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K^+/H^+ ANTIPORTER FUNCTIONS AS A REGULATOR OF CYTOPLASMIC pH IN A MARINE BACTERIUM, *VIBRIO ALGINOLYTICUS*

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The marine bacterium, *Vibrio alginolyticus*, regulates the cytoplasmic pH at about 7.8 over the pH range 6.0–9.0. By the addition of diethanolamine (a membrane-permeable amine) at pH 9.0, the internal pH was alkalized and simultaneously the cellular K^+ was released. Following the K^+ exit, the internal pH was acidified until 7.8, where the K^+ exit leveled off. The K^+ exit was mediated by a K^+/H^+ antiporter that is driven by the outwardly directed K^+ gradient and ceases to function at the internal pH of 7.8 and below. The Na^+-loaded cells assayed in the absence of KCl generated inside acidic ΔpH at alkaline pH due to the function of an Na^+/H^+ antiporter, but the internal pH was not maintained at a constant value. At acidic pH range, the addition of KCl to the external medium was necessary for the alkalization of cell interior. These results suggested that in cooperation with the K^+ uptake system and H^+ pumps, the K^+/H^+ antiporter functions as a regulator of cytoplasmic pH to maintain a constant value of 7.8 over the pH range 6.0–9.0.

Introduction

All the bacteria living in a variety of environmental pH range regulate their cytoplasmic pH at around neutrality (for a review, see Ref. 1). Thus, the regulation of cytoplasmic pH is apparently essential for the growth of bacteria and enables the cells to adapt to environmental pH changes. In bacteria, protons are translocated outwards across the cytoplasmic membrane via primary proton pumps linked to electron transport and ATP hydrolysis. These pumps have been suggested to play a role in controlling internal pH in cooperation with an electrical K^+ transport [2–6]. On the other hand, the operation of cation/proton antiporter has been suggested for the acidification of internal pH [3,7–10]. Mutants defective in a Na^+/H^+ antiporter have been isolated from *Bacillus alcalophilus* [11] and *Escherichia coli* [12], which have lost the adaptability to alkaline pH in addition to the ability to extrude Na^+. Zilberstein et al. [13] concluded that in *E. coli*, the cooperative action of the proton pumps and the Na^+/H^+ antiporter constitutes the pH homeostasis mechanism at alkaline pH range. A participation of K^+/H^+ antiporter in the regulation of internal pH has also been suggested in *E. coli* [8–10]. However, a precise mechanism of the pH regulation by these antiporters still remains unsolved.

Earlier studies in this laboratory [14] demonstrated that in a marine bacterium, *Vibrio alginolyticus*, intracellular K^+ is released at alkaline pH in the presence of membrane-permeable amines

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Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide.

and that the K^+ exit is mediated via an electroneutral K^+/H^+ antiporter that ceases to function at an internal pH of 7.8 and below. Since the activity of this antiporter is dependent on the internal pH, it is very likely that this antiporter functions as a regulator of cytoplasmic pH. Therefore, we studied further the role of cation/proton antiporters in the pH regulation and came to the conclusion that the K^+/H^+ antiporter is able to function as a regulator of cytoplasmic pH in *V. alginolyticus*.

Materials and Methods

Growth of cells. V. alginolyticus 138-2 was grown and harvested as previously described [14].

Preparation of Na^+- and K^+-loaded cells. The cells washed with 0.4 M NaCl contained about 470 mM K^+ and 70 mM Na^+, which were referred to as normal cells.

Na^+-loaded cells were prepared according to the procedure described previously [14]. The Na^+-loaded cells contained about 400 mM Na^+ and less than 1 mM K^+.

For the preparation of K^+-loaded cells, the cells were treated twice in the medium containing 0.4 M KCl, 50 mM Tricine-KOH (pH 8.2) and 10 mM $MgCl_2$ for 5 min at 25 °C. Since the cells become fragile in the KCl medium [15], 10 mM $MgCl_2$ was added to the incubation mixture to prevent any lysis during treatments. The K^+-loaded cells contained about 480 mM K^+ and less than 1 mM Na^+.

Determinations of intracellular cations, ΔpH and $\Delta\psi$. Intracellular cation concentrations were determined by the filtration method as described previously [14]. The cell concentration was adjusted to about 0.4 mg protein/ml in a total volume of 200 μl.

