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POSSIBLE MEDIATION OF G-PROTEINS IN COLD-SENSORY TRANSDUCTION IN *PARAMECIUM MULTIMICRONUCLEATUM*

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Summary

The possible involvement of G-proteins in cold-sensory transduction was examined using voltage-clamped *Paramecium multimicronucleatum* into which non-hydrolyzable guanosine nucleotide analogues had been applied intracellularly. Guanosine-5′-*O*-3-thiotriphosphate, guanosine-5′-*O*-2-thiodiphosphate and aluminium fluoride all reduced the transient inward current in response to cooling, suggesting the possibility that G-proteins mediate cold-sensory transduction. Internal application of a Ca^{2+} chelator, EGTA, also reduced the current response. In addition to their effect on reducing the cold-sensory response, application of these chemicals modulated both the resting potential and the membrane conductance. Possible correlations between G-protein activity and the regulation of intracellular Ca^{2+} levels are discussed.

Key words: cold-sensory response, G-protein, membrane current, *Paramecium multimicronucleatum*, thermoreception.

Introduction

The reception of thermosensory information is found in many living organisms (Hensel, 1974; Eckert *et al.* 1988), but the way in which intracellular transduction of such information occurs remains unknown. In the ciliated protozoan *Paramecium*, it has been shown that both warm and cold stimuli induce transient depolarization of the surface membrane, which triggers spike-like depolarizations and leads to directional changes in swimming (Hennessey *et al.* 1983; Nakaoka *et al.* 1987; Tominaga and Naitoh, 1992). In particular, cold-sensitive receptors are distributed on the anterior portion of the cell (Nakaoka *et al.* 1987), and their sensitivity is sufficiently high that they respond to an extremely slow rate of temperature decrease from the temperature at which the cell has been cultured (Nakaoka and Oosawa, 1977; Inoue and Nakaoka, 1990).

Heterotrimeric guanine-nucleotide-binding proteins (G-proteins) are involved in the pathways of a variety of sensory transduction processes in which the sensory response is amplified (Shepherd, 1991). In this study, we have examined whether G-proteins are involved in the cold-sensory response. *Paramecium multimicronucleatum* cells were voltage-clamped, and guanine nucleotide analogues were introduced into them. The results suggest the possibility that the cold-sensory transduction process in this organism involves some changes in G-protein activity.

Materials and methods

Cells

Paramecium multimicronucleatum were cultured in a hay infusion inoculated with *Klebsiella pneumoniae*. The culture temperature was kept constant at 25 °C by incubation in a water bath. Cells at the stationary phase were collected by low-speed centrifugation and suspended in a solution containing 0.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} $CaCl_2$, 4 mmol l^{-1} KCl and 2 mmol l^{-1} Tris–HCl (pH 7.2). This solution was used as the experimental medium and, after suspension, cells were left for at least 1 h at 25 °C to enable them to adapt to these conditions. To carry out the experiments in the absence of action potentials, the cells were deciliated by incubation in experimental medium containing 5 % ethanol for 2–3 min before returning them to the experimental medium (Inoue and Nakaoka, 1990).

Intracellular recording

Methods of intracellular recording were similar to those described previously (Nakaoka and Iwatsuki, 1992). The cells were placed in a glass vessel mounted on an inverted microscope. Two electrodes were inserted from the upper side of the cell, one for injecting current and the second for recording the intracellular voltage. The electrodes usually contained 1 mol l^{-1} KCl and had a resistance of 20–50 MΩ. The temperature around the cell was controlled by changing the temperature of the water flowing beneath the vessel and was monitored with a thermistor probe placed close to the cell surface. The time constant of the thermistor was 0.4 s. The cells were initially voltage-clamped at 25 °C, then the temperature was lowered to 21 °C at an initial rate of 0.18 °C s^{-1} for 1 min, before returning the temperature to 25 °C at the same rate. The amplitudes of current responses were obtained from the

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maximal current changes that occurred during a 20 s period after the temperature drop. Membrane conductance was measured from the current evoked by application of a voltage step (–5 mV for 0.4 s) to the resting membrane. The resting potential was measured as the voltage difference between the internal and external electrodes after nulling the tip potentials in the bath.

