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Cold-induced Decrease of K^+ Conductance and Its Inhibition by a Calmodulin Antagonist, W-7, in *Paramecium tetraurelia*

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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 the cell caused an inward current. The amplitude of the current was increased as the membrane potential was made more positive than the 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 a decrease in K^+ conductance. The cold-induced inward current was reversibly inhibited by the 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 in K^+ conductance induced by cooling is Ca^{2+}-dependent and is inhibited by W-7.**

Paramecium cells accumulate in regions with temperatures close to the temperature of their culture, the optimum temperature region (6). Cells respond to temperature changes, both cooling and heating, by increasing the frequency of ciliary reversal, i.e., thermal avoidance behavior (9). 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 (2). 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 (5, 7, 10), while that caused by heating is generated by an increase in the Ca^{2+} conductance.

In a previous study, we found that some calmodulin mutants did not induce inward currents in response to cooling (8). 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*.

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MATERIALS AND METHODS

Cells. "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 the 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, and 1 mM HEPES-Tris (pH 7.2).

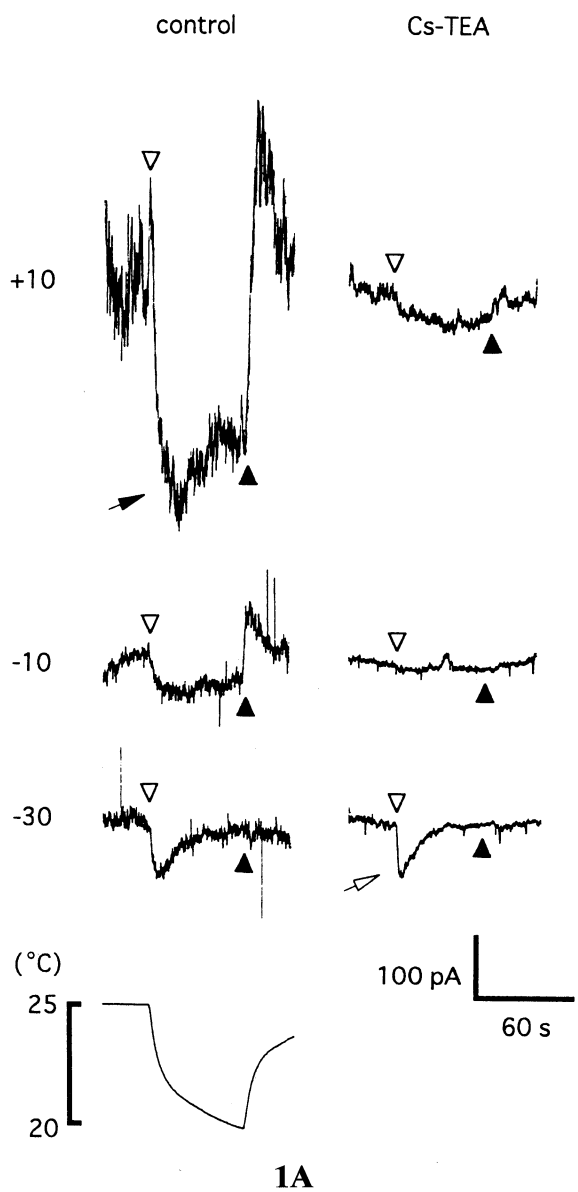
Intracellular recording. The membrane currents of the *Paramecium* were recorded using a method described by Nakaoka and Iwatsuki (11). The capillary microelectrodes used for the voltage clamp contained 1 M KCl or 1 M CsCl, and had a tip resistance of about 50 MΩ. The cells were deciliated by incubation in a standard solution containing 5% ethanol for 0.5–1 min and then returned to various solutions (13). 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 the 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 (10). The cold-induced membrane current from the resting level, ΔIm, was measured at 10 sec after the start of cooling.

