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Identification of the Ca^{2+} Conductance Responsible for K^+-induced Backward Swimming in *Paramecium caudatum*

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Abstract. Membrane potential responses of *Paramecium caudatum* to an application of K^+-rich solution were examined to understand the mechanisms underlying K^+-induced backward swimming. A wild-type cell impaled by a microelectrode produced action potentials followed by a sustained depolarization in response to an application of a K^+-rich test solution. After termination of the application, a prolongation of the depolarization (depolarizing after-potential) took place. Behavioral mutants incapable of exhibiting K^+-induced backward swimming did not show depolarizing afterpotentials. Upon short application of K^+-rich solution, the timing and duration of the ciliary reversal of the wild-type cell coincided well with the K^+-induced depolarization. The duration of the depolarizing afterpotential decreased as the duration of the application increased. The depolarizing afterpotential recovered slowly after it had been suppressed by a preceding application of the K^+-rich solution. By injection of an outward current into the wild-type cell, the action potentials were evoked normally during the period when the K^+-induced depolarizing afterpotential was suppressed. We concluded that the prolongation of the depolarizing membrane potential response following the application of the K^+-rich solution represents the Ca^{2+} conductance responsible for the K^+-induced backward swimming in *P. caudatum* and that the characteristics of the K^+-induced Ca^{2+} conductance are distinct from those of the Ca^{2+} conductance responsible for the action potentials.

Key words: *Paramecium* — Membrane excitability — Ca^{2+} conductance — Chemoreception — Ciliary reversal — Behavioral mutant

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Introduction

The swimming behavior of *P. caudatum* is under the control of membrane electric events (Eckert, 1972; Naitoh, 1974). Free-swimming *Paramecia* show avoidance responses when they swim into a physical obstacle (Naitoh and Eckert, 1969, Machemer & Ogura, 1979) or are exposed to certain chemicals (Jennings, 1906; Van Houten, 1992; Oami, 1996a, b). The avoidance responses take place due to changes in beating activities of the cilia on the surface of *Paramecium* cells. If the direction of the effective stroke reverses (ciliary reversal), the cells show backward swimming. The ciliary reversal is produced by an activation of the Ca^{2+}-dependent reversal mechanism in the cilia (Naitoh & Kaneko, 1972). An increase in the ciliary Ca^{2+} concentration takes place in association with the Ca^{2+}-dependent depolarizing action potential.

In addition to the action potential-mediated backward swimming, it is well known that cells of *Paramecium* show prolonged backward swimming when transferred to a K^+-rich solution (Naitoh, 1968, Nakazato & Naitoh, 1993). The cells immersed in the K^+-rich solution exhibit long-lasting continuous backward swimming. K^+-induced backward swimming is, under certain circumstances, more than two orders of magnitude longer than that associated with the action potential. The action potential-associated backward swimming and the K^+-induced backward swimming have been assumed to be induced by the Ca^{2+} influx through the same voltage-gated Ca^{2+} channels. Therefore, measurements of the K^+-induced backward swimming have been used as a simple method for evaluating the membrane excitability of *Paramecium*. However, the mechanisms underlying the K^+-induced ciliary reversal have not been determined.

In this paper, we examined the membrane electric events controlling the K^+-induced backward swimming in *P. caudatum*. To identify the membrane conductance responsible for K^+-induced backward swimming, we employed mutant cells of *P. caudatum* (caudatum non-reversal, CNR; Takahashi et al., 1985), which do not show backward swimming in the K^+-rich solution. The results obtained indicate that K^+-induced backward swimming is controlled by a Ca^{2+} conductance whose kinetics are different from those of the Ca^{2+} conductance responsible for the action potentials. It is concluded that K^+-induced backward swimming is controlled by a novel Ca^{2+} conductance activated by the K^+-rich solution. Some of these results have been presented orally and in abstract form elsewhere (Oami & Takahashi, 1999).

