Cold-sensory response in *Paramecium* membrane

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Abstract

The cold-sensitive responses of the ciliated protist *Paramecium* was examined by analysis of swimming behavior, electrophysiological recording, and image analysis of the concentration of intracellular calcium. A *Paramecium* cell responded to cold stimuli, by increasing frequency of ciliary reversal and membrane depolarization. Comparisons of the cold-sensitive responses between wild type and calmodulin mutants of *Paramecium tetraurelia* show that *cam*^{12} mutant reduced both responses in the swimming and the membrane potential. Under voltage clamp conditions, cooling to the wild-type cell induced a transient inward current whose amplitude increased with the rate of temperature drop. The *cam*^{12} mutant also reduced the amplitude of the current response. The amplitude of the cold-induced inward current increased with positive shifts of the potential, and it was significantly blocked by using CsCl-filled electrodes and tetraethyammonium in the bath solution, suggesting that it was accompanied mainly by decrease of K conductance. On the other hand, the addition of a calmodulin antagonist, W-7, or the microinjection of a Ca^{2+} chelator, EGTA, reduced the amplitude of the cold-induced inward current of wild type. These results suggest that the cold-induced decrease of K conductance in *Paramecium* was modulated by Ca^{2+}/calmodulin.

Cooling of *Paramecium multimicronucleatum* or *P. tetraurelia* induced not only a decrease of K conductance but also an increase of Ca conductance. Under image analysis of the concentration of intracellular calcium, $[Ca^{2+}]_i$, cooling of a cell caused a transient increase in $[Ca^{2+}]_i$ at the anterior region of the cell. On the voltage-clamped cell at resting potential, cooling induced a transient inward current under conditions

where K current suppressed. The amplitude of the current decreased as the membrane potential was made more positive than resting potential and it was 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 cold-sensitive Ca conductance that are permeable to Ca^{2+}, Ba^{2+} and Sr^{2+} in the anterior soma membrane. Moreover, cold-activated Ca current was reversibly inhibited by amiloride or some divalent cations in a concentration-dependent manner. Based on these properties, I suggest that Ca current activated by cooling is mediated by a novel, cold-activated Ca conductance that is distinct from depolarization-, hyperpolarization-, chemorepellent-, mechano- or heat-activated Ca conductances in *Paramecium*.

要旨

繊毛虫ゾウリムシの冷刺激感受性反応について調べるために、行動解析、電気生理学的測定、細胞内 Ca^{2+}濃度測定を行った。ゾウリムシは、冷刺激に対して繊毛打逆転による遊泳方向変換と膜電位の脱分極応答を引き起こす。野生株とカルモジュリン変異株の冷刺激に対する反応を比較したところ、カルモジュリン変異株である *cam*^{12} がこれらの反応をほとんど示さないことが分かった。膜電位固定下で、野生株に冷刺激を与えると、一過性の内向き電流を引き起こすが、*cam*^{12}ではこの電流応答も小さくなっていた。冷刺激に対する内向き電流応答は、静止電位より脱分極側で大きくなったことと、K 電流抑制条件下ではその大部分が抑制されたことから、主にKコンダクタンスの減少によって引き起こされていると考えられる。一方、外液にカルモジュリン阻害剤であるW－７を加えたり、細胞内にCa キレーターである EGTA を注入すると野生株でみられる内向き電流応答は抑制された。これらの結果は、冷刺激に対するKコンダクタンスの減少が、Ca^{2+}/calmodulin によって調節されることを示唆している。

ゾウリムシは、冷刺激に対して K コンダクタンスの減少だけでなく、Ca コンダクタンスの増加も引き起こす。細胞内 Ca^{2+}濃度、$[Ca^{2+}]_i$の測定下で冷刺激を与えると、細胞の前端部で一過性の$[Ca^{2+}]_i$の増加が観察された。静止電位付近で膜電位を固定し、K 電流を抑制して冷刺激を与えると一過性の内向き電流が観察された。この電流応答は、静止電位より脱分極側ではその振幅は小さくなり、外液の Ca^{2+}を取り除くと消失した。また、この電流応答は、外液の Ca^{2+}を Co^{2+}、Mg^{2+}、Mn^{2+}に置き換えると消失するが、Ba^{2+}、Sr^{2+}に置き換えてもその振幅には影響がなかった。これらの結果は、ゾウリムシが細胞の前端部に Ca^{2+}、Ba^{2+}、Sr^{2+}に透過性のある冷刺激感受性 Ca コンダクタンスを持っていることを示唆している。さらに、この Ca コンダクタンスは、外液のアミロライドや Ni^{2+}などの２価カチオンによって抑制される。これらの性質は、冷刺激感受性 Ca コンダクタンスが、これまでにゾウリムシにおいて知られている脱分極、過分極、化学刺激、機械刺激、温度上昇刺激に対して活性化される Ca コンダクタンスとは異なる新しいタイプのものであることを示唆している。

CONTENTS

GENERAL INTRODUCTION

Many organisms have thermal sensation, from bacteria to unicellular organisms and higher organisms (Jennings, 1906; Hensel, 1974; Maeda & Imae, 1979). However, the mechanism of thermoreception remains unclear. In the recently study, the thermoreception is closely related to the chemoreception that has been reported. The *Escherichia coli* four chemosensory receptors all containing two transmembrane domains also function as thermosensory receptors (Nara et al., 1996; Nishiyama et al., 1997): they fall into two classes, namely warm receptors (Tsr, Tar, and Trg) and cold receptors (Tar in the presence of attractants and Tap). In the nematode *Caenorhabditis elegans*, it was suggested that a cyclic nucleotide-gated channel was required for thermosensation and chemosensation and cGMP was an important intracellular messenger in sensory transduction (Komatsu et al., 1996). In sensory neuron, the capsaicin, the main pungent ingredient in hot chilli peppers, receptor was activated by heating that was reported (Caterina et al., 1997). This receptor is a cation channel containing six transmembrane domains and has high Ca^{2+} permeability.

The ciliated protists *Paramecium* and *Blepharisma* also have the thermo-sensory mechanism. They accumulate in regions with temperature close to the temperature of their culture, the optimum temperature region, because they respond to temperature changes from the temperature of the culture both cooling and heating, by increasing frequency of ciliary reversal, i.e., thermal avoidance behavior (Fig. 1; Jennings, 1906; Nakaoka & Oosawa, 1977; Matsuoka et al., 1991; Kuriu et al., 1994). Both cooling and heating induce slow transient depolarizations of the membrane potential, which

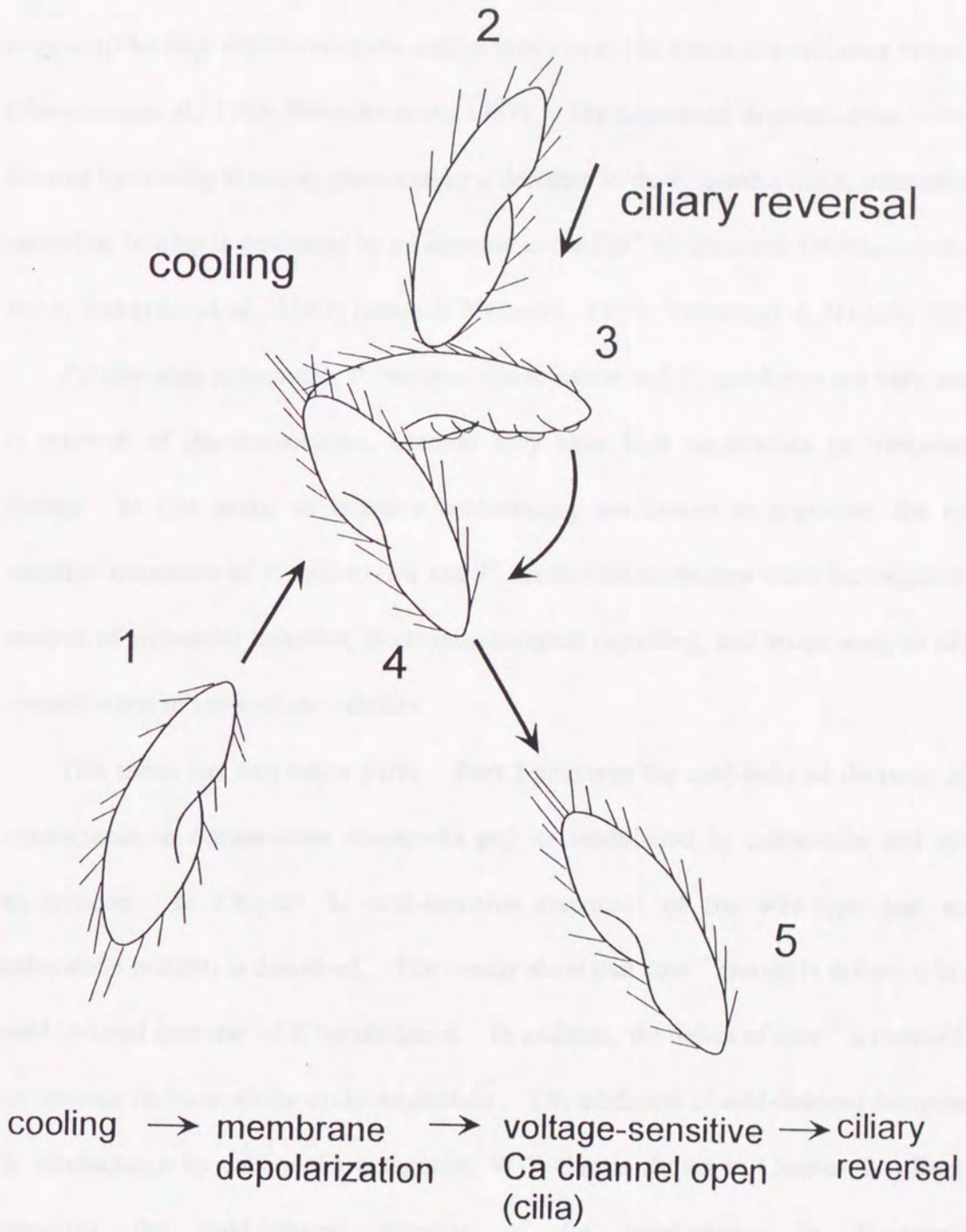

Fig. 1. Thermal avoidance behavior in *Paramecium*. A *Paramecium* cell responds to cooling (or warming) from the temperature which it has been adapted, by producing transient ciliary reversal, to cause directional change in the swimming behavior.

trigger spike-like depolarizations and in turn cause the thermal avoidance behavior (Hennessey et al., 1983; Nakaoka et al., 1987). The membrane depolarization which is induced by cooling is mainly generated by a decrease in the K^+ conductance, whereas that caused by heating is generated by an increase in the Ca^{2+} conductance (Hennessey et al., 1983; Nakaoka et al., 1987; Inoue & Nakaoka, 1990; Tominaga & Naitoh, 1992).

Paramecium tetraurelia, *P. multimicronucleatum* and *P. caudatum* are very useful in research of thermoreception, because they have high sensitivities to temperature change. In this study, to examine cold-sensory mechanism in organism, the cold-sensitive responses of *P. tetraurelia* and *P. multimicronucleatum* were investigated by analysis of swimming behavior, electrophysiological recording, and image analysis of the concentration of intracellular calcium.

This thesis has two major parts. **Part I** concerns the cold-induced decrease of K conductance in *Paramecium tetraurelia* and its modulation by calmodulin and cyclic nucleotides. In *Chapter* 1, cold-sensitive responses of the wild-type and some calmodulin mutants is described. The results show that *cam*^{12} mutant is defective in the cold-induced decrease of K conductance. In addition, the defect of *cam*^{12} is restored by an increase on intracellular cyclic nucleotide. The inhibition of cold-induced decrease of K conductance by calmodulin antagonist, W-7, that is shown in *Chapter* 2. **Part II** concerns the cold-induced increase of Ca conductance in *Paramecium multimicronucleatum*. *P. multimicronucleatum* has higher sensitivity to cooling than *P. tetraurelia*. In *P. multimicronucleatum*, cooling of a cell induced not only a decrease of K conductance but also an increase of Ca conductance. In *Chapter* 3, using image analysis of the concentration of intracellular calcium, $[Ca^{2+}]_i$, and electrophysiological

recording, cooling of a *Paramecium* cell 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+} that are shown. In *Chapter* 4, some properties of Ca conductance activated by cooling, including Ca^{2+}-dependent desensitization and inactivation are described.

PART I

COLD-INDUCED DECREASE OF K CONDUCTANCE IN *Paramecium tetraurelia*

CHAPTER 1 DEFECT OF COLD-SENSITIVE RESPONSE IN CALMODULIN MUTANTS OF *Paramecium* AND THE RESTORATION BY CYCLIC NUCLEOTIDE

Abstract. Wild type and calmodulin mutants (*cam*) of *Paramecium tetraurelia* were examined for cold-sensitive responses. Among mutants tested, cam^{12} and cam^{13} mutants, which have substitutions in N-terminal lobe of calmodulin molecule reduced both responses in the swimming and the membrane potential. Under voltage clamp conditions, the cooling stimulus to the wild-type cell induced a transient inward current whose amplitude increased with the rate of temperature drop. The cam^{12} cell did not induced inward currents in response to cooling with a rate slower than -0.4 °C/s. The reduced current response of cam^{12} mutant was restored by an external application of a phosphodiesterase inhibitor, theophylline. Also, an intracellular injection of hydrolysis-resistant cyclic nucleotides, either 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) or 8-bromoguanosine 3',5'-cyclic monophosphate (8-Br-cGMP), restored the current response. Such restoration was accompanied by shifts of the resting potential to hyperpolarized levels and by an increase in the membrane conductance. The results suggest the possibility that calmodulin and cyclic nucleotide regulate K^+ channels responsive to the cooling stimulus.

Introduction

Thermosensory receptors are ubiquitous in many organisms, and they are specified as warm and cold receptors. However, the mechanisms of thermosensory-transduction are not well understood. The ciliated protozoan, *Paramecium* has warm and cold receptors. Warming above the temperature to which the cells have been adapted, and cooling below this temperature induces transient increases in the frequency of directional changes in the swimming behavior (Hennessey & Nelson, 1979; Nakaoka & Oosawa, 1977). Both warming and cooling stimuli induce slow transient depolarizations of the membrane potential, which trigger spike-like depolarizations and in turn cause the directional changes in the swimming. It has been shown that the depolarization induced by warming is accompanied by an increase in the membrane conductance for Ca^{2+} (Hennessey et al, 1983; Nakaoka et al., 1987; Tominaga & Naitoh, 1992), and the depolarization induced by cooling is accompanied primarily by a decrease in the membrane conductance for K^+ (Inoue & Nakaoka, 1990; Nakaoka et al, 1987).

Among many behavioral mutants of *Paramecium*, some have substitutions of amino acid residues in the calmodulin molecule (Kink et al.,1990; Ling et al., 1994). There are two groups; mutants having substitutions in the C-terminal lobe of calmodulin molecule carry defects in the interaction of calmodulin with a Ca^{2+}-dependent K^+ channel. Mutants having substitutions in the N-terminal lobe prevent the interaction of calmodulin with a Ca^{2+}-dependent Na^+ channel. The latter is directly activated by the binding of Ca^{2+}-calmodulin (Saimi & Ling, 1990; Saimi & Ling, 1995). The role of calmodulin in exocytosis has also been defined through analyses of these calmodulin mutants (Kerboeuf

et al., 1993).

In the present study, we compare the cold-sensitive responses between the wild type and the calmodulin mutants. The results show that *cam*^{12} and *cam*^{13} mutants are defective in the cold-sensitive response. In addition, the defect of *cam*^{12} is restored either by an external application of cyclic nucleotides. These results suggest that calmodulin and cyclic nucleotides are included in the cold-sensory transduction chain.

Materials and Methods

Cell culture

Paramecium tetraurelia, wild type (stock 51 s) and the following calmodulin mutants supplied by Dr. C. Kung (Madison, Wisc. USA) were used; Pantophobiac type mutants that show a strong backward swimming in Na^+ solution were *cam*^{1} and *cam*^{2}. Fast-2 type mutants that show no backward swimming in Na^+ solution were *cam*^{12} and *cam*^{13} (Kink et al., 1990). Wild and mutant cells were cultured in hay infusion inoculated with *Klebsiella pneumoniae*. Culture temperature was kept constant at 25 °C by incubating culture vessels in a water bath. Stationary cells were collected by low speed centrifugation and suspended in a standard solution. The cell suspension was left for 1 h or more at 25 °C prior to the experiments.

Solutions

The standard solution contained 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4 mM KCl, 1 mM HEPES (pH 7.2, adjusted with $Tris^+$). In some cases of current measurement, 10 mM NaCl was added to the standard solution or $MgCl_2$ was omitted from the standard solution. Theophylline (Wako, Japan) was added to the standard solution in a final concentration of 5 mM. The solution for intracellular injections contained either 1 mM 8-bromo-cAMP or 1 mM 8-bromo-cGMP and 2 mM HEPES-Tris (pH 7.2, adjusted with KOH). Chemicals used for the microinjection were obtained from Sigma.

Behavioral assays

Cells suspended in the standard solution were placed in a glass vessel whose temperature was controlled by water flow beneath it (Nakaoka & Oosawa, 1977). An abrupt change of the temperature was achieved by switching a valve connected to outlets of two different temperature-regulating units. The temperature of the cell suspension was measured with a thermistor placed in the vessel. The time constant of the thermistor was 0.4 s. Swimming behavior of cells was monitored with a video camera mounted just above the vessel and was recorded by a video-tape recorder. Swimming tracks of 1 s duration were obtained from the video record by using an image processor (Image Sigma-2, Avionics, Japan). Among the swimming tracks of 70-150 cells, those that changed the swimming direction were counted and this fraction was taken as frequency of directional changes.

Intracellular recording

Membrane potential and membrane current were measured by the methods described previously (Nakaoka & Iwatsuki, 1992). Microelectrodes used for the current clamp contained 0.1 M KCl and the tip resistance were 150-200 MΩ. Voltage clamp electrodes contained 1 M KCl and the resistances were about 50 MΩ. For measurements in the absence of depolarizing spikes, cells were deciliated by incubation in the standard solution containing 5 % ethanol for 0.5-1 min and then returned to the standard solution (Inoue & Nakaoka, 1990). Intracellular measurements of the deciliated cells were made in a vessel mounted on an inverted microscope. The temperature of the vessel was controlled by a water flow beneath it, and was changed by

switching the water flow. The temperature was measured with a thermo-couple placed near the cell. The time constant of the thermo-couple was 0.1 s.

Microinjection

The arrangement for pressure injections was a modification of a method described by Oosawa and Yamagishi (1989). The tip diameter of the microcapillary used for injection was about 1 μm. An air pressure pulse of 10-30 psi x 0.1-0.5 s was applied for the injection (Picosprizer, General Valve, USA). Injected volumes were 10-20 pl.

Assay of cyclic nucleotides

The cells were washed with the standard solution and concentrated to 5×10^4 cells/ml. After standing at 25 °C for about 10 min, the cells were disrupted by addition of HCl to a final concentration of 0.1 M, then neutralized by the addition of KOH. Cell debris were removed by centrifugation (10^4 g $\times$ 10 min) and the supernatant was used for the measurements of cAMP and cGMP with a radio-immuno assay kit (Yamasa, Japan). For estimation of intracellular concentrations of cyclic nucleotides, the cell volume was calculated to be 100 pl from the microscopic observation of the cell size.

Results

Swimming responses to cooling

When the temperature of cell vessel started to drop from 25 °C with an initial rate of cooling, -0.13 °C/s, wild-type *P. tetraurelia* rapidly increased the frequency of directional changes in the swimming, attained a peak value after 10-20 s, and decrease gradually thereafter (Fig. 1-1). Calmodulin mutants, cam^{1} and cam^{2} increased transiently the frequency of directional changes, which were similar to the wild-type cell. On the other hand, cam^{12} and cam^{13} mutants showed almost no or only a small increase in the frequency of directional changes upon cooling.

