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Spontaneous fluctuation of the resting membrane potential in *Paramecium*: amplification caused by intracellular Ca^{2+}

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SUMMARY

The ciliated protozoan *Paramecium* spontaneously changes its swimming direction in the absence of external stimuli. Such behavior is based on resting potential fluctuations, the amplitudes of which reach a few mV. When the resting potential fluctuation is positive and large, a spike-like depolarization is frequently elicited that reverses the beating of the cilia associated with directional changes during swimming. We aimed to study how the resting potential fluctuation is amplified. Simultaneous measurements of the resting potential and intracellular Ca^{2+} ($[Ca^{2+}]_i$) from a deciliated cell showed that positive potential fluctuations were frequently accompanied by a small increase in $[Ca^{2+}]_i$. This result suggests that Ca^{2+} influx through the somatic membrane occurs during the resting state. The mean amplitude of the resting potential fluctuation was largely decreased by either an intracellular injection of a calcium chelater (BAPTA) or by an extracellular addition of Ba^{2+}. Hence, a small increase in $[Ca^{2+}]_i$ amplifies the resting potential fluctuation. Simulation analysis of the potential fluctuation was made by assuming that Ca^{2+} and K^+ channels of surface membrane are fluctuating between open and closed states. The simulated fluctuation increased to exhibit almost the same amplitude as the measured fluctuation using the assumption that a small Ca^{2+} influx activates Ca^{2+} channels in a positive feedback manner.

Key words: spontaneous fluctuation, membrane potential, calcium, *Paramecium*.

INTRODUCTION

Animals alter their behavior in response to external stimuli. However, sometimes they spontaneously alter their behavior in the absence of external stimuli. A typical example of spontaneous behavior alteration is observed in the ciliated protozoan *Paramecium*. The cell swimming in a homogeneous environment spontaneously changes its swimming direction (Jennings, 1906; Naitoh and Eckert, 1974; Oosawa and Nakaoka, 1977). Such a change of swimming direction is caused by a transient reversal of the cilia beating on the cell surface. The frequency of the directional change increases or decreases in response to external stimuli (Nakaoka and Oosawa, 1977; Van Houten, 1992). However, spontaneous directional changes usually occur in homogeneous conditions. In the homogeneous condition, the mean time intervals between successive directional changes are in the range of 5–10 s.

Spontaneous changes in the swimming direction are caused by an electric signal in the paramecium cell that initiates the reversal of ciliary beating (Machemer, 1988; Naitoh and Eckert, 1974). The resting membrane potential, which on average is about –25 mV, is not kept constant but instead displays a random amplitude fluctuation of about 1–3 mV (Majima, 1980; Moolenaar et al., 1976). When the resting potential fluctuation reaches a positive level, a spike-like depolarization of much larger amplitude is frequently generated. This spike-like depolarization is accompanied by the opening of Ca^{2+} channels localized in the ciliary membrane (Dunlap, 1977; Ogura and Takahashi, 1976) and the simultaneous influx of Ca^{2+} into the intraciliary spaces (Plattner et al., 2006), which induces the reversal of ciliary beating on the cell surface. The probability of spike generation is found to increase approximately exponentially with a positive shift of the resting potential fluctuation (Toyotama, 1981). The fluctuation of resting potential is therefore important for the generation of spontaneous changes in swimming direction.

In an electrophysiological study using ciliated *Paramecium* cells, Moolenaar et al. showed that the amplitude of membrane potential fluctuation increases with an increase in the motive force of the K^+ current that forms the membrane potential difference between resting and equilibrium potentials for K^+ (Moolenaar et al., 1976). Therefore, the membrane potential fluctuation was interpreted to be mainly due to the fluctuation of K^+ channels. However, Majima indicated that Ca^{2+} current also makes contributions to the membrane potential fluctuation after experiments with various concentrations of K^+ and Ca^{2+} (Majima, 1980). The membrane potential fluctuation increased with an increase in the motive force of the Ca^{2+} current. In normal conditions, the K^+ concentration inside the cell is much larger than that outside, and so K^+ ions flow from inside to outside. Conversely, the Ca^{2+} concentration is much larger outside than inside the cell and so Ca^{2+} ions flow into the cell. Subsequently, Oosawa has presented a theoretical treatment on the mechanism for the generation of resting potential fluctuations (Oosawa, 2001). He indicates that the amplitude of a resting potential fluctuation is proportional to the circulating current, which consists of the outward K^+ and inward Ca^{2+} currents.

Resting potential fluctuations in *Paramecium* are based on the opening and closing fluctuations of ionic channels in the surface membrane. However, if the opening and closing fluctuations were only random, a resting potential fluctuation with large amplitude

would not be generated. Some feedback regulation to amplify the fluctuation seems to be involved in the generation process. In the present study, we have analyzed the resting potential fluctuations using deciliated cells that have no voltage-sensitive Ca^{2+} channels and elicit no spike-like depolarizations. Simultaneous measurements of both the resting membrane potential and the intracellular Ca^{2+} ($[Ca^{2+}]_i$) have shown that a membrane depolarization is accompanied by a small increase in $[Ca^{2+}]_i$. Some treatments to reduce $[Ca^{2+}]_i$ were found to decrease the amplitude of resting potential fluctuations. Based on these results, we assume that $[Ca^{2+}]_i$ affects the regulation of the Ca^{2+} and K^+ channels in the surface membrane. Simulation analysis was successful only with the assumption that $[Ca^{2+}]_i$ activates Ca^{2+} channels in a positive feedback manner.

