

Inhibition of Anodic Galvanotaxis of Green Paramecia by T-Type Calcium Channel Inhibitors

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Calcium ion (Ca^{2+}) is one of the key regulatory elements for ciliary movements in the *Paramecium* species. It has long been known that members of *Paramecium* species including green paramecia (*Paramecium bursaria*) exhibit galvanotaxis which is the directed movement of cells toward the anode by swimming induced in response to an applied voltage. However, our knowledge on the mode of Ca^{2+} action during green paramecia anodic galvanotactic response is still largely limited. In the present study, quantification of anodic galvanotaxis was carried out in the presence and absence of various inhibitors of calcium signaling and calcium channels. Interestingly, galvanotactic movement of the cells was completely inhibited by a variety of Ca^{2+} -related inhibitors. Such inhibitors include a Ca^{2+} chelator (EGTA), general calcium channel blockers (such as lanthanides), inhibitors of intracellular Ca^{2+} release (such as ruthenium red and neomycin), and inhibitors of T-type calcium channels (such as NNC 55-0396, 1-octanol and Ni^{2+}). However, L-type calcium channel inhibitors such as nimodipine, nifedipine, verapamil, diltiazem and Cd^{2+} showed no inhibitory action. This may be the first implication for the involvement of T-type calcium channels in protozoan cellular movements.

Key words: Calcium Signaling, Galvanotaxis, Inhibitor, *Paramecium bursaria*

Introduction

Galvanotaxis is the directed movement of cells induced in response to an applied voltage or an intrinsic locomotor response to an electrical stimulus. A wide variety of aerobic and anaerobic ciliates often swims towards the cathode when they are exposed to a constant DC field (Van Hoek *et al.*, 1999). It has long been established that members of the *Paramecium* species exhibit a different galvanotaxis (Ludloff, 1895), and more specifically, paramecia align with an electric field or voltage gradient and swim toward the anode if the electric field is sufficiently strong (Jennings, 1906; Ogawa *et al.*, 2005).

Voltage-gated Ca^{2+} channels play a critical role in the control of selective Ca^{2+} flow down their electrochemical gradient in response to a change in the membrane potential in various cells including protozoan cells (Berridge *et al.*, 2000). Ca^{2+} entered into the cytosolic space may regulate diverse processes such as controls in waveforms of cilia (Naitoh and Kaneko, 1972) and flagella (Schmidt and Eckert, 1976).

The ciliary reversal in the *Paramecium* species depends on the intracellular increase in Ca^{2+} concentration (Iwadate, 2003) and Ca^{2+} influx through voltage-gated Ca^{2+} channels on the ciliary membrane (Gonda *et al.*, 2004). Recent electrophysiological experiments (with patch-clamping technique) have demonstrated that some *Paramecium* species possess calcium channels which are voltage-dependently gated and involved in ciliary movements (Gonda *et al.*, 2004). However, involvement of calcium channels in galvanotactic migration has not been tested and thus we have no information on the specific types of calcium channels involved, if any, in the galvanotactic responses.

Paramecium bursaria, well known as green paramecia, is widely habitable in freshwaters such as rivers, lakes, ponds and other wetlands (Kosaka, 1991). *P. bursaria* is a unique photosynthetic *Paramecium* species possessing several hundred cells of *Chlorella*-like endosymbiotic green algae in its cytoplasm (Kadono *et al.*, 2004a). According to a co-evolution model, this symbiotic relationship between algal cells and the ciliate has been devel-

oped as a consequence of host cells' adaptation to the oxidative stress due to algal photosynthesis (Kawano *et al.*, 2004) and it has been considered that the capability of directed movement of the host *Paramecium* cells towards the photosynthetically better environments may be one of the factors benefiting the symbiotic algae (Kadono *et al.*, 2004b). From such view, it is important to understand the signaling mechanism governing the directed migration of the *P. bursaria* cells, and the authors proposed here that galvanotactic movement of *P. bursaria* can be a good model system for studying the tuning mechanism for the locomotive machinery within *P. bursaria*.

In the present study, involvement of calcium channels in the galvanotactic gathering of green paramecia (*Paramecium bursaria*; F1-1b strain, syngen 1, mating type I) cells around the anode was examined by treating the cells of *P. bursaria* with various pharmacological inhibitors of the calcium-related cellular signal transductions.