Membrane potential responses to cooling

The resting potentials of cells used in this study (wild type, cam^{1}, cam^{2}, cam^{12}, cam^{13} mutants) were in the range from -22 to -26 mV at 25 °C. When the temperature of experimental vessel was reduced from 25 °C, with an initial rate of cooling, -0.13 °C/s, wild-type cells and cam^{1} and cam^{2} mutants showed transient depolarizations (Fig. 1-2). The amplitudes of the depolarization (mean $\pm$ *SD*) were 4.0 $\pm$ 1.5 mV (n = 15), 5.0 $\pm$ 2.8 mV (n = 5), 3.2 $\pm$ 0.3 mV (n = 7) for wild-type cells, cam^{1} and cam^{2} mutant, respectively. On the other hand, depolarizations in cam^{12} and cam^{13} mutants were not distinguished from fluctuations of the resting potential (Fig. 1-2). Based on these results, we hereafter compared the cold-sensitive response between wild-type cell and cam^{12} mutant.

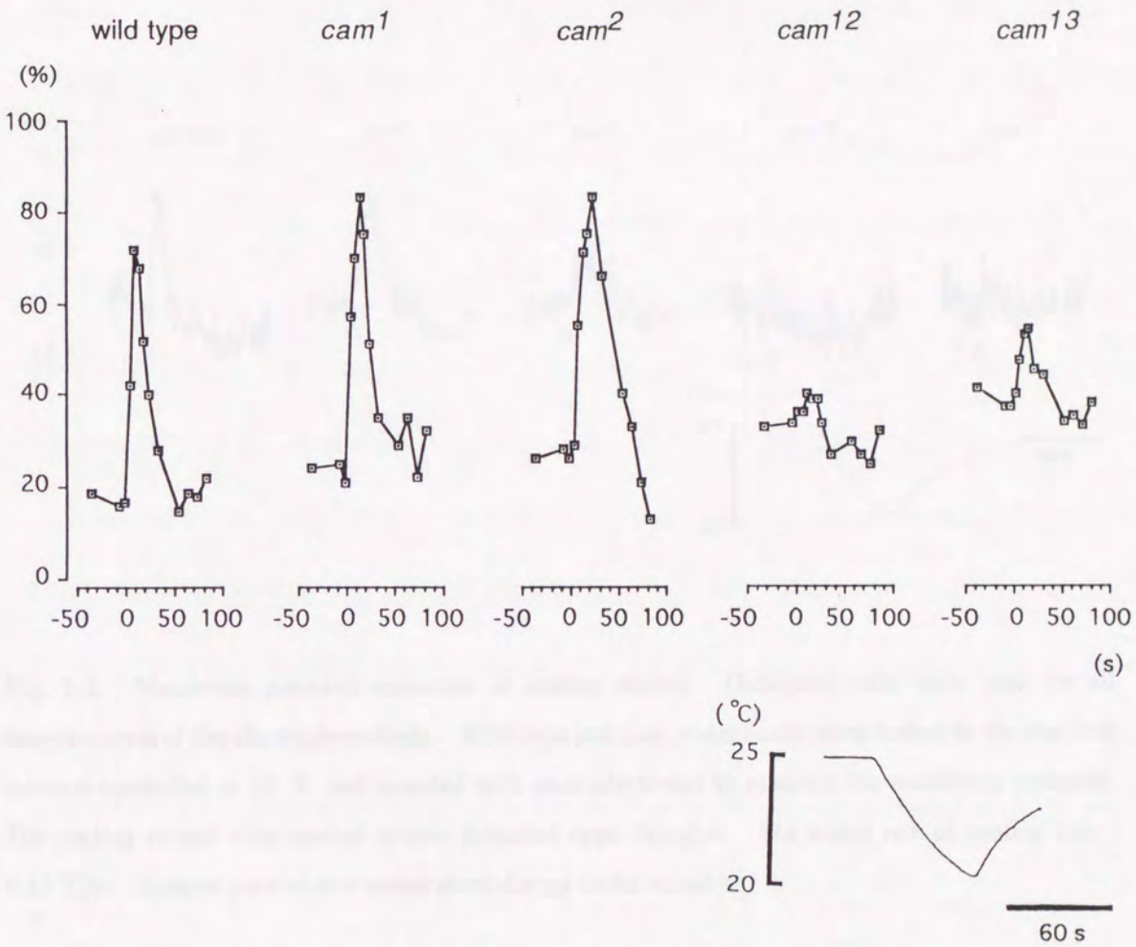

Fig. 1-1. Behavioral response to cooling stimuli. Wild type and *cam* mutant cells were placed in the vessel of 25 °C, then subjected to the initial rate of cooling, -0.13 °C/s at 0 s on the abscissa. The swimming responses are expressed by % of cells showing directional changes in the swimming (see Materials and Methods). The bottom trace gives the temperature change in the vessel.

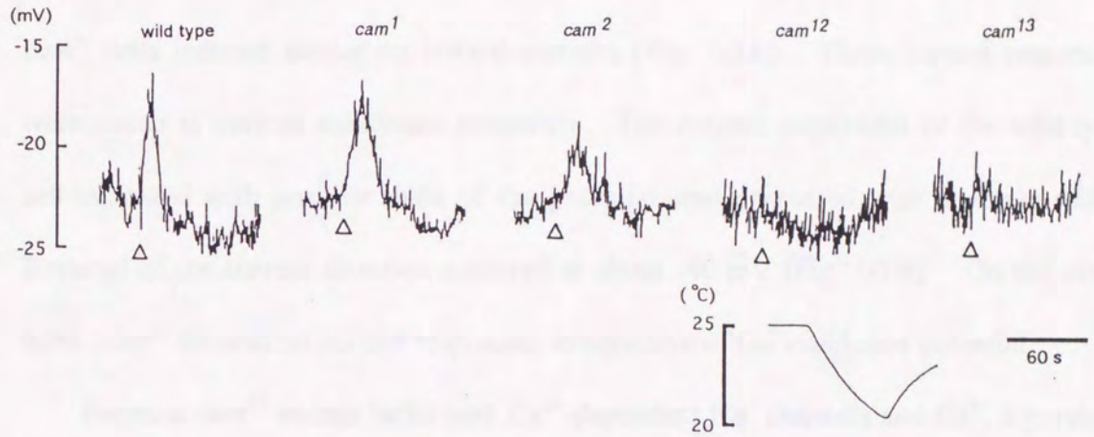

Fig. 1-2. Membrane potential responses to cooling stimuli. Deciliated cells were used for all measurements of the electrophysiology. Wild type and *cam* mutant cells were bathed in the standard solution controlled at 25 °C and impaled with microelectrodes to measure the membrane potential. The cooling stimuli were started at time indicated open triangles. The initial rate of cooling was -0.13 °C/s. Bottom trace shows temperature change in the vessel.

Voltage dependence of cold-sensitive response

Under the voltage clamp, cooling stimuli with an initial rate of cooling, -0.34 °C/s, were applied. In the wild-type cells, a transient inward current occurred that peaked 10-20 s after the start of cooling. However, the same rate of cooling stimulation applied to the *cam*^{12} cells induced almost no inward currents (Fig. 1-3A). These current responses were tested at various membrane potentials. The current amplitudes of the wild-type cell increased with positive shifts of the potential, and decreased with negative shifts. Reversal of the current direction occurred at about -40 mV (Fig. 1-3B). On the other hand, *cam*^{12} showed no current responses, irrespective of the membrane potential.

Because *cam*^{12} mutant lacks both Ca^{2+}-dependent Na^{+} channels and Ca^{2+} dependent Mg^{2+} channels (Kink et al., 1990; Kung et al., 1992), the current response upon cooling of the wild-type cells might include Na^{+} or Mg^{2+} current. So, the current response of the wild-type cell was tested in the medium containing 10 mM NaCl or in the medium without $MgCl_2$. However, neither the amplitude nor the reversal potential of the current responses changed from those seen in the standard solution (Fig. 1-3B).

Effect of cooling rate on the current response

When the initial rate of cooling from 25 °C was slower than -0.34 °C/s, the wild-type cell responded with an inward current, whereas *cam*^{12} did not show such current (Fig. 1-4A). When the rate of cooling was higher than -0.46 °C/s, *cam*^{12} mutant began to display a current response whose amplitude of which was smaller than that of the wild type (Fig. 1-4A, B). Therefore, *cam*^{12} mutant cells retained an attenuated sensitivity to the cooling stimulus as compared to the wild-type cell.

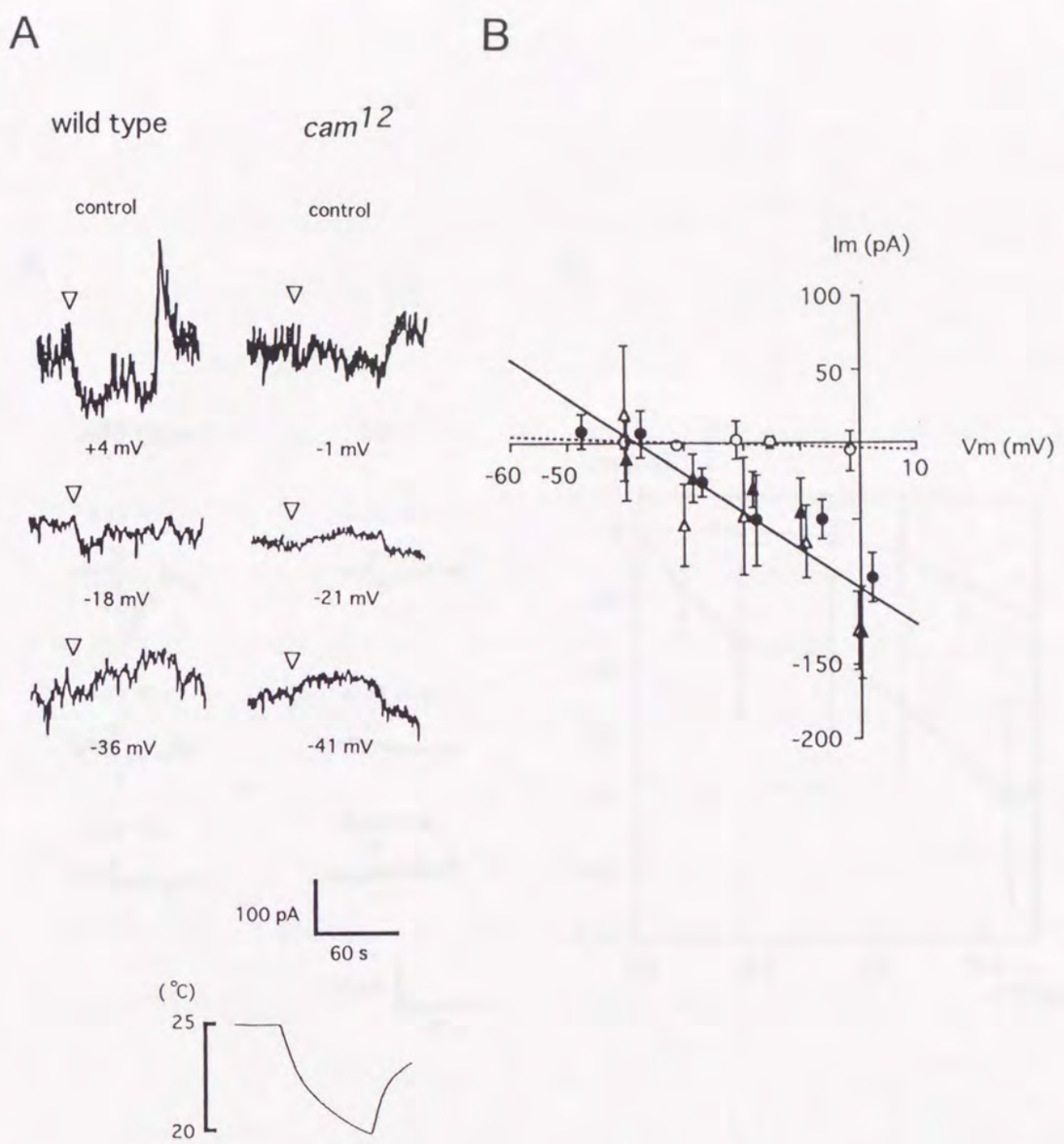

Fig. 1-3. Current responses to cooling stimuli. Wild type and *cam*^{12} mutant cells were voltage clamped at various potentials in the experimental solution at 25 °C, and cooling stimuli were applied. The initial rate of cooling was -0.34 °C/s. (A) Current responses of wild type and *cam*^{12} mutant cells. The membrane potential was clamped at various levels indicated at the left side of the current traces, and the cooling was started at open triangles. Bottom trace gives temperature change in the vessel. (B) Voltage dependence of the current response. From the current records at various potentials, amplitudes of the maximal current changes at 10 s after the start of cooling were obtained. The amplitudes of wild type (filled circle, open and closed triangles) and *cam*^{12} mutant (open circle) cells are plotted as a function of the membrane potential. Open triangles are amplitudes in the 10 mM NaCl solution. Closed triangles are amplitudes in the solution without $MgCl_2$. Each point represents the means ± SD of 3-7 cells.

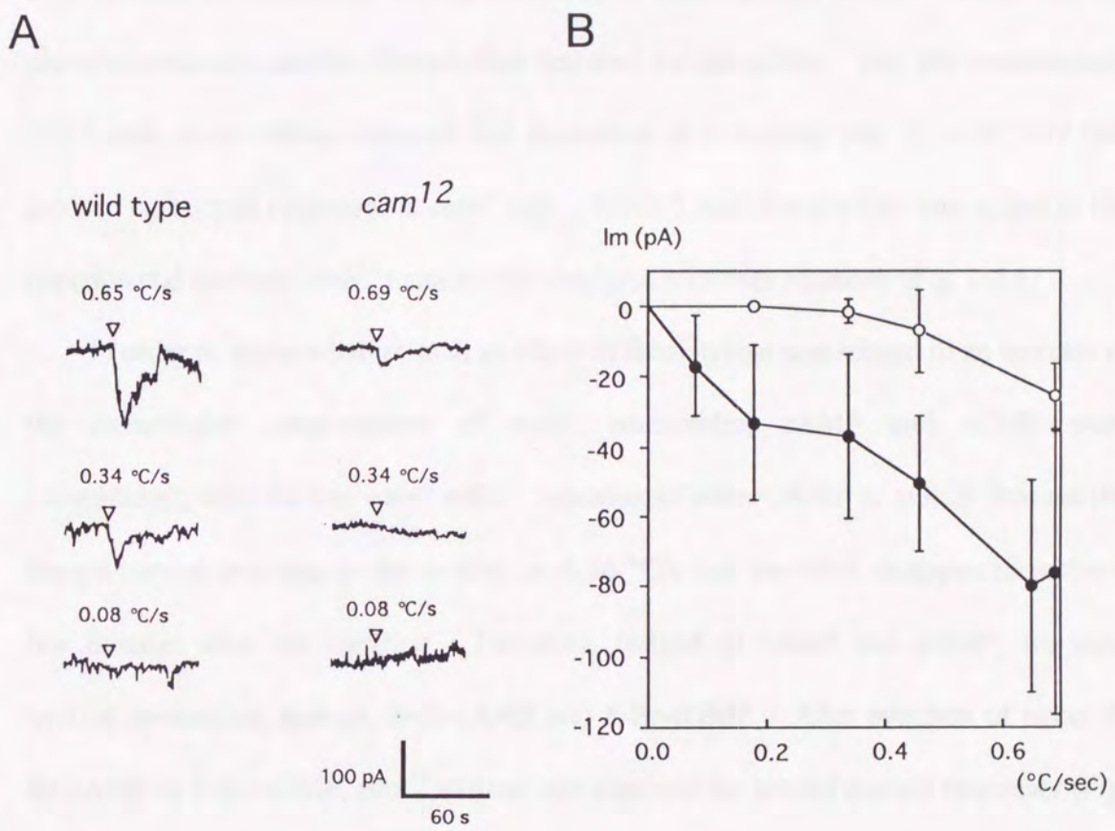

Fig. 1-4. Effect of cooling rate on the current responses. Wild type and *cam*^{12} cells were voltage clamped at -25 mV in the standard solution of 25 °C, and various rates of cooling stimuli were applied. (A) Current responses induced by various rate of cooling. Initial cooling rates and the start of cooling (open triangle) are shown on top of the traces. (B) Relationship between the cooling rate and the amplitude of current response. From the current responses induced by various cooling rates, amplitudes of the maximal current changes at 10 s after the start of cooling were obtained. The amplitudes of wild type (closed circle) and *cam*^{12} mutant (open circle) cells are plotted as a function of the cooling rate. Data are means $\pm$ SD of 4-8 cells.

Effects of theophylline and cyclic nucleotide-injection

In an attempt to restore the cooling sensitivity of *cam*^{12} mutant cells, we found that the phosphodiesterase inhibitor theophylline restored the sensitivity. For this examination, *cam*^{12} cells were voltage clamped and stimulated at a cooling rate of -0.34 °C/s that induced no current responses in *cam*^{12} cell. When 5 mM theophylline was added to the experimental medium, *cam*^{12} mutant cells displayed a current response (Fig. 1-5A).

In order to know whether such an effect of theophylline was related to an increase in the intracellular concentration of cyclic nucleotides, cAMP and cGMP were intracellularly injected into *cam*^{12} cells. Injection of either cAMP or cGMP induced the inward current response to the cooling at -0.34 °C/s, but the effect disappeared within a few minutes after the injection. Therefore, instead of cAMP and cGMP, we used hydrolysis-resistant analogs, 8-Br-cAMP and 8-Br-cGMP. After injection of either 8-Br-cAMP or 8-Br-cGMP, *cam*^{12} mutant cells regained the inward current responses (Fig. 1-5A).

Amplitudes of the current responses at various membrane potentials were then compared under those conditions which caused current responses upon cooling at -0.34 °C/s (Fig. 1-5B). The current amplitudes elicited after addition of theophylline were slightly larger than those restored by the injections of 8-Br-cAMP and 8-Br-cGMP, and amplitudes caused by both 8-Br-cAMP and 8-Br-cGMP were almost similar to that of the wild-type cell. All these current responses were reversed at about -40 mV. The results suggest that addition of theophylline or cyclic nucleotide-injection can restore the cooling sensitivity of *cam*^{12} mutant.