MATERIALS AND METHODS

Cells

Paramecium multimicronucleatum cells were cultured in a hay infusion inoculated with *Klebsiella pneumoniae*. The culture temperature was kept constant by incubation in a water bath at 25°C. *P. multimicronucleatum* cells in the stationary phase, 7–14 days after inoculation, were collected by low-speed centrifugation (~200 ***g***) and suspended in a standard solution containing 1 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} KCl and 2 mmol l^{-1} Tris-HCl (pH 7.2). In order to adapt to the solution, the cell suspension was left for 1–2 h prior to examination.

Intracellular recording of membrane potential

In order to avoid spike-like depolarizations elicited from the ciliary membranes, the cells were deciliated by incubation in a standard solution containing 6% ethanol and gently pipetted for 0.5–1 min and transferred to standard solution without ethanol. The method of membrane potential recording was similar to that described previously (Nakaoka et al., 1987a). The electrode was filled with 0.1 mol l^{-1} KCl and the resistance was 100–150 MΩ. The deciliated cells were placed in the glass vessel mounted on an inverted microscope (IX70; Olympus, Tokyo, Japan) and two electrodes were inserted from above, one for recording potential and one for the injection of current. The membrane potential recordings were performed at 25°C in current clamp conditions with a two-electrode voltage clamp amplifier (MEZ-7200 and CEZ-1200; Nihon Kohden, Tokyo, Japan). The recordings were sampled at 20 Hz and stored on a personal computer (PC-9801; NEC, Tokyo, Japan) using an A/D interface board (ADXM-98; Canopus, Kobe, Japan).

In order to evaluate membrane potential fluctuation, the records of membrane potential that were in the range between –22 mV and –27 mV were passed through a low-pass filter at 0.12 Hz, and the filtered potential record was subtracted from the original one. Since the subtracted record could be interpreted as the potential fluctuations around the mean resting potential, the potential fluctuations from zero were displayed as an amplitude histogram. Most histograms showed a roughly symmetrical distribution with the maximum count at a potential of zero. The full width at half maximum (FWHM) was adopted as a measure of membrane potential fluctuation.

Simultaneous recordings of $[Ca^{2+}]_i$ and membrane potential fluctuations

Image analysis of $[Ca^{2+}]_i$ change was performed using fluo-3, a calcium indicator dye (Dojindo, Kumamoto, Japan). A solution of fluo-3 dissolved in water was microinjected into the deciliated cells, which were held with microelectrodes for membrane potential recording on an inverted microscope stage equipped with a dichroic mirror (U-MWIB; Olympus; excitation 460–490 nm; emission 515 infinity). A fluorescence image was obtained with an EB-CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) and was displayed on a monitor. On a part of the monitor screen, the synchroscope cursor image showing the membrane potential recorded by another CCD camera (WV-BD400; Panasonic, Osaka, Japan) was simultaneously displayed. The screen image was stored on a digital videotape at 30 frames s^{-1}. The stored images were displayed by frame, and successive changes in the fluorescence intensity, which represented $[Ca^{2+}]_i$, and the cursor position, which represented membrane potential, were measured using NIH image (NIH, Bethesda, MD, USA). Fluorescence intensity was measured either for the whole cellular area or for the anterior or posterior halves of the fluorescent image by enclosing each area. To ascertain the accuracy of the fluorescence measuring system, a fluorescent image of a glass capillary containing fluo-3 was recorded and the fluctuation of the fluorescence intensity was measured. The maximum fluctuation from the mean fluorescence intensity was 0.5%.

Microinjection

Microinjection of either fluo-3 (1.8 mmol l^{-1} solution) or BAPTA (10 mmol l^{-1} solution) was performed by means of an air pressure pulse (3×10^5 Pa, 0.1 s; Basic Picosprizer, General Valve, Brookshire, TX, USA), through a microcapillary with a tip diameter of ~1 μm (Kuriu et al., 1996). The injected volumes (10–50 pl) were between 2 and 10% of the cell volume, which was assumed to be 500 pl.

Simulation analyses

The data for resting potential fluctuation were numerically simulated using the computational models for membrane potential fluctuation in *Paramecium*. The model was based on that of Oosawa, who proposed that the fluctuation of ion channel gates produces the fluctuation seen in the intracellular electric potential (Oosawa, 2001). In order to study the effects of intracellular Ca^{2+} on membrane potential fluctuation, we modified the original Oosawa model in such a way that the intracellular Ca^{2+} ions could activate the ion channel gates, increasing the probability of their opening. Detailed descriptions of the models are given in Appendix 1.

RESULTS

Simultaneous measurements of resting potential and $[Ca^{2+}]_i$

Both the synchroscope image showing resting membrane potential and the fluorescence image indicating intracellular Ca^{2+} were simultaneously recorded from a deciliated cell as a composite video image (Fig. 1A). From the video images, successive changes in resting potential and fluorescence intensity were measured. Initially, we analyzed the fluorescence changes in about one-third of the deciliated cellular image, e.g. anterior, posterior or middle part of the cell. However, those fluorescence changes showed almost no correlation with the membrane potential changes. Correlated changes were found when the fluorescence of a whole or a half cell area was measured (Fig. 1B). The fluorescence intensity of a whole cell area tended to increase with the spontaneous potential change up to a depolarized level. Such tendencies in the recordings are indicated as horizontal bars in Fig. 1Bi,ii. Calculations of the cross-correlation between the resting membrane potential and the fluorescence intensity indicated that the depolarization occurs at 0.41±0.14 s (mean ± s.d., N=6 cells) prior to the fluorescence increase. Therefore, the Ca^{2+} influx through the Ca^{2+}-permeable channels of soma membranes seems to diffuse into the intracellular space in the resting state.