Materials and Methods

Organisms

As previously reported (Kadono *et al.*, 2006), the green paramecia strain F1-1b (syngen 1, mating type I) was produced by conjugation of KN-15 strain (syngen 1, mating type I) and BWK-4 strain (syngen 1, mating type IV) in our laboratory in spring of 2003. The KN-15 and BWK-4 strains originally collected from the Kinokawa River (Wakayama, Japan) and Lake Biwa (Shiga, Japan), respectively, in 2002, were provided by Prof. T. Kosaka (Hiroshima University, Japan).

P. bursaria strains were cultured in a medium made of the yeast extract-based nutrition mixture EBIOS (1 tablet/l; Asahi Food & Healthcare, Tokyo, Japan), after inoculation with the food bacterium *Klebsiella pneumoniae*, under a light cycle of 12 h light and 12 h dark with *ca.* 3500 lux (30 cm from the light source) of natural-white fluorescent light at 23 °C. EBIOS tablets (250 mg each) contain 94.2% (w/w) dry yeast homogenates and 5.5% (w/w) carbohydrates. Concomitant minerals carried over by yeast cells, namely potassium 1.9% (w/w), phosphate 1.6% (w/w), calcium 0.26% (w/w), magnesium 0.25% (w/w), sodium 0.16% (w/w) and other minerals, can be found in the tablets (Kadono *et al.*, 2006). The bacterized EBIOS medium was prepared by inoculating the medium

with *K. pneumoniae* one day prior to the use in ciliate culture.

Chemicals

Calcium chelator, ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) was obtained from Dojindo, Kumamoto, Japan. Calcium ionophore A23187 and KT5720 were purchased from Calbiochem (San Diego, CA, USA). Thapsigargin, LaCl_3 , GdCl_3 , AlCl_3 , $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, nimodipine, verapamil, CdCl_2 , ruthenium red, KN-62, and dimethyl sulfoxide (DMSO) were from Wako (Osaka, Japan). Trifluoperazine was obtained from MP Biochemicals (Irvine, CA, USA). NNC 55-0396, 1-octanol, nifedipine, (+)-*cis*-diltiazem, flunarizine, neomycin, and other chemicals used were all purchased from Sigma (St. Louis, MO, USA).

Galvanotactic migration assay

Cells of *P. bursaria* (100 cells randomly isolated from the culture by pipetting) were suspended in 2 ml of culture medium and pre-treated with vari-

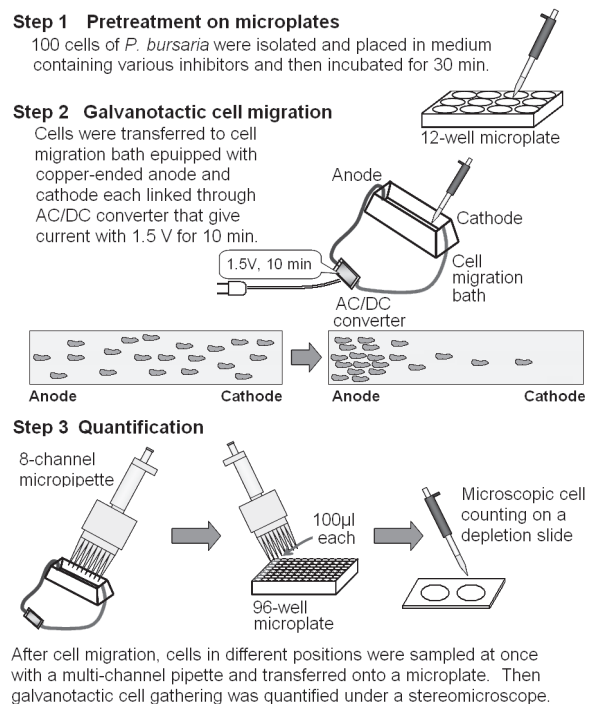


Fig. 1. Quantification of galvanotactic cell migration (gathering) of *P. bursaria*.

ous inhibitors in 12-well microplates (Fig. 1, Step 1). Then cells were transferred to the cell migration bath (with V-shaped cross-section, 70 mm in length) equipped with both a copper-ended anode and a cathode connected through an AC/DC converter that allows galvanotactic cell migration by generating an electric field with 1.5 V for 10 min (Fig. 1, Step 2). After cell migration, cells in different positions were sampled at once with a multi-channel pipette (Eppendorf Research® M 8-channels, 30–300 µl), thus dividing the cellular population into 8 fractions (fraction 1, anode-attracted cells; fraction 8, cathode-attracted cells), and each fraction (100 µl, sampled with each channel) was transferred to each well on a 96-well microplate (Fig. 1, Step 3). Then galvanotactically altered localizations of the cells were quantified by counting the cells in each well under a stereomicroscope (SMZ645; Nikon, Tokyo, Japan) after re-transferring each fraction onto a depletion slide. All experiments were repeated at least three times and mean population in each fraction was expressed as the percentage to the total population (with error bar, S. D.).

