JOURNAL OF BACTERIOLOGY, Apr. 1997, p. 2524–2528 Vol. 179, No. 8
0021-9193/97/$04.00+0

Calcium Is Required for Swimming by the Nonflagellated Cyanobacterium *Synechococcus* Strain WH8113

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Received 4 November 1996/Accepted 5 February 1997

The marine cyanobacterium *Synechococcus* strain WH8113 swims in the absence of any recognizable organelles of locomotion. We have found that calcium is required for this motility. Cells deprived of calcium stopped swimming, while addition of calcium completely restored motility. No other divalent ions tested could replace calcium. Terbium, a lanthanide ion, blocked motility even when calcium was present at 10^5-fold-higher concentrations, presumably by occupying calcium binding sites. Calcium chelators, EGTA or EDTA, blocked motility, even when calcium was present at 25-fold-higher concentrations, presumably by acting as calcium ionophores. Finally, motility was blocked by verapamil and nitrendipine, molecules known to block voltage-gated calcium channels of eukaryotic cells by an allosteric mechanism. These results suggest that a calcium potential is involved in the mechanism of motility.

In 1985, Waterbury et al. discovered a cyanobacterium that swam at speeds up to 25 μm/s (34). The cells were rod-shaped and moved without any apparent change in shape. They had no flagella or other external appendages that might be responsible for cell movement. The fine structure of the cell envelope of motile strains appeared to be identical to that of nonmotile strains (37). These results have been confirmed by many laboratories, including our own (28). The lack of appendages leaves the cell surface as the only structure with which the cells can generate thrust.

Willey et al. demonstrated that sodium motive force is the most probable energy source for motility (36). Sodium was specifically required, and changes in speed correlated with changes in sodium motive force, not proton motive force or ATP concentration. The authors suggest that a threshold of sodium motive force is required for motility, since cells failed to swim at a sodium motive force less than −120 mV. Here, we show the situation to be more complex, since motility is also calcium dependent.

The calcium requirement is particularly interesting in light of a polypeptide of 130 kDa found only in motile strains of *Synechococcus* (4). This protein has one EF-hand and one hemolysin-type calcium binding site as well as 12 GGXGXD calcium binding motifs (as determined by its gene sequence). Deletion of the gene encoding this protein rendered cells unable to translocate but, surprisingly, did not affect their ability to rotate (4).

Previously, we showed that *Synechococcus* does not use ions for propulsion via a self-electrophoretic mechanism (28). The cell surface must flow in bulk or propagate longitudinal or transverse waves (7). If bulk flow is confined to the cell surface, it cannot be incompressible (30). The present work raises the intriguing possibility that wave motion is coordinated or driven by local changes in calcium concentration.

MATERIALS AND METHODS

Strains and growth conditions. *Synechococcus* strain WH8113 was used for all experiments. The cells were grown to middle or late exponential phase in SN medium (75% natural seawater in deionized H_2O supplemented with 15 μM EDTA, 9 mM $NaNO_3$, 90 μM K_2HPO_4, and 100 μM Na_2CO_3 [pH 8.0]) (35) at 22°C under Sylvania warm white fluorescent tubes. Light intensity was adjusted to 10 μE m^{-2} s^{-1} by placing a single layer of fiberglass screen material between the light bulbs and the cells. Cells were harvested by centrifugation (5 min, 4,000 × *g*, 4°C) in Eppendorf tubes coated with bovine serum albumen (BSA). The tubes were prepared by filling them with 1% BSA in distilled water, incubating them at room temperature for 5 min, and rinsing them three or more times with distilled water. This procedure prevented cells from sticking to the polyethylene, which rendered them nonmotile. Cells were then resuspended in AN medium, an artificial seawater (250 mM NaCl, 30 mM $MgSO_4$, 20 mM $MgCl_2$, 10 mM KCl, 15 μM EDTA, 3 mM $NaNO_3$, 90 μM K_2HPO_4, 200 μM Na_2CO_3, 3 mM $NaHCO_3$ [pH 8.3]) (35) supplemented with 1% BSA, which prevented cells from sticking to the coverslip. Concentrations of $CaCl_2$ and inhibitors are given for individual experiments. Cells were examined within a few minutes of addition of inhibitors. Stocks of EGTA and EDTA were adjusted to pH 8.3 prior to use. The addition of these chelators to AN medium caused a slight decrease in pH, which remained in a range that did not affect motility. For chelator recovery experiments, cells were exposed to chelator for 5 min and then washed three times by centrifugation with fresh AN medium. For experiments with terbium, which precipitates in AN medium, cells were resuspended in a mixture of 250 mM NaCl, 10 mM KCl, and 10 mM $CaCl_2$. Cells swam about 25% more slowly in this medium than they did in AN medium.

Measurement of cell swimming speed. Glass slides and coverslips were treated with Rain-X (a commercial mixture of siloxanes and silicones; Unelko Corp., Scottsdale, Ariz.). This treatment made the glass uniformly hydrophobic and prevented adsorption of calcium or ionic inhibitors to the glass. Similar results were obtained with *n*-dodecyltriethoxysilane, but Rain-X was easier to use. The Rain-X procedure was omitted when the hydrophobic calcium channel blockers were tested. Chambers were constructed by placing 8 μl of cell suspension on a slide and covering it with a 22- by 22-mm coverslip. The coverslip was sealed with a 1:1:1 mixture of petrolatum, lanolin, and paraffin to prevent flow due to evaporation. The slides were then examined by dark-field microscopy with a 570-nm long-pass filter (Schott Glass Technologies, Duryea, Pa.) placed over the field-limiting diaphragm to prevent photodamage to the cells. The depth of the chamber was slightly greater than the depth of focus of the 10× objective used; cells in the field remained in focus for the duration of the experiment. Time exposures were made by accumulating video frames from a charge-coupled device camera (C2400-75i) in an Argus 20 image processor (Hamamatsu Corp., Tokyo, Japan). Moving cells produced a bright track on a black background (Fig. 1).