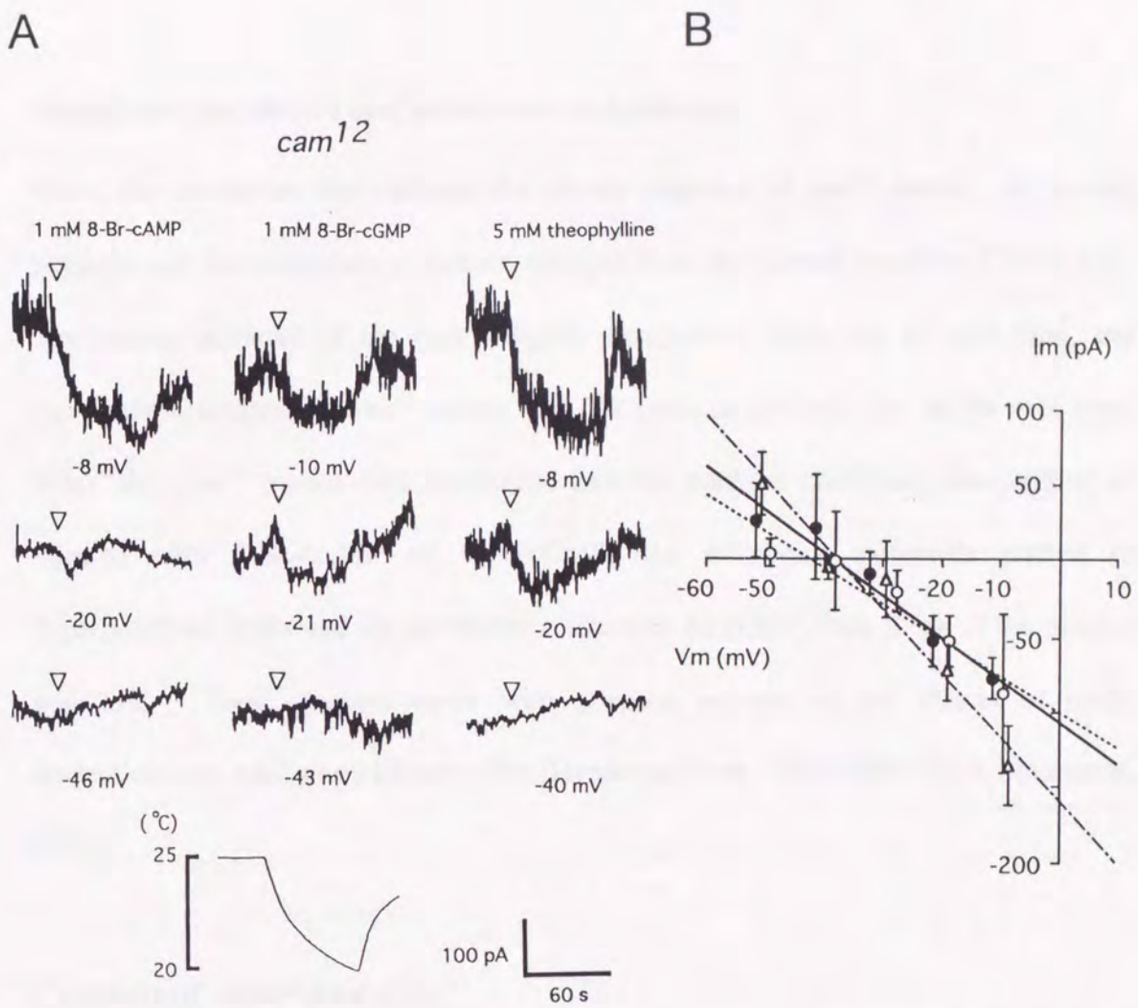

Fig. 1-5. Effects of theophylline, 8-Br-cAMP and 8-Br-cGMP on the cold-sensitive current of *cam*^{12} mutant. Deciliated *cam*^{12} cells were voltage-clamped at various potentials in the solution of 25 °C, and cooling stimuli at -0.34 °C/s were applied. (A) Current responses at various potentials. For the test of theophylline, 5 mM theophylline was added into the external standard solution. For the tests of 8-Br-cAMP and 8-Br-cGMP, these chemicals were injected into the cell. Membrane potentials are indicated beneath the current traces. The start of cooling is indicated by open triangles. The bottom trace shows temperature change in the vessel. (B) Voltage dependence of the current responses restored by theophylline, 8-Br-cAMP and 8-Br-cGMP. Following treatment of these chemicals, current responses at various membrane potentials were recorded. Amplitudes of the maximal current changes at 10 s after the start of cooling were obtained from records following treatment with theophylline (open triangle), 8-Br-cAMP (open circle) or 8-Br-cGMP (closed circle). Points are means $\pm$ SD from 3-5 cells.

Membrane potentials and membrane resistances

Under the conditions that restored the current response of *cam*^{12} mutant, the resting potential and the membrane resistance changed from the control condition (Table 1-1). The resting potential of the *cam*^{12} slightly depolarized from that of wild type, and membrane resistance of *cam*^{12} mutant was 1.2 times larger than that of the wild type. When the *cam*^{12} mutant was transferred into the medium containing theophylline or injected with 8-Br-cAMP or 8-Br-cGMP, the membrane potentials shifted to hyperpolarized levels and the membrane resistances decreased from those of the original condition. These changes agree with previous reports on the effects of cyclic nucleotides and analogs on *Paramecium* (Hennessey et al., 1985; Nakaoka & Machemer, 1990).

Contents of cAMP and cGMP

Because the intracellular injections of cyclic nucleotide analogs restored the cooling-sensitive response of *cam*^{12} mutant, cyclic nucleotide levels of *cam*^{12} cell might be reduced from the level of wild-type cell. So, we compared the contents of cyclic nucleotides between wild type and *cam*^{12} mutant cells. Contents of cAMP (mean $\pm$ *SD*) were 16.1 $\pm$ 2.0 pmoles/10^5 cells (n = 6) and 15.9 $\pm$ 2.0 pmoles/10^5 cells (n = 5) for wild type and *cam*^{12} mutant cells, respectively. Contents of cGMP (mean $\pm$ *SD*) were 8.4 $\pm$ 0.8 pmoles/10^5 cells (n = 3) and 7.6 $\pm$ 0.5 pmoles/10^5 cells (n = 2) for wild type and *cam*^{12} mutant cells, respectively. These measurements suggest that cAMP and cGMP concentrations of the *cam*^{12} mutant are similar to those of wild-type cells.

Table 1-1. Resting potentials and membrane resistances in wild type and *cam*^{12} mutant of *Paramecium*.

Cell type	Treatment	Vrest (mV)	Rm (MΩ)♠	n
wild	Control	-26.1 ± 4.0	71.5 ± 11.8	30
cam^{12}	Control	-24.9 ± 4.9	85.8 ± 15.9	24
cam^{12}	Theophylline*	-28.9 ± 6.4	69.6 ± 14.9	14
cam^{12}	8-Br-cGMP#	-31.6 ± 5.0	58.0 ± 9.1	5
cam^{12}	8-Br-cAMP#	-32.2 ± 6.0	58.0 ± 10.4	5

♠ input resistance obtained by application of current pulse (-0.1 nA x 0.5 s) at the resting potential.

* 5 mM theophylline was added into the standard solution.

injected into the cell.

Values represent mean ± S D of n cells.

Most measurements except theophylline treatment were done in the standard solution at 25° C.

Discussion

Cold-sensitive responses of the wild type and calmodulin mutants

Comparisons of the cold-sensitive responses between wild type and calmodulin mutants show that these responses are reduced in *cam*^{12} and *cam*^{13} mutants. *Cam*^{12} mutant cells display the inward current response only when the rate of cooling was faster than -0.46 °C/s, whereas wild-type cells responds to cooling already at rates as low as -0.08 °C/s (Fig. 1-4B). The present results suggest that calmodulin is involved in the sensitivity to the cooling stimulus.

It has been shown that *cam*^{1} and *cam*^{2} mutants have a defect in generating the Ca^{2+}-dependent K^+ current, and *cam*^{12} and *cam*^{13} mutants can generate neither the Ca^{2+}-dependent Na^+ current and the Ca^{2+}-dependent Mg^{2+} current (Kink et al., 1990; Kung et al., 1992; Saimi & Kung, 1994). These findings suggest that, firstly, the Ca^{2+}-dependent K^+ current is unlikely to be involved in the inward current response upon cooling, because *cam*^{1} and *cam*^{2} mutants responded to the cooling stimulus as well as wild-type cells. Secondly, the assumption that the reduced sensitivity of *cam*^{12} might be related to the defect in the inward Na^+ current or the Ca^{2+}-dependent Mg^{2+} current is not realistic, because an external addition of Na^+ or removal of external Mg^{2+} changed neither the amplitude of current response nor the reversal potential of the cooling-sensitive current.

Previous reports have shown that the transient depolarization upon cooling is accompanied primarily by a decrease in the membrane conductance for K^+ at the resting potential (Inoue & Nakaoka, 1990; Nakaoka et al., 1987). In the present study, the amplitudes of the current responses were decreased by hyperpolarization and reversed at

approximately -40 mV, which is almost the equilibrium potential for K^+ (Oertel et al., 1978; Oka et al., 1986). Therefore, the present results also suggest that the cooling stimulus induces transient changes in the membrane conductance for K^+. Comparison of the membrane resistances at the resting state, shows that *cam*^{12} mutant input resistances is 1.2 times larger than that of the wild type (Table 1-1). The reduced cooling sensitivity of *cam*^{12} mutant might be related to this increase in membrane input resistance. Because most of the resting conductance is based on open K^+ channels (Machemer, 1988), a reduction of the conductance carried by K^+ at rest will increase the membrane resistance of *cam*^{12} mutant and presumably reduces the cooling sensitivity. Such considerations are in line with *cam*^{12} mutant defect in a K^+ current activated by hyperpolarization (R.R. Preston, personal communication).

Restoration of the cooling-sensitive response in cam^{12}

Application of theophylline or injection of cyclic nucleotide analogs into *cam*^{12} cells caused the membrane resistance to decrease to 81 % or 67 %, and these treatments shifted the membrane potential to hyperpolarized levels (Table 1-1). Such changes in the *cam*^{12} membrane accompanied the restoration of the cold-sensitive current whose reversal potentials were almost the same as that of the wild-type cell (Fig. 1-5B). These results suggest that the decrease resting conductance of *cam*^{12} membrane is elevated by some effect of cyclic nucleotides, and the increased conductance may be a part of the cooling sensitive response.

Restoration of the cooling-sensitivity following cyclic nucleotide-injection might suggest that *cam*^{12} has a reduced level of cyclic nucleotides. In fact, it has been shown

that calmodulin from *Paramecium* activates guanylate cyclase (Schultz et al., 1987). However, measurement of cyclic nucleotide contents shows no clear differences between wild type and cam^{12} mutant cells. Therefore, the calmodulin mutation of cam^{12} might reduce rather the sensitivity of cyclic nucleotide-dependent reactions, e.g. a protein kinase or channel activation.

In the case of olfactory- and photo-receptor cells, cyclic nucleotides directly activate ion channels (Fesenko et al., 1985; Nakamura & Gold, 1987), and these cyclic nucleotide-activated channels are in addition regulated by an interaction with calmodulin (Chen & Yau, 1994; Hsu & Molday, 1993; Liu et al., 1994). Cooling-sensitive currents in *Paramecium* were induced after injection of hydrolysis-resistant cyclic nucleotides and they appear dependent on calmodulin. Such a resemblance leads us to propose that the cooling-sensitive channels are directly activated by cyclic nucleotides. Following the injection of hydrolysis resistant cyclic nucleotide analogs, these concentrations are assumed to be almost constant during the cooling stimulus, while the current response is induced. This consideration rather supports the view that the cooling-sensitive channels are indirectly regulated by cyclic nucleotides, e.g. through activation of cyclic nucleotide-dependent protein kinases.

In conclusion, our data suggest that calmodulin and cyclic nucleotides regulate the cold-sensitive response in *Paramecium* mainly based on reduced K^+ channel activity. The reduced sensitivity of cam^{12} mutant cells is probably related to a reduced number of available temperature-sensitive K^+ channels which are open at the resting potential. The causes for the channel activity to be transiently suppressed upon cooling remain to be determined.

CHAPTER 2 COLD-INDUCED DECREASE OF K^+ CONDUCTANCE AND ITS INHIBITION BY A CALMODULIN ANTAGONIST, W-7, IN *Paramecium tetraurelia*

Abstract. Under voltage clamp, *Paramecium tetraurelia* was used to examine the cold-induced inward current and its inhibition by a calmodulin antagonist, W-7 [N-(6 aminohexyl)-5-chloro-1-naphthalenesulphonamide]. Cooling of these cells caused an inward current. The amplitude of the current was increased as the membrane potential was made more positive than resting potential, and it was significantly blocked by using CsCl-filled electrodes and tetraethylammonium in the bath solution, suggesting that the current was accompanied mainly by decrease of K^+ conductance. The cold-induced inward current was reversibly inhibited by external application of W-7 in a concentration-dependent manner. EGTA-microinjection into the cell also reduced the current. These results indicate that the decrease of K^+ conductance induced by cooling is Ca^{2+} dependence and is inhibited by W-7.

Introduction

Paramecium cells accumulate in regions with temperature close to the temperature of their culture, the optimum temperature region (Jennings, 1906). Cells respond to temperature changes, both cooling and heating, by increasing frequency of ciliary reversal, i.e., thermal avoidance behavior (Nakaoka & Oosawa, 1977). Electrophysiological recording has revealed that temperature changes induce a transient depolarization of the membrane potential. This depolarization causes the opening of voltage-dependent Ca^{2+} channels in the ciliary membrane, and the Ca^{2+} influx in the cilia triggers the thermal avoidance behavior (Hennessey et al., 1983). The membrane depolarization which is induced by cold stimuli is generated by a decrease in the K^+ conductance and an increase in the Ca^{2+} conductance (Nakaoka et al., 1987; Inoue & Nakaoka, 1990; Kuriu et al., 1996), while that caused by heating is generated by an increase in the Ca^{2+} conductance (Hennessey et al., 1983; Nakaoka et al., 1987; Tominaga & Naitoh, 1992).

In the previous study, we found that some calmodulin mutants did not induce inward currents in response to cooling (Kuriu et al., 1997a). Thus, in the present study, we examined the effect of a calmodulin antagonist, W-7 [N-(6 aminohexyl)-5-chloro-1-naphthalenesulphonamide], on the cold-induced inward current of *Paramecium tetraurelia*.

Materials and methods

Cell

"Wild type" refers to trichocyst nondischarge mutation nd-6 (nd6/nd6), a mutant of stock 51s of *Paramecium tetraurelia* with no observed defect in behavior or electrophysiology. Cells were cultured in a hay infusion inoculated with *Klebsiella pneumoniae*. The temperature of the culture was kept constant at 25 °C by incubation in a water bath. Cells at stationary phase (5 - 8 days after inoculation) were collected by low-speed centrifugation and suspended in a standard solution containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4 mM KCl and 1 mM HEPES-Tris (pH 7.2). Cells were preincubated in this solution at 25 °C for 1 h or more prior to examination. W-7 (Biomol, PA, USA) and W-5 ([N-(6 aminohexyl)-1-naphthalenesulphonamide], Seikagaku Kogyo, Tokyo, Japan) were dissolved in ethanol (W-7) or distilled water (W-5) and added to the standard solution with a concentration of organic solvent not exceeding 0.2 % (v/v). The TEA (tetraethylammonium) solution contained 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM TEA-Cl, 1 mM HEPES-Tris (pH 7.2).

Intracellular recording

The membrane currents of *Paramecium* were recorded using a method described by Nakaoka and Iwatsuki (1992). The capillary microelectrodes used for voltage clamp contained 1 M KCl or 1 M CsCl, and had a tip resistance about 50 MΩ. The cells were deciliated by incubation in a standard solution containing 5 % ethanol for 0.5 - 1 min and then returned to various solutions (Ogura & Machemer, 1984). The deciliated cells

were placed in a glass vessel mounted on an inverted microscope. The temperature of the vessel was controlled at 25 °C by water flow beneath it. Temperature changes of the vessel were made by altering this water flow, and were monitored with a thermocouple probe placed near the cell (Nakaoka et al., 1987). The cold-induced membrane current from the resting level, Im, was measured at 10 sec after the start of cooling.

EGTA-injection

The pressure injection was performed by a modification of the method described by Oosawa and Yamagishi (1989). The solution for injection was injected into a *Paramecium* cell by means of an air pressure pulse (10-30 psi, 0.1-0.5 sec: Picosprizer, General Valve, USA). The tip diameter of the microcapillary used for injection was about 1 μm. The injected volumes (10-20 pl) were between 10 % and 20 % of the cell volume, which assumed to be 100 pl. The injected solution contained 50 mM EGTA and 1 mM HEPES (pH 7.2, adjusted with KOH).

Results

Decrease of K^+ conductance and increase of Ca^{2+} conductance induced by cooling

The cooling from 25 °C in the wild-type *Paramecium tetraurelia* induced an inward current (Fig. 2-1A). The amplitude of the inward current elicited by the cooling was increased as the membrane potential was made more positive than resting potential (Fig. 2-1A, B). The K^+ current of *Paramecium* was suppressed by the use of a voltage clamp 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 significantly blocked by using CsCl-filled electrodes and tetraethylammonium in the bath solution, suggesting that K^+ carried the current (Fig. 2-1A, B). In *P. multimicronucleatum*, cooling of the voltage-clamped cell under conditions where K^+ currents are suppressed elicited a transient current (Kuriu et al. 1996). *P. tetraurelia* also showed a transient inward current under conditions where K^+ currents was suppressed (Fig. 2-1A, open arrow). This current was lost upon when the extracellular Ca^{2+} was replaced with an equimolar concentration of Mg^{2+} (data not shown), suggesting that Ca^{2+} carried the current. The amplitude of the cold-induced Ca^{2+} current of *P. tetraurelia* was smaller than that of *P. multimicronucleatum* (*P. tetraurelia*, -39.8 $\pm$ 20.3 pA , n = 8, Vm = -30 mV; *P. multimicronucleatum*, -208.0 $\pm$ 23.2 pA, n = 5, Vm = -30 mV; Kuriu et al., 1996).

Effects of calmodulin antagonists and EGTA

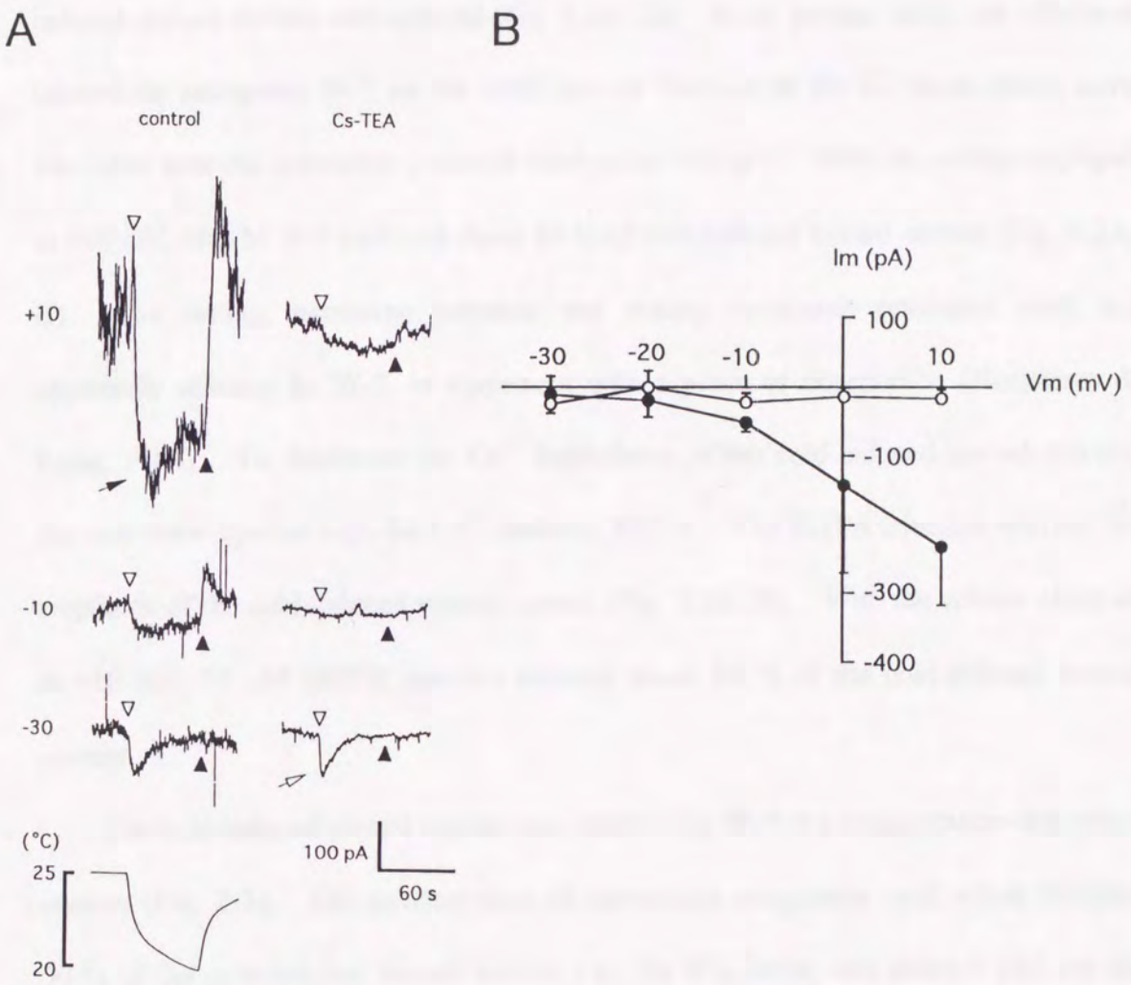

Fig. 2-1 (A) Cold-induced membrane currents in standard solution (left traces), and the inhibition of the current when the K^+ current was suppressed (right traces). The K^+ current was suppressed by the use of a voltage clamp containing 1 M CsCl in the pipette, and by including 10 mM TEA-Cl in the bath solution (Hinrichsen and Saimi, 1984). The filled arrow indicates the K^+ current, and open arrow shows the Ca^{2+} current. The membrane potential was clamped at various levels indicated at the left side of the current trace. The open triangles and closed triangles indicate the start of cooling and the end of cooling, respectively. The bottom trace is a record of temperature. (B) The I-V relationships of the cold-induced membrane current. Closed circles, in standard solution; open circles, under K^+ current suppressed. Each point is the mean $\pm$ SD of 3-9 cells.