Assessing the lethal concentrations of various inhibitors

Prior to the galvanotactic cell migration assays, cells of *P. bursaria* (100 cells) were suspended in 2 ml of culture medium and incubated with various concentrations of pharmacological inhibitors and then used in the galvanotactic cell migration assays for 12 h. Rate of cell death was assessed by counting the live cells under a microscope as described above. Then, lethal concentration for each chemical was worked out. By this way, we could avoid the use of lethal concentrations of inhibitors in the galvanotactic cell migration assays.

Results and Discussion

Quantification of cell migration

With a newly developed method, galvanotactic migration of *P. bursaria* towards the anode (fraction 1) was successfully demonstrated (Fig. 2). While no cell gathering in fraction 1 was observed without application of the electric field (Fig. 2A, left), high accumulation of the cells in fraction 1 (anode) was observed by exposing the cells to the electric field (1.5 V) for 10 min (Fig. 2A, right). The differences in cellular populations between

fraction 1 (anode) and other fractions (2–8) were all significant (see error bars).

Effect of voltage on cell migration

We tested the effect of an electric field with different voltages on the galvanotactic movement of *P. bursaria* cells (Fig. 2B). As the applied 10 minutes lasting voltage increased from 0 V to 0.5 V, 1.5 V, or 3 V, attraction of cells by the anode became greater. No migration was observed with 0 V application (as in Fig. 2A, left), slight increase without leaching significant level was observed with 0.5 V, significant increase in cellular population in the anode fraction was observed with 1.5 V, and much greater percentage of the cells was observed with 3 V application. However, we could obtain only partial recovery of live cells after exposure to a 3 V electric field while apparent rate of anode-attracted cells (% of total live cells collected) was high. This indicates that certain portion of the cells exposed to high voltage was damaged or killed. By 10 min exposure to a 3 V electric field, one third of the cells was killed while no decrease in cellular population was observed with 0.5 and 1.5 V applications (data not shown). Therefore further tests were carried out by applying 1.5 V to the cells.

Effect of cell density on cell migration

We also tested the effect of cell density in the cell migration bath on the galvanotactic movement of *P. bursaria* cells (Fig. 2C). With 100 cells (in 2 ml medium), accumulation of the cells in fraction 1 (anode) was optimal. It seemed that the anode-directed cell migration was inhibited in high cell density (Fig. 2C) since cells were likely aggregated under the electric field if the cell density was too high (confirmed by microscopic observation).

Time required for cell gathering

We examined the time required for the completion of anode-directed cell migration with *ca.* 100 *P. bursaria* cells (in 2 ml) under an 1.5 V electric field (Fig. 2D). The cells started being accumulated around the anode (fraction 1) within 1–3 min of DC field application. After 1 min of DC field application, the populational cell density in the cathode fraction (fraction 8) was shown to be lowered. Significant accumulation of the cells in the anode fraction (fraction 1) was observed only after 5 min of continuous application of the DC