Images were printed on a Sony video printer, digitized, and transferred to a personal computer with a hand scanner (Scanman 256; Logitech Inc., Fremont, Calif.). Images of cell tracks were measured by tracing with a mouse by use of Sigma Scan Image software (Jandel Scientific Corp., San Rafael, Calif.) calibrated with an image of an objective micrometer. The lengths of 50 to 300 tracks were averaged for each speed measurement. Error bars are standard errors of the mean speeds. In cases requiring only a qualitative assessment of motility, cells were examined by eye with phase-contrast microscopy. To assess motility in the absence of photosynthesis, cells were examined with infrared phase-contrast microscopy. The microscope was illuminated with a standard 100-W tungsten-halogen bulb with a Schott 850-nm long-pass filter placed over the field-limiting

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FIG. 1. Tracks of *Synechococcus* strain WH8113 in AN medium plus BSA. Sixty-four frames were accumulated for a total elapsed time of 2.13 s.

diaphragm. Images were detected with a Hamamatsu charge-coupled device camera (C2400-75i) with the infrared filter removed.

RESULTS

Calcium is required for outer membrane stability. The addition of EDTA, and to a lesser extent EGTA, in excess of the calcium concentration or repeated washing of cells in calcium-free medium disrupted the outer membrane. This damage could be observed as an irreversible reduction in swimming speed (see below), by changes in the morphology of the cell surface, as shown in thin-section electron micrographs, or from the appearance of carotenoid-containing subcellular particles in the medium, as detected by sucrose gradient ultracentrifugation (data not shown). The disruption of the outer membrane by EDTA has been observed in another motile *Synechococcus* strain, WH8102 (4), as well as in a nonmotile freshwater *Synechococcus* species (29).

Calcium is required for motility. Careful resuspension of the cells in calcium-free medium rendered them nonmotile, but their outer membrane remained intact; only repeated washing and resuspension damaged the cells. The addition of calcium restored motility to the level of untreated cells (Fig. 2). This effect was specific: no other divalent ion tested could substitute for calcium, and many inhibited motility (Table 1). The plot of motility restoration was a steep sigmoid, indicating that the binding or effect of calcium is highly cooperative. The slope of the Hill plot was 7.4 (Fig. 2, inset).

Lanthanide ions inhibit motility. Trivalent lanthanide ions have an ionic radius similar to that of calcium and are known calcium analogs (6, 20, 25). These ions bind to calcium-binding proteins at the ion-binding site (20, 25). We found that terbium ion at 700 nM totally inhibited motility of cells in the presence of 10 mM calcium (Fig. 3). The linear nature of this inhibition suggests that there are multiple force generators that act independently and can be inhibited in an all-or-none fashion. Terbium acted as a competitive inhibitor, outcompeting 10^5-fold-higher concentrations of calcium (Fig. 4). We are currently purifying the terbium-binding protein by monitoring terbium luminescence (6).

EGTA and EDTA inhibit motility. We found that the inhibition of motility by the calcium chelators EGTA and EDTA was not due to the sequestration of calcium. The cells live in seawater, which contains approximately 10 mM calcium and 50 mM magnesium (19), as does AN medium, yet their motility was completely inhibited by 2.2 mM EGTA or 3.2 mM EDTA (Fig. 5). Raising the calcium concentration to 50 mM did not alter this effect (data not shown). The addition of the chelators lowered the pH of the medium from 8.3 to 7.6 in the case of EGTA (2.2 mM) and to 7.9 in the case of EDTA (3.2 mM). In

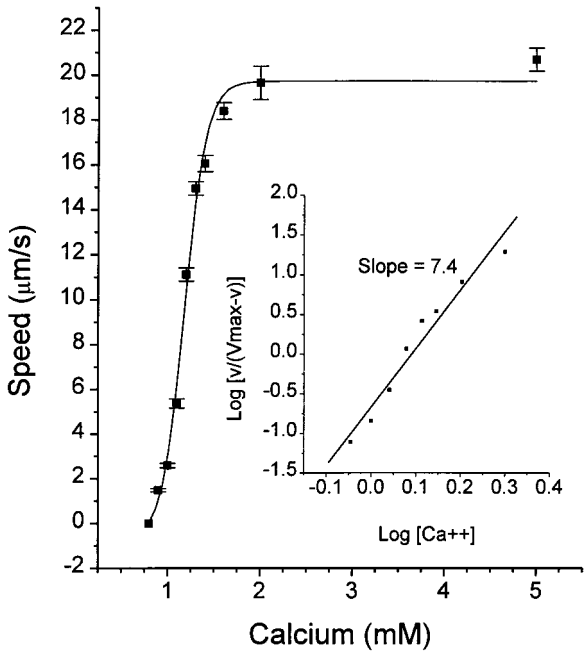

FIG. 2. Effect of calcium concentration on swimming speed. Cells were aliquoted, pelleted by centrifugation, and resuspended in AN medium plus 1% BSA without calcium. Calcium was added to each tube to give the desired concentration, and then the cells were assayed for swimming speed. Inset, Hill plot of the same data set.

TABLE 1. Effect of divalent ions on motility[a