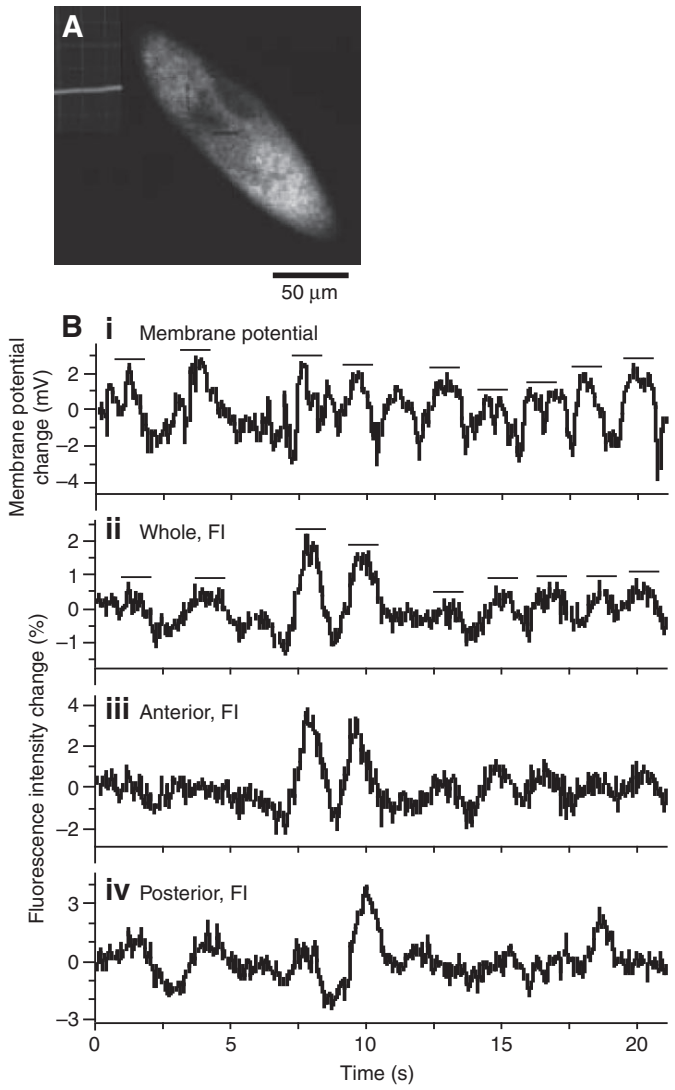

Fig. 1. Simultaneous measurements of the resting potential and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). (A) A composite image from a synchroscope and a fluorescence microscope. Both images from the synchroscope and fluorescence microscope indicate resting potential and $[Ca^{2+}]_i$, respectively, and are joined to form a single image. The bottom right of the fluorescence cellular image is the anterior side of the cell. (B) Fluctuations of resting potential and fluorescence intensity (FI). (i) Fluctuation of resting potential. Time average of membrane potential is shown as zero. (ii) Fluctuation of fluorescence intensity measured across the whole cell area. Fluorescence intensity was normalized using the time average of fluorescence intensity for the whole cell area. Changes from the average are shown. (iii) Fluctuation of fluorescence intensity measured across the anterior half of the cell. Fluorescence intensity was normalized using the time average of fluorescence intensity of the anterior half of the cell. (iv) Fluctuation of fluorescence intensity measured across the posterior half of the cell. Fluorescence intensity was normalized using the time average of fluorescence intensity of the posterior half of the cell. Above data represent data obtained from a single cell.