Intracellular application of chemicals

Two methods were used for the intracellular application of reagents. In the first of these, chemicals were diffused through two microelectrodes for voltage-clamping. 10 mmol l^{-1} guanosine-5′-*O*-3-thiotriphosphate (GTP-γS), 10 mmol l^{-1} guanosine-5′-*O*-2-thiodiphosphate (GDP-βS), 0.1 mol l^{-1} EGTA, 10 μmol l^{-1} aluminium fluoride (AlF_4^-; consisting of 10 μmol l^{-1} $AlCl_3$ and 20 mmol l^{-1} NaF), 10 mmol l^{-1} adenosine-5′-triphosphate (ATP) or 10 mmol l^{-1} adenosine-3′,5′-cyclic monophosphate (cyclic AMP) was added to the KCl electrodes and diffused into the cell. The second technique involved injection of chemicals through an additional glass microcapillary pipette using a pressure pulse of 30 Pa for 0.3 s (Picospritzer II, General Valve Co., Fairfield, NJ, USA). The capillary was filled with 5 mmol l^{-1} Hepes (pH 7.0 adjusted with KOH) containing 5 mmol l^{-1} GTP-γS, 5 mmol l^{-1} GDP-βS, 25 mmol l^{-1} EGTA or 30 mmol l^{-1} LiCl. The volume injected was between 5 and 10 % of the cell volume, which was assumed to be 5×10^{-10} l. Both GTP-γS and GDP-βS (Boehringer-Mannheim) were in the form of lithium salts; ATP and cyclic AMP (Sigma) were sodium salts. EGTA (Dojindo, Japan) was neutralized with KOH.

Results

Current response to temperature change under control conditions

When the membrane potential of cells at 25 °C was clamped at the resting level (approximately –21 mV) and the cells were subjected to a temperature drop for 1 min, an inward current was transiently induced at the time of maximum cooling (Fig. 1A). This observation was in agreement with previous results showing that the membrane transiently depolarized upon cooling (Nakaoka *et al.* 1987). Returning the temperature to 25 °C caused the inward current to disappear rapidly and sometimes induced a transient outward current (not illustrated).

When the holding potential was shifted to a more depolarized level than the resting potential, the amplitude of the inward current in response to cooling was increased. When the holding potential was shifted to a hyperpolarized level, the inward current decreased and disappeared at approximately –40 mV. Further hyperpolarization elicited only a slow outward current. The amplitude of the inward current was almost linearly proportional to the holding potential (Fig. 1B).