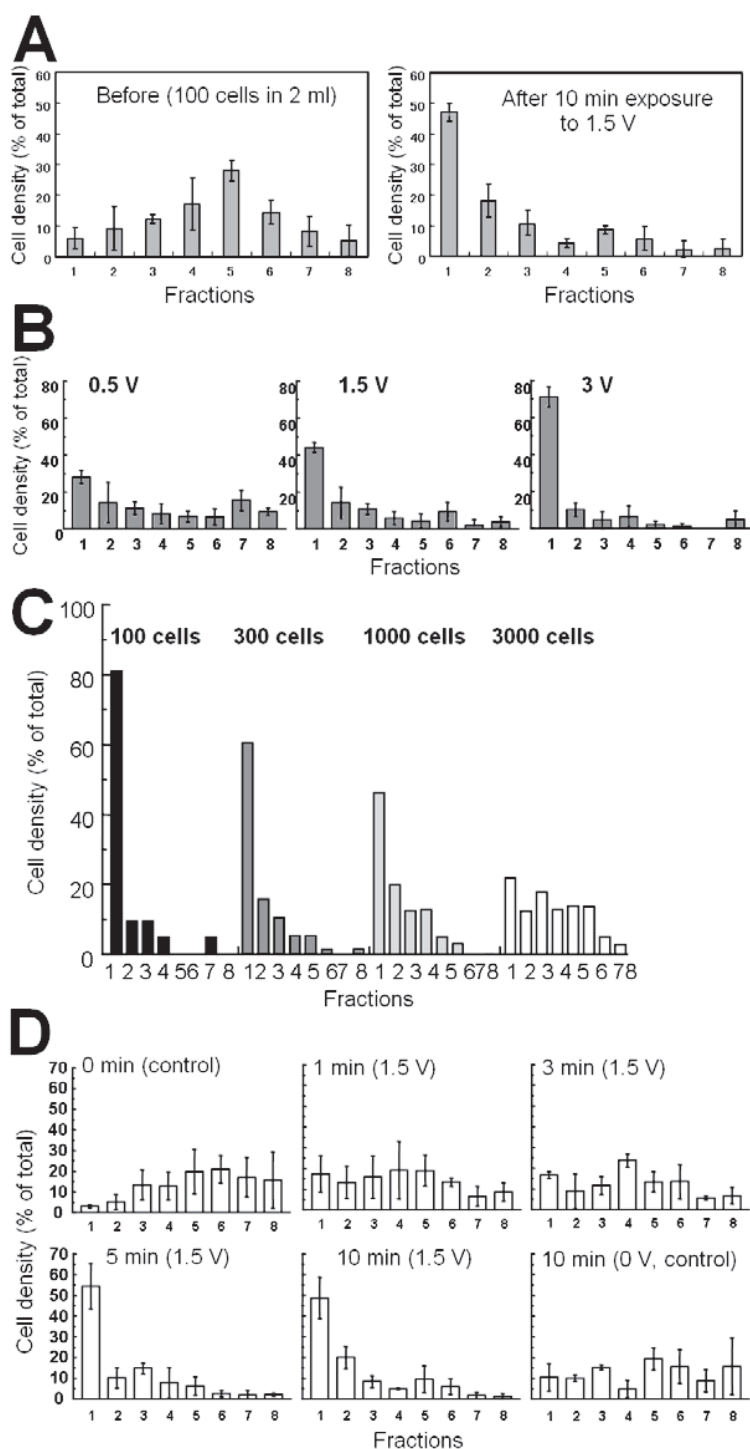


Fig. 2. Quantification of cell migration with the novel assay procedure. (A) Typical populational distribution profiles of *P. bursaria* cells with (left) and without (right) electric field (1.5 V, 10 min). (B) Effect of voltage on cell migration towards the anode. (C) Effect of cell density on cell migration towards the anode. (D) Time course of galvanotactic cell migration.

electric field. With 10 min continuous treatment by the DC field, no further increase in the anode-attracted cells was observed, suggesting that 5–10 min are sufficient for completion of cell migration across the total distance (70 mm), and the responses observed in these time points were maximal. Further experiments with various inhibitors were demonstrated in the apparatus by the appli-

cation of 1.5 V DC electric field for 10 min to 100 cells in 2 ml medium.

Effect of a Ca^{2+} chelator and Ca^{2+} channel blockers

Effects of a calcium chelator and three inorganic calcium channel blockers on inhibition of galvano-