The transmembrane electrical potential ($\Delta\psi$) and the inside alkaline ΔpH were determined from the equilibrium distribution of tetra[^{3}H]phenylphosphonium and [^{14}C]acetylsalicylic acid, respectively. To the reaction mixture, 20 μM each of the radioactive probes was included and the uptake was followed by the same procedure as used for the determination of cation concentrations [14]. The amount of radioactivity retained on the filter in the absence of cells served as the blank. The cell concentration was adjusted to about 0.4 mg protein/ml in a total volume of 200 μl.

The inside acidic ΔpH was determined from the distribution of di[^{14}C]methylamine. Since the dimethylamine accumulated inside the cells was easily washed out of the cells, a glass-fiber filter was employed to avoid the washing procedure as described by Zilberstein et al. [13]. The reaction was carried out at the cell concentration of 0.4 mg protein/ml in a total volume of 10 ml in the presence of 20 μM di[^{14}C]methylamine. At time intervals, 1.9 ml aliquot was filtered on a glass filter (GF/F Whatman, 25 mm diameter) and the radioactivity was determined. The amount of radioactivity retained on the filter in the absence of cells served as the blank.

Calculations of $\Delta\psi$ and ΔpH were performed as previously described [6]. The protonmotive force (ΔP) was calculated from the equation, $\Delta P = \Delta\psi - Z\Delta\text{pH}$, where $Z = 2.3\ RT/F$.

Materials. Tetra[^{3}H]phenylphosphonium bromide was a generous gift from H.R. Kaback. Other radioactive materials were purchased from New England Nuclear. Other reagents used were of analytical grade.

Results

Relationship between K^+ exit and internal pH

Fig. 1 shows the effect of diethanolamine on the K^+ exit and the internal pH at pH 9.0. When the K^+-loaded cells were preincubated for 5 min in 0.4 M NaCl containing 50 mM Tricine-NaOH (pH 9.0), the internal pH was maintained at 7.8 and only a slow release of cellular K^+ was observed. The addition of 20 mM diethanolamine-hydrochloride (pH 9.0), a membrane-permeable amine, at zero-time, induced an alkalization of cell interior and a simultaneous increase in K^+ exit. Following the K^+ exit, the internal pH was acidified and again became 7.8, where the K^+ exit leveled off. The second addition of diethanolamine also induced K^+ exit which ceased at the internal pH of about 8.0. During the K^+ exit, no bulk entry of Na^+ was detected and the magnitude of $\Delta\psi$ was unaffected. Furthermore, the addition of 10 mM cyanide showed no significant effect on the K^+ exit.

Similar results were obtained by using the nor-

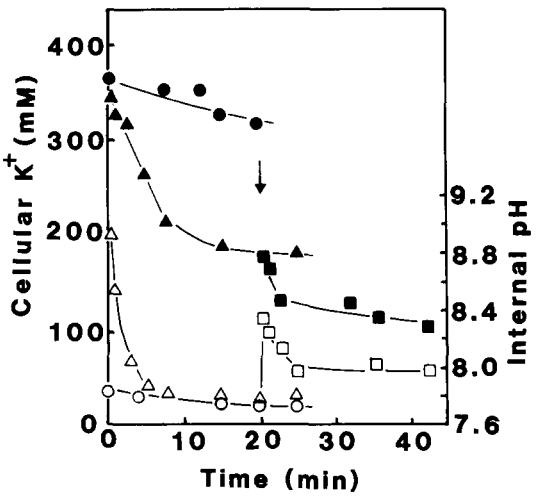

Fig. 1. Effect of diethanolamine on the K^+ exit and the internal pH at pH 9.0. The reaction mixture contained 0.4 M NaCl, 50 mM Tricine-NaOH (pH 9.0) and 10 mM $MgCl_2$. The K^+-loaded cells were suspended in the reaction mixture and preincubated for 5 min at 25 °C. At zero time and 20 min, respectively, 20 mM diethanolamine-hydrochloride (pH 9.0) was added and the cellular K^+ (closed symbols) and the internal pH (open symbols) were determined as described under Materials and Methods. ○, ●, In the absence of diethanolamine; △, ▲, after the first addition of diethanolamine; □, ■, after the second addition of diethanolamine.