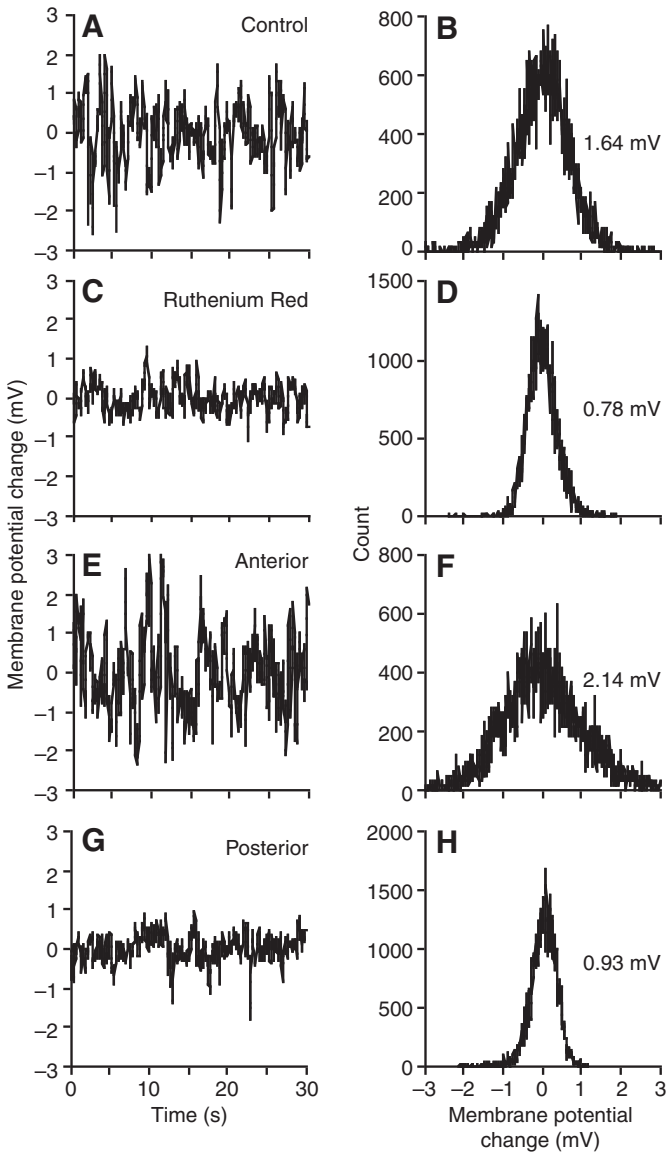

Fig. 2. Effects of Ca^{2+} inflow on the fluctuations of membrane potential. The fluctuation of membrane potential at the resting level was recorded for 5 min, and a fluctuation histogram from the mean potential level was calculated. (A) Fluctuation of membrane potential and (B) a fluctuation histogram using standard solution as the control. (C) Membrane potential fluctuation and (D) a fluctuation histogram in the presence of 5 μmol l^{-1} Ruthenium Red. (E) Membrane potential fluctuation and (F) a fluctuation histogram recorded from the anterior fragment of the cell. (G) Membrane potential fluctuation and (H) a fluctuation histogram recorded from the posterior fragment of the cell. Fluctuation amplitudes corresponding to the full width at half maximum (FWHMs) of the distributions are shown on the right side of the histograms.

When the fluorescence image of a cell was divided into anterior and posterior halves, the fluorescence intensities of the anterior and posterior halves sometimes increased at the same time, while others increased independently (Fig. 1Biii,iv). Therefore, it seems that intracellular Ca^{2+} sometimes increases simultaneously in an area exceeding half of the cell.

Contribution of $[Ca^{2+}]_i$ to resting potential fluctuation

In the control, deciliated *Paramecium* cells exhibited spontaneous fluctuation of their resting potential, the amplitudes of which were in the range of a few mV (Fig. 2A). When the potential fluctuations from the resting level were expressed as an amplitude histogram, the distribution was almost symmetric, with the resting level

showing the highest value (Fig. 2B). From such a distribution, we set the FWHM as a fluctuation amplitude at resting potential. In the control, the fluctuation amplitude was 1.64±0.17 mV (N=7 cells).

To understand the contribution of Ca^{2+} influx to the resting membrane potential fluctuation, some channel blockers were tested for their effects on the frequency of directional changes during swimming. Among those tested, the addition of Ruthenium Red, a possible blocker of Ca^{2+}-permeable channels, was found to reduce the frequency of directional changes. Then, we tested Ruthenium Red against the resting potential fluctuation of a deciliated cell. After the addition of 5 μmol l^{-1} Ruthenium Red, the potential fluctuation was attenuated, and the fluctuation amplitude was decreased to 0.78±0.22 mV (N=5 cells; Fig. 2C,D).

It has been shown that Ca^{2+}-permeable channels responsible for mechanical and thermal stimulation are localized in the anterior part of the *Paramecium* cell (Kuriu et al., 1996; Naitoh and Eckert, 1969; Nakaoka et al., 1987b; Ogura and Machemer, 1980). Based on this, we dissected deciliated cells into anterior and posterior fragments with a glass microelectrode, and each fragment was tested for its membrane potential fluctuation. The resting potential recorded from the anterior fragments elicited larger fluctuations than those from the posterior fragments (Fig. 2E,G). The fluctuation amplitudes of the anterior and posterior fragments were 2.14±0.08 mV (N=7 fragments) and 0.93±0.23 mV (N=5 fragments), respectively (Fig. 2F,H). These results suggest that Ca^{2+} influx at the resting state is important for the generation of membrane potential fluctuations.

In order to test the contribution of intracellular Ca^{2+} to the fluctuation, the intracellular Ca^{2+} was reduced by injection of BAPTA. After the injection, the resting potential fluctuation was decreased, and the fluctuation amplitude decreased to 0.42±0.12 mV (N=6 cells) (Fig. 3A,B).

The external addition of Ba^{2+} to the ciliated *Paramecium* induced frequent and continuous backward swimming referred to as the 'barium dance'. It has been shown that Ba^{2+} influx through Ca^{2+} channels abolishes the Ca^{2+}-dependent regulation of channels (Brehm and Eckert, 1978; Brehm et al., 1980). Addition of 5 mmol l^{-1} $BaCl_2$ to the deciliated *Paramecium* largely reduced the resting potential fluctuation, and the fluctuation amplitude was 0.40±0.08 mV (Fig. 3C,D). Hence, Ca^{2+}-dependent regulation seems to contribute to the amplification of potential fluctuation.

Simulation analysis of membrane potential fluctuation

Paramecium membrane is permeable to most cations, although it is most permeable to K^+ (Naitoh and Eckert, 1968; Naitoh and Eckert, 1974). Present experiments were performed in a solution containing 2 mmol l^{-1} K^+ and 1 mmol l^{-1} Ca^{2+}. Intracellular K^+ and Ca^{2+} concentration are, respectively, ~20 mmol l^{-1} (Naitoh and Eckert, 1974; Oka et al., 1986) and ~10^{-8} mol l^{-1} (Erxleben et al., 1997; Kuriu, 1996). Based on these circumstances, we have assumed that K^+ and Ca^{2+} channels of somatic membrane contribute to the resting potential fluctuation. In order to consider how a small increase in $[Ca^{2+}]_i$ leads to an amplification of resting potential fluctuations, three assumptions were made about the effects of $[Ca^{2+}]_i$ on the regulation of K^+ and Ca^{2+} channels (Cases I–III), and the fluctuations of the membrane potential were numerically simulated in each case using computational models. In these simulations, Ca^{2+} ions that could activate the ion channels were limited to those that stayed in the specific zones near the cell membrane, which we called 'hot spots' (Chay, 1993), and the Ca^{2+} concentration in the hot spots was denoted by $[Ca^{2+}]_s$. In Case I, in which the internal Ca^{2+} exerts no effects on the regulation of Ca^{2+} and K^+ channels, the simulated fluctuation of the resting potential