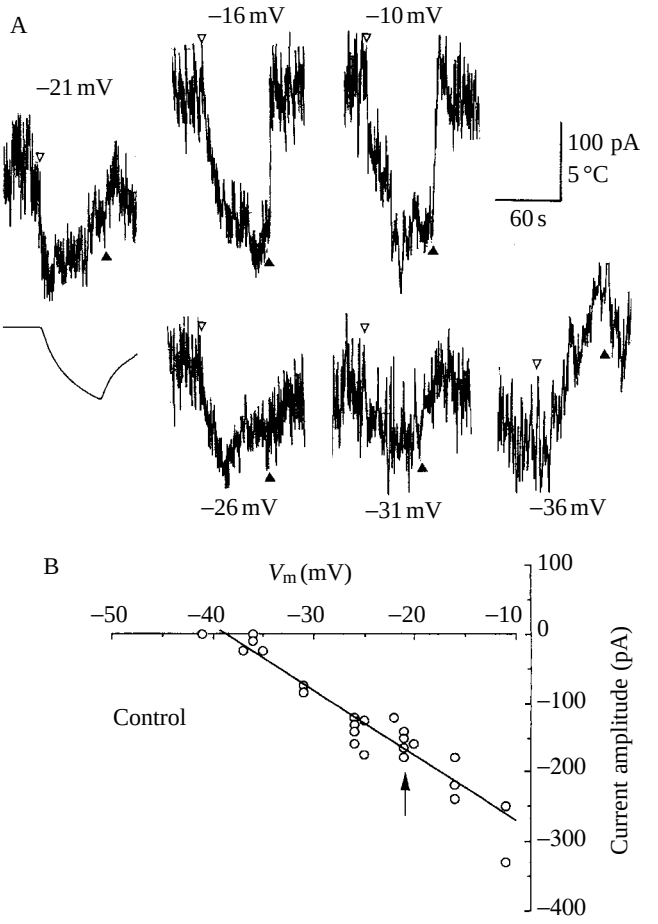

Fig. 1. Membrane currents in response to a cooling stimulus. *Paramecium multimicronucleatum* was voltage-clamped at various potentials and a cooling stimulus consisting of a temperature drop from 25 °C was applied. (A) Current responses at various potentials are shown for a typical cell; recordings were made from six cells. Clamped potentials are indicated above or below the current recordings. The two traces on the left-hand side show changes in the temperature (lower) and current (upper) when the cell is clamped at the resting potential (–21 mV). The point at which the temperature drops is indicated by an open triangle, the commencement of a return to 25 °C is marked by a filled triangle. (B) Voltage-dependence of the current response. The amplitudes of the current response are plotted as a function of the membrane potential (V_m). The line is a regression line (y=–365.1–9.413x, r^2=0.893). The arrow indicates the mean value for the resting potential. Data were obtained from six cells.

The linear slope of the current–voltage plot was –9.41 pA mV^{-1}.

Effects of GTP-γS

We examined the effects of chemicals diffusing into the cell from the electrodes on the cold-sensory response, soon after the voltage-clamp had been established for the cell.

GTP-γS (10 mmol l^{-1}), which may activate G-proteins, caused a large reduction in the inward current elicited in response to cooling (Fig. 2). This effect was particularly

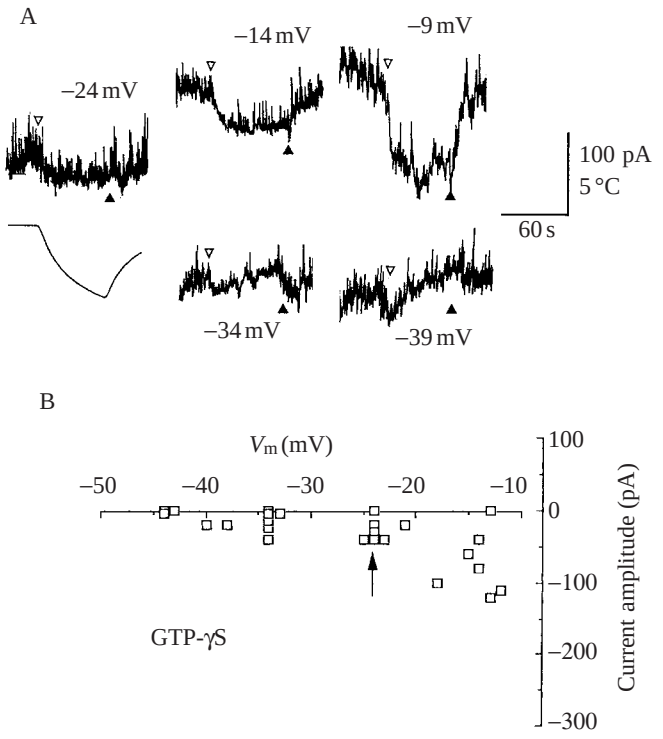

Fig. 2. The effect of GTP-γS on the current response. A few minutes after the application of 10 mmol l^{-1} GTP-γS, a cooling stimulus consisting of a drop in temperature from 25 °C was applied to the cell. (A) Representative current responses at various potentials are shown from one of the six cells examined. Clamped potentials are indicated above or below the recordings. Traces on the left-hand side show changes in the temperature (lower) and current (upper) of cells clamped at the resting potential (−24 mV). Open triangles indicate the point at which the temperature drop begins; filled triangles show when warming is again initiated. (B) Voltage-dependence of the current response. The amplitudes of the current response are plotted as a function of membrane potential (V_m). The arrow indicates the mean value for the resting potential. Data were obtained from six cells.

evident at the hyperpolarized holding potential below the resting potential (−24 mV). At depolarized potentials above the resting one, an inward current (whose amplitude was much smaller than that before the injection) was elicited. However, when the concentration of GTP-γS in the electrodes was decreased from 10 mmol l^{-1} to 1 mmol l^{-1}, a reduction in inward current was not observed.