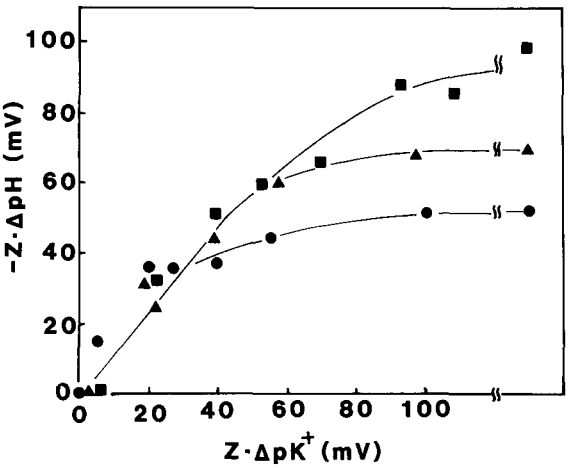

Fig. 2. Effect of K^+ chemical potential on the generation of inside acidic ΔpH. The reaction mixture contained 50 mM Tris-HCl buffer and varied concentrations of KCl and NaCl to maintain the total concentration at 0.4 M. The normal cells were suspended in the reaction mixture and incubated for 5–7 min at 25 °C, where the cellular K^+ and the internal pH reached a steady-state level. Then, the cellular K^+ and the ΔpH were determined as described under Materials and Methods. The external pH was adjusted to 8.6 (●), 9.0 (▲) and 9.6 (■). The results of ΔpH and ΔpK^+ are expressed in mV. The points shown at the extreme right of the figure represent the values obtained in the absence of K^+ in the external medium.

mal cells which contained about 470 mM K^+ and 70 mM Na^+. As shown in our previous paper [14], the permeable amine is accumulated in exchange for K^+ in the ratio 1 : 1. Since the permeable amine distributes according to the pH gradient across the membrane, these results may be explained as follows: the alkalization induced by the permeable amine allows the K^+ exit via a K^+/H^+ antiporter, which continues to function until the internal pH becomes about 7.8.

Effect of K^+ chemical potential on the acidification of cytoplasm at alkaline pH

In contrast to the case of NaCl medium, the K^+-containing cells suspended in 0.4 M KCl containing 50 mM Tricine-KOH (pH 9.0) generated no inside acidic ΔpH even after 20 min incubation. Therefore, the effect of outwardly directed K^+ chemical gradient (ΔpK^+) on the acidification of cytoplasm was determined at the external pH of 8.6, 9.0 and 9.6. As shown in Fig. 2, the cytoplasm was not acidified in the absence of ΔpK^+ and the internal pH was identical to the respective external pH. With the increase in ΔpK^+ directed outwards, the magnitude of inside acidic ΔpH increased and then reached a constant value, where the internal pH became 7.7, 7.8 and 7.9 at the external pH of 8.6, 9.0 and 9.6, respectively. Within the internal pH range above 8.0 where the K^+/H^+ antiporter is functioning, the magnitude of ΔpH was well-balanced by the magnitude of ΔpK^+. These results strongly suggested that the outwardly directed K^+ gradient was utilized to generate inside acidic ΔpH by means of the K^+/H^+ antiporter. This means that the antiporter functions as a transformer to convert the energy of chemical K^+ gradient into the ΔpH. In this case, since the function of the antiporter was regulated by the internal pH, the cytoplasm was never acidified in excess of pH 7.7.

At acidic pH range, we have shown in the previous paper [6] that the K^+-containing cells incubated in the KCl medium generate inside al-

kaline ΔpH to maintain the internal pH at about 7.8. Since there is no significant ΔpK^+ under those conditions, the presence of ΔpK^+ is not essential for the regulation of cytoplasmic pH at acidic pH range, which will be discussed later.

Energetic parameters of the normal and the Na^+-loaded cells in response to the external pH

We have demonstrated that K^+ is required for the generation of inside alkaline ΔpH so that the internal pH remains constant at about 7.8 [6]. However, the inside acidic ΔpH at alkaline pH has not been determined in our previous experiments. As shown in Fig. 3, when the normal cells were assayed in the presence of 10 mM KCl, the internal pH was regulated from 7.6 to 7.8 over the pH range 6.0–9.0 and the ΔpH reversed from inside alkaline to inside acidic at pH about 7.7. Thus, it is apparent that this bacterium has a capacity to control the cytoplasmic pH over the pH range examined. In the presence of 10 mM KCl, the Na^+-loaded cells and the K^+-loaded cells gave the same results as the normal cells.