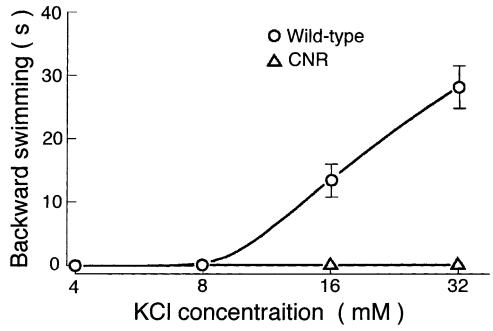

Fig. 1. Effects of KCl concentration on the duration of the backward swimming exhibited by the cells of *Paramecium caudatum*. Duration of backward swimming was plotted against the K^+ concentration in the test solution. Circles, wild-type cells; triangles, CNR mutant cells. Each symbol represents the mean and its standard error of 10–20 measurements with different cells.

Materials and Methods

Cells of *Paramecium caudatum* (27a G3 belonging to syngen 3) were cultured in a bacterialized (*Klebsiella pneumoniae*) hay infusion medium. In some experiments, the behavioral mutant cells defective in voltage-gated Ca^{2+} channels (caudatum non-reversal, *cnrD* (18 D 504); Takahashi et al., 1985) were used. cnrD is the least leaky non-excitable mutant among four strains of CNR mutants (Takahashi et al., 1985). Cells in the early stationary growth phase were collected and washed three times with a reference solution that consisted of (mM) 4 KCl, 1 $CaCl_2$, and 1 Tris-HCl buffer (pH 7.4), and cells were kept in the solution for more than 30 minutes prior to the experimentation. To change K^+ concentration in the test solution, the KCl concentration of the reference solution was changed.

To examine K^+-induced backward swimming, cells were transferred by a pipette from the reference solution to the test solution with varying K^+ concentration. After transfer to the K^+-rich solution (e.g., 32 mM K^+), the wild-type cells swam backward (backward swimming), then gyrated at the same position (whirling) and finally resumed forward swimming. Duration of the backward swimming was determined by measuring the net backward locomotion of the cell.

Conventional electrophysiological techniques were employed for examining membrane potential responses to the K^+-rich test solution and to an electric current pulse (Oami, 1996a, b).

Test solution was applied to the cell through a pipette by increasing the hydrostatic pressure (3–5 mm H_2O) inside the pipette (internal diameter of tip about 200 μm). The opening of the pipette was first placed 3–4 mm below the cell. It was then raised to the level of the cell (about 200 μm distant from the cell). The external solution was continuously exchanged with reference solution (flow rate about 6 ml/min) to minimize the accumulation of the test solution around the cell. External reference solution moved about 250 μm/sec in the direction identical to that of flow of the test solution around the cell.

All experiments were done at room temperature ranging from 20 to 24°C.

Results

In the first series of experiments we measured the duration of the K^+-induced backward swimming of the cells of *P. caudatum* in test solutions with varying

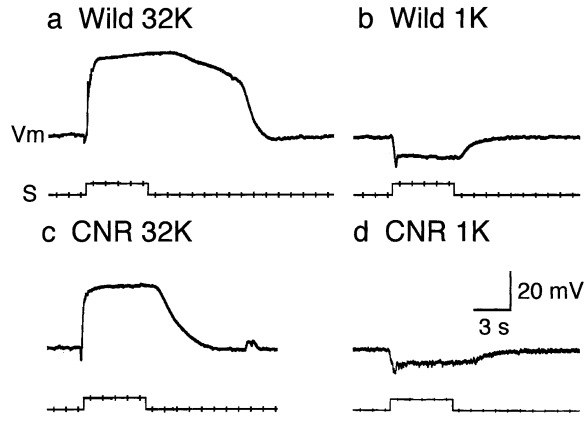

Fig. 2. Membrane potential responses of *P. caudatum* in response to an application of test solution with different K^+ concentrations. The upper trace in each pair of recordings shows the membrane potential and the lower trace, timing and duration of the application of test solution. *a* and *b*, wild type cell; *c* and *d*, *cnrD* cell. The applied test solution contained 32 mM KCl in *a* and *c* and 1 mM in *b* and *d*.

K^+ concentration. The wild-type cells showed whirling but not continuous backward swimming when they were transferred from a reference solution (4 mM KCl) to a test solution containing 8 mM KCl. Raising the KCl concentration to 16 mM, the cells showed backward swimming followed by whirling. The duration of the backward swimming increased in the test solution containing 32 mM KCl. CNR did not show backward swimming in the test solution with any of the K^+ concentrations (Fig. 1).