Injection of the Ca^{2+} chelator, EGTA. The pressure injection was performed by a modification of the method described by Oosawa and Yamagishi (14). 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: Picospritzer, 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 was 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. 1A). The amplitude of the inward current elicited by the cooling was increased as the membrane potential was made more positive than the resting potential (Fig. 1A, B). The K^+ current of the *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 (4). The cold-in-



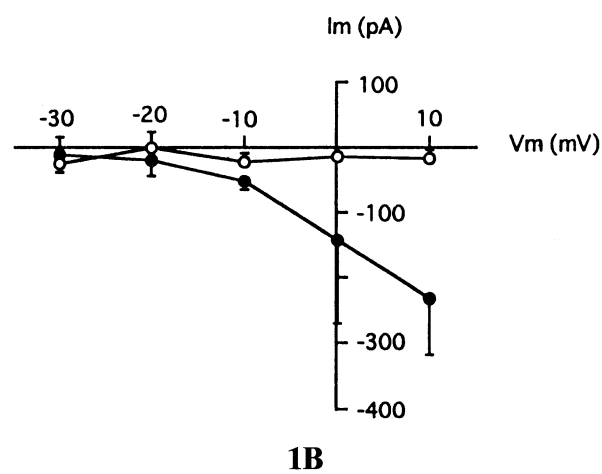


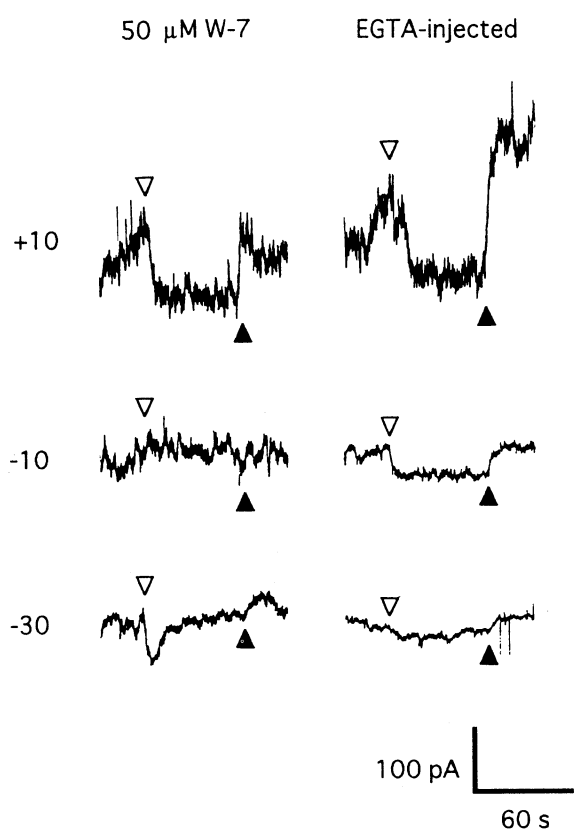
Fig. 1. (A) Cold-induced membrane current in standard solution (left traces), and the inhibition of the current when the K^+ current was suppressed (right traces). The K^+ current was suppressed by the use of a voltage clamp containing 1 M CsCl in the pipette, and by including 10 mM TEA-Cl in the bath solution (4). The filled arrow indicates the K^+ current, and the open arrow shows the Ca^{2+} current. The membrane potential was clamped at the various levels indicated at the left side of the current trace. The open triangles and the closed triangles indicate the start of cooling and the end of cooling, respectively. The bottom trace is a record of the temperature. (B) The I–V relationships of the cold-induced membrane current. Closed circles, in standard solution; open circles, with the K^+ current suppressed. Each point is the mean±SD of 3–9 cells.

duced inward current was significantly blocked by using CsCl-filled electrodes and tetraethylammonium in the bath solution, suggesting that K^+ carried the current (Fig. 1A, B). In *P. multimicronucleatum*, cooling of the voltage-clamped cell under conditions where the K^+ current was suppressed elicited a transient Ca^{2+} current (7). *P. tetraurelia* also showed a transient inward current under conditions where the K^+ current was suppressed (Fig. 1A, open arrow). This current was lost 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 ± 20.3 pA, n=8, Vm=-30 mV; *P. multimicronucleatum*, -208.0 ± 23.2 pA, n=5, Vm=-30 mV; 7).