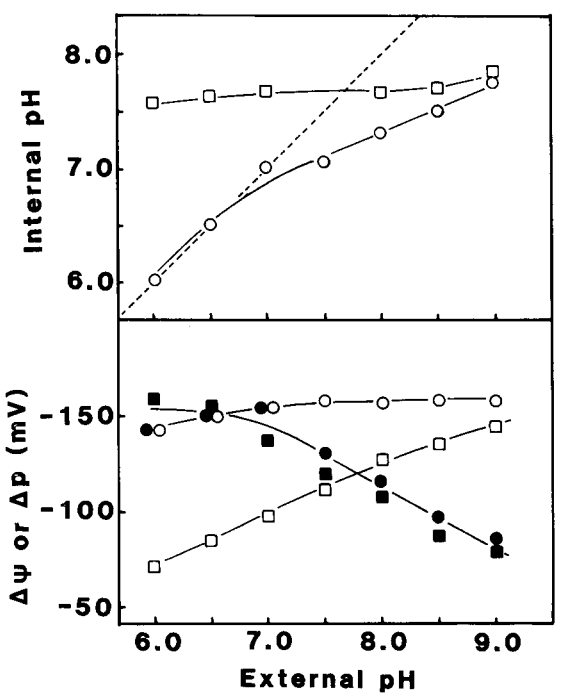

Fig. 3. The internal pH, $\Delta\psi$ and ΔP in the normal and the Na^+-loaded cells as a function of external pH. The reaction mixture contained 0.4 M NaCl, 20 mM glycerol and 50 mM Tricine-NaOH buffer (pH 8.0–9.0), or 0.3 M NaCl and 0.1 M sodium phosphate buffer (pH 6.0–8.0). The normal cells (squares) and the Na^+-loaded cells (circles) were employed for the determination of $\Delta\psi$ and ΔpH. The normal cells were assayed in the presence of 10 mM KCl. Values presented are an average of three experiments. Closed symbols represent ΔP. The dotted line drawn in the upper figure represents where $\Delta pH = 0$.

As described in our previous paper [6], the internal pH of the Na^+-loaded cells assayed in the absence of KCl was not maintained at a constant value and no inside alkaline ΔpH was generated (Fig. 3). Furthermore, even the K^+-containing normal cells could not generate inside alkaline ΔpH at acidic pH range in the absence of KCl (data not shown). These results indicated that the addition of KCl to the external medium was essential for the generation of inside alkaline ΔpH.

At alkaline pH range, the Na^+-loaded cells generated inside acidic ΔpH in the absence of KCl, but the internal pH was not regulated at a constant value. On the other hand, the normal cells were able to regulate the cytoplasmic pH by generating inside acidic ΔpH. In this case, it is important to note that the presence of outwardly directed K^+ gradient is essential, but the addition of KCl to the external medium is not necessary (see Figs. 1 and 2).

As shown in Fig. 3, the $\Delta\psi$ generated by the normal cells increased with the external pH, whereas the Na^+-loaded cells generated almost the same magnitude of $\Delta\psi$ from pH 6.0 to 9.0. These results are essentially the same as reported in our previous paper [6]. On the other hand, the magnitude of ΔP calculated from $\Delta\psi$ and ΔpH at the respective pH gave nearly the same value with the normal and the Na^+-loaded cells (Fig. 3), suggesting that the ΔP generated at the respective pH was little affected by the cationic conditions of the cells. The magnitude of ΔP, however, was dependent on the external pH and decreased from -150 mV at pH 6.0 to -80 mV at pH 9.0.