To examine the membrane potential responses underlying the K^+-induced ciliary reversal, we applied the K^+-rich solution to cells impaled by a microelectrode. Figure 2 shows representative membrane potential responses exhibited by wild-type and CNR cells. In response to an application of a test solution containing 32 mM K^+, the wild-type cell

showed depolarizing membrane potential responses consisting of an initial action potential(s) and subsequent sustained depolarization (Fig. 2*a*). The CNR cell showed sustained depolarization but not action potentials upon the application (Fig. 2*c*). The wild-type and CNR cells exhibited sustained membrane hyperpolarization to a test solution with reduced KCl concentration (Fig. 2*b*, *d*). It should be noted that the duration of the depolarization exhibited by the wild-type cell is longer than the duration of application of the test solution due to apparent prolongation of the depolarization. The CNR cell did not show prolongation following the K^+-induced depolarization. In the following section, we refer to this prolongation of the response as the depolarizing afterpotential.

To examine the relationship between the K^+-induced membrane potential responses and the ciliary reversal of the cell, we recorded both phenomena simultaneously. A test solution containing 16 mM K^+ was employed for the examination of the responses. When the duration of application of the test solution was short (e.g., 6 sec), the timing and the duration of the ciliary reversal always coincided with the K^+-induced sustained depolarization, including the depolarizing afterpotential (Fig. 3*a*). During a prolonged application of the test solution, ciliary reversal terminated even though the application continued. After termination of the ciliary reversal, the membrane repolarized rapidly, and the depolarizing afterpotential was not observed (Fig. 3*b*, *c*).

We next examined the effects of the duration of the application of K^+-rich solution on the relaxation process of the membrane potential responses following the application. Figure 4*A* shows overlapped recordings of the relaxation process of the membrane potential responses following application of the test solution containing 32 mM K^+ for various times. When the application was shorter than 3 sec, membrane depolarization continued for about 10 sec after termination of the application. With increasing application duration, the prolongation of the depolarization became shorter. The duration of the prolongation decreased to a steady level of about 3 sec when the duration of application was longer than 80 sec. This value is similar to that exhibited by the CNR mutant cells following the application of 32 mM K^+ (e.g., 2.8 sec in the record shown in Fig. 6). Therefore, it is probable that the depolarizing afterpotential is reduced or absent after prolonged exposure of the cell to the K^+-rich solution.

The amplitude of the K^+-induced sustained membrane depolarization during the application decreased slightly when the duration of the application was increased.

To express the degree of decrease in prolongation quantitatively, we measured the duration of the depolarizing afterpotential. Duration was measured from the time when the application was terminated

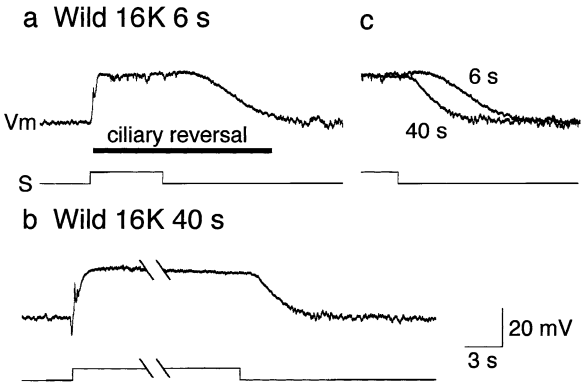

Fig. 3. Simultaneous recordings of the membrane potential responses and the ciliary reversal of the wild-type cell of *P. caudatum* in response to an application of a test solution containing 16 mM K^+. The upper trace in each pair of recordings shows the membrane potential and the lower trace, timing and duration of the application of test solution. The bar below the voltage trace in *a* indicates the timing and the duration of the ciliary reversal exhibited by the cell. The duration of the application was 6 sec in *a* and 40 sec in *b*; *c* shows the overlapped recordings of the recovery phase of the K^+-induced membrane potential responses shown in *a* and *b*, adjusted so that the timing of termination of the application becomes identical.

to the time when the depolarizing response decayed to its half level. To obtain the net duration of the prolongation, the measured value was subtracted by the minimum stable value obtained when the application was longer than 160 sec and expressed as a value relative to the maximum value. As shown in Fig. 4*B*, the duration of the prolongation declined more or less exponentially with increasing duration of application of the K^+-rich solution. Maximum duration of the depolarizing afterpotential changed among the cells examined. However, the time course of the shortening in the duration of the depolarizing afterpotential was not significantly affected.