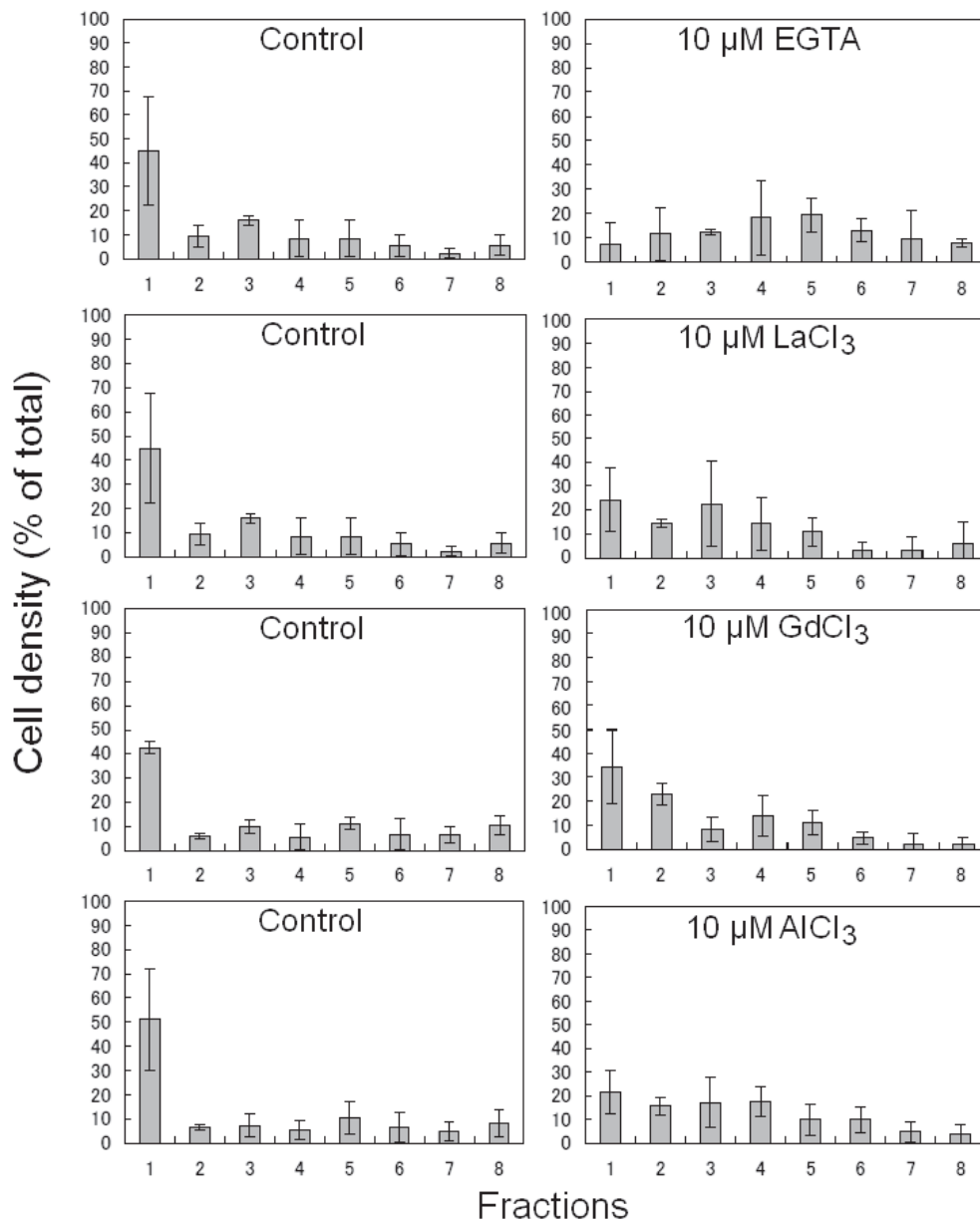


Fig. 3. Inhibition of galvanotactic migration of *P. bursaria* cells by EGTA, LaCl₃, GdCl₃ and AlCl₃.

tactic cell migration were tested (Fig. 3). In the presence of 10 μM EGTA, La^{3+} , Gd^{3+} or Al^{3+} , attraction of cells around the anode (fraction 1) was significantly lowered. These data suggest that the electrically stimulated migration of the *P. bursaria* cells towards the anode possibly requires the uptake of extracellular calcium ions (removable by EGTA) via Ca^{2+} channels (sensitive to La^{3+} , Gd^{3+} and Al^{3+}) as the secondary trigger of cellular (ciliary) movements.