Relationship between Na^+ exit and internal pH

The Na^+-loaded cells generated inside acidic ΔpH at alkaline pH in the absence of KCl (see Fig. 3). Therefore, the effect of diethanolamine on the generation of ΔpH was examined. As shown in Fig. 4, the internal pH of the Na^+-loaded cells incubated in 0.4 M NaCl containing 50 mM Tri-

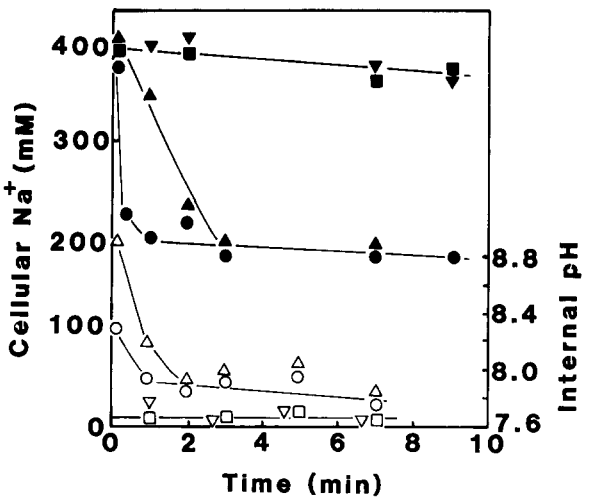

Fig. 4. Effect of diethanolamine on the Na^+ exit and the internal pH in the Na^+-loaded cells. The reaction mixture contained 0.4 M NaCl, 50 mM Tricine-NaOH (pH 9.0) and 20 mM glycerol. The Na^+-loaded cells were suspended in the absence (circles and squares) or presence (triangles and inverted triangles) of 50 μM HQNO and preincubated for 5 min at 25 °C. At zero-time, 20 mM diethanolamine-hydrochloride (pH 9.0) was added (circles and triangles), and the cellular Na^+ (closed symbols) and the internal pH (open symbols) were determine at time intervals.

cine-NaOH (pH 9.0) was from 7.6 to 7.7 without significant extrusion of cellular Na^+ (squares). By the addition of diethanolamine, the internal pH was alkalized and, at the same time, Na^+ was extruded from the cells against its concentration gradient (circles). Following the Na^+ exit, the internal pH was acidified and became about 7.8. Although these experiments were performed at pH 9.0, the internal pH varied in response to the external pH (see Fig. 3).

V. alginolyticus extrudes Na^+ by two different mechanisms at alkaline pH [16]. The one is the Na^+/H^+ antiporter which is driven by ΔP, and the other is the respiration-coupled primary Na^+ pump which is independent of ΔP. 2-Heptyl-4-hydroxyquinoline *N*-oxide (HQNO) acts as a strong inhibitor of primary Na^+ pump, but the cells are able to generate ΔP in its presence due to the HQNO-insensitive respiration [17,18]. Therefore, the effect of HQNO was examined. In the presence of 50 μM HQNO, the rate of Na^+ exit was slightly retarded but the internal pH was acidified to the same extent as observed in its absence (Fig. 4, triangles). In the absence of diethanolamine, HQNO had no effect on the internal pH (Fig. 4, inverted triangles). These results indicated that the acidification of cytoplasm was mediated by the function of Na^+/H^+ antiporter which was driven by ΔP. This means that the Na^+-loaded cells have a capacity to acidify the cytoplasm even in the absence of K^+. The internal pH, however, was not maintained at a constant value as shown in Fig. 3.

Discussion

The present results show that the K^+/H^+ antiporter is strongly dependent on the internal pH and functions only at the internal pH above 7.8 (Fig. 1). When the internal pH is alkalized above 7.8, this antiporter begins to function and H^+ enters inside the cells in exchange for K^+ until the internal pH becomes about 7.8. The outwardly directed K^+ gradient is utilized for the generation of inside acidic ΔpH (Fig. 2). With the Na^+-loaded cells, the function of Na^+/H^+ antiporter acidifies cytoplasm even in the absence of K^+ (Fig. 4), but the cytoplasmic pH is not maintained at a constant value (Fig. 3).

The presence of K^+ is essential for the regulation of internal pH over the pH range 6.0–9.0 (Fig. 3). At acidic pH range, the addition of KCl to the external medium is necessary for the alkalization of cell interior. This may be due to the electrical entry of K^+, which allows more H^+ to be extruded from the cells by the primary H^+ pumps. For the bulk entry of K^+, the extrusion of a counter-cation is required. The extrusion of Na^+ via the Na^+/H^+ antiporter acidifies cytoplasm, thus alleviating excessive alkalization due to the K^+ entry. However, the alkalization of cell interior must be inevitable for the massive accumulation of K^+. Under those conditions, when the cytoplasmic pH exceeds 7.8, the K^+/H^+ antiporter will begin to function to acidify until pH 7.8.

