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Cold-sensitive Ca^{2+} Influx in *Paramecium*

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Abstract. The concentration of intracellular calcium, $[Ca^{2+}]_i$, in *Paramecium* was imaged during cold-sensitive response by monitoring fluorescence of two calcium-sensitive dyes, Fluo-3 and Fura-Red. Cooling of a deciliated *Paramecium* caused a transient increase in $[Ca^{2+}]_i$ at the anterior region of the cell. Increase in $[Ca^{2+}]_i$ was not observed at any region in Ca^{2+}-free solution. Under the electrophysiological recording, a transient depolarization of the cell was observed in response to cooling. On the voltage-clamped cell, cooling induced a transient inward current under conditions where K^+ currents were suppressed. These membrane depolarizations and inward currents in response to cooling were lost upon removing extracellular Ca^{2+}. The cold-induced inward current was lost upon replacing extracellular Ca^{2+} with equimolar concentration of Co^{2+}, Mg^{2+} or Mn^{2+}, but it was not affected significantly by replacing with equimolar concentration of Ba^{2+} or Sr^{2+}. These results indicate that *Paramecium* cells have Ca^{2+} channels that are permeable to Ca^{2+}, Ba^{2+} and Sr^{2+} in the anterior soma membrane and the channels are opened by cooling.

Key words: Thermoreception — Ca^{2+} response — Ca^{2+} channel — Cold-sensitive — Fluo-3 — *Paramecium*

Introduction

Many organisms have thermal sensation (Jennings, 1906; Hensel, 1974). However, the mechanism of thermoreception remains unknown. A *Paramecium* responds to cooling below the temperature to which it has been adapted, and to warming above this temperature, by producing transient changes in the frequency of directional changes in swimming (Nakaoka & Oosawa, 1977). The temperature change induces a slow transient depolarization of the membrane potential and this depolarization triggers the opening of the voltage-dependent Ca^{2+} channel in the ciliary membrane and thus initiates the action potential. The Ca^{2+} influx increases the intraciliary concentration of Ca^{2+} and this Ca^{2+} causes the directional changes in swimming (Hennessey et al., 1983). Previous measurements of the membrane potential suggested that a transient depolarization in response to cooling is induced by a decrease of K^+ conductance and the depolarization in response to warming is induced by an increase of Ca^{2+} conductance (Hennessey et al., 1983; Nakaoka et al., 1987; Inoue & Nakaoka, 1990).

In the present study, using image analysis of the concentration of intracellular calcium, $[Ca^{2+}]_i$, and electrophysiological recording, we show that cooling of a deciliated *Paramecium* induced a transient increase of $[Ca^{2+}]_i$ at the anterior region, and this $[Ca^{2+}]_i$ increase was caused by influx of external Ca^{2+}. Thus cooling of a *Paramecium* cell induced not only a decrease of K^+ conductance but also an increase of Ca^{2+} conductance.

Materials and Methods

Cell Culture

Paramecium multimicronucleatum was cultured in a hay infusion inoculated with *Klebsiella pneumoniae.* The culture temperature was kept constant at 25°C by incubation in a water bath. *Paramecium* cells at the stationary phase were collected by low speed centrifugation and suspended in an adaptation solution containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4 mM KCl and 1 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid)-Tris [Tris(hydroxymethyl)aminomethane] (pH 7.2). Cells were preincubated in this solution at 25°C for 1 hr or more prior to examination.

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Deciliation

The cells were deciliated by incubation in an adaptation solution containing 5% ethanol for 0.5–1 min and then transferred to the various experimental solutions (Ogura & Machemer, 1984).

Solutions

When the responses of $[Ca^{2+}]_i$ imaging or membrane potential under current clamp were recorded, the deciliated cells were transferred to the standard solution which contained 1 mM $CaCl_2$, 4 mM KCl and 1 mM HEPES-Tris (pH 7.2) or the Ca^{2+}-free solution which contained 1 mM $MgCl_2$, 4 mM KCl and 1 mM HEPES-Tris (pH 7.2). The solution used to bathe the deciliated cells during voltage clamp (Ca^{2+}/TEA^+ solution) contained 1 mM $CaCl_2$, 10 mM TEA-Cl (tetraethylammonium chloride) and 1 mM HEPES-Tris (pH 7.2). In some experiments, 1 mM $CaCl_2$ was replaced by equimolar concentration of $BaCl_2$, $SrCl_2$, $CoCl_2$, $MgCl_2$ or $MnCl_2$.