Effect of T-type calcium channel-specific inhibitors

Effects of three specific inhibitors for T-type calcium channels, namely NNC 55-0396, 1-octanol and Ni^{2+} were tested (Fig. 4). By treating the cells with a newly developed selective inhibitor of T-type calcium channels, NNC 55-0396 (Huang *et al.*, 2004) at 1 μM , the galvanotactic cell attraction of the anode fraction was significantly inhibited (Fig. 4A). Responses to metals can be good measures of channel involvement. For instance, Ni^{2+} and Cd^{2+} can be used as the pair of calcium channel inhibitors specific to T-type and L-type channels, respectively (Plasman *et al.*, 1990). Pre-treatment of the cells with 1 μM Ni^{2+} , a T-type calcium channel blocker, resulted in the inhibition of the galvanotactic anode-specific accumulation of the cells (Fig. 4B) while Cd^{2+} , an L-type channel blocker, which was highly toxic to *P. bursaria* cells, showed no significant inhibition of the galvanotactic behaviour of *P. bursaria* cells at 1 μM (data not shown). 1-Octanol is another commonly used inhibitor of T-type calcium channels (Todorovic and Lingle, 1998). As expected, 1-octanol also interfered with the galvanotactic cell migration (Fig. 4C), but the concentrations required for a significant decrease in anode-attracted cells were much higher (10 μM –1 mM) than with NNC 55-0396 and Ni^{2+} (1 μM). Interestingly, in the presence of high concentration of 1-octanol (1 mM), the cells were likely distributed on both ends of the migration apparatus (fraction 1 and 8, anode and cathode, respectively).

Flunarizine (up to 3 μM tested as non-lethal concentrations), a putative inhibitor of both L-type and T-type Ca^{2+} channels (Loikkanen *et al.*, 2003), showed no inhibitory effect on the galvanotactic accumulation of *P. bursaria* cells around the anode (data not shown). While flunarizine non-selectively inhibits voltage-gated Ca^{2+} channels in the

low μM range, it also inhibits a variety of other ion channels permeable for not only Ca^{2+} but also K^{+} and Na^{+} (Trepakova *et al.*, 2006). Among the Ca^{2+} channel blockers, flunarizine is known as one of the most potent non-specific inhibitors of ATPases (Santos *et al.*, 1994). We assume that the modes of action of flunarizine and other T-type channel inhibitors may largely differ and thus differed in inhibitory impacts on the anodic galvanotactically migrating *P. bursaria*.

Effects of L-type calcium channel-specific inhibitors

In contrast to the actions of non-lethal concentrations of T-type channel-specific inhibitors (Fig. 4) and general channel inhibitors (Fig. 3), all L-type calcium channel inhibitors tested here showed no inhibitory action against the galvanotactic cell migration. Such L-type channel-targeted pharmacological inhibitors failed to inhibit the galvanotactic migration of *P. bursaria* at non-lethal concentrations tested were (1) nimodipine (3, 10, 30 μM), (2) nifedipine (5, 50, 500 μM), (3) verapamil (1, 3, 10 μM), (4) (+)-*cis*-diltiazem (100 μM) and (5) CdCl_2 (0.1–1 μM) (data not shown).

Effects of Ca^{2+} release inhibitors

According to Sipido *et al.* (1998), the T-type Ca^{2+} current triggers the Ca^{2+} release from the internal Ca^{2+} stores such as sarcoplasmic reticulum in guinea-pig ventricular myocytes, when the T-type Ca^{2+} current was artificially elicited (albeit less efficiently than L-type Ca^{2+} current does). In that case, both the T-type current and the induced Ca^{2+} release were shown to be sensitive to Ni^{2+} . Thus by analogy to such report, we assume that in *P. bursaria* cells, exposed to the electric field, the likely T-type Ca^{2+} channel-mediated Ca^{2+} entry may trigger further release of Ca^{2+} from the intracellular stores. Here, the effects of two pharmacological inhibitors of Ca^{2+} release from the internal Ca^{2+} stores on galvanotactic migration of *P. bursaria* towards the anode were also examined (Fig. 5).

Neomycin is known to inhibit the Ca^{2+} release from internal Ca^{2+} stores by blocking the phospholipase C-catalyzed production of 1,4,5-inositol triphosphate in various organisms despite its specificity has been sometimes questioned (Prentki *et al.*, 1986). We observed that treatment of the *P. bursaria* cells with 10 μM neomycin successfully blocked the galvanotropic cell migration (Fig. 5).