At the alkaline pH range, the addition of KCl to the external medium is not necessary, but the presence of ΔpK^+ directed outwards is essential for the generation of inside acidic ΔpH (Fig. 2). The presence of ΔpK^+, however, is not essential at acidic pH range. In this case, when the cytoplasmic pH exceeds 7.8, the K^+/H^+ antiporter

may still be able to function, due to the presence of the inwardly directed H^+ gradient. Therefore, it is reasonable to conclude that in cooperation with the K^+ uptake system and H^+ pumps, the K^+/H^+ antiporter functions as the regulator of cytoplasmic pH over the pH range 6.0–9.0.

We previously pointed out that the effect of K^+ on the generation of pH-dependent $\Delta\psi$ and ΔpH cannot be explained from the pH dependence of the K^+ uptake system [6]. Once the cytoplasmic pH is regulated at about 7.8 by means of the K^+/H^+ antiporter, the pH-dependent $\Delta\psi$ in the presence of K^+ may be simply determined by the magnitude of ΔP that can be generated at the respective external pH, because the respiratory chain can only achieve the same magnitude of ΔP as in the absence of K^+ (Fig. 3). With the Na^+-loaded cells, the electrical movement of ion(s) across the membrane may be minimal due to the absence of K^+. This might be the reason why the magnitude of $\Delta\psi$ was maintained at a high and a constant level over the pH range 6.0–9.0 (Fig. 3). Therefore, the internal pH of the Na^+-loaded cells seems to be determined by the magnitude of ΔP at respective external pH.

Brey et al. [8,9] postulated that the K^+/H^+ antiporter regulates cytoplasmic pH in *E. coli*. By using inverted membrane vesicles, the optimum pH has been estimated to be 7.8–8.2. They considered that the antiporter reaches its maximum activity at the internal pH of 8.0, where the H^+ entry approximates to the H^+ extrusion by the H^+ pumps, thereby preventing a rapid rise in the internal pH. In the intact cells of *V. alginolyticus*, the optimum pH of the K^+/H^+ antiporter, as measured from the initial rate of K^+ exit, was 9.6 with only a slight activity at pH 7.8 [14]. Therefore, our proposed mechanism is considerably different from that of Brey et al.

Our conclusion is consistent with the proposal of Kroll and Booth [19], who mentioned that the cycling of K^+ involving separate entry and exit routes constitutes an intrinsic part of cytoplasmic pH regulation. However, it must be emphasized that the cytoplasmic pH is determined by the pH dependence of the K^+/H^+ antiporter, that is, the exit route of K^+. Further studies with *E. coli* cells are under progress.

It is apparent that the Na^+/H^+ antiporter constitutes a part of the pH homeostasis mechanism, since the mutants defective in the antiporter are unable to grow at alkaline pH [11,12]. However, this does not necessarily mean that the Na^+/H^+ antiporter functions as a regulator of cytoplasmic pH. As discussed above, the cytoplasm may easily be alkalized in the absence of the Na^+/H^+ antiporter. Thus, it is very likely that the mutants defective in this antiporter are dramatically reduced in their capacity to adapt to alkaline external pH. The primary function of the Na^+/H^+ antiporter may be to extrude Na^+ over the pH range 6.0–9.0.

Recently, Kobayashi et al. [5] demonstrated that in *Streptococcus faecalis* that has no respiratory chain, the cytoplasmic pH is regulated by the pH dependence of H^+-ATPase activity, which becomes very low at pH above 8.0 and thus the cytoplasmic pH is regulated at around 8.0. It is very interesting to note that bacteria having respiratory chain regulate cytoplasmic pH by the acidification mechanism, whereas those having no respiratory chain regulate by the alkalization mechanism. These mechanisms are consonant to the differences in the bioenergetics of these bacteria. It should be emphasized that in both cases, the activity of pH regulator is precisely controlled by the cytoplasmic pH.

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