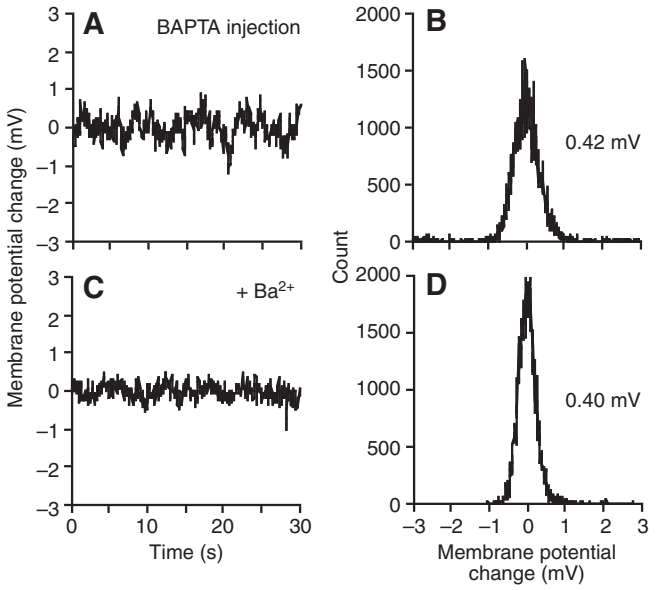

Fig. 3. Effects of $[Ca^{2+}]_i$ on the fluctuations of membrane potential. (A) Membrane potential fluctuation and (B) a fluctuation histogram recorded from a cell injected with BAPTA. (C) Membrane potential fluctuation and (D) a fluctuation histogram in the presence of 5 mmol l^{-1} $BaCl_2$. Fluctuation amplitudes corresponding to the full width at half maximum (FWHMs) of the distributions are shown on the right side of the histograms.

was much smaller than the measured fluctuation in the control (Fig. 4A,B). Therefore, a model where the fluctuations are caused by the random opening and closing of Ca^{2+} and K^+ channel gates is insufficient to explain the observed amplitude of membrane potential fluctuations. In Case II, in which only the K^+ channels are activated by $[Ca^{2+}]_s$, the simulated fluctuation (Fig. 4C,D) was even smaller than in Case I. Finally, in Case III, in which $[Ca^{2+}]_s$ activates both Ca^{2+} and K^+ channels with the dissociation constants of 1 μmol l^{-1} and 8 μmol l^{-1}, respectively, the amplitude of simulated fluctuation (Fig. 4E,F) was almost the same as the measured fluctuation (Fig. 2A,B). These simulations suggest that the $[Ca^{2+}]_s$-dependent activation of channels plays an essential role in the increase of the amplitude of resting potential fluctuations. In Case III, the simulation data also exhibit correlated changes of membrane potential and intracellular Ca^{2+} concentration (Fig. 5) just as the experimental data indicate (Fig. 1B). The simulated membrane potential changes are, however, followed by those of intracellular Ca^{2+} concentration with a time delay of 0.05 s on the average, while the experimentally observed time delay is 0.41 s. This disagreement should be attributed to the simplified description of the model for the diffusion process of Ca^{2+}, wherein the time needed for Ca^{2+} to diffuse spatially over the whole cell is neglected.

DISCUSSION

Paramecium cells in homogeneous conditions spontaneously change their direction of swimming. Such behavior is based on a spontaneous fluctuation of resting potential, the amplitudes of which are 1–3 mV. However, it is uncertain how such a large fluctuation in the resting potential is generated. This study noted the contribution of $[Ca^{2+}]_i$ to the amplification of membrane potential fluctuations.

Ca^{2+} influx during the resting potential

Simultaneous measurements of resting potential and $[Ca^{2+}]_i$ fluctuations show that a membrane depolarization of a few mV was