Application of AlF_4^-, which may activate G-proteins, also reduced the current response (Fig. 3). This reduction was very similar to that caused by GTP-γS.

Effect of GDP-βS

After the cell had been perfused with 10 mmol l^{-1} GDP-βS, which is thought to inhibit G-protein activity, the inward current in response to cooling was reduced (Fig. 4). At a depolarized potential above the resting potential (−18 mV), this reduction was more pronounced than that resulting from application of either GTP-γS or AlF_4^-. When the GDP-βS

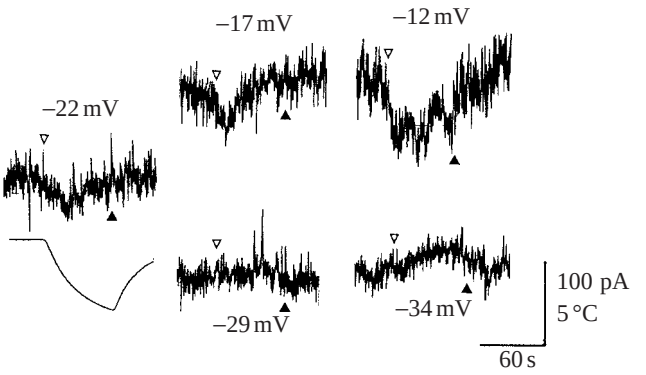

Fig. 3. Current responses at various potentials after application of AlF_4^-. A few minutes after application of 10 μmol l^{-1} AlF_4^-, a temperature drop from 25 °C was applied to the cell. Typical recordings from one of the four cells tested are shown. Clamped potentials are indicated above or below the current recordings. The traces on the left-hand side show changes in temperature (lower) and current (upper) in the cell clamped at the resting potential (−22 mV). The point at which the temperature drop begins is marked by an open triangle; the filled triangles indicate when the temperature begins to rise again.

concentration was reduced from 10 mmol l^{-1} to 1 mmol l^{-1}, the effects on the inward current virtually disappeared.

Effect of EGTA

Intracellular application of EGTA, which should decrease the Ca^{2+} concentration within the cell, also caused a reduction of the inward current produced in response to cooling (Fig. 5). The results of this experiment also showed that the application of EGTA reduced the spontaneous fluctuations in current observed before the application of a cooling stimulus at various holding potentials (Fig. 5A).

Other chemicals

Since the GTP-γS and GDP-βS used in the above experiments were lithium salts, the effect of intracellular application of LiCl was tested. In the presence of LiCl, no changes were detected in the inward current produced in response to cooling. Furthermore, neither ATP nor cyclic AMP had any effect on the current response (data not shown).

Resting potential and membrane conductance

The chemicals that reduced the current response modulated both the resting potential and the membrane conductance (Table 1). Compared with the control condition, application of both GDP-βS and EGTA changed the resting potential to depolarized levels and decreased membrane conductance. In contrast, application of GTP-γS caused almost no changes in the resting potential and an increase in membrane conductance.

Discussion

When the surface membrane of *Paramecium multimicronucleatum* was held under voltage-clamp