We examined the recovery of the depolarizing afterpotential after it had been suppressed by a preceding prolonged application (160 sec) of the K^+-rich test solution. The K^+-rich solution was applied twice to the cell, and duration of the depolarizing afterpotential in response to the second application was then measured by changing the interval between applications. The durations of the first and second applications were kept constant at 160 sec and 5 sec, respectively. As shown in Fig. 5*A*, the duration of the depolarizing afterpotential in response to the second application increased as the interval between applications increased. It became maximal when the interval was longer than 200 sec.

Figure 5*B* shows the relative duration of the depolarizing afterpotential upon the second application plotted against the interval between applications. The

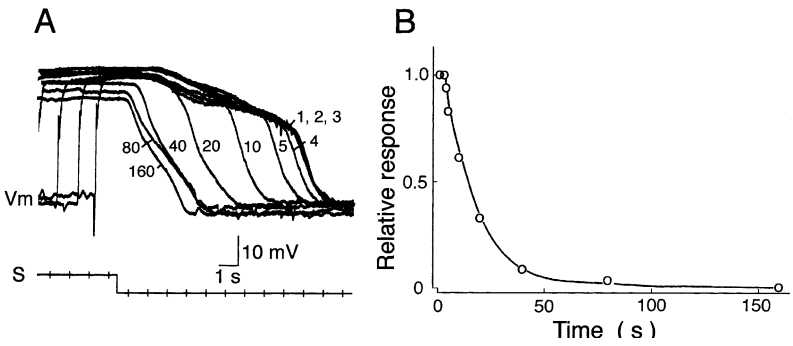

Fig. 4. (*A*) Effects of the duration of the application of the K^+-rich solution on the recovery phase of the K^+-induced membrane potential responses of the wild-type cell of *P. caudatum*. The duration of the application was changed from 1 sec to 160 sec, while the KCl concentration was kept constant at 32 mM. Records were overlapped so that the timing of the termination of the application became identical. (*B*) Measurements of the duration of the prolongation of the depolarization (depolarizing afterpotential) following the application plotted against the duration of the application of the test solution. The duration of the prolongation was measured at the half-decaying point and expressed as a relative value normalizing the maximum response as 1 and the minimum value as 0.

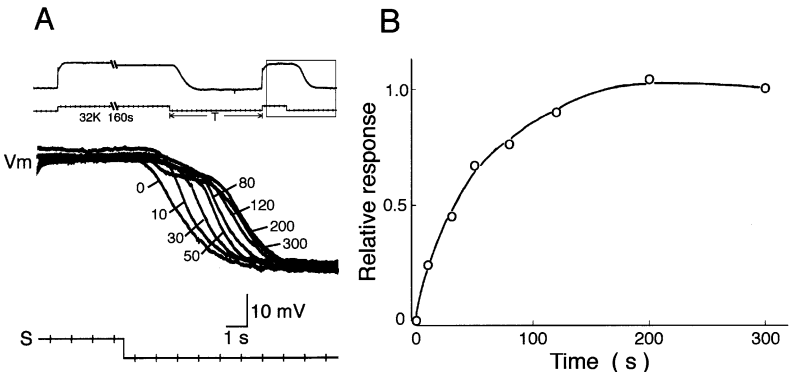

Fig. 5. Recovery of the prolongation of the depolarizing response following the preceding application of K^+-rich solution in the wild-type cell of *P. caudatum*. (*A*) A test solution containing 32 mM K^+ was applied twice to the cell, and the effects of the interval between stimulations on the recovery phase of the membrane potential responses to the second application were examined. The duration of the first application was 160 sec, and that of the second application was 5 sec. Records were overlapped so that the timing of the termination of the application became identical. The inset shows the protocol of the application. (*B*) Measurements of the duration of the prolongation of the depolarization induced upon a second application of the K^+-rich solution plotted against the interval between the applications of the test solution. The duration was expressed as in Fig. 4*B*.

