fig. 1). The uptake of K^+ allows more H^+ to be extruded by the primary H^+ pumps and thus alkalinizes the cell interior (Fig. 2). The K^+/H^+ antiporter is active at pH_{in} values above 7.6; thus it may be driven by the inwardly directed H^+ gradient when the pH_{in} is alkalinized above 7.6 at acidic pH_{out} , even in the absence of a ΔpK . The K^+/H^+ antiporter may regulate pH_{in} even at acidic pH_{out} . The difference in the K^+ requirement for the pH homeostasis observed at acidic and alkaline pH_{out} can be explained by postulating the K^+/H^+ antiporter functions as the regulator of pH_{in} over the pH_{out} range 6.0–9.0.

The Na^+/H^+ antiporter is also capable of acidifying the cell interior. Castle *et al.* (1986a, b) studied the coupling between ΔpNa and inside alkaline ΔpH in respiring *E. coli* and concluded that the magnitude of ΔpNa is determined by the magnitude of ΔpH at pH_{out} below 7.2. Thus, the Na^+/H^+ antiporter is mainly driven by the inside alkaline ΔpH generated as a result of pH homeostasis. At alkaline pH_{out} , since the direction of ΔpH is reversed, the inside acidic ΔpH is no longer effective as a driving force for Na^+ extrusion and an electrogenic Na^+/H^+ antiporter is generally considered to be driven by the protonmotive force, ΔP . Nakamura *et al.* (1984) showed that the generation of an inside acidic ΔpH by Na^+ -loaded cells at pH_{out} above 7.0 may be due to the homeostasis of ΔP at the respective pH_{out} . When collapsed by the addition of diethanolamine, the inside acidic ΔpH of Na^+ -loaded cells returns to the respective original value concomitant with the extrusion of Na^+ (Fig. 3), clearly indicating that acidification of the cell interior is mediated by an electrogenic Na^+/H^+ antiporter. In *E. coli*, the Na^+/H^+ antiporter has been shown to function at pH_{in} values above 6.5–7.0 (Bassilana *et al.*, 1984a, b; Nakamura *et al.*, 1986; Taglicht *et al.*, 1991). These properties are suited to the extrusion of Na^+ over a wide pH_{out} range in response to ΔP .

In medium of high K^+ concentration, where the regulation of pH_{in} by the K^+/H^+ antiporter becomes difficult at alkaline pH_{out} due to the decrease in ΔpK , cell

growth is supported by addition of Na^+ , even at alkaline pH_{out} (Fig. 4). In this case, cells containing higher internal Na^+ concentrations are able to adapt more easily to these growth conditions (Fig. 5). Moreover, the pH_{in} of such cells is acidified. The role of Na^+ in cell growth at alkaline pH_{out} is apparently related to the acidification of the cell interior through the electrogenic Na^+/H^+ antiporter. Na^+ is required for the growth of *E. coli* especially at alkaline pH_{out} (McMorrow *et al.*, 1989).

The presence of a K^+/H^+ antiporter similar to that of *V. alginolyticus* has been reported in *Nitrosomonas europaea* and *Nitrosobacter agilis* (Kumar & Nicholas, 1984), and in the green alga *Chlorella fusca* (Tromballa, 1987). An ATP-driven K^+/H^+ antiporter that expels K^+ by exchange for H^+ and is active only at alkaline pH_{in} was reported in *Streptococcus faecalis* (Kakinuma & Igarashi, 1988). In *E. coli*, two kinds of K^+ efflux system (*kefB* and *C*, formerly named *trkB* and *C*) have been cloned (Munro *et al.*, 1991). Although *KefB* and *KefC* were shown to function as a K^+/H^+ antiporter, a third system named *KefA* was considered to function as the regulator of pH_{in} (Booth *et al.*, 1985; Douglas *et al.*, 1991). Therefore, it is necessary to further define a true pH_{in} regulator among these multiple K^+/H^+ antiporters.

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