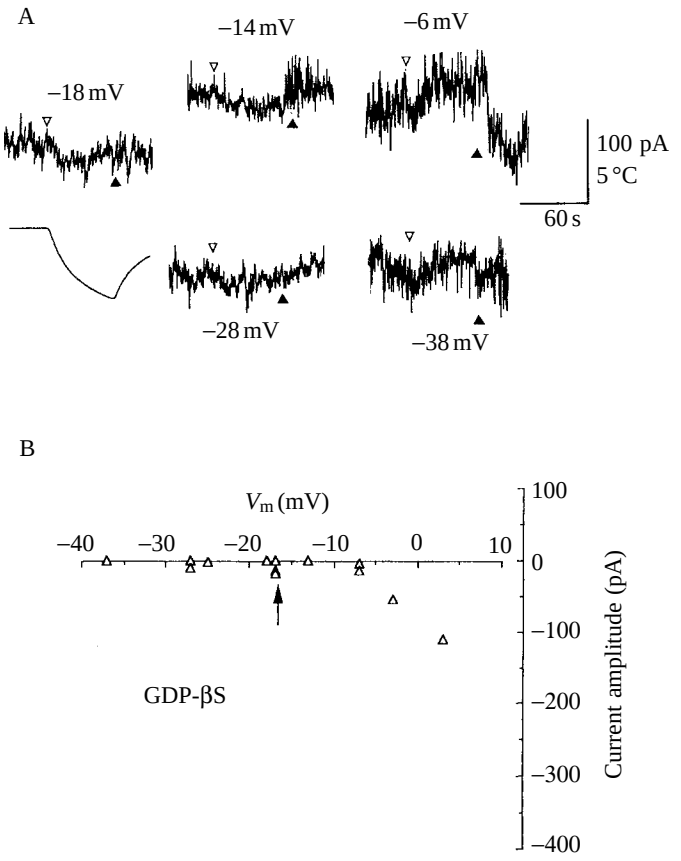

Fig. 4. Current response after application of GDP-βS. A few minutes after application of 10 mmol l^{-1} GTP-βS, the cooling stimulus, consisting of a temperature drop from 25 °C, was applied. (A) Representative current responses at various potentials from one of the four cells tested. Clamped potentials are indicated above or below the current recordings. Traces on the left-hand side show the changes in temperature (lower) and current (upper) that occur in a cell clamped at the resting potential (−18 mV). Open triangles indicate the point at which the temperature drop begins, filled triangles indicate the beginning of a return to 25 °C. (B) Voltage-dependence of the current response. The amplitudes of the current response are plotted as a function of membrane potential (V_m). The arrow indicates the mean value for the resting potential. Data were obtained from four cells.

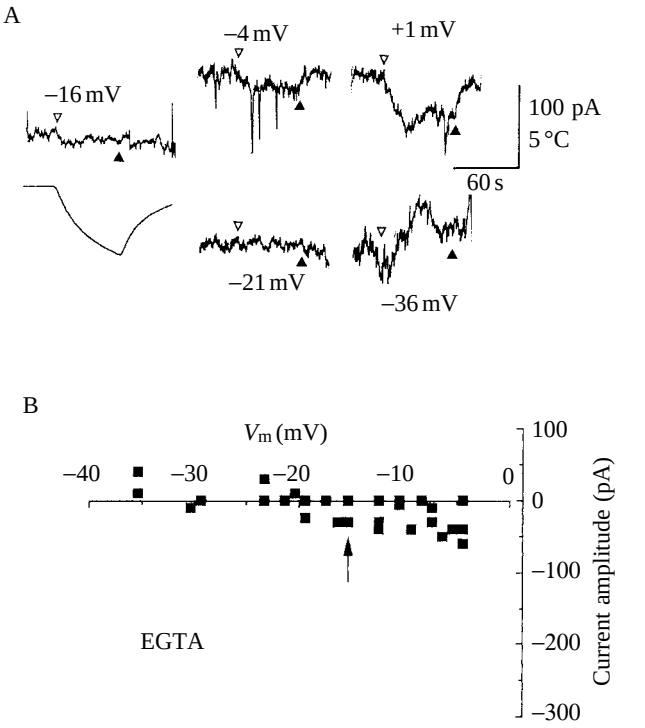

Fig. 5. Current response after application of EGTA. A few minutes after application of 0.1 mol l^{-1} EGTA, a temperature drop from 25 °C was applied to the cell. (A) Representative current responses at various potentials for one of six cells tested. Clamped potentials are indicated above or below the current recordings. Traces on the left-hand side show temperature (lower) and current (upper) changes in a cell clamped at the resting potential (−16 mV). The open triangles indicate the start of the temperature drop, the filled triangles indicate the point at which the temperature begins to rise again. (B) Voltage-dependence of the current response. The amplitudes of the current response are plotted as a function of the membrane potential (V_m). The arrow indicates the mean value for the resting potential. Data were obtained from six cells.

conditions, the application of a cooling stimulus elicited an inward current. The amplitude of the inward current was linearly decreased by negative shifts of the holding potential and disappeared at approximately −40 mV, which is almost the equilibrium potential for K^+ (Oka *et al.* 1986; Machemer, 1988). From the linear slope of the change in current amplitude with potential, the change in membrane conductance in response to a cooling rate of 0.18 °C s^{-1} was estimated to be 9.4 nS, and this value was approximately half of the resting conductance at 25 °C (Table 1, control). These results are in agreement with the conclusion drawn from a previous current-clamp study that it is mainly the membrane conductance for K^+ that changes in response to cooling (Nakaoka *et al.* 1987) and, since *Paramecium multimicronucleatum* membranes depolarize in response to cooling, K^+ conductance must decrease under these conditions. This, in turn, suggests that K^+ channels which respond to a cooling stimulus are open before the application of the stimulus.