When the cell was transferred to the standard solution containing 50 μM W-7, cold-induced inward current was reduced (Fig. 2-2A, B). In the present study, the effects of calmodulin antagonist W-7 on the cold-induced decrease in the K^+ conductance were estimated with the membrane potential clamped at +10 mV. With the voltage clamped at +10 mV, 50 μM W-7 inhibited about 80 % of cold-induced inward current (Fig. 2-2A, B). The resting membrane potential and resting membrane resistance were not apparently affected by W-7, in agreement with a previous observation (Hennessey & Kung, 1984). To determine the Ca^{2+} dependence of the cold-induced inward current, the cells were injected with the Ca^{2+} chelator, EGTA. The EGTA injection reduced the amplitude of the cold-induced inward current (Fig. 2-2A, B). With the voltage clamped at +10 mV, 50 μM EGTA injection reduced about 80 % of the cold-induced inward current.

The cold-induced inward current was inhibited by W-7 in a concentration-dependent manner (Fig. 2-3). The concentration of calmodulin antagonists used which inhibited 50 % of the cold-induced inward current, i.e., the IC_{50} value, was about 7 μM and the inhibition was nearly complete with 100 μM W-7. W-5, the dechlorinated analogue of W-7, also inhibited cold-induced inward current, although the IC_{50} was higher than that of W-7 (about 115 μM for W-5, Fig. 2-3).

W-7 reversibly inhibited the cold-induced inward current (Fig. 2-4). With the voltage clamped at +10 mV, the net outward current before cooling was about 1 nA and cooling of the cell caused the inward current (or decrease in net outward current). The perfusion with the standard solution containing 100 μM W-7 caused a decrease of net outward current before cooling, and the cold-induced inward current was not observed.

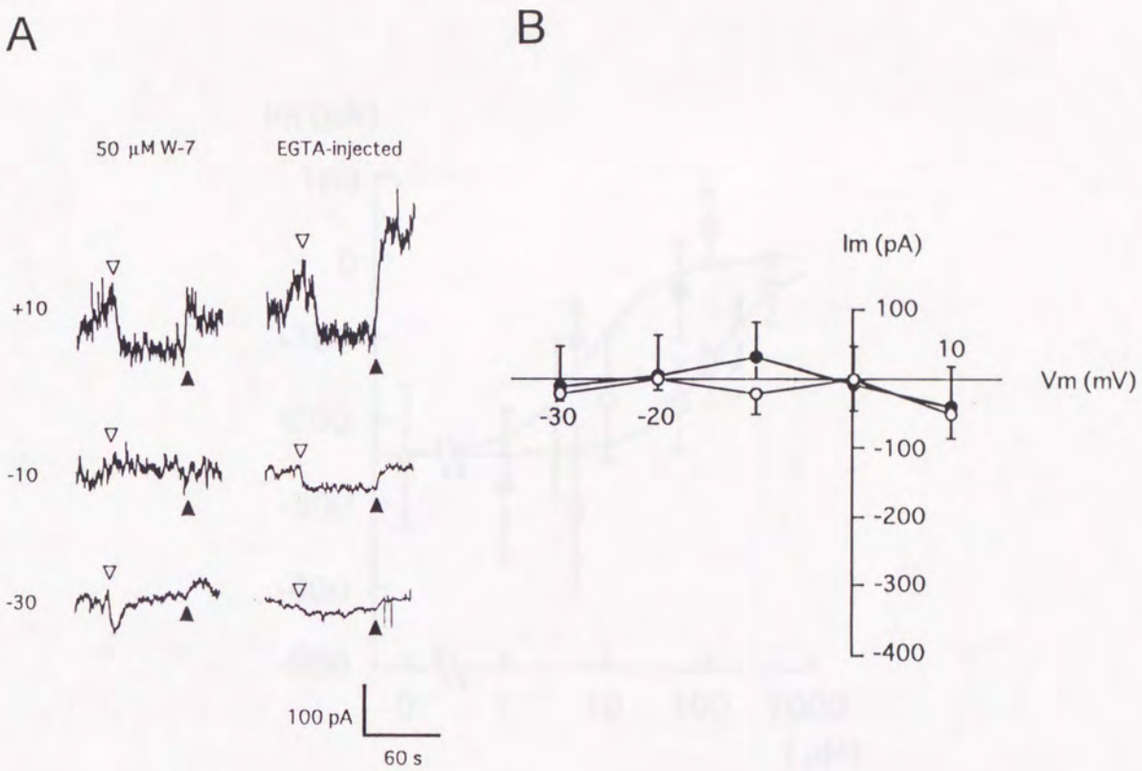

Fig. 2-2 (A) Inhibitions of the cold-induced inward currents by the application of 50 μM W-7 (left traces) and the microinjection of 50 mM EGTA (right traces). The membrane potential was clamped at the various levels indicated at the left side of the current trace. The open triangles and closed triangles indicate the start of cooling and the end of cooling, respectively. (B) The I-V relationships of the cold-induced membrane current. Closed circles, in standard solution containing 50 μM W-7; open circles, in standard solution with the microinjection of EGTA. Each point is the mean ± SD of 3-5 cells.

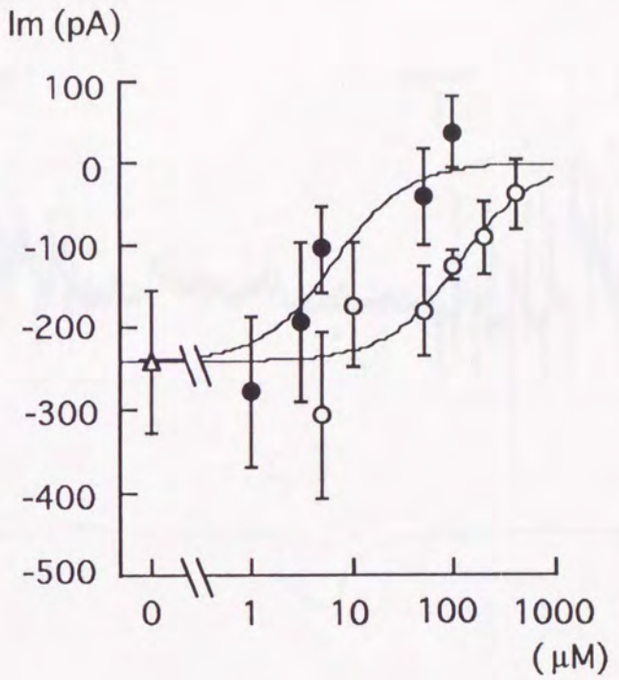

Fig. 2-3 Concentration effects of W-7 (closed circles) and W-5 (open circles) on the cold-induced inward currents. Each point is the mean ± SD of 3-5 cells. Ordinate: ΔIm value were measured under voltage clamp at +10 mV. Abscissa: concentration of W-7 or W-5 on a logarithmic scale. Interpolation from the dose-response curves shown gave estimated IC_{50} values of 7 μM for W-7 and 115 μM for W-5. The dose-response curves were described by the Hill equation,

$$\Delta Im = A / \{1 + (C / IC_{50})^n\},$$

where A is the mean of ΔIm in the standard solution, and *n* is the Hill coefficient. These values were 241 and 1.2, respectively, when the experimental data were fitted to the Hill equation. C is the calmodulin antagonist concentration, and IC_{50} is the concentration of the calmodulin antagonist producing half of the current response.

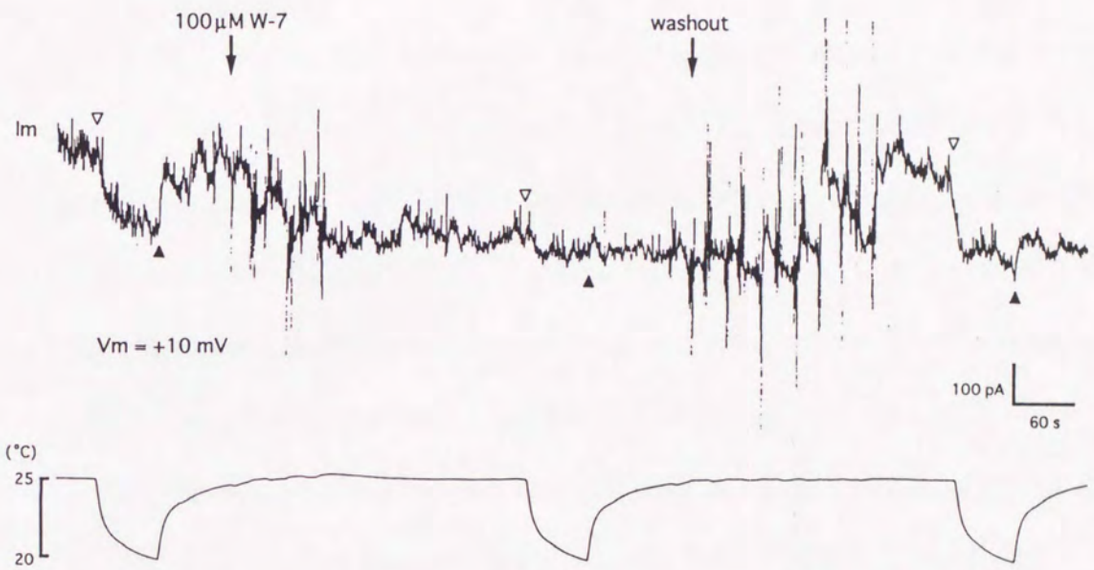

Fig. 2-4 Reversible inhibition of cold-induced inward current by 100 μM W-7. The upper trace is the membrane current under voltage clamp at +10 mV and is representative of results obtained in 2 additional cells. The bottom trace is a record of temperature. The open triangles and closed triangles indicate the start of cooling and the end of cooling, respectively.

Subsequently, when the cell was returned to the standard solution, the net outward current was increased to the initial level and the cold-induced inward current was again observed.

Discussion

Inhibition of cold-induced inward current by a calmodulin antagonist, W-7

The cold-induced inward current was significantly blocked by the use of CsCl-filled electrodes and TEA in the bath solution, suggesting that the current was accompanied mainly by a decrease in the K^+ conductance (Fig. 2-1). This current was reversibly inhibited by the external application of W-7 in a concentration-dependent manner (Figs. 2-2, 2-3 and 2-4). With the voltage clamped at +10 mV, the addition of W-7 also caused a decrease in the net outward current before cooling (Fig. 2-4). Therefore, W-7 may act to close the cold-sensitive K^+ channel before cooling.

The cold-induced inward current was also reduced by the injection of EGTA (Fig. 2-2). Moreover, in the previous study, the calmodulin mutant (*cam12*) which has substitutions in N-terminal lobe of the calmodulin molecule reduced inward currents in response to cooling (Kuriu et al., 1997a). These findings suggest that the cold-induced inward current is modulated by Ca^{2+}/calmodulin. W-7 has been reported to block various channels, although it has often been used as calmodulin antagonist. In *Paramecium*, Ca^{2+} channel located in ciliary membrane is directly blocked by W-7 (Ehrlich et al., 1988). In addition, W-7 inhibited the cold-induced inward current at lower concentrations than the bovine calmodulin-dependent phosphodiesterase activity (Nelson et al., 1983). The concentration of W-7 which inhibit 50 % of the cold-induced inward current, the IC_{50}, was 7 μM, and the IC_{50} of W-5 was 115 μM (Fig. 2-3), whereas the concentration of calmodulin antagonists which inhibit 50 % of the bovine calmodulin-dependent phosphodiesterase activity was found to be about 30 μM for W-7 and about 240 μM for

W-5 (Nelson et al., 1983). Thus, there is also a possibility that W-7 acts directly on the cold-sensitive K^+ conductance.

Cold-induced decrease of K^+ conductance and increase of Ca^{2+} conductance.

In *Paramecium tetraurelia* and *P. multimicronucleatum*, the cooling of the cells induced a decrease in K^+ conductance (Nakaoka et al, 1987; Inoue and Nakaoka, 1990; Kuriu et al., 1997a). *P. multimicronucleatum* exhibited not only a cold-induced decrease in K^+ conductance but also a cold-induced increase in Ca^{2+} conductance (Kuriu et al., 1996). In the present study, *P. tetraurelia* showed the inward Ca^{2+} current under conditions where K^+ current was suppressed (Fig. 2-1A). Therefore, the cold-induced inward current of *P. tetraurelia* was a complex of a decrease in the K^+ conductance and an increase in the Ca^{2+} conductance, as observed in *P. multimicronucleatum*. The cold-induced increase in Ca^{2+} conductance causes an increase in the intracellular Ca^{2+} concentration (Kuriu et al., 1996). Intracellular Ca^{2+} increased by cooling may modulated the cold-sensitive K^+ channel via calmodulin.

PART II

COLD-INDUCED INCREASE OF Ca CONDUCTANCE IN *Paramecium multimicronucleatum*

CHAPTER 3 COLD-SENSITIVE Ca^{2+} INFLUX IN *Paramecium*

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.

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 warning 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.

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). 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 (Fig. 3-1; 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

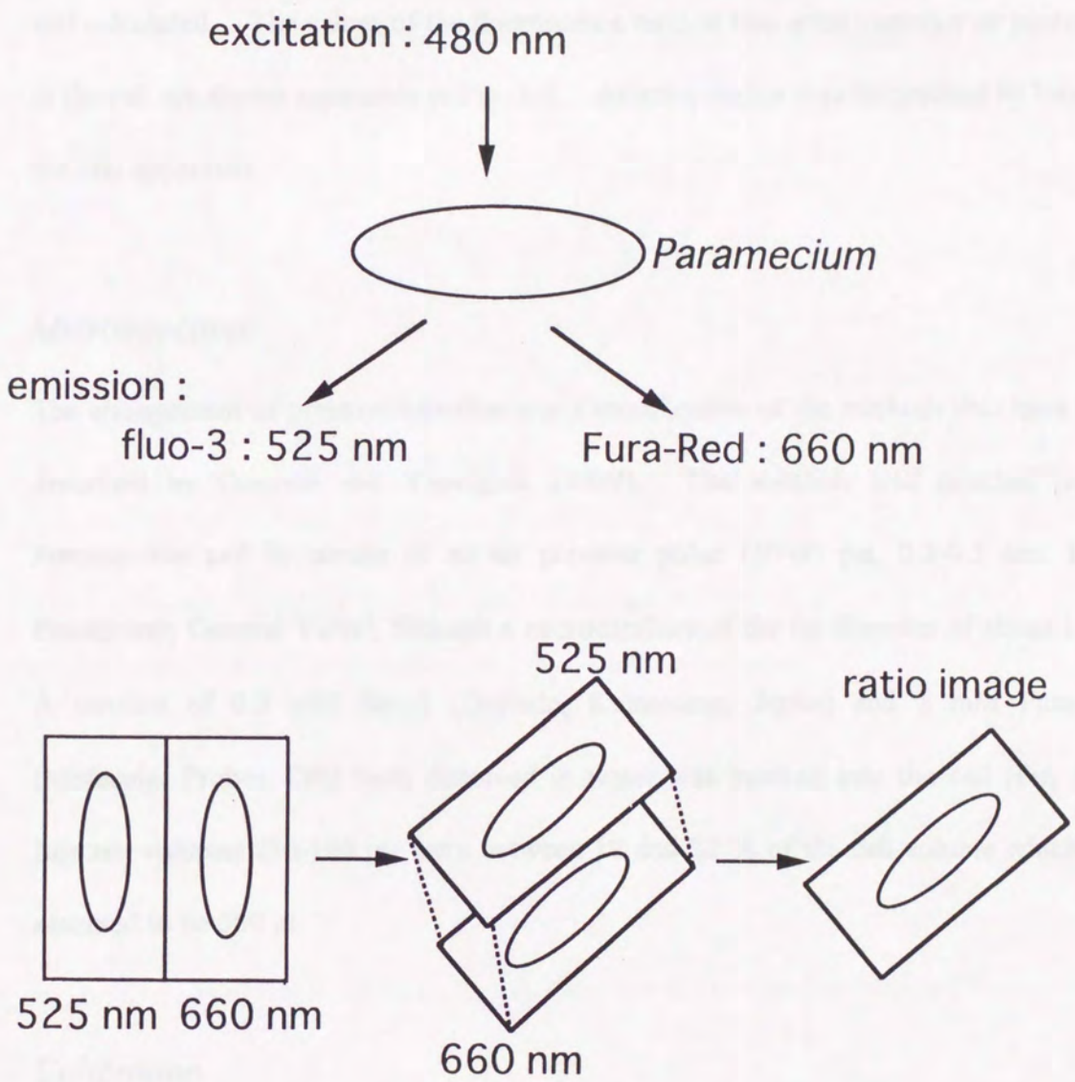

Fig. 3-1. A scheme of the method for the image analysis of $[Ca^{2+}]_i$ change.

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. 3-5. 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 (Fig. 3-2). 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.

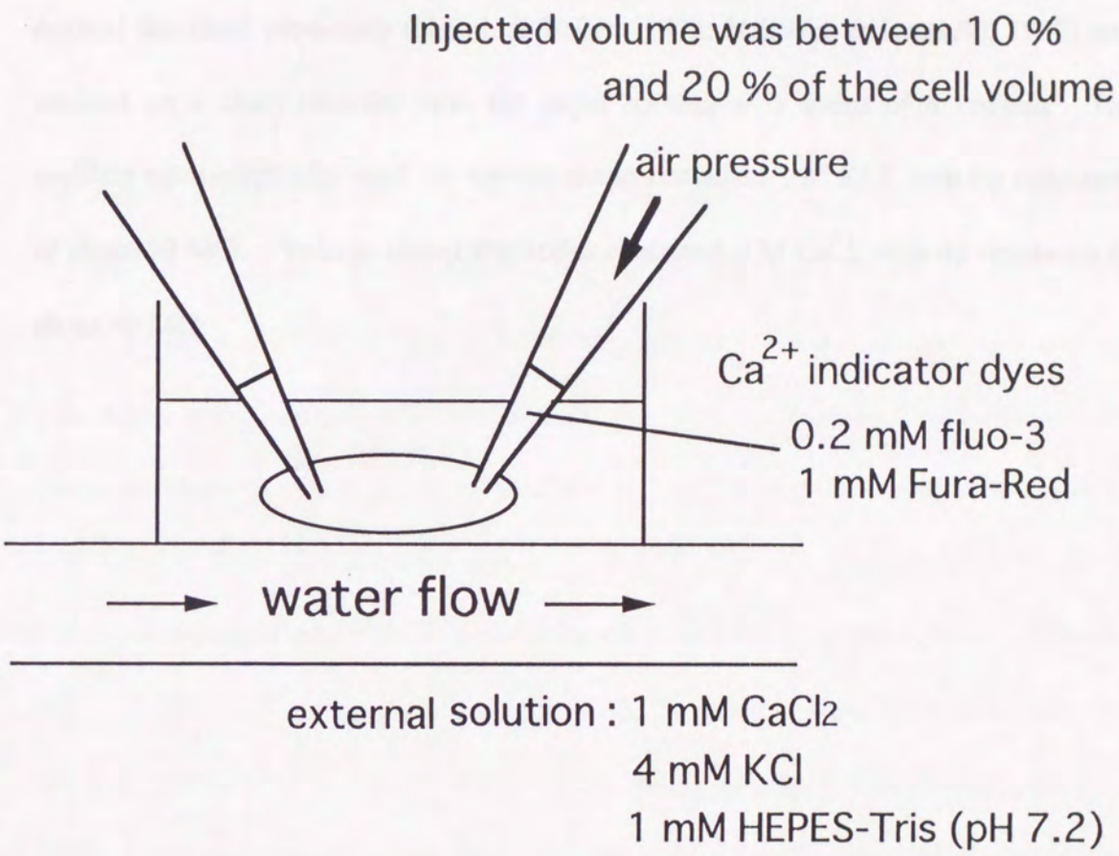

Fig. 3-2. A scheme of the method for the microinjection of Ca^{2+} indicator dyes into the cell.