Temperature Change

The deciliated cells were placed in a glass vessel mounted on an inverted microscope. The temperature was varied by switching the water flow beneath the vessel, and was monitored with a thermocouple probe placed near the specimen in the vessel (Nakaoka et al., 1987).

Image Analysis of $[Ca^{2+}]_i$ Change

Image analysis of the $[Ca^{2+}]_i$ change was performed using a mixture of the calcium indicator dyes, fluo-3 and Fura-Red (Lipp & Niggli, 1993; Novak & Rabinovitch, 1994) with a $[Ca^{2+}]_i$ image analysis system ARGUS-50/CA (Hamamatsu Photonics, Japan). The principal advantage of using mixture of the fluo-3 and Fura-Red is the ability to make measurements as a ratio of fluorescence intensity at two wavelengths. This ratiometric approach yields the value of $[Ca^{2+}]_i$ which is independent of the amount of dye contained within the cell; in addition it minimizes the effect of variations in emission intensity due to variations of illumination intensity, emission collection efficiency, and effective cell thickness in determining actual $[Ca^{2+}]_i$. The set of filters used for fluo-3 and Fura-Red was as follows: a bandpass excitation filter centered at 480 nm (half bandpass 20 nm); a long-pass emission filter centered at 520 nm; a fluo-3 emission filter centered at 525 nm (half bandpass 10 nm) and a Fura-Red emission filter centered at 660 nm (half bandpass 10 nm). Fluorescence images at 525 nm for fluo-3 and 660 nm for Fura-Red upon excitation at 480 nm were stored in a computer (PC/AT compatible) with time intervals of 3 sec and then fluo-3/Fura-Red ratio was calculated. The values of the fluorescence ratio at two areas (anterior or posterior) of the cell are shown separately in Fig. 2. Anterior region was determined by locating the oral apparatus.

Microinjection

The arrangement of pressure injection was a modification of the methods that have been described by Oosawa and Yamagishi (1989). The solution was injected into a *Paramecium* cell by means of an air pressure pulse (30–60 psi, 0.3–0.5 sec: Basic Picosprizer, General Valve), through a microcapillary of the tip diameter of about 1 μm. A mixture of 0.2 mM fluo-3 (Dojindo, Kumamoto, Japan) and 1 mM Fura-Red (Molecular Probes, OR) both dissolved in water was injected into the cell. Injected volumes (50–100 pl) were between 10 and 20% of the cell volume which was assumed to be 500 pl.

Calibration

Calibration was done as follows: the mixed solution of fluo-3 (20 μM, final concentration) and Fura-Red (100 μM, final concentration) were added to calcium calibration buffer solutions (Calcium Calibration Buffer Kit #2, Molecular Probes, OR) which contained 1, 17, 38, 65, 100, 150, 225, 351, 602, 1350 nM Ca^{2+} in 100 mM KCl, 10 mM MOPS, pH 7.2. Our results are given in terms of both fluo-3/Fura-Red ratio and Ca^{2+} concentration.

Intracellular Recording

Membrane potentials and membrane currents of *Paramecium* were recorded using a method described previously (Naitoh & Eckert, 1972; Nakaoka & Iwatsuki, 1990) and retained on a chart recorder with the paper moving at a speed of 4 cm/min. The capillary microelectrodes used for current clamp contained 1 M KCl, with tip resistance of about 50 Mohm. Voltage clamp electrodes contained 1 M CsCl, with tip resistance of about 50 Mohm.