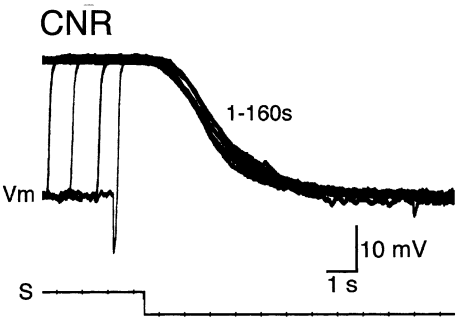

Fig. 6. Effects of the duration of the application of the K^+-rich solution on the recovery phase of the K^+-induced membrane potential responses of the CNR mutant cell of *P. caudatum*. The duration of the application of a test solution containing 32 mM KCl was changed from 1 sec to 160 sec. Other explanations are as in Fig. 4*A*.

depolarizing afterpotential recovered more or less exponentially as the interval between the applications increased.

Figure 6 shows the effects of duration of the application of K^+-rich solution on the K^+-induced membrane depolarization of a CNR mutant cell. A test solution containing 32 mM K^+ was applied to the cell for various durations. As described previously, CNR mutants did not produce prolongation of the depolarizing response after termination of the application. This repolarizing phase following the application did not change with increasing duration of the application. The relaxation phase of the depolarization was short irrespective of the duration of the application. The values were comparable to those obtained in the wild-type cells upon prolonged (e.g., more than 160 sec) application of the test solution. The amplitude of the K^+-induced depolarization during the application did not change with increasing duration of application.

To examine the effects of an application of K^+-rich solution on the depolarizing Ca^{2+} action potential, the current-evoked action potentials were compared before and immediately after a prolonged

Fig. 7. Effects of the application of the K^+-rich solution on the current-evoked action potential in the wild-type cell. Action potentials were evoked by injecting an outward current into the cell. (*A*) Action potentials evoked without preceding application of the K^+-rich solution. (*B*) Action potentials evoked immediately after termination of the 160-sec application of the solution containing 32 mM K^+. Upper traces, membrane potential responses; middle traces, timing and duration of the application of the K^+-rich solution; lower traces, injected current.

application of the K^+-rich solution. Figure 7 shows representative results obtained with a single cell. Without a preceding application of the K^+-rich solution, the wild-type cell produced a depolarizing action potential in response to the injection of an outward current pulse. The action potential was not affected much when it was evoked after the 160-sec application of the test solution containing 32 mM K^+.

Discussion

In the present study, we revealed the depolarizing membrane potential responses closely associated with the K^+-induced ciliary reversal. It is suggested that the depolarizing afterpotential represents the response underlying the K^+-induced ciliary reversal since the CNR mutant, incapable of exhibiting the backward swimming, produced only steady depolarization associated with the application of the K^+-rich solution, but not the depolarizing afterpotential (Fig. 2).

The steady depolarization during the application of K^+-rich solution was caused mainly by a shift in the equilibrium potential for K^+ ions. After the application of the test solution, the equilibrium potential for K^+ is assumed to recover to the original negative level before application. The depolarizing afterpotential is produced most probably by the depolarizing ion conductance persisting after termination of the application. It is assumed that the prolongation of the depolarization is produced by Ca^{2+} conductance persisting after termination of the application, because only the equilibrium potential for Ca^{2+} ions is expected to be positive. The repolarization phase of the potential responses most probably represents the deactivation process of this conductance. During the K^+-induced backward swimming, the reversal mechanism in the cilia is activated by the Ca^{2+} ions driven into the cilia through the K^+-induced Ca^{2+} conductance.

Thus, in the wild-type cells, it is suggested that the membrane depolarization induced by K^+-rich solution consists of both K^+ conductance and the Ca^{2+} conductance activated by the application of K^+-rich solution. The slight decrease in the amplitude of the membrane depolarization during the application of the K^+-rich solution (Fig. 4) is attributable to the time-dependent inactivation of the K^+-induced Ca^{2+} conductance.

The duration of the depolarizing afterpotential became shorter with increasing duration of application of the K^+-rich solution, as shown in Fig. 4. If we assume that the duration of the depolarizing afterpotential is dependent on the amount of Ca^{2+} conductance remaining at the end of application, the figure might represent the inactivation time course of the conductance. It should be noted that the time course of the inactivation of the Ca^{2+} conductance is comparable to that of backward swimming in the free-swimming cells in K^+-rich solution. From Fig. 4*B*, it is suggested that about 80 percent of the K^+-induced Ca^{2+} conductance was inactivated when the free-swimming cell began whirling, following the backward swimming (about 28 sec after the transfer; Fig. 1*A*).