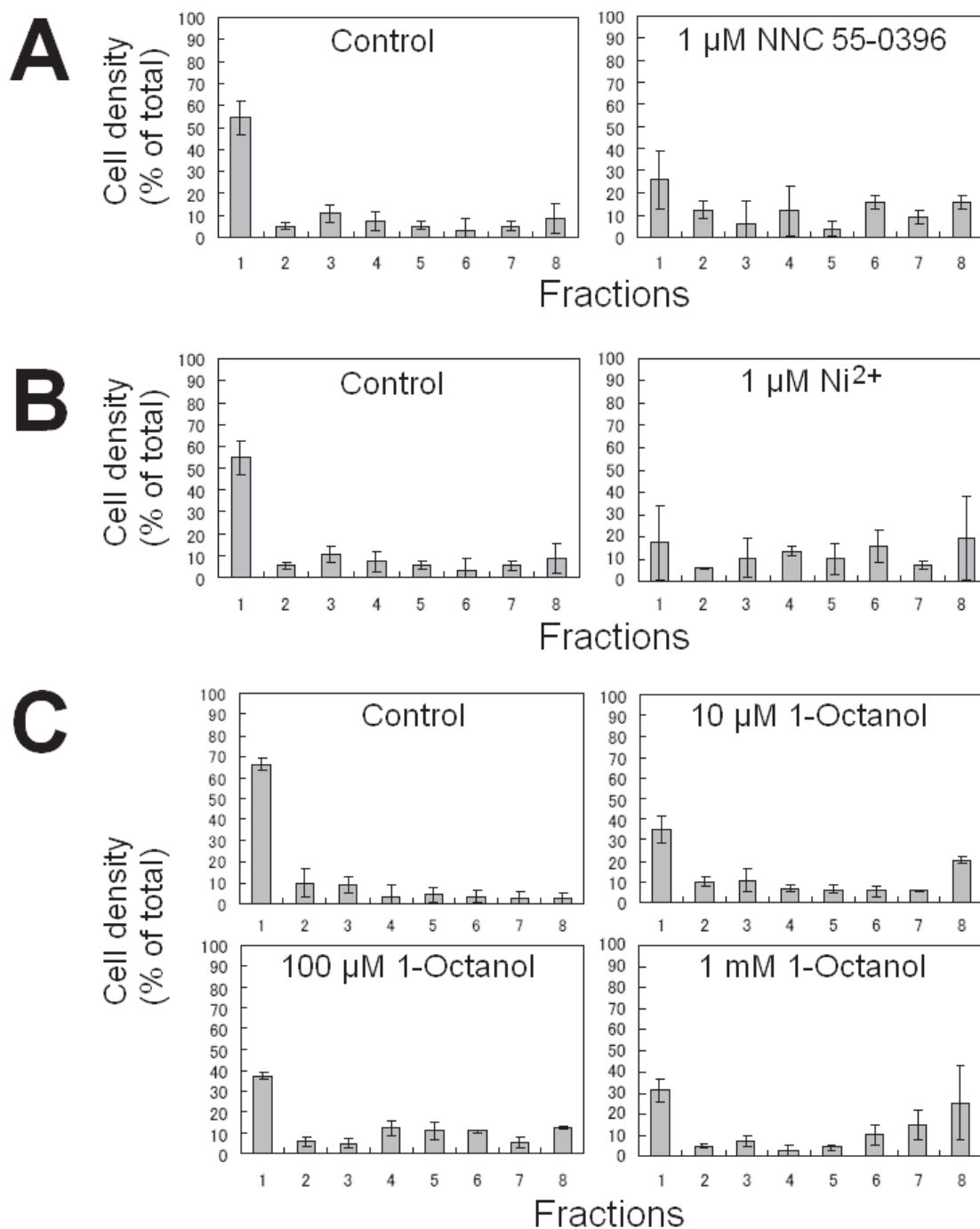


Fig. 4. Effects of T-type channel inhibitors on galvanotactic cell attraction by the anode. (A) Effect of NNC 55-0396. (B) Effect of Ni²⁺. (C) Effect of 1-octanol.