Although the non-hydrolyzable guanosine analogues GTP-γS and GDP-βS affect G-protein activity in opposite directions (the former increases and the latter decreases G-protein activity), both reduced the current response upon cooling. Such effects indicate that cold-sensory transduction may include some transient changes in G-protein activity. These changes in G-protein activity might modulate effector systems, presumably resulting in the transient closing of K^+ channels in response to cooling.

Two possible mechanisms can be suggested for the way in which G-protein regulation of the K^+ channel activity occurs: first, a direct interaction with a G-protein subunit, which changes the K^+ channel activity (Brown and Birnbaumer, 1990; Hille, 1992); second, changes in G-protein activity

Table 1. *Effects of chemicals on the current response, resting potential and resting conductance of* Paramecium multimicronucleatum *exposed to a decrease in temperature*

Chemicals	Concentration* (mmol l^{-1})	Current response amplitude (pA)	Resting membrane potential† (mV)	Resting membrane conductance† (nS)
Control (*N*=6)		−156±20	−20.8±1.9	19.1±4.1
GTP-γS (*N*=6)	10	−24±18	−23.5±2.5	21.5±2.2
GDP−βS (*N*=4)	10	−14±17	−16.5±1.8	14.9±6.8
EGTA (*N*=6)	100	−18±12	−15.0±2.0	13.4±2.8

*Concentration in the microelectrodes for voltage-clamping.
†Measurements were performed at 25 °C.
Values are given as means ± S.D.
Measurements were made a few minutes after exposure to each chemical.

modulate the effector system that produces cyclic nucleotides or raises the intracellular Ca^{2+} concentration, and this in turn affects the channel activity (Simon *et al.* 1991; Kaupp and Koch, 1992; Berridge, 1993). Although the present study does not indicate which of these two mechanisms is involved in regulating K^+ channels in *Paramecium multimicronucleatum*, the inhibitory effects of EGTA on the cooling-sensitive response suggest that Ca^{2+} levels contribute to the regulating effect. Intracellular application of EGTA, which reduces the intracellular Ca^{2+} concentration, decreased the membrane conductance and depolarized the membrane potential from the control value. Such changes in the membrane seem to be caused by a $[Ca^{2+}]$-dependent decrease in K^+ channel activity. Similar changes in the membrane conductance and the resting potential were induced by the application of GDP-βS. It follows therefore, that GDP-βS might decrease the activity of K^+ channels through a decrease in the intracellular $[Ca^{2+}]$.

In support to this hypothesis, we have recently found that a mutant of *Paramecium tetraurelia* (*cam*^{12}) with a substitution of an amino acid residue in the calmodulin molecule (Kink *et al.* 1990) shows a reduced sensitivity to the cooling stimulus and that this mutant has a decreased resting membrane conductance for K^+ (T. Kuriu, Y. Oosawa, Y. Watanabe and Y. Nakaoka, in preparation). These results support the view that K^+ channels whose activities are regulated by Ca^{2+} are involved in the response to the cooling stimulus.

Although in several *Paramecium* species, including *P. multimicronucleatum*, a β-subunit-like gene of the heterotrimeric G-protein has been found (Forney and Rodkey, 1992), a G-protein α-subunit has not yet been identified. Nevertheless, the cortical alveoli of *Paramecium tetraurelia* have been shown to act as a submembranous Ca^{2+} storage compartment and the alveolar membranes have Ca^{2+}-pumping activity (Stelly *et al.* 1991, 1995; Lange *et al.* 1995). However, the regulatory mechanism controlling intracellular Ca^{2+} homeostasis has not been established.

The results of the present study indicate that G-proteins are involved in the transduction of cold-sensory information; however, it remains to be determined how they transmit the cooling stimulus and regulate the activity of ion channels.

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