The fact that the current-evoked action potential was not affected by the preceding application of the K^+-rich solution (Fig. 7) indicates that the inactiva-

tion of the present K^+-induced Ca^{2+} conductance does not affect the voltage-gated Ca^{2+} conductance responsible for the action potential. Therefore, it is suggested that the present K^+-induced Ca^{2+} conductance is different from the Ca^{2+} conductance responsible for the action potential, at least in its inactivation kinetics. As a matter of fact, the time course of the inactivation of the present K^+-induced Ca^{2+} conductance (about 20 sec for half-decay upon application of 32 mM K^+; Fig. 4) differs about two orders of magnitude from that of the Ca^{2+} conductance responsible for the action potential (within several tens of millisecs; Brehm & Eckert, 1978). The time course of recovery from the inactivation also differs significantly (Fig. 6).

The present experiments revealed that the CNR mutants do not show activation of the Ca^{2+} conductance responsible for the K^+-induced backward swimming. It has been reported that CNRs have a defect in the voltage-gated Ca^{2+} channel function responsible for the action potential (Takahashi et al., 1985). Because the CNR used in the present study has a mutation in a single point, there is a possibility that the voltage-gated Ca^{2+} channels responsible for the action potentials have important roles for the regulation of the K^+-induced Ca^{2+} conductance. Therefore, both types of conductance might be produced by the same channel, or alternatively, they might be produced independently by distinct ion channels and these hypothetical ion channels need the same regulatory factor for activation, a factor in which CNRs are defective in. At present, the former possibility is more likely, because many mutants defective in voltage-gated Ca^{2+} channel functions with mutations on different genes (three Pawn mutants in *P. tetraurelia* and four CNR mutants in *P. caudatum*) have defects in their response to a K^+-rich solution (Ramanathan et al., 1988; Takahashi, 1988). Recently, genes of Pawn mutants of *P. tetraurelia* have been cloned (Heynes et al., 1998). These molecular biological techniques employed for determination of the molecules responsible for the regulation of channel functions (Kung et al., 2000) must be useful to understand the mechanisms underlying the regulation of the present K^+-induced Ca^{2+} conductance.

It is not clear whether the present K^+-induced Ca^{2+} conductance is activated by membrane depolarization or other mechanisms, such as the binding of the K^+ ion to its hypothetical receptor site on the membrane. Because the depolarizing afterpotential was not observed in association with the current-evoked membrane depolarization, the present K^+-induced Ca^{2+} conductance cannot be activated directly by the membrane depolarization. Jahn (1962) first found the close relationship between the duration of the backward swimming exhibited by the cells in response to the K^+-rich solution and the $[K]/[Ca]^{1/2}$ ratio in the stimulation medium. He emphasized the importance of the Ca^{2+} ions bound on the surface of *Paramecium* cell. By measuring the binding of radioactive Ca^{2+} to the cell, Naitoh and Yasumasu (1967) confirmed Jahn's prediction. Naitoh (1968) finally concluded that the duration of the backward swimming was primarily dependent on the $[K]/[Ca]^{12}$ ratio, and the liberation of the Ca^{2+} from the cell surface was the first step in the initiation of the reversal. Therefore, it would be interesting to investigate whether the present K^+-induced Ca^{2+} conductance is activated according to the theory described by these previous authors.

Paramecium responds to various organic chemicals in the external solution with specific receptor systems (Hennessey, Frego & Francis, 1994; Hennessey, Kim & Satir, 1995; VanHouten 1992). In addition to these specific receptor systems for organic chemicals, the present study revealed that *Paramecium* possesses a receptor system for inorganic ions. Freshwater ciliates are supposed to be exposed to the changes in the chemical environment of the water surrounding them. Ionic composition and concentration are major factors of the chemical environment in the external solution. Because the behavioral responses of *Paramecium* are under the control of membrane electric events, changes in ionic concentration responsible for membrane electrogenesis have a large influence on the regulation mechanisms of the behavior. In *Paramecium*, K^+ ions, the major determinant of the resting potential, and Ca^{2+} ions, the major determinant of the action potential, are the most important ion species for membrane electrogenesis. *Paramecium* can avoid the drastic changes in external K^+ concentration by behavioral responses controlled by the receptor system found in the present study.

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