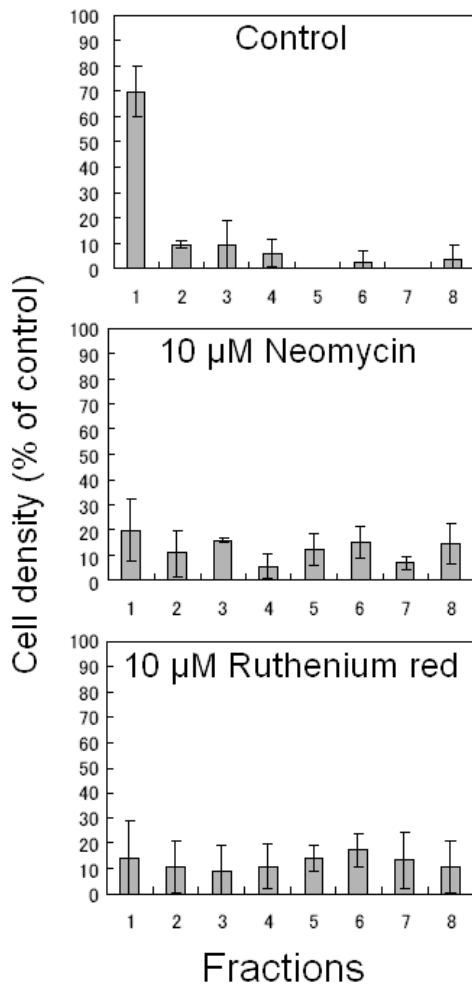


Fig. 5. Effects of Ca^{2+} release inhibitors. *P. bursaria* cells were treated without (top) or with Ca^{2+} release inhibitors, 10 μM neomycin (middle) or 10 μM ruthenium red (bottom), prior to the galvanotactic demonstrations. Anodic galvanotaxis was scored as described above.

However, the effect of neomycin should be taken with care since some reports have described that neomycin likely inhibits the high potassium-stimulated backward swimming coupled with inward Ca^{2+} currents (Gustin and Hennessey, 1988), and the lysozyme-induced chemorepulsion (directed cell migration) coupled with Ca^{2+} influx (Hennessey *et al.*, 1995) in *Paramecium* species.

In addition to neomycin, 10 μM ruthenium red strongly inhibited the anodic migration of *P. bursaria* cells (Fig. 5). Ruthenium red is an inhibitor of the mitochondrial Ca^{2+} uniporter active in both

mammalian cells (Parkash *et al.*, 2006) and protozoan cells including those of ciliates (Kim *et al.*, 1984).

In conclusion, we understand that Ca^{2+} -induced Ca^{2+} release from organelles in DC field-exposed *P. bursaria* is likely to occur.

Effects of other signaling inhibitors

Since the involvement of calcium signaling mechanisms in galvanotactic cell movement towards the anode was suggested by the actions of EGTA, lanthanides, and T-type channel-specific inhibitors, effects of other signaling-related inhibitors were examined.

A23187 (50 μM –1 mM), a calcium ionophore, showed no inhibitory or stimulatory action towards anodic galvanotaxis in *P. bursaria*, suggesting that uniform influx of Ca^{2+} itself without any directional gradient is not sufficient for induction of directed cell migration. Trifluoperazine (an inhibitor of calmodulin) and KN-62 (an inhibitor of calmodulin kinases) showed no inhibitory action against anodic galvanotaxis in *P. bursaria*, suggesting that calmodulin is not involved in galvanotactic

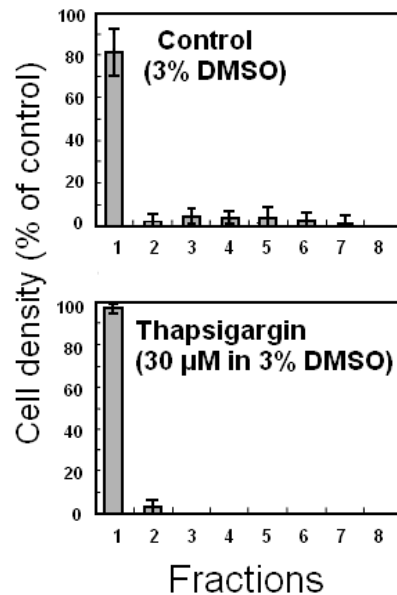


Fig. 6. Enhancement of anodic galvanotaxis by treating *P. bursaria* cells with thapsigargin. Since thapsigargin was first dissolved in DMSO, control experiments were conducted in the presence of 3% DMSO.

ciliary controls. Involvement of protein kinase A was also denied by the use of KT5720, a specific inhibitor of protein kinase A.

Treatment with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase, significantly enhanced the anodic cell migration (Fig. 6). Since thapsigargin was first dissolved in DMSO, control experiments were conducted in the presence of 3% DMSO (which is at non-lethal level). Generally, presence of 1–3% of DMSO enhanced the

anodic cell migration for unknown reason. Presence of 30 μM thapsigargin further enhanced the migration of the cells. Note that no cells remained in the fractions 3–8 in the presence of thapsigargin, while in the control (3% DMSO-treated), cells could be observed even in fraction 7 (adjacent to the cathode). Effect of thapsigargin further confirms the importance of the calcium signaling machinery for enabling the anodic cell migration of the DC field-exposed *P. bursaria*.

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