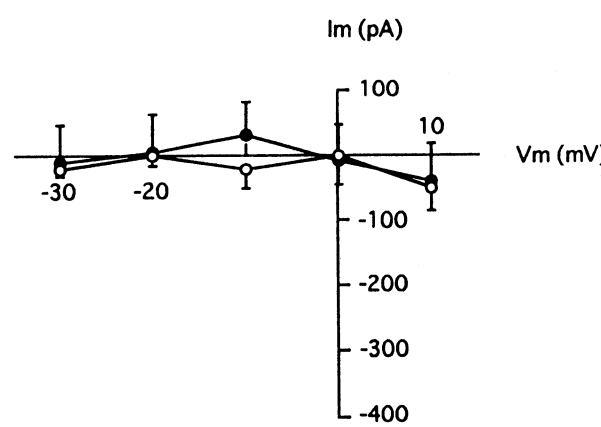
Effects of calmodulin antagonists and EGTA. When the cell was transferred to the standard solution containing 50 μM W-7, the cold-induced inward current was reduced (Fig. 2A, B). In the present study, the effects of the calmodulin antagonist W-7 on the cold-induced decrease in the K^+ conductance were estimated with the membrane potential clamped at +10 mV. With the voltage clamped at +10 mV, 50 μM W-7 inhibited about 80% of the cold-induced inward current (Fig. 2A, B). The resting membrane potential and resting membrane resistance apparently were not affected by W-7, in agreement with a previous observation (3). 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. 2A, B). With the voltage clamped at +10 mV, the 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. 3). The concentration of the calmodulin antagonists used which inhibited 50% of the cold-induced inward current, i.e., the IC_{50} value, was 7 μM and the inhibition was nearly complete with 100 μM W-7. W-5, the dechlorinated analogue of W-7, also inhibited the cold-induced inward current, although the IC_{50} was higher than that of W-7 (about 115 μM for W-5, Fig. 3).

W-7 reversibly inhibited the cold-induced inward current (Fig. 4). With the voltage clamped at +10 mV, the net outward current before cooling was about 1 nA and the 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 de-



2A



2B

Fig. 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 the 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$\pm$SD of 3–5 cells.

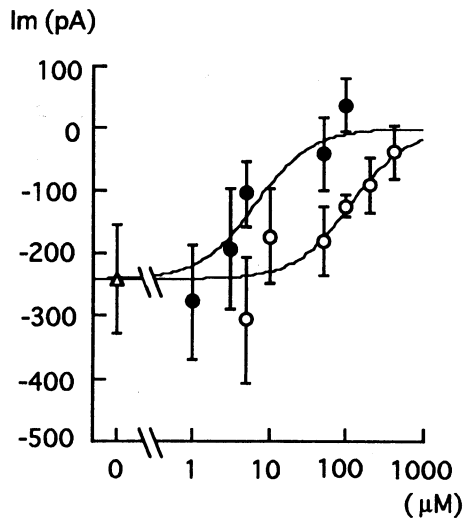


Fig. 3. Concentration effects of W-7 (closed circles) and W-5 (open circles) on the cold-induced inward current. Each point is the mean ± SD of 3–5 cells. Ordinate: ΔIm value were measured under voltage clamp at +10 mV. Abscissa: concentration of W-7 or W-5 on a logarithmic scale. Interpolation from 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.

crease of net outward current before cooling, and the cold-induced inward current was not observed. 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 currents by the 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. 1). This current was reversibly inhibited by the external application of W-7 in a concentration-dependent manner (Figs. 2, 3 and 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. 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). Moreover, in the previous study, the calmodulin mutant (*cam*^{12}) which has substitutions in the N-terminal lobe of the calmodulin molecule reduced inward currents in response to cooling (8). 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