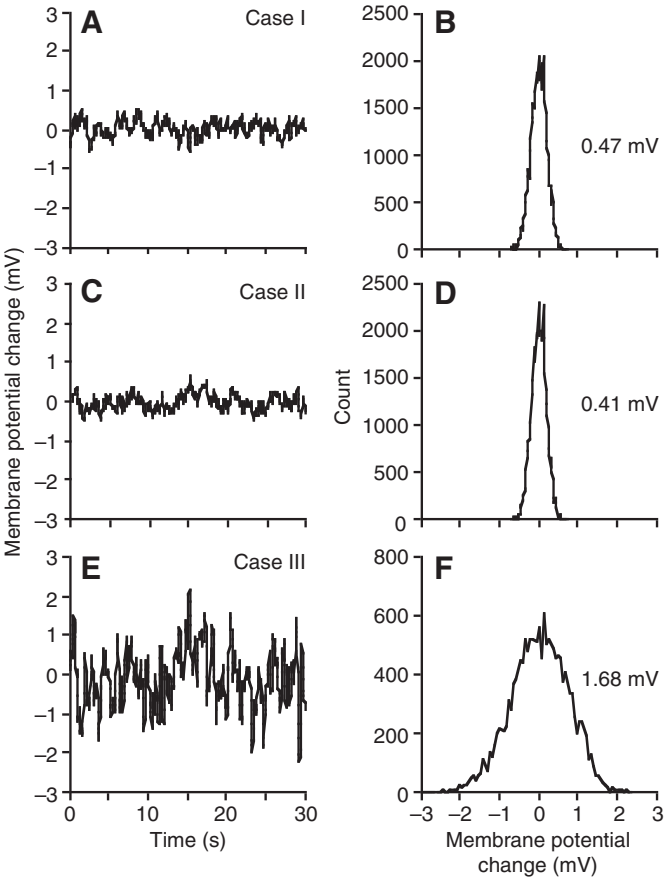

Fig. 4. Simulation of membrane potential fluctuations with a computational model as described in Appendix 1. Three cases of Ca^{2+} sensitivity of ion channels are assumed. The parameter values used in the simulation are given in Appendix 1. (A) Membrane potential fluctuation and (B) a fluctuation histogram in Case I, in which neither K^+ nor Ca^{2+} channels are assumed to have Ca^{2+} sensitivity. (C) Membrane potential fluctuation and (D) a fluctuation histogram in Case II, in which only K^+ channels are assumed to have Ca^{2+} sensitivity. (E) Membrane potential fluctuation and (F) a fluctuation histogram in Case III, in which both K^+ and Ca^{2+} channels are assumed to have Ca^{2+} sensitivity. Fluctuation amplitudes corresponding to the FWHMs of the distributions are shown on the right side of the histograms.

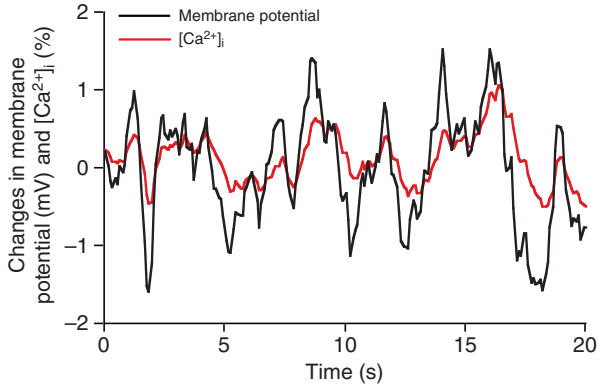

Fig. 5. Simulation of simultaneous changes of membrane potential and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) for Case III of the computational model as described in Appendix 1. Membrane potential fluctuations $\delta V(t)$ and fluctuation ratios of intracellular Ca^{2+} concentration $\delta\rho_i(t)$ are defined as $\delta V(t) \equiv V(t) - \langle V(t) \rangle$ and $\delta\rho_i(t) \equiv [\rho_i(t) - \langle\rho_i(t)\rangle]/\langle\rho_i(t)\rangle$, respectively, where $\langle V(t) \rangle$ and $\langle\rho_i(t)\rangle$ are long time averages of membrane potential $V(t)$ and intracellular Ca^{2+} concentration $\rho_i(t)$, respectively. Fluctuation ratios of intracellular Ca^{2+} concentration $\delta\rho_i(t)$ are indicated as changes in $[Ca^{2+}]_i$ in the ordinate.

frequently accompanied by a small increase in $[Ca^{2+}]_i$ about 0.4 s later (Fig. 1Bi,ii). This result implies that the opening of Ca^{2+} channels in the surface membrane leads to a Ca^{2+} influx and a diffusion into the intracellular space. Although the gating characteristics of Ca^{2+} channels are not yet clear, the present measurements suggest that these Ca^{2+} channels go through opening and closing fluctuations at the resting potential. Since the resting potential of *Paramecium* cells is depolarized not only by an increase in extracellular K^+ but also by an increase in extracellular Ca^{2+} (Naitoh and Eckert, 1968; Nakaoka et al., 1987b), the cell membrane is permeable to both K^+ and Ca^{2+}. Therefore, the Ca^{2+} channels that affect the resting potential seem to be related to the $[Ca^{2+}]_i$ fluctuation. In addition to these channels, the Ca^{2+}-permeable channels that are sensitive to mechanical (Naitoh and Eckert, 1969; Ogura and Machemer, 1980) and thermal (Kuriu et al., 1996; Nakaoka et al., 1987b) stimulation may also contribute to the $[Ca^{2+}]_i$ fluctuation.

The fluctuation of $[Ca^{2+}]_i$ is not uniform across the whole cell area. Temporal fluctuations of $[Ca^{2+}]_i$ in the anterior half are different from those in the posterior half. However, sometimes the fluctuations of both the anterior and posterior halves increase simultaneously (Fig. 1Biii,iv). These characteristics of $[Ca^{2+}]_i$ fluctuations imply that a small increase in $[Ca^{2+}]_i$ occurs collectively across a considerable area within the cell and that such an increase in $[Ca^{2+}]_i$ may be caused by a cooperative activation of the Ca^{2+} channels in the somatic surface membrane.

$[Ca^{2+}]_i$ -dependent activation of resting potential fluctuation

In most animal cells, the amplitudes of potential fluctuations at resting state are much smaller than 1 mV. However, some secretory cells and heart cells exhibit oscillatory action potentials with simultaneous intracellular Ca^{2+} oscillations (Chay, 1993; Kass and Tsien, 1982; Li et al., 1995). In those cells, Ca^{2+} influx plays a key role in generating the oscillations. Some experiments in the present study have shown that the Ca^{2+} influx through the somatic membrane is important for the generation of potential fluctuations with large amplitudes. Firstly, the external addition of Ruthenium Red, a possible blocker of Ca^{2+}-permeable channels, decreased the amplitude of the potential fluctuation (Fig. 2C,D). Secondly, the recordings of the dissected cell showed that the anterior fragment, in which Ca^{2+} channels sensitive to mechanical and thermal stimulation are localized, generated much larger fluctuations than the posterior fragment (Fig. 2E–H). Thirdly, an intracellular injection of BAPTA to reduce $[Ca^{2+}]_i$ resulted in a decrease of the fluctuation amplitude (Fig. 3A,B). Therefore, the Ca^{2+} influx and the increase in $[Ca^{2+}]_i$ play a key role in the amplification of resting potential fluctuations.