Results

$[Ca^{2+}]_i$ Imaging

Cooling from 25 to 20°C caused a transient increase of $[Ca^{2+}]_i$ at the anterior region of the deciliated cell in standard solution (Fig. 1*B*). In Ca^{2+}-free solution, increase of $[Ca^{2+}]_i$ was not observed at any region (Fig. 1*C*). Figure 1*A* shows $[Ca^{2+}]_i$ image at 5 sec after cooling (maximum response; frame 3 in Fig. 1*B* and *C*). The values of the fluorescence ratio at two areas (anterior or posterior) of the cell are shown in Figure 2. The transient increase of $[Ca^{2+}]_i$ in response to cooling attained a peak value at about 5 sec from the start of cooling (Fig. 2*A*). Return from 20 to 25°C did not cause an increase of $[Ca^{2+}]_i$ at any region. In a buffer solution that contained 17 nM Ca^{2+}, 20 μM fluo-3 and 100 μM Fura-Red, the value of fluo-3/Fura-Red ratio was not changed significantly (about −0.1 as a ratio value) by temperature change from 25 to 20°C. Therefore, this temperature change did not affect significantly the ratio value when calcium concentration was constant.

Electrophysiological Recording

A *Paramecium* cell responded to cooling with a transient membrane depolarization (Fig. 3). This depolarization had a peak value about 5 sec from the start of cooling in standard solution. In Ca^{2+}-free solution, a small underlying hyperpolarization was observed (Fig. 3). Cooling of the cell which was voltage clamped at −30 mV (near resting potential in standard solution) elicited a transient inward current (Fig. 4*A*). In this experiment, the K^+ currents were suppressed by the use of a voltage clamp

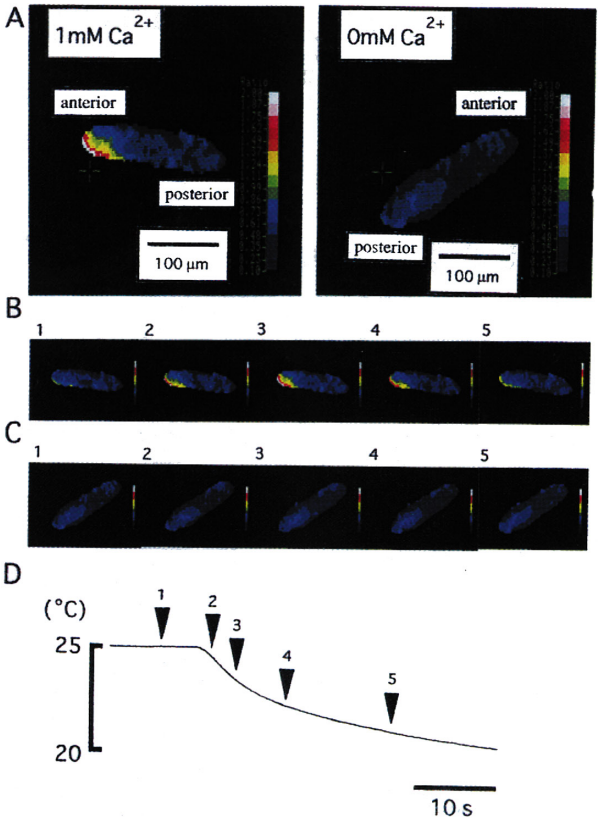

Fig. 1. (*A*) $[Ca^{2+}]_i$ responses of *Paramecium* cells in standard solution (left image) and in Ca^{2+}-free solution (right image) at arrowhead 3 in (*D*). (*B*) Ratio images of $[Ca^{2+}]_i$ response in standard solution, and (*C*) in Ca^{2+}-free solution. (*D*) A record of temperature. The arrowheads in (*D*) indicate the time points where the ratio images were obtained in (*B*) and (*C*). These images are representative of results obtained in 35 additional cells in standard solution and in 25 additional cells in Ca^{2+}-free solution.