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 MΩ. Voltage clamp electrodes contained 1 M CsCl, with tip resistance of about 50 MΩ.

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. 3-3 and 3-4B). In Ca^{2+}-free solution, increase of $[Ca^{2+}]_i$ was not observed at any region (Fig. 3-4C). Figure 3-4A shows $[Ca^{2+}]_i$ image at 5 sec after cooling (maximum response; frame 3 in Fig. 3-4B and C). The values of the fluorescence ratio at two areas (anterior or posterior) of the cell are shown in Figure 3-5. 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. 3-5A). 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. 3-6). 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. 3-6). Cooling of the cell which was voltage clamped at -30 mV (near resting potential in standard solution) elicited a transient inward current (Fig. 3-7A). In

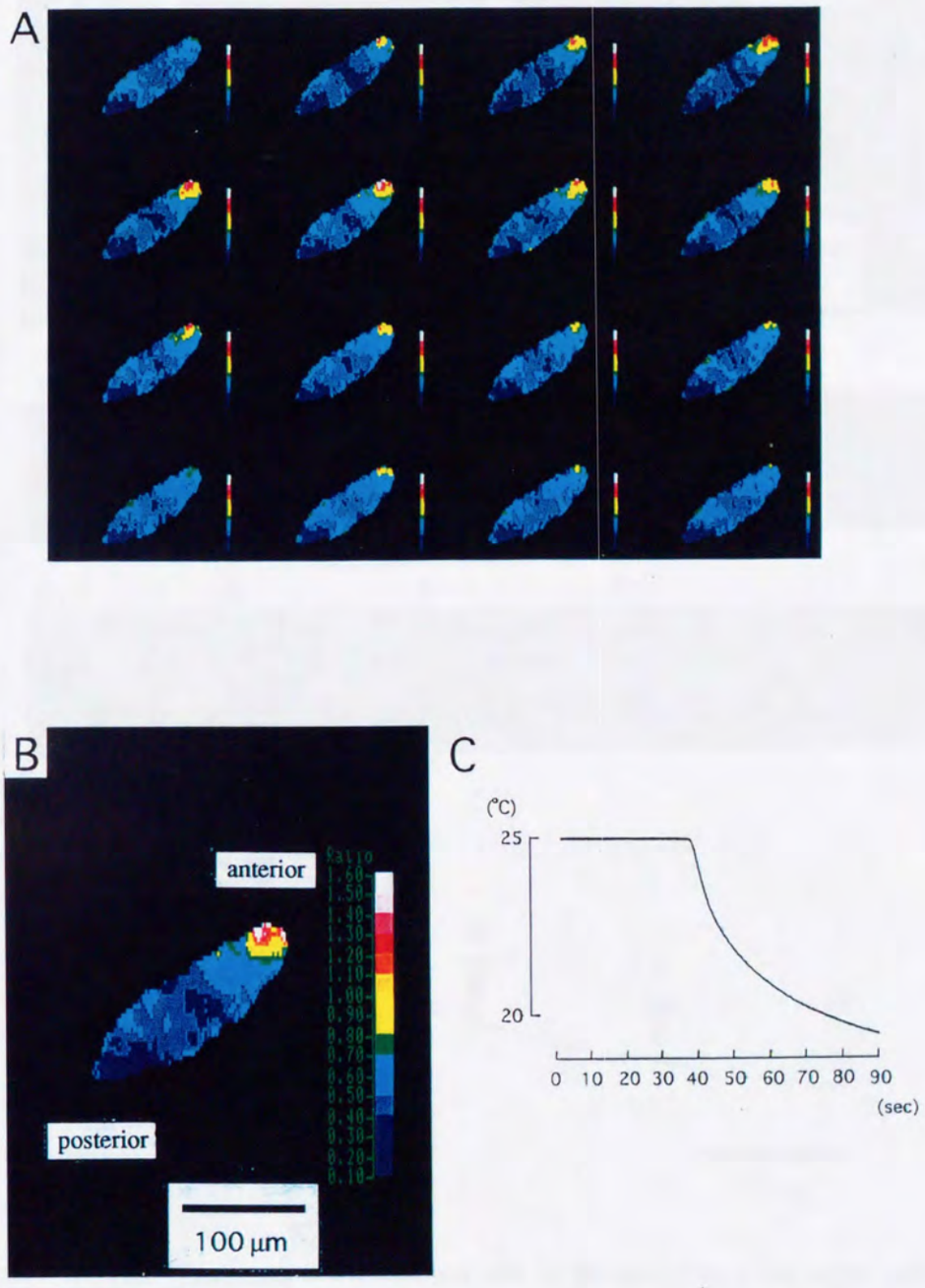

Fig. 3-3. (A) $[Ca^{2+}]_i$ response of *Paramecium* to cooling in standard solution. Ratio images at 3 s intervals after cooling are arranged from left to right and from top to bottom. First image was sampled before cooling. In the figure, red (to white) parts indicate the regions in which fluo-3/Fura-Red ratio increased, i.e., in which $[Ca^{2+}]_i$ increased. (B) $[Ca^{2+}]_i$ response at 12 s after cooling (frame 6 in Fig. 1A). (C) A record of temperature.

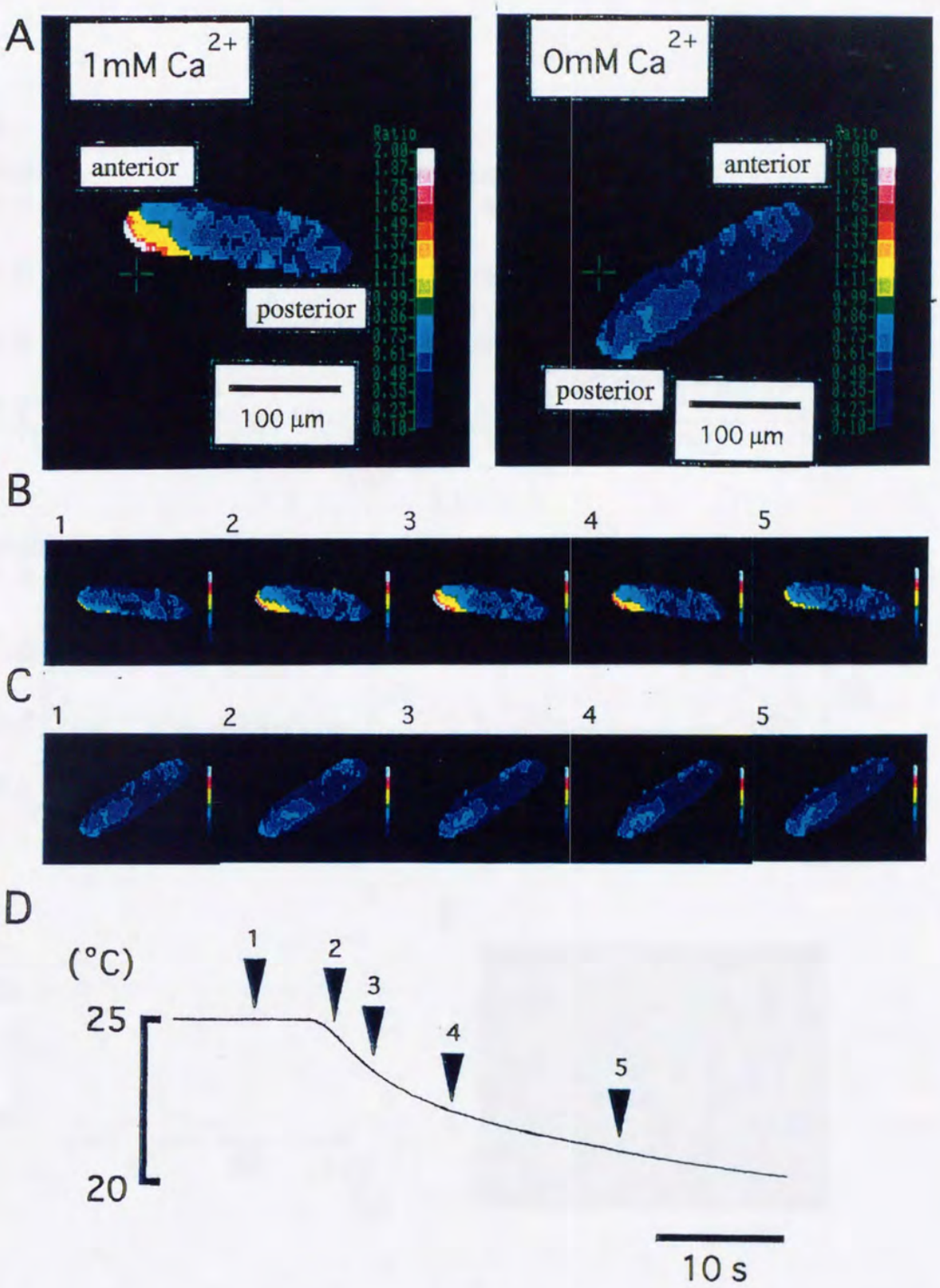

Fig. 3-4. (A) $[Ca^{2+}]_i$ responses of *Paramecium* cells in standard solution (left image) and in Ca^{2+}-free solution (right image) at arrowhead 3 in (D). (B) Ratio images of $[Ca^{2+}]_i$ response in standard solution, and (C) in Ca^{2+}-free solution. (D) A record of temperature. The arrowheads 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.

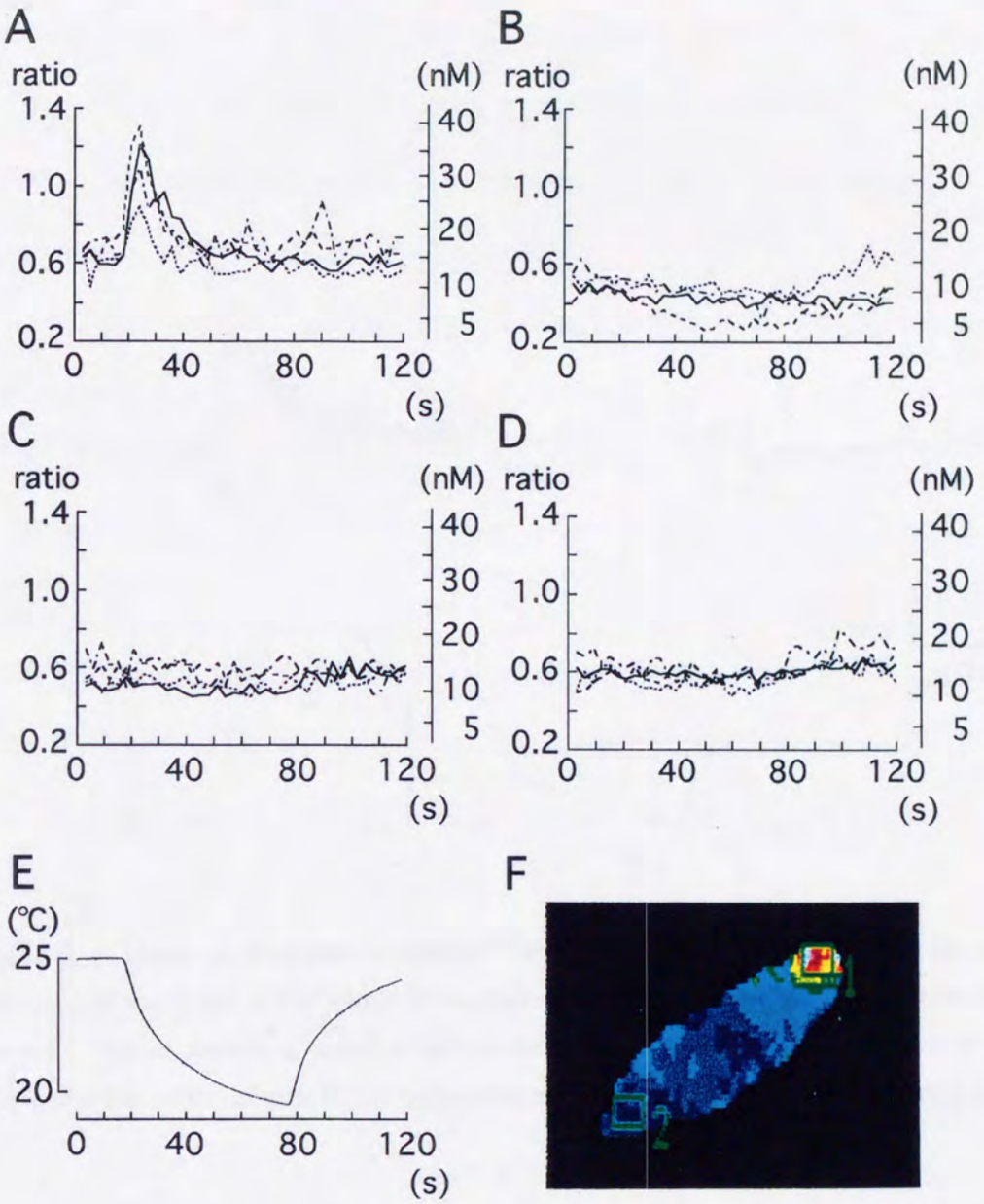

Fig. 3-5. $[Ca^{2+}]_i$ responses in standard solution (A, anterior; C, posterior; data from four different cells), and in Ca^{2+}-free solution (B, anterior; D, posterior; data from four different cells). (E) A record of temperature. Anterior (1) and posterior (2) areas that were calculated in (A) to (D) are shown in (F).

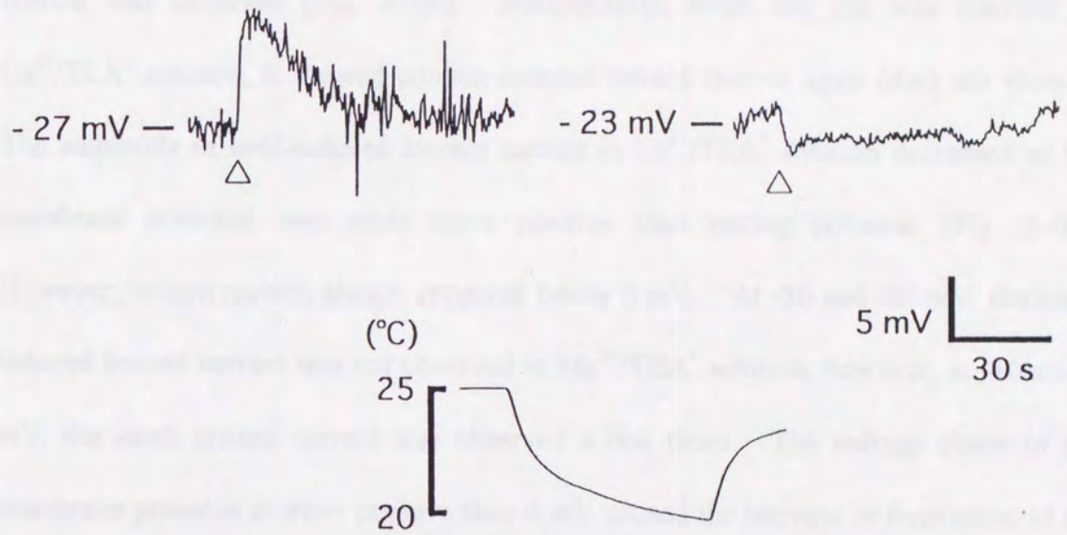

Fig. 3-6. Changes in membrane potential of *Paramecium* cells elicited by cooling in standard solution (left trace) and in Ca^{2+}-free solution (right trace). Open arrowheads indicate the start of cooling. Bottom trace is a record of temperature. These traces are representative of results obtained in four additional cells in standard solution and in two additional cells in Ca^{2+}-free solution.

this experiment, the K^+ currents were suppressed by the use of a voltage clamp 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. 3-7A). 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. 3-4B). 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. 3-8). 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 $\pm$ 23.2 pA (n = 5, mean $\pm$ SD), -214 $\pm$ 67.0 pA (n = 4), -222 $\pm$ 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,

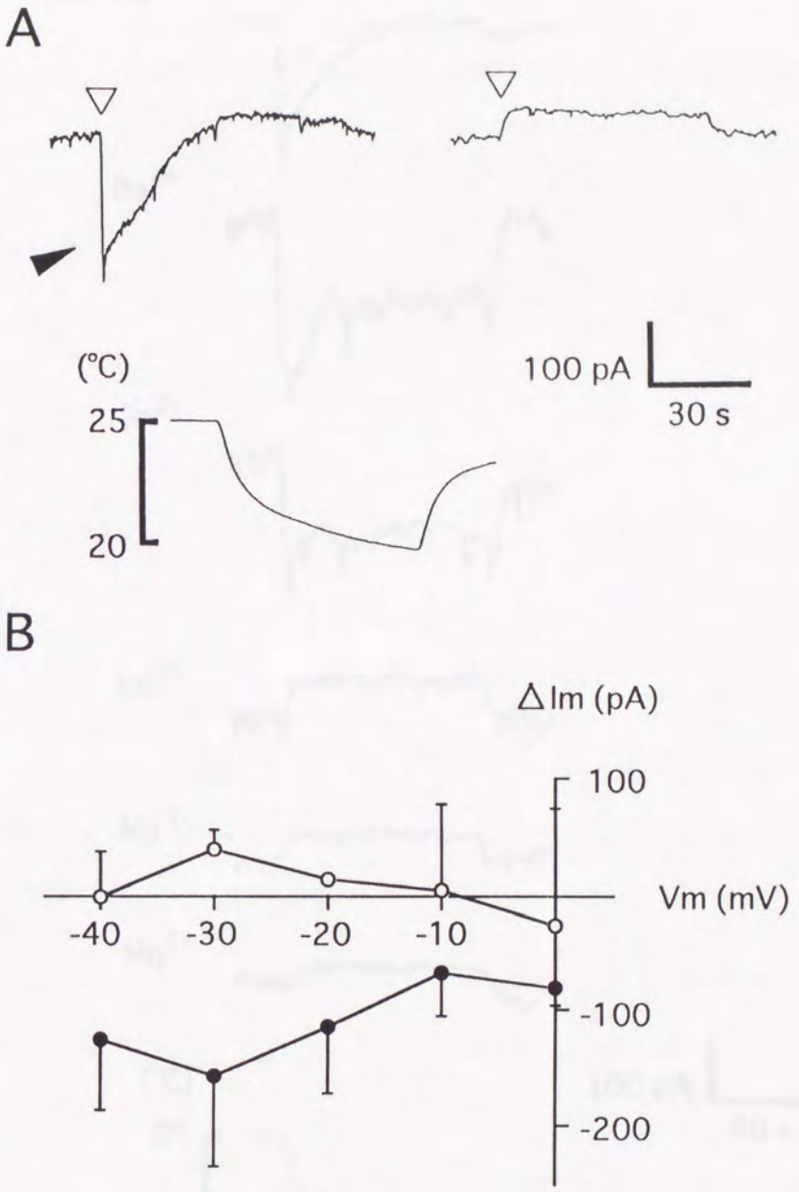

Fig. 3-7. (A) Cold-induced membrane currents in Ca^{2+}/TEA^{+} solution (left trace), and in a solution replaced 1 mM $CaCl_2$ with 1 mM $MgCl_2$ (right trace). Holding potential was -30 mV. Open arrowheads indicate the start of cooling, and filled arrowhead indicates the cold-induced Ca^{2+} current. 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 s 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.