External addition of Ba^{2+} attenuated the fluctuation amplitude (Fig. 3C,D). Ba^{2+} is considered to be able to permeate through Ca^{2+} channels, and internal Ba^{2+} competitively interacts with the Ca^{2+}-sensitive sites of Ca^{2+} and K^+ channels (Brehm et al., 1978; Kas and Tsien, 1982). Interaction of these sites with Ba^{2+} consequently inhibits the $[Ca^{2+}]_i$-dependent regulation of channels. Thus, this result also suggests that the $[Ca^{2+}]_i$-dependent regulation of the channels is essential for the amplification of resting potential fluctuations.

Simulation analysis of resting potential fluctuation

Fluctuations at resting potential are essentially caused by the opening and closing fluctuations of the ion channel gates that are distributed on the somatic membrane. These ion channels are thought to be Ca^{2+} and K^+ channels. However, in the conditions of Case I, in which the internal Ca^{2+} had no effect on these channels, the simulated fluctuation of resting potential was much smaller than the measured fluctuation in the control (Fig. 4A,B). Therefore, one needs another mechanism to enhance the fluctuation caused by stochastic gating of the channels. The most natural mechanism for such an enhancement may be the regulation of channels by intracellular Ca^{2+}. Therefore, the effects of intracellular Ca^{2+} on the regulation of channels are required for the amplification of resting potential fluctuation.

Intracellular Ca^{2+} gives positive and negative effects on the activity of cation channels. In *Paramecium*, some K^+ channels are activated by Ca^{2+} (Saimi and Martinac, 1989; Saitow et al., 1997). On the other hand, several Ca^{2+}-permeable TRP channels are activated by Ca^{2+} concentrations of 0.3–1 μmol l^{-1} and inhibited at higher Ca^{2+} levels (Harteneck, 2005; Minke, 2006; Zhu, 2005). Although TRP channels have not yet been found in *Paramecium*, Ca^{2+}/calmodulin-activated inward current has been shown (Erxleben and Plattner, 1994). Two possible mechanisms for the interaction between Ca^{2+} and cation channels were tested using the computer simulation. In Case II, in which internal Ca^{2+} activates the gating of only K^+ channels, the simulated fluctuation was even smaller than that of Case I (Fig. 4C,D). Such a decrease in fluctuation is caused in Case II because the fluctuating depolarization associated with a temporary increase of Ca^{2+} inflow always activates K^+ channels, and the resultant K^+ outflow immediately acts with the membrane potential to return it to its former state. In Case III, both the Ca^{2+} and K^+ channels are activated by the $[Ca^{2+}]_s$ staying in the specific zone near the surface membrane with dissociation constants for Ca^{2+} and K^+ channels being 1 μmol l^{-1} and 8 μmol l^{-1}, respectively. In this case, a small temporary increase of Ca^{2+} inflow first leads to activation of the Ca^{2+} channels, which brings about a further increase of Ca^{2+} inflow, thereby amplifying the membrane depolarization. This positive-feedback process is repeated until the $[Ca^{2+}]_s$ in the specific zone increases sufficiently to activate K^+ channels, which in turn causes the membrane hyperpolarization. Due to this interplay between the membrane potential and $[Ca^{2+}]_s$, the simulated membrane potential and intracellular Ca^{2+} concentration vary interdependently (Fig. 5). The simulated fluctuation exhibits a marked increase, being almost the same amplitude as the measured fluctuation (Fig. 4E,F). To increase the fluctuation amplitude, it is therefore assumed that it is the Ca^{2+} channels whose activities are upregulated by an increase in $[Ca^{2+}]_i$. However, such Ca^{2+} channels have not yet been identified by experiments using *Paramecium* cells. We are now trying to find mutant cells that show no spontaneous directional changes in their swimming and elicit attenuated potential fluctuations during the resting state.

In summary, various experimental results in this study have shown that a small increase in $[Ca^{2+}]_i$ caused by Ca^{2+} inflow through the surface membrane plays a key role in the generation of resting potential fluctuations with large amplitudes. We assumed that the increase in $[Ca^{2+}]_i$ activates either Ca^{2+} or K^+ channels on the somatic membrane. Simulation analyses with such assumptions have indicated that the regulation of the Ca^{2+} channels by $[Ca^{2+}]_i$ is essential for the generation of the observed large fluctuations of resting potential. The actual Ca^{2+} sensitivities of these channels, especially Ca^{2+} channels, are yet to be determined.

APPENDIX 1

The model used for the simulations of membrane potential fluctuations was constructed on the basis of the model proposed by Oosawa (Oosawa, 2001). In the Oosawa model, it is assumed that the resting potential of the cell membrane is produced by two kinds of cation currents across the membrane, i.e. the current I_A of cation A^+ and the current I_B of cation B^+, and the membrane potential $V(t)$ at time t is determined from the equation:

$$C\frac{dV(t)}{dt} = I_A + I_B \,, \tag{1}$$

where C is the capacitance of the membrane. The membrane currents I_A and I_B are given by:

$$I_{A(B)} = -G_{A(B)}n_{A(B)}(V(t) - V_{A(B)}) \,, \tag{2}$$

where $G_{A(B)}$ is the conductance of a single $A^+(B^+)$ channel, $n_{A(B)}(t)$ is the number of the $A^+(B^+)$ channels that are open at time t, and $V_{A(B)}$ is the equilibrium potential defined by:

$$V_{A(B)} = \left(\frac{kT}{e_{A(B)}}\right)\ln\left(\frac{[A^+(B^+)]_o}{[A^+(B^+)]_i}\right) , \tag{3}$$

where k, T, and $e_{A(B)}$ are the Boltzmann constant, the absolute temperature, and the electric charge of the cation $A^+(B^+)$, respectively; $[A^+(B^+)]_i$ and $[A^+(B^+)]_o$ represent the concentrations of cation $A^+(B^+)$ inside and outside the cell, respectively. Here, we postulate that $[A^+]_i > [A^+]_o$, while $[B^+]_i < [B^+]_o$. $n_{A(B)}(t)$ is assumed to be a stochastic variable that fluctuates around the constant average $\langle n_{A(B)} \rangle$ according to a Poisson distribution. Thus, Eqn 1 is a Langevin-type equation, wherein the opening and closing fluctuations of the channels act as a random force.

In application of the Oosawa model to *Paramecium* cells, we identified the cations A^+ and B^+ as K^+ and Ca^{2+} ions (Naitoh et al., 1972) and have modified the model by introducing Ca^{2+} sensitivity into these ion channels; we consider that Ca^{2+} ions that can activate the ion channels are limited to those that stay in the specific zones near to the cell membrane, which we call 'hot spots' (Chay, 1993), and therefore have assumed that $\langle n_{A(B)} \rangle$ depends on the concentration $\rho_s(t)$ of Ca^{2+} ions in the hot spots as:

$$< n_{K(Ca)} > = N_{K(Ca)}\left\{1 - R_{K(Ca)} + R_{K(Ca)}\frac{\rho_s(t)}{K_{K(Ca)} + \rho_s(t)}\right\} , \tag{4}$$

where $N_{K(Ca)}$ and $R_{K(Ca)}$ are constant parameters, and $K_{K(Ca)}$ is the dissociation constant of the Ca^{2+} ion from the $K^+(Ca^{2+})$ channel. We note that $R_{K(Ca)}$ represents the rate of the number of the Ca^{2+}-sensitive $K^+(Ca^{2+})$ channels and therefore takes the value $0 \le R_{K(Ca)} \le 1$. We describe the change of $\rho_s(t)$ by the following diffusion-type equation (Chay, 1993):

$$\frac{d\rho_s(t)}{dt} = \frac{I_{Ca}}{2F\sigma S} - k\rho_s(t) \,, \tag{5}$$

where F and k are the Faraday constant and diffusion constant, respectively, and S and σ are the area of the membrane surface and the effective thickness of the hot spots, respectively. On the other hand, the change of the intracellular Ca^{2+} concentration $\rho_i(t)$ observed by the measurement of fluorescence intensity is assumed to obey the following equation (Chay, 1993):

$$\frac{d\rho_i(t)}{dt} = \eta\{k\rho_s(t) - k_p[\rho_i(t) - \rho_{rest}]\} \,, \tag{6}$$

where the first term on the right-hand side is the influx of Ca^{2+} ions from the hot spots; the second term is the efflux of Ca^{2+} ions due to $\rho_i(t)$-dependent pumping of Ca^{2+} ions out of the cell; the third term is the leaking flux of Ca^{2+} ions responsible for keeping $\rho_i(t)$ around the resting level ρ_{rest}; κ_p is a constant and η also is a constant representing a measure of the buffer strength. Eqns 5 and 6 are coupled to Eqn 1 through Eqns 2 and 4, and these simultaneous equations describe the temporal evolution of the membrane and intracellular system in a closed form.

Based on the above equations, we performed numerical simulations of the membrane potential fluctuations, wherein $n_{K(Ca)}(t)$ in Eqn 2 was calculated from Eqn 4 using Poissonian random integers generated by a computer. To clarify the effects of the Ca^{2+} sensitivity of the ion channels on the fluctuations of the membrane potential, we calculated the membrane potential for the case that neither the K^+ nor Ca^{2+} channels have Ca^{2+} sensitivity (Case I), the case that only K^+ channels have Ca^{2+} sensitivity (Case II), and the case that both K^+ and Ca^{2+} channels have Ca^{2+} sensitivity (Case III). The values of the parameters are chosen to reproduce the average resting potential (–25 mV) and the full width at half maximum (FWHM) of the distribution of the membrane potential in each case, i.e. the FWHM obtained from the control data in Case III and that obtained from the BAPTA-applied data in Cases I and II. In Case III, the data of the cross-correlation between the fluctuations of the membrane potential and the intracellular Ca^{2+} concentration are also used to fix the values of k_p and η.

The parameter values whereby the best fits were obtained are as follows:

Case I: G_k/C=0.012 (s^{-1}), G_{Ca}/C=0.018 (s^{-1}), V_k=–57 (mV), V_{Ca}=120 (mV), R_K=0, R_{Ca}=0, N_K=220, N_{Ca}=32.

Case II: R_K=0.8, R_{Ca}=0, N_K=1000, N_{Ca}=32, K_K=8 ($\mu mol\,l^{-1}$), F=96,500 ($C\,mol^{-1}$), k=80 (s^{-1}), C/S=1.7 $\mu F\,cm^{-2}$, $\sigma=5\times10^{-5}$ (cm); the values of the other parameters are the same as Case I.

Case III: R_K=0.8, R_{Ca}=1, N_K=1000, N_{Ca}=1000, K_K=8 ($\mu mol\,l^{-1}$), K_{Ca}=1 ($\mu mol\,l^{-1}$), k_p=80 (s^{-1}), ρ_{rest}=0.1 ($\mu mol\,l^{-1}$), η=0.005; the values of the other parameters are the same as Case II.

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