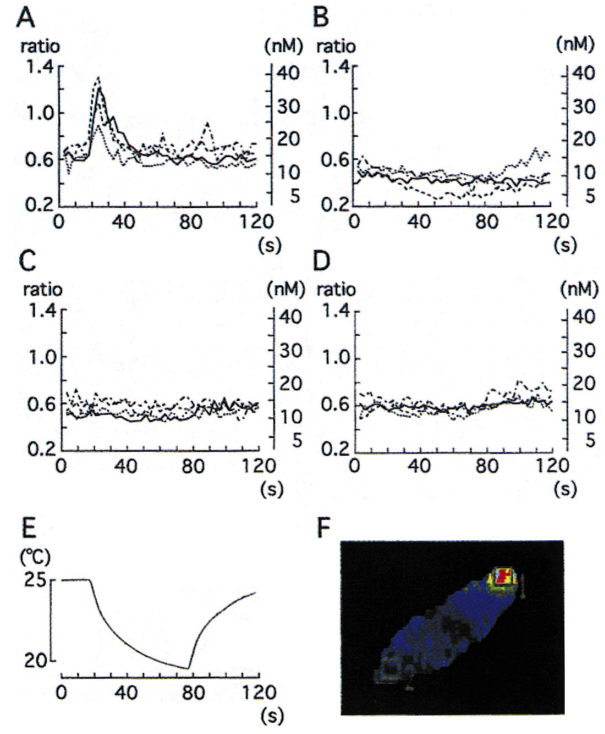

Fig. 2. $[Ca^{2+}]_i$ responses in standard solution (*A*, anterior; *C*, posterior; data from four different cells), and in Ca^{2+}-free solution (*B*, anterior; *D*, posterior; data from four different cells). (*E*) A record of temperature. Anterior (1) and posterior (2) areas that were calculated in (*A*) to (*D*) are shown in (*F*).

electrode containing 1 M CsCl, and by including 10 mM TEA-Cl in the bath solution (Hinrichsen & Saimi, 1984). The cold-induced inward current was lost in a solution replaced 1 mM $CaCl_2$ with 1 mM $MgCl_2$ (Mg^{2+}/TEA^+ solution), where a small underlying outward current was observed (Fig. 4*A*). Subsequently, when the cell was returned to Ca^{2+}/TEA^+ solution, it showed cooling induced inward current again (*data not shown*). The amplitude of cold-induced inward current in Ca^{2+}/TEA^+ solution decreased as the membrane potential was made more positive than resting potential (Fig. 4*B*). However, inward current always appeared below 0 mV. At −30 and −20 mV, the cold-induced inward current was not observed in Mg^{2+}/TEA^+ solution, however, at −10 and 0 mV, the small inward current was observed a few times. The voltage clamp of the membrane potential at more positive than 0 mV caused the increase in fluctuation of the membrane current, and the cold-induced inward current was indistinguishable from its underlying fluctuation.

The ability of other divalent cations to cause the cold-induced inward current was tested. The cold-induced inward current was lost in the solutions replaced extracellular Ca^{2+} in Ca^{2+}/TEA^+ solution with equimolar concentration of Co^{2+}, Mg^{2+} or Mn^{2+}, but it was not affected significantly by replacing Ca^{2+} with equimolar concentrations of Ba^{2+} (Ba^{2+}/TEA^+ solution) or Sr^{2+} (Sr^{2+}/TEA^+ solution) (Fig. 5). The cells transferred to Ca^{2+}/TEA^+, Ba^{2+}/TEA^+ or Sr^{2+}/TEA^+ solution showed the inward currents whose peak amplitude from the resting level were respectively, −208.0 ± 23.2 pA (n = 5, mean ± *SD*), −214.0 ± 67.0 pA (n = 4), −222.0 ± 20.7 pA (n = 4) at −30 mV. However, in the Ca^{2+}/TEA^+ solution, the inward current decayed within about 40 sec after the start of cold stimuli, while the decay was slowed when the extracellular Ca^{2+} was replaced by either Ba^{2+} or Sr^{2+} (Fig. 5).

Discussion

Cold-Induced Ca^{2+} Influx and Membrane Depolarization

Cooling from 25 to 20°C caused a transient increase in $[Ca^{2+}]_i$ at the anterior region of a deciliated *Paramecium*