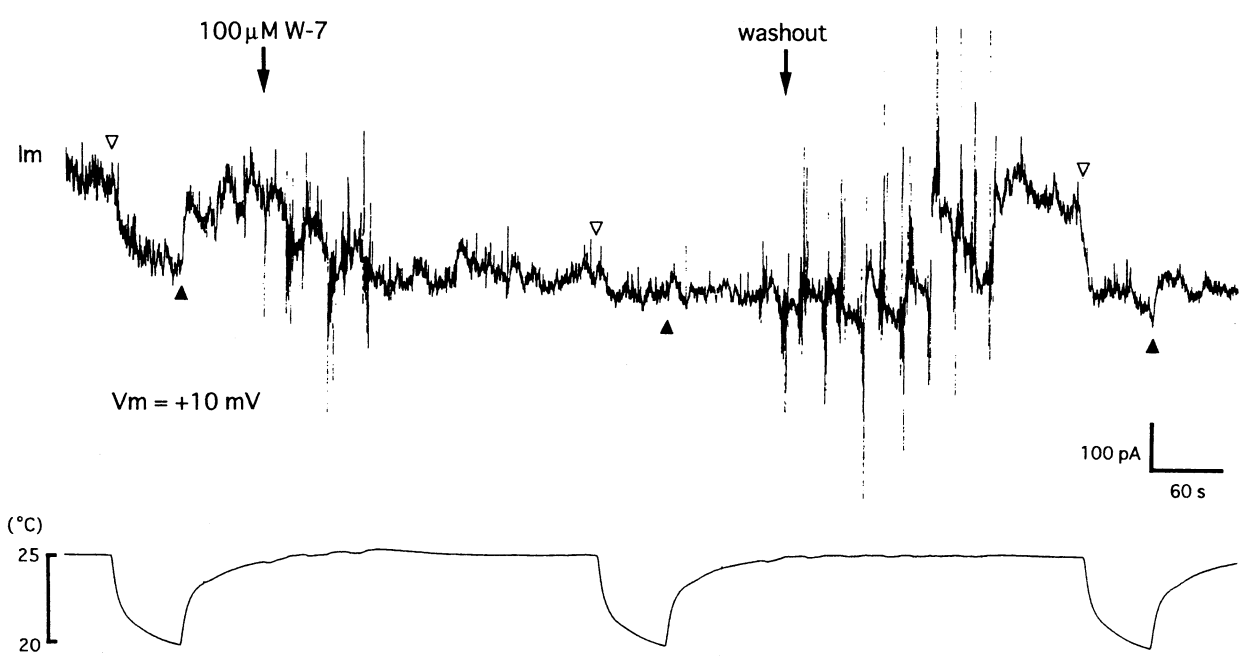


Fig. 4. Reversible inhibition of cold-induced inward current by 100 μM W-7. The upper trace is the membrane current under voltage clamp at +10 mV and is representative of results obtained in 2 additional cells. The bottom trace is a record of the temperature. The open triangles and the closed triangles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

been used as calmodulin antagonist. In *Paramecium*, the Ca^{2+} channel located in the ciliary membrane is directly blocked by W-7 (1). In addition, W-7 inhibited the cold-induced inward current at lower concentrations than the bovine calmodulin-dependent phosphodiesterase activity (12). The concentration of W-7 which inhibited 50% of the cold-induced inward current, the IC_{50}, was 7 μM, and the IC_{50} of W-5 was 115 μM (Fig. 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 (12). Thus, it is also possible that W-7 acts directly on the cold-sensitive K^+ conductance.

Cold-induced decrease in K^+ conductance and increase in Ca^{2+} conductance. In *Paramecium tetraurelia* and *P. multimicronucleatum*, the cooling of the cells induced a decrease in K^+ conductance (5, 8, 10). *P. multimicronucleatum* exhibited not only a cold-induced decrease in K^+ conductance but also a cold-induced increase in Ca^{2+} conductance (7). In the present study, *P. tetraurelia* showed the inward Ca^{2+} current under conditions where the K^+ current was suppressed (Fig. 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 (7). Intracellular Ca^{2+} increased by cooling may modulate the cold-sensitive K^+ channel via calmodulin.

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