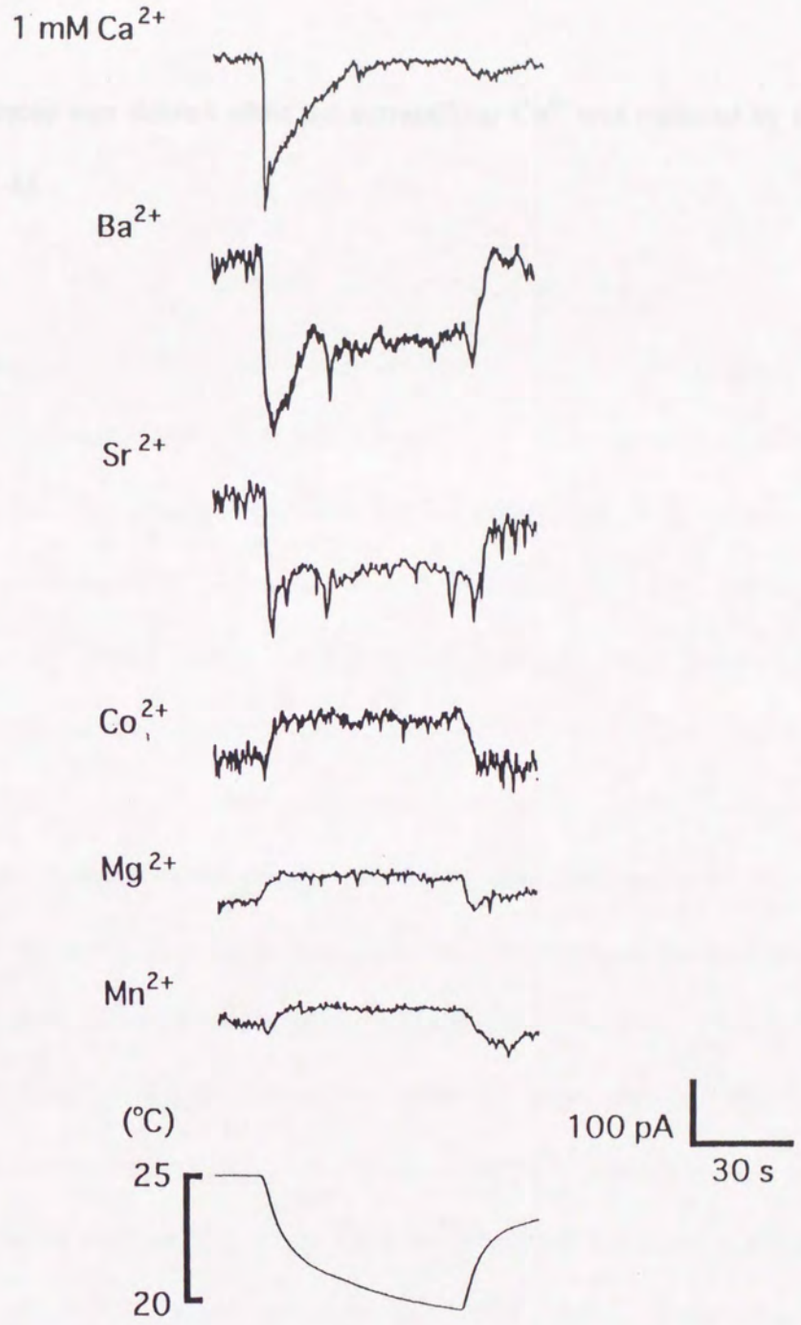

Fig. 3-8. Divalent cation selectivity of cold-induced inward current. Currents elicited by cooling in Ca^{2+}/TEA^{+} solution, and upon replacing 1 mM $CaCl_2$ with equimolar concentrationof $BaCl_2$, $SrCl_2$, $CoCl_2$, $MgCl_2$ or $MnCl_2$. Representative recording are shown from different cells in each solution. Bottom trace is a record of temperature.

while the decay was slowed when the extracellular Ca^{2+} was replaced by either Ba^{2+} or Sr^{2+} (Fig. 3-8).

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* cell (Figs. 3-3, 3-4 and 3-5). The membrane potential response of the cell to cooling was a transient depolarization (Fig. 3-6). These increases of $[Ca^{2+}]_i$ and membrane depolarization in response to cooling were lost upon removing extracellular Ca^{2+} (Figs. 3-4, 3-5 and 3-6), 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. 3-7A). 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. 3-6). 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. 3-8). 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., 1992b). 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. 3-8). 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, 1978), while hyperpolarization-activated Ca^{2+} channel located in soma membrane was impermeable to these ions (Preston et al., 1992a). 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.

CHAPTER 4 CALCIUM CURRENT ACTIVATED BY COOLING IN *Paramecium*

Abstract. Cooling of deciliated *Paramecium* cell induced a transient Ca current ($I_{Ca, cold}$) under conditions where K currents were suppressed. The amplitude of $I_{Ca, cold}$ was depended on the rate of temperature drop. It was appeared as the cooling rate was faster than -0.2 °C / 5 s and was saturated with the cooling rate was faster than approximately -1.7 °C / 5 s. The amplitude of $I_{Ca, cold}$ increased by the addition of Ca^{2+} to the bath solution in a concentration-dependent manner, whereas Ni^{2+}, Co^{2+}, Mn^{2+} or Mg^{2+} reversibly inhibited $I_{Ca, cold}$ in a concentration-dependent manner with apparent dissociation constants (K_D) of 0.52, 0.66, 0.67 or 2.17 mM, respectively. $I_{Ca, cold}$ was also inhibited reversibly by amiloride, with a K_D of 0.32 mM. On the other hand, $I_{Ca, cold}$ was desensitized by repetitive cooling. The amplitude of $I_{Ca, cold}$ by the second cooling was smaller than that by the first cooling when the interval was short, but recovered as the interval increased. Replacing extracellular Ca^{2+} with equimolar Sr^{2+} or Ba^{2+} did not significantly affect the amplitude of the current response to cooling, but it accelerated the rate of recovery from desensitization and slowed the decay of the current response. These results suggested that desensitization and inactivation on $I_{Ca, cold}$ involved a Ca^{2+}-dependent pathway.

Introduction

The mechanism of thermoreception in organisms remains unknown. The ciliated protists *Paramecium multimicronucleatum, P. tetraurelia*, and *P. caudatum* are very useful in research of thermoreception, because they have high sensitivities to temperature change. *Paramecium* cell remarkably responds to temperature change below the temperature to which it has been adapted, by producing the directional changes in swimming, i.e., avoidance behavior (Nakaoka & Oosawa, 1977). In the electrophysiological recording, cooling of the *Paramecium* cell induces a slow transient depolarization of the membrane potential (Nakaoka et al., 1987). This depolarization triggers the opening of the voltage-dependent Ca^{2+} channel in the ciliary membrane and the increase of Ca^{2+} concentration in cilia causes the avoidance behavior (Hennessey et al., 1983). The depolarization induced by cooling involves both increase of Ca conductance and decrease of K conductance (Nakaoka et al., 1987; Inoue and Nakaoka, 1990; Kuriu et al., 1996). Recent studies suggest the possibility that cold-induced decrease of K conductance is modulated by Ca^{2+}/calmodulin and change of intracellular Ca^{2+} level via G-protein (Kuriu et al., 1997a, 1997b; Nakaoka et al., 1997).

In the present study, we report some properties of a Ca conductance activated by cooling, including Ca^{2+}-dependent desensitization and inactivation.

Materials and Methods

Cell

Paramecium multimicronucleatum was cultured in a hay infusion inoculated with *Klebsiella pneumoniae* at 25 °C (Kuriu et al., 1996). *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-hydroxyehylpiperazine - N' - 2 - ethanesulfonicacid) - Tris [Tris (hydroxymethyl) aminomethane] (pH 7.2). Cells were preincubated in this solution at 25 °C for 1 hr or more prior to examination. The cells were deciliated by incubation in an adaptation solution containing 5 % ethanol for 0.5-1 min (Ogura & Machemer, 1980). During electrical recording, 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 Ca/TEA solutions which contained 0.05, 0.1, 0.5, 1 or 5 mM $CaCl_2$, 10 mM TEA-Cl (tetraethylammonium chloride) and 1 mM HEPES, and Tris or TEA-OH was added to adjust the pH 7.2. In some experiments, 1 mM $CaCl_2$ was replaced by equimolar concentration of $SrCl_2$ or $BaCl_2$.

Electrophysiological recording

Membrane currents of *Paramecium* were recorded using a method described previously (Naitoh & Eckert, 1972; Nakaoka & Iwatsuki, 1990). The capillary microelectrodes used for voltage clamp contained 1 M KCl or CsCl with tip resistance of about 50 MΩ.

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). Currents and temperature changes were sampled at 100 Hz after being filtered at 10 Hz through a 8-pole Bessel filter (model 902, Frequency Device, Haverhill, MA).

Data analysis

Data are presented as means ± SD or SE. The dose-suppression curves of divalent cations or amiloride were described by the Hill equation

$$I / I_{max} = 1 - C^n / (C^n + K_D{}^n),$$

where I and I_{max} are values for the amplitude of cold-sensitive Ca^{2+} current in the presence and absence, respectively, of Ni^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+} or amiloride. C is the divalent cation or amiloride concentration, n is Hill coefficient, and K_D is the concentration of divalent cation or amiloride producing half of the maximal current.

Results

Separation of $I_{Ca,\ cold}$ from the cold-induced inward current

Paramecium multimicronucleatum induced an inward current to cooling from 25 °C in standard solution (Fig. 4-1A). The amplitude of the inward current elicited by the cooling was increased as the membrane potential was made more positive than -30 mV (near resting potential in standard solution)(Fig. 4-1B). This current was significantly blocked at 0 mV or -10 mV by using CsCl-filled electrodes and tetraethylammonium in the bath solution, suggesting that K^+ mainly carried it (Fig. 4-1A, B). Cs^+ diffusing from the electrodes inhibits the K currents internally, whereas TEA inhibits K currents externally (Hinrichsen & Saimi, 1984). As reported previously (Kuriu et al., 1996), cooling of the voltage-clamped cell under conditions where K currents were suppressed elicited Ca current (Fig. 4-1A). The amplitude of $I_{Ca,\ cold}$ decreased as the membrane potential was made more positive than -30 mV (Fig. 4-1B). Estimation of the reversal potential on $I_{Ca,\ cold}$ was difficult, because the voltage clamp of membrane potential at more positive than 0 mV caused the increase in fluctuation of the membrane current, and $I_{Ca,\ cold}$ was indistinguishable from its underlying fluctuation.

Effects of the cooling rate on $I_{Ca,\ cold}$

The amplitude of $I_{Ca,\ cold}$ was depended on the cooling rate (Fig. 4-2). Cooling rate at the beginning of temperature drop from 25 °C was increased by lowering the temperature of switched water flow. $I_{Ca,\ cold}$ was appeared as the initial rate of cooling was made

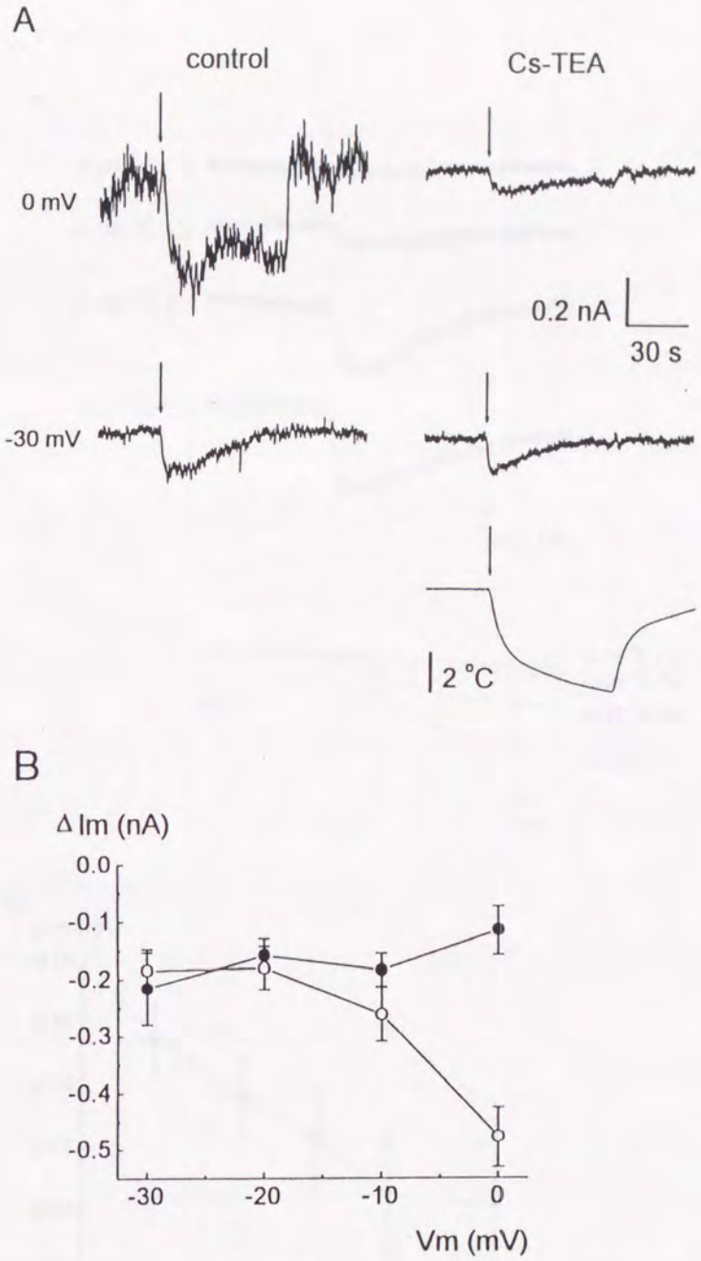

Fig. 4-1 Isolation of $I_{Ca,\ cold}$. (A) Currents activated by cooling of *Paramecium* in standard solution with 1 M KCl electrodes (left trace) and in the condition where the K currents were suppressed (right trace). The K current was suppressed by the use of a voltage clamp electrode containing 1 M CsCl, and by including 10 mM TEA-Cl in the bath solution (Ca/TEA solution). $I_{Ca,\ cold}$ was clearly observed under conditions where the K currents were suppressed. Initial rates of cooling were -1.7 °C / 5 s as that of previous study (Kuriu et al., 1996). Both standard solution and Ca/TEA solution were contained 1 mM $CaCl_2$. The voltage clamped potentials are indicated at the left side of the current trace, and the arrows indicate the start of cooling. The bottom trace is a record of the temperature. (B) The I-V relationships of the cold-induced inward current. ΔIm is the amplitude of cold-induced inward current. Open circles, in standard solution; closed circles, where the K currents were suppressed. Each point is the mean ± SD of 3-5 cells.

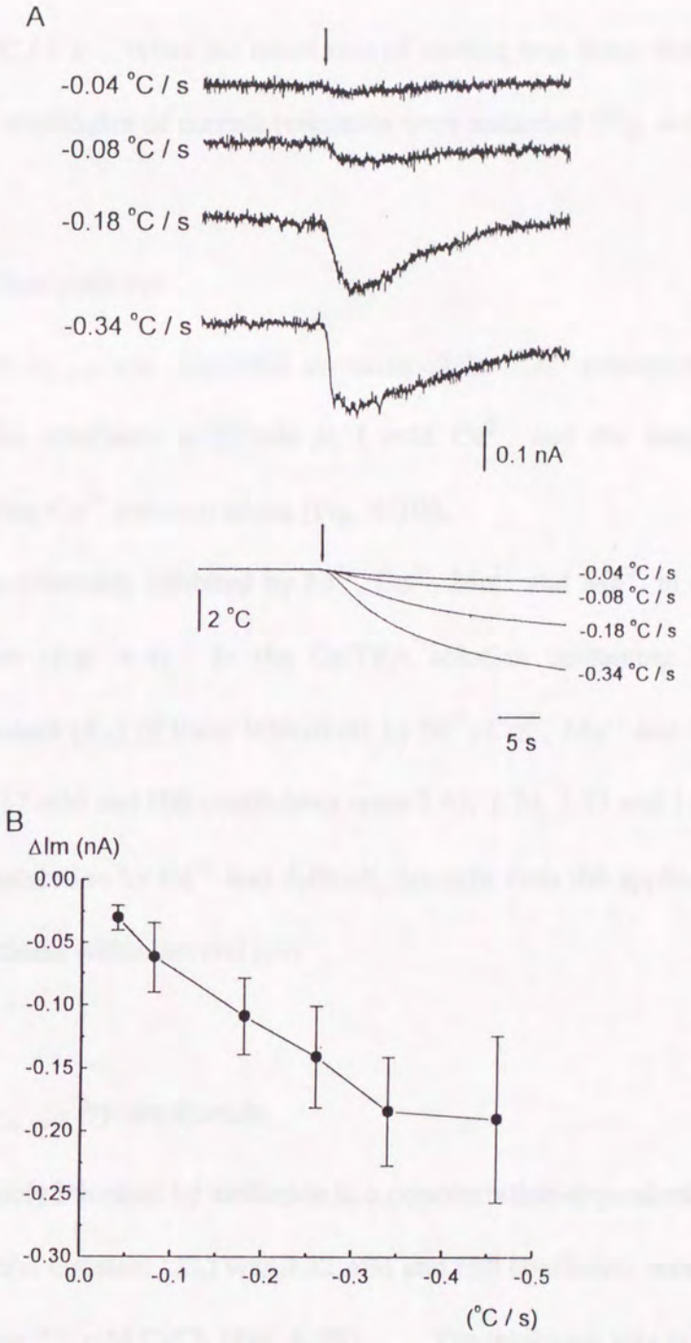

Fig. 4-2 Effect of cooling rate on $I_{Ca,\ cold}$. (A) Cells were voltage clamped at -30 mV in Ca/TEA solution containing 1 mM $CaCl_2$, and various rates of cooling stimuli were applied. $I_{Ca,\ cold}$ gradually declined as being slower rates of cooling. Initial rates of cooling are indicated at the left of each current trace and the right of each temperature trace. The voltage clamp electrodes were contained 1 M CsCl in this and subsequent figures. (B) Relationship between the cooling rate and the amplitude of $I_{Ca,\ cold}$. The amplitudes of $I_{Ca,\ cold}$ (ΔIm) are plotted as a function of the cooling rate. Each point is the mean $\pm$ SD of 3-7 cells.

faster than -0.2 °C / 5 s. When the initial rate of cooling was faster than approximately -1.7 °C / 5 s, the amplitudes of current responses were saturated (Fig. 4-2B).

Effects of divalent cations

The amplitude of $I_{Ca,\ cold}$ was depended on extracellular Ca^{2+} concentration (Fig. 4-3). $I_{Ca,\ cold}$ showed the maximum amplitude at 1 mM Ca^{2+}, and the amplitude gradually declined as lowering Ca^{2+} concentrations (Fig. 4-3B).

$I_{Ca,\ cold}$ was reversibly inhibited by Ni^{2+}, Co^{2+}, Mn^{2+} and Mg^{2+} in a concentration-dependent manner (Fig. 4-4). In the Ca/TEA solution containing 0.1 mM $CaCl_2$, dissociation constants (K_D) of these inhibitions by Ni^{2+}, Co^{2+}, Mn^{2+} and Mg^{2+} were 0.52, 0.66, 0.67 and 2.17 mM and Hill coefficients were 2.65, 2.74, 1.73 and 1.60, respectively. Investigation of inhibition by Cd^{2+} was difficult, because even the application of 0.1 mM Cd^{2+} caused cell death within several min.

Inhibition of $I_{Ca,\ cold}$ by amiloride

$I_{Ca,\ cold}$ was reversibly inhibited by amiloride in a concentration-dependent manner (Fig. 4-5). Dissociation constant (K_D) was 0.32 mM and Hill coefficient was 5.60 in Ca/TEA solution containing 0.1 mM $CaCl_2$ (Fig. 4-5B). The inhibition was nearly complete by 1 mM amiloride.

Effects of the repetitive cooling stimulus on $I_{Ca,\ cold}$

Fig. 4-6 shows effects of the repetitive cooling on $I_{Ca,\ cold}$. Cooling from 25 °C was

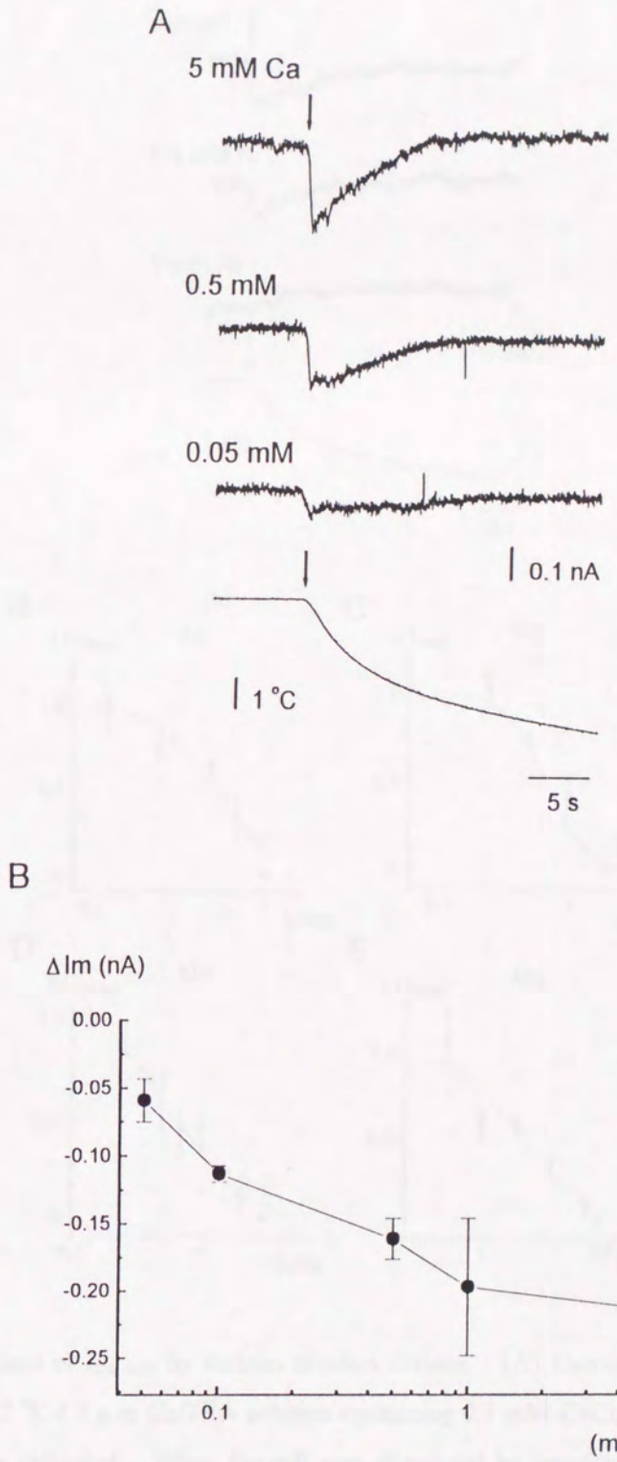

Fig. 4-3 Ca^{2+} concentration dependence of $I_{Ca,\ cold}$. (A) Currents elicited by cooling with initial rates of -1.3 °C / 5 s in Ca/TEA solution containing $CaCl_2$ at the concentrations indicated. Holding potential was -30 mV. (B) The amplitude of $I_{Ca,\ cold}$ (ΔIm) plotted as a function of the external Ca concentration. Each point is the mean ± SD of 3 cells.

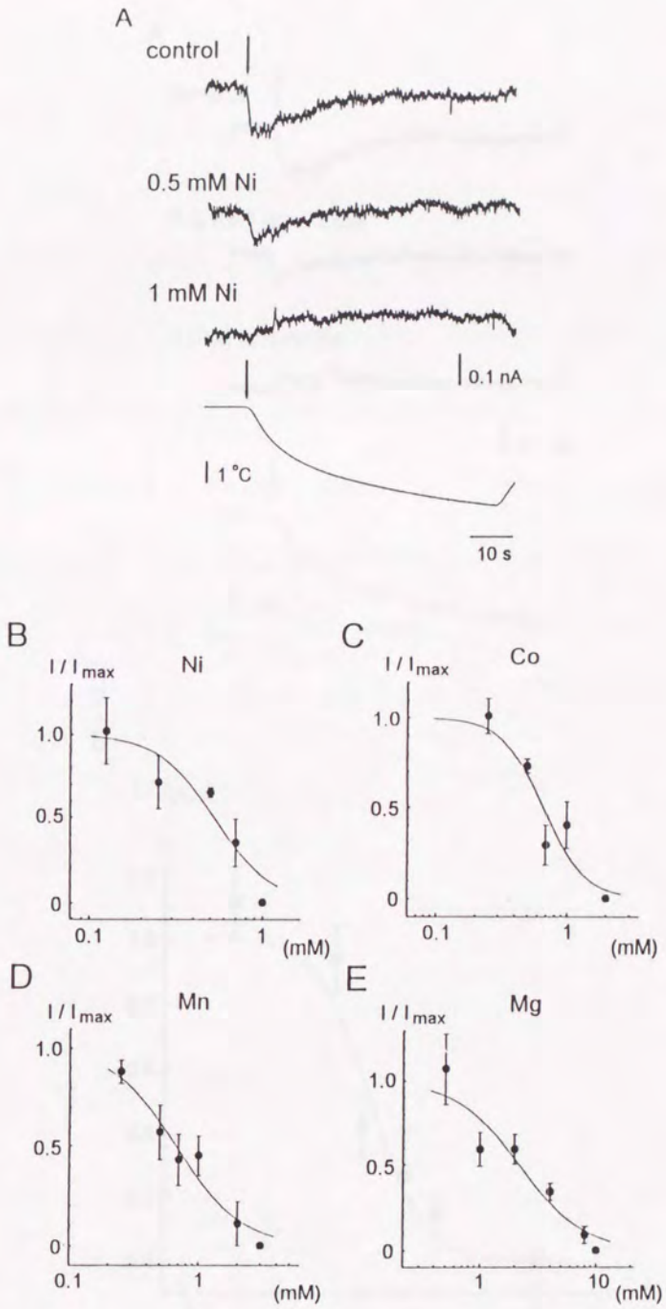

Fig. 4-4 Inhibitions of $I_{Ca,\ cold}$ by various divalent cations. (A) Currents elicited by cooling with initial rates of -1.3 °C / 5 s in Ca/TEA solution containing 0.1 mM $CaCl_2$ supplemented with Ni^{2+} at the concentrations indicated. When the cell was stimulated by repetitive cooling, various divalent cations were applied between a first and a second cooling. Time interval of repetitive cooling was 10 min. Holding potential was -30 mV. Concentration dependence of Ni^{2+} (B), Co^{2+} (C), Mn^{2+} (D) and Mg^{2+} (E) on the inhibitions of $I_{Ca,\ cold}$. The amplitude of $I_{Ca,\ cold}$ in the presence of divalent cation relative to control current-amplitudes (I/I_{max}) is plotted as a function of divalent cation concentration. The inhibition curves were described by the Hill equation (see Materials and Methods). Each point is the mean $\pm$ SE of 3-4 cells.

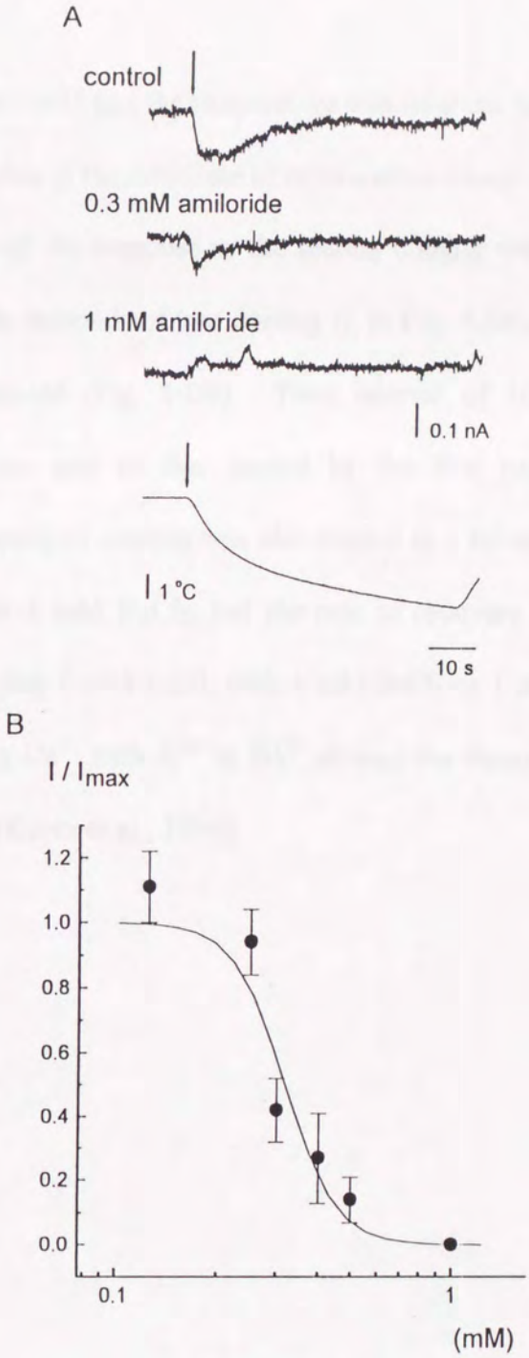

Fig. 4-5 Inhibition of $I_{Ca,\ cold}$ by amiloride. (A) Currents elicited by cooling with initial rates of -1.3 °C / 5 s in Ca/TEA solution containing 0.1 mM $CaCl_2$ supplemented with amiloride at the concentrations indicated. When the cell was stimulated by repetitive cooling, amiloride was applied between a first and a second cooling. Time interval of repetitive cooling was 10 min. Holding potential was -30 mV. (B) Concentration dependence of amiloride inhibitions of $I_{Ca,\ cold}$. The amplitude of $I_{Ca,\ cold}$ in the presence of amiloride relative to control current-amplitudes (I/I_{max}) is plotted as a function of amiloride concentration. The inhibition curve was described by the Hill equation. Each point is the mean ± SE of 3-7 cells.

applied to the cell for 1 min and the temperature was returned to 25 °C. Subsequently, a second cooling stimulus of the same rate of temperature change and duration was applied. The peak amplitude of the response to the second cooling was smaller than that to the first cooling when the intervals of two cooling (t, in Fig. 4-6A) was short, but recovered as the interval increased (Fig. 4-6B). Time interval of 10 min was produced the response of the same size as that caused by the first cooling (Fig. 4-6B). The desensitization to repetitive cooling was also caused in a solution replaced 1 mM $CaCl_2$ with 1 mM $SrCl_2$ or 1 mM $BaCl_2$, but the rate of recovery from desensitization was accelerated by replacing 1 mM $CaCl_2$ with 1 mM $SrCl_2$ or 1 mM $BaCl_2$ (Fig. 4-6A, B). In addition, replacing Ca^{2+} with Sr^{2+} or Ba^{2+} slowed the decay of $I_{Ca,\ cold}$ (Fig. 4-6A) as reported previously (Kuriu et al., 1996).

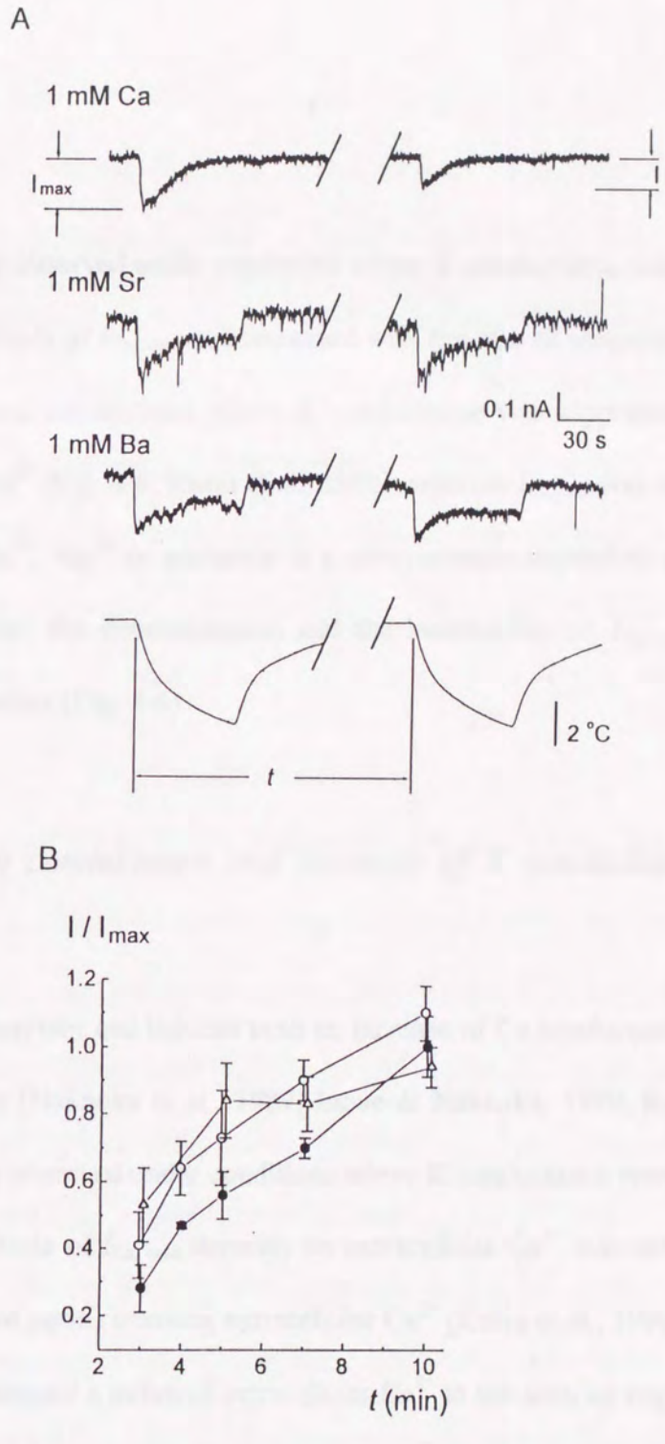

Fig. 4-6 The desensitization by the repetitive cooling in cold-activated membrane current. (A) The traces show a current induced by cooling in Ca/TEA solution containing 1 mM $CaCl_2$. Initial rates of cooling were −1.7 °C / 5 s. Holding potential was -30 mV. Time intervals between first and second cooling, *t*, were 5 min. (B) The amplitude of the current relative to control values (I/I_{max}) plotted as a function of time interval in Ca/TEA solution containing 1 mM $CaCl_2$(closed circles), and in a solution replacing 1 mM $CaCl_2$ with equimolar $SrCl_2$ (open circles) or $BaCl_2$ (open triangles). Each point is the mean ± SE of 3-4 cells.

Discussion

$I_{Ca,\ cold}$ was clearly observed under conditions where K conductance was suppressed (Fig. 4-1). The amplitude of $I_{Ca,\ cold}$ was increased with the rate of temperature drop (Fig. 4-2). Cold-activated conductance where K conductance was suppressed was permeable to Ca^{2+}, Sr^{2+} or Ba^{2+} (Fig. 4-6; Kuriu et al., 1996), whereas $I_{Ca,\ cold}$ was reversibly inhibited by Ni^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+} or amiloride in a concentration-dependent manner (Figs. 4-4 and 4-5). Further, the desensitization and the inactivation on $I_{Ca,\ cold}$ were likely to depend on Ca^{2+} influx (Fig. 4-6).

Increase of Ca conductance and decrease of K conductance induced by cooling

Cooling of *Paramecium* cell induces both an increase of Ca conductance and a decrease of K conductance (Nakaoka et al., 1987; Inoue & Nakaoka, 1990; Kuriu et al., 1996). $I_{Ca,\ cold}$ was clearly observed under conditions where K conductance were suppressed (Fig. 4-1). The amplitude of $I_{Ca,\ cold}$ depends on extracellular Ca^{2+} concentrations (Fig. 4-3) and $I_{Ca,\ cold}$ was lost upon removing extracellular Ca^{2+} (Kuriu et al., 1996). Moreover, a cooling stimuli caused a influx of extracellular Ca^{2+} at the anterior region of a deciliated *Paramecium* cell (Kuriu et al., 1996). These results indicate that $I_{Ca,\ cold}$ is mediated by a cold-activated Ca conductance located on the anterior soma membrane.

When the membrane potential was made more positive than resting potential (-30 mV), the amplitude of the inward current elicited by cooling in standard solution was

increased, while the amplitude of $I_{Ca,\ cold}$ was decreased (Fig. 4-1). And under voltage clamped at -30 mV, the amplitude of the current response was not significantly affected by suppression of K conductance (Fig. 4-1). These results suggest that increase of Ca conductance triggers the cold-induced depolarization at resting potential and decrease of K conductance mainly enhances the cold-induced depolarization at more positive membrane potential than the resting potential. In addition, cold-induced decrease of K conductance may be modulated by $I_{Ca,\ cold}$. Actually, the possibilities that cold-induced decrease of K conductance was modulated by Ca^{2+}/calmodulin and change of intracellular Ca^{2+} level were reported (Nakaoka et al., 1997; Kuriu et al., 1997a, 1997b).

$I_{Ca,\ cold}$ is mediated by a Ca permeable ion channel or a Ca transporter

$I_{Ca,\ cold}$ is due to change in Ca conductance, consistent with either the opening of a Ca channel or the activation of a Ca transporter. Ni^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+} and Mg^{2+} can block conventional voltage-gated Ca channels at 10 μM to 20 mM concentrations (Hille, 1992). Thus, the ability of these divalent cations to inhibit $I_{Ca,\ cold}$ upon their addition to the bath solution was tested. $I_{Ca,\ cold}$ was reversibly inhibited by Ni^{2+}, Co^{2+}, Mn^{2+} or Mg^{2+} in a concentration-dependent manner (Figs. 4-4 and 4-5). Moreover, cold-activated Ca conductance is permeable to Sr^{2+} and Ba^{2+} (Fig. 4-6; Kuriu et al., 1996). These properties of $I_{Ca,\ cold}$ are similar to conventional voltage-gated Ca channel currents. On the other hand, $I_{Ca,\ cold}$ was also reversibly inhibited by amiloride in a concentration-dependent manner (Fig. 4-5). Amiloride inhibit not only various channels, i.e., T- and L-type Ca channel (Tang et al., 1988; Garcia et al., 1990), Na channel (Ismailov et al., 1995), mechanosensitive cation channel (Lane et al., 1991) but also Na/Ca exchanger, or

Na/H antiporter (Benos, 1982; Kleyman & Cragoe, 1988; Antolini et al., 1993). However, Na/Ca exchanger exchange 3 Na^+ for each Ca^{2+}, so that Na/Ca exchange would generate outward current under the Na-free conditions in the Ca/TEA solution (discussed by Preston et al., 1992a). Therefore, $I_{Ca,\ cold}$ is probably mediated by a Ca permeable ion channel not a Na/Ca exchanger, although there is also possibility that $I_{Ca,\ cold}$ is mediated by other types of Ca transporters.

$I_{Ca,\ cold}$ *is specifically activated by cooling*

$I_{Ca,\ cold}$ was inhibited by amiloride (Fig. 4-5). In *Paramecium,* amiloride has been previously described as a blocker of hyperpolarization-activated Ca conductance in soma membrane (Preston et al., 1992a). However, hyperpolarization-activated Ca conductance is different from cold-activated Ca conductance because hyperpolarization-activated Ca conductance is impermeable to Sr^{2+} or Ba^{2+}, whereas cold-activated Ca conductance is permeable to these ions (Fig. 4-6; Kuriu et al., 1996). On the other hand, depolarization-activated Ca conductance is permeable to Sr^{2+} or Ba^{2+} (Brehm & Eckert, 1978). However, depolarization-activated Ca conductance is also distinct from cold-activated Ca conductance, because depolarization-activated Ca conductance is located on the ciliary membrane, while cold-activated Ca conductance is located on the anterior soma membrane (Kuriu et al., 1996). In addition, $I_{Ca,\ cold}$ was not significantly affected by 0.1 mM of W-7 (data not shown) which have been known as the blocker of the depolarization-activated Ca conductance in *Paramecium* (Hennessey & Kung, 1984; Ehrlich et al., 1988), and 1 mM amiloride inhibits depolarization-activated Ca conductance by only 27 % (Preston et al., 1992a).

Chemorepellent (lysozyme) activates a receptor-operated Ca conductance (Hennessey et al., 1995). This conductance is not affected by amiloride and is permeable to Mg^{2+} and Mn^{2+}. Similarly, mechano-receptor Ca conductance and heat-activated Ca conductance are also permeable to these divalent cations (Tominaga & Naitoh, 1994). The cold-activated Ca conductance is impermeable to Mg^{2+} and Mn^{2+} (Kuriu et al., 1996). Thus, these chemorepellent-operated Ca conductance, mechano-receptor Ca conductance and heat-activated Ca conductance which are apparently nonselective are different from the cold-activated Ca conductance.

Based on these facts, we suggest that $I_{Ca,\ cold}$ is distinct from depolarization-, hyperpolarization-, chemorepellent-, mechano- or heat-activated Ca currents in *Paramecium,* and is specifically activated by cooling.

Desensitization and inactivation of $I_{Ca,\ cold}$

$I_{Ca,\ cold}$ was desensitized by repetitive cooling (Fig. 4-6). The amplitude of $I_{Ca,\ cold}$ to the second cooling was small when the interval was short, but recovered as the interval increased (Fig. 4-6B). The desensitization to repetitive cooling was also caused in a solution replaced extracellular Ca^{2+} with equimolar Sr^{2+} or Ba^{2+}, but the rate of recovery from desensitization was accelerated by replacing with Sr^{2+} or Ba^{2+} (Fig. 4-6B). Thus desensitization on $I_{Ca,\ cold}$ may involve both a Ca^{2+}-dependent and Ca^{2+}-independent pathway. On the other hand, inactivation of $I_{Ca,\ cold}$ is also likely to depend on Ca^{2+} influx. Substituting Sr^{2+} or Ba^{2+} for Ca^{2+} caused that the decay of the inward current was slowed (Fig. 4-6A; Kuriu et al., 1996), suggesting that the decay of cold-activated inward current resulted from Ca-dependent inactivation of cold-activated Ca conductance. Similarly,

Ca^{2+} influx lead to inactivation of depolarization- or hyperpolarization-activated Ca conductance (Brehm & Eckert, 1978; Preston et al., 1992b). Thus it seems that Ca^{2+} plays a important role of the desensitization and the inactivation on $I_{Ca,\ cold}$. Further investigation is needed to understand the mechanisms of the desensitization and the inactivation on cold-sensitive response.

GENERAL DISCUSSION

The cold-induced decrease of K conductance in *Paramecium* was modulated by Ca^{2+}/calmodulin (*Chapters* 1 and 2). In addition, it seems that cyclic nucleotides, cAMP or cGMP, also affected cold-induced decrease of K conductance (*Chapter* 1). On the other hand, the cooling of *Paramecium* induced not only a decrease of K conductance but also an increase of Ca conductance (*Chapter* 3). The cold-sensitive Ca conductance is distinct from the depolarization-, hyperpolarization-, chemorepellent-, mechano- and heat-activated Ca conductances in *Paramecium*, and is specifically activated by cooling (*Chapter* 4).

Cold-induced decrease of K conductance

A calmodulin mutant, *cam*^{12}, is defective in the cold-induced decrease of K conductance (Fig. 1-3). Comparison of the membrane resistances at the resting state, it is seen that *cam*^{12} mutant input resistance is 1.2 times larger than that of the wild type (Table 1-1). The reduced cooling sensitivity of *cam*^{12} mutant might be related to this increase in membrane input resistance. Because most of the resting conductance is based on open K channel (Machemer, 1988), a reduction of the conductance carried by K^+ at rest will increase the membrane resistance of *cam*^{12} mutant and presumably reduces the cooling sensitivity. On the other hand, the addition of a calmodulin antagonist, W-7, caused a decrease in the net outward current before cooling and inhibited cold-induced decrease of K conductance (Fig. 2-4). Therefore, the inhibition of function of calmodulin by mutation on *cam*^{12} or W-7 probably acts to close the cold-sensitive K channel before

cooling.

Application of theophylline or injection of cyclic nucleotide analogs into *cam*^{12} cells caused the membrane resistance to decrease to 81 or 67 % (Table 1-1), and these treatment accompanied the restoration of the cold-induced decrease of K conductance (Fig. 1-5). These results suggest that the decreased resting conductance of *cam*^{12} membrane is elevated by some effect of cyclic nucleotides, and the increased conductance is a part of the cooling sensitive response (Fig. 1). There is a possibility that the cold-induced decrease of K conductance inhibited by W-7 in wild type is also restored by application of theophylline or injection of cyclic nucleotide analogs.

Restoration of the cold-induced decrease of K conductance by an increase of intracellular cyclic nucleotide suggests that cyclic nucleotide may regulate the cold-sensitive K channel. In the case of olfactory- and photo-receptor cells, cyclic nucleotides directly activate ion channels (Fesenko et al., 1985; Nakamura & Gold, 1987), and these cyclic nucleotide-activated channels are also regulated by an interaction with calmodulin (Hsu & Molday, 1993; Chen & Yau, 1994; Liu et al., 1994).

In conclusion, calmodulin and cyclic nucleotides probably act to increase the cold-sensitive K conductance before cold-stimuli. This K conductance is reduced by cooling, but its causes remain to be determined. The cold-induced decrease of K conductance may be resulted from the decrease of intracellular Ca^{2+} or/and cyclic nucleotide concentration by cooling. However, it seems unlikely that cold-induced decrease of K conductance is caused by decrease in intracellular Ca^{2+} concentration, because cooling of a cell causes an increase of intracellular Ca^{2+} concentration (Fig. 3-3). Thus there are possibilities that intracellular cyclic nucleotide concentration is reduced by cooling, or

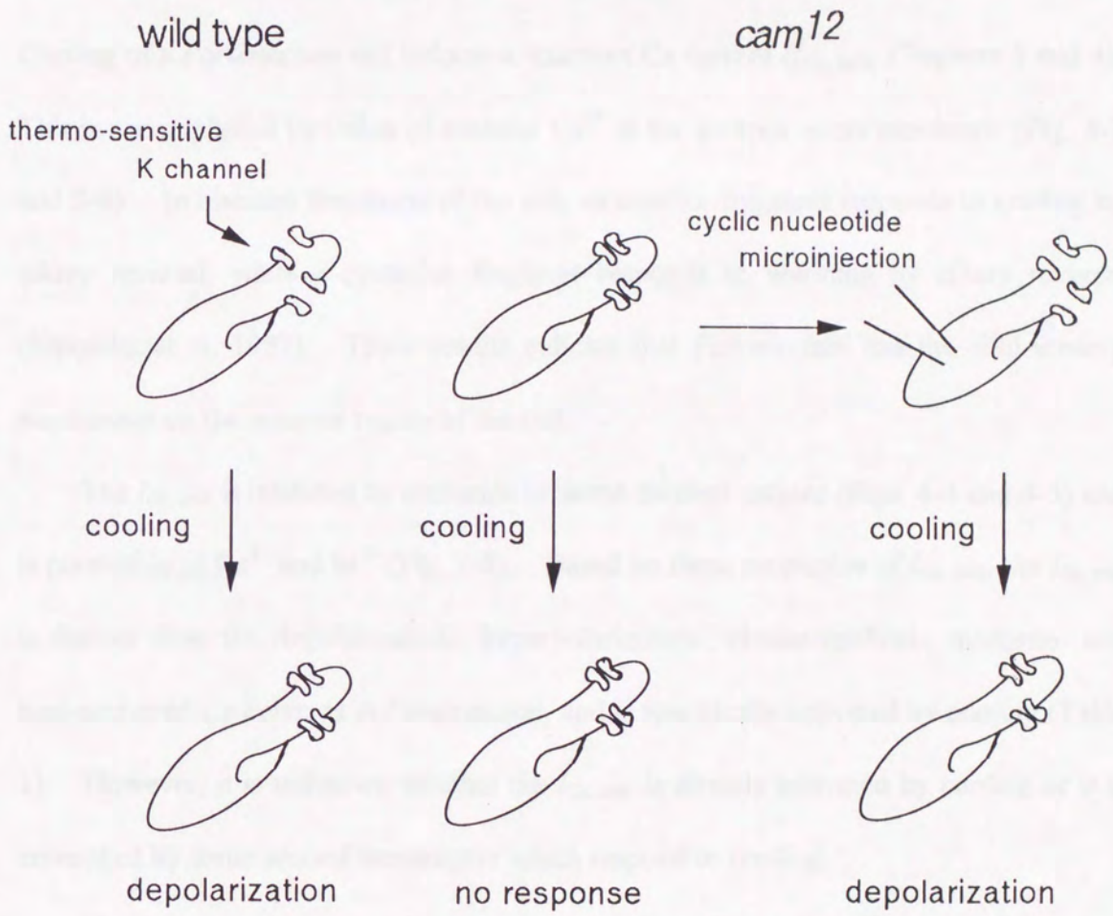

Fig. 1. Shematic diagram showing possible mechanisms for cold-sensitive responses of wild type, *cam*^{12} and *cam*^{12} which was raise intracellular levels of cyclic nucleotide.

cold-sensitive K channel is directly inactivated by cooling.

Cold-induced increase of Ca conductance

Cooling of a *Paramecium* cell induces a transient Ca current ($I_{Ca,\ cold}$; *Chapters* 3 and 4). This is accomplished by influx of external Ca^{2+} at the anterior soma membrane (Fig. 3-3 and 3-4). In bisected fragments of the cell, an anterior fragment responds to cooling by ciliary reversal, while a posterior fragment responds to warming by ciliary reversal (Nakaoka et al, 1987). These results indicate that *Paramecium* has the cold-sensory mechanism on the anterior region of the cell.

The $I_{Ca,\ cold}$ is inhibited by amiloride or some divalent cations (Figs. 4-4 and 4-5) and is permeable to Ba^{2+} and Sr^{2+} (Fig. 3-8). Based on these properties of $I_{Ca,\ cold}$, the $I_{Ca,\ cold}$ is distinct from the depolarization-, hyperpolarization-, chemorepellent-, mechano- and heat-activated Ca currents in *Paramecium*, and is specifically activated by cooling (Table 1). However, it is unknown whether the $I_{Ca,\ cold}$ is directly activated by cooling or it is controlled by some second messengers which respond to cooling.

The $I_{Ca,\ cold}$ is desensitized by repetitive cooling (Fig. 4-6). The rate of recovery from desensitization is accelerated by replacing 1 mM Ca^{2+} with equimolar Sr^{2+} or Ba^{2+} (Fig. 4-6). On the other hand, the inactivation of the $I_{Ca,\ cold}$ is likely to depend on Ca^{2+} influx. Substituting Sr^{2+} or Ba^{2+} for Ca^{2+} caused the decay of the inward current to be slowed down (Figs. 3-8 and 4-6). Thus it seems that Ca^{2+} plays an important role of the desensitization and the inactivation of the $I_{Ca,\ cold}$. Moreover, intracellular Ca^{2+} increased by cooling may modulate the cold-sensitive K channel via calmodulin. In conclusion, I suggest that the intracellular Ca^{2+} which is increased by cooling induces the inactivation

Table 1. Calcium currents in *Paramecium*.

stimulus	localization	ion selectivity	amiloride	W-7
cold	soma	Ca^{2+}, Ba^{2+}, Sr^{2+} >> Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}	block	unblock
depolarization	cilia	Ca^{2+}, Ba^{2+}, Sr^{2+} >> Mg^{2+}	unblock	block
hyperpolarization	soma	Ca^{2+} >> Ba^{2+}, Sr^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}	block	----------
chemorepellent	soma	Ca^{2+}, Mg^{2+}, Mn^{2+} (non selective)	unblock	unblock
mechanical	soma	Ca^{2+}, Mg^{2+}, Mn^{2+} (non selective)	----------	----------
heat	soma	Ca^{2+}, Mg^{2+}, Mn^{2+} (non selective)	----------	----------

References

Kuriu et al. (1996), Brehm & Eckert (1978), Hennessey & Kung (1984), Preston et al. (1992), Tominaga & Naitoh (1994), Hennessey et al. (1995)

of cold-sensitive Ca channels and the activation of cold-sensitive K channel via calmodulin, and the membrane potential is repolarized (Fig. 2).

Finally, some additional information in cold-sensitive Ca current will be described.

The *cam*^{12} mutant has defects in not only cold-induced decrease of K conductance but also cold-induced increase of Ca conductance. Cooling of *cam*^{12} did not induce $I_{Ca, cold}$ under condition where K current suppressed. However, it seems unlikely that the defect of $I_{Ca, cold}$ is resulted in calmodulin mutation on *cam*^{12}, because the amplitude of $I_{Ca, cold}$ is unaffected by application of W-7 or EGTA-injection. In fact, *cam*^{12} has multiple defects (Preston et al., 1991). Further investigation of the $I_{Ca, cold}$ in *cam*^{12} is needed.

I tried to investigate cold-sensitive Ca channel at single channel level using patch clamp technique which was developed by Saimi & Martinac (1989). However, it was not succeeded, because it was difficult to examine a Ca channel under patch clamp in Ca or Ba solution. Recently, I found that $I_{Ca, cold}$ was permeable to Na^+. The amplitude of $I_{Ca, cold}$ is increased by addition of 10 mM NaCl in Ca/TEA solution, and the increase also appeared under condition where a cell was injected EGTA. Moreover, the current response is examined on replacing extracellular $CaCl_2$ with 10 mM NaCl. These results suggest that Na^+ can flow through the cold-sensitive Ca channel in response to cooling. Therefore, it is probably possible that cold-sensitive Ca channel is recorded under patch clamp in Na solution.

In the recently study, the thermoreception is closely related to the chemoreception that has been reported (see GENERAL INTRODUCTION). There is possibility that $I_{Ca, cold}$ is also activated by some chemical stimuli like menthol. It is very interesting to

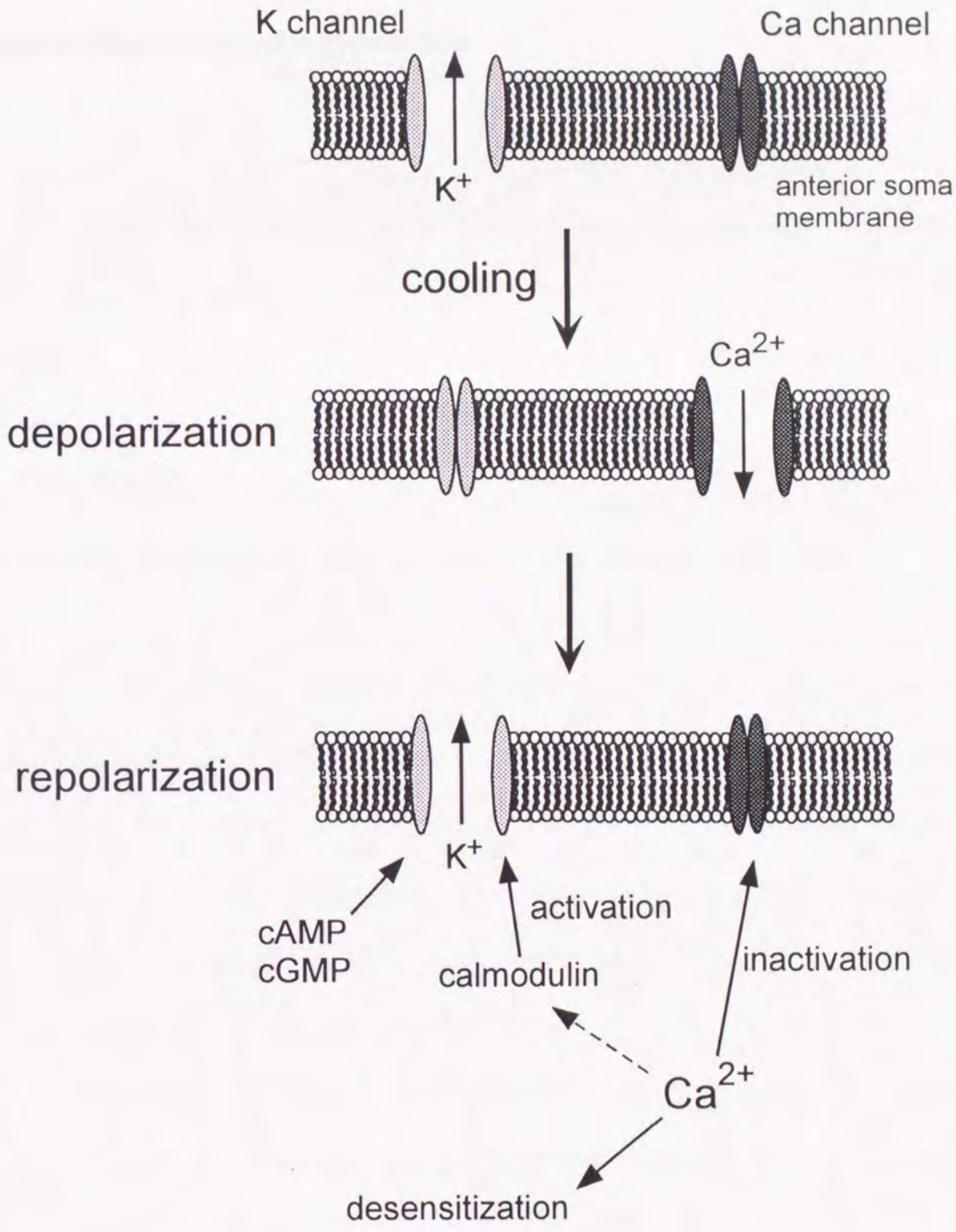

Fig. 2. Schematic diagram of cold-sensitive response in *Paramecium*. Cooling of a *Paramecium* cell induces the close of K channel and the open of Ca channel. Ca^{2+} influx through the Ca channels causes an increase of intracellular Ca^{2+} concentration. Intracellular Ca^{2+} plays a important role of the desensitization and the inactivation of the $I_{Ca,\ cold}$. Moreover, intracellular Ca^{2+} increased by cooling may modulate the cold-sensitive K channel via calmodulin. The membrane repolarization is presumably induced by Ca^{2+}-induced inactivation on Ca channel and Ca^{2+}-induced activation on K channel via calmodulin.

examine effects of menthol in *Paramecium*.

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