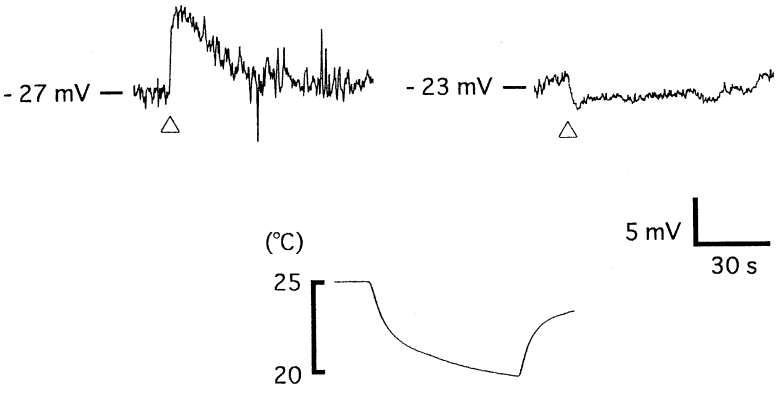

Fig. 3. Changes in membrane potential of *Paramecium* cells elicited by cooling in standard solution (left trace) and in Ca^{2+}-free solution (right trace). Open arrowheads indicate the start of cooling. Bottom trace is a record of temperature. These traces are representative of results obtained in four additional cells in standard solution and in two additional cells in Ca^{2+}-free solution.

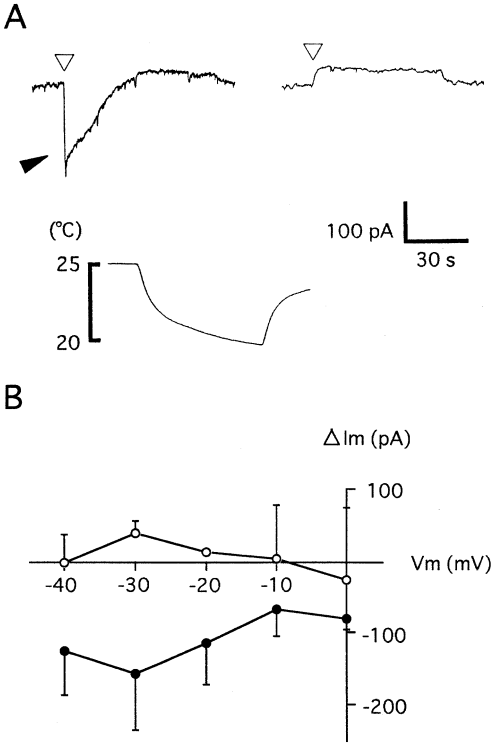

Fig. 4. (*A*) Cold-induced membrane currents in Ca^{2+}/TEA^{+} solution (left trace), and in a solution replaced 1 mM $CaCl_2$ with 1 mM $MgCl_2$ (right trace). Holding potential was −30 mV. Open arrowheads indicate the start of cooling, and filled arrowhead indicates the cold-induced Ca^{2+} current. Bottom trace is a record of temperature. (*B*) The *I–V* relationships of the cold-induced membrane current. The cold-induced membrane current from the resting level, ΔIm, was measured at 5 sec after the start of cooling. Closed circles, in Ca^{2+}/TEA^{+} solution; open circles, in a solution replaced 1 mM $CaCl_2$ with 1 mM $MgCl_2$. Each symbol is the mean (± SD) of measurements from 3–5 cells.

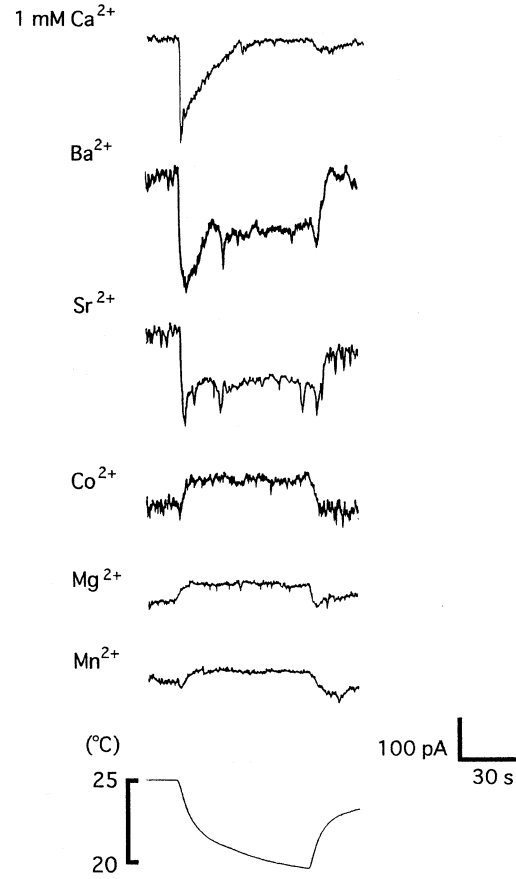

Fig. 5. Divalent cation selectivity of cold-induced inward current. Currents elicited by cooling in Ca^{2+}/TEA^{+} solution, and upon replacing 1 mM $CaCl_2$ with equimolar concentration of $BaCl_2$, $SrCl_2$, $CoCl_2$, $MgCl_2$ or $MnCl_2$. Representative recording are shown from different cells in each solution. Bottom trace is a record of temperature.

cell (Figs. 1 and 2). The membrane potential response of the cell to cooling was a transient depolarization (Fig. 3). These increases of $[Ca^{2+}]_i$ and membrane depolarization in response to cooling were lost upon removing extracellular Ca^{2+} (Figs. 1–3), suggesting that they were accomplished by influx of external Ca^{2+} through cold-sensitive Ca^{2+} channels at the anterior soma membrane.

Previous measurements of the membrane potential indicated that the reversal potential of the cold-induced depolarization was more negative than the resting potential and was dependent on extracellular K^{+} concentration

(Nakaoka et al., 1987; Inoue & Nakaoka, 1990). Based on these results, it was thought that cold-induced depolarization was primarily attributed to decrease of K^+ conductance. In the present study, we found that cooling of the voltage-clamped cell induced Ca^{2+} current under conditions where K^+ current was suppressed (Fig. 4*A*). Thus the cold-induced depolarization involves both decrease of K^+ conductance and increase of Ca^{2+} conductance. In addition, membrane depolarization in response to cooling was triggered by increase of Ca^{2+} conductance rather than decrease of K^+ conductance because the depolarization was lost upon removing extracellular Ca^{2+} (Fig. 3). Decrease of K^+ conductance may serve to enhance and prolong the cold-induced depolarization.

Inactivation of Cold-Induced Ca^{2+} Current

The Ca^{2+} current was induced by cooling of the voltage-clamped cell and subsequently decayed within about 40 sec (Fig. 5). This decay was slowed when the extracellular Ca^{2+} was replaced by either Ba^{2+} or Sr^{2+}. In *Paramecium*, Ca^{2+} influx leads to inactivation of depolarization- or hyperpolarization-activated Ca^{2+} channels (Brehm & Eckert, 1978; Preston et al., 1992*b*). The decay of cold-induced inward current may also result from Ca^{2+}-dependent inactivation of cold-sensitive Ca^{2+} channels.

Ion Selectivity of Cold-Sensitive Ca^{2+} Channel

The cold-induced inward current was not affected in amplitude significantly by replacing Ca^{2+} with equimolar concentration of Ba^{2+} or Sr^{2+}, but it was lost upon replacing extracellular Ca^{2+} with equimolar concentration of Co^{2+}, Mg^{2+} or Mn^{2+}, where a small underlying outward current was observed (Fig. 5). This outward current may represent Cl^- influx or Cs^+ efflux. Depolarization-activated Ca^{2+} channel located in ciliary membrane was permeable to Ba^{2+} and Sr^{2+} (Brehm & Eckert, 1987), while hyperpolarization-activated Ca^{2+} channel located in soma membrane was impermeable to these ions (Preston et al., 1992*a*). Ion selectivity of depolarization-activated Ca^{2+} channel was similar to that of cold-sensitive Ca^{2+} channel, but depolarization-activated Ca^{2+} channel was different from cold-sensitive Ca^{2+} channel because the cold-sensitive Ca^{2+} current were seen in deciliated cells. Heat-sensitive, mechanosensitive or lysozyme (chemorepellent)-sensitive Ca^{2+} channels in *Paramecium* were permeable to Mg^{2+} and Mn^{2+} (Tominaga & Naitoh, 1994; Hennessey et al., 1995). Therefore, the cold-sensitive Ca^{2+} channel may be different from these channels.

It is unknown whether this Ca^{2+} channel is directly controlled by cooling or the Ca^{2+} channel is controlled by some second messengers which respond to cooling. Further investigation of the operation of this Ca^{2+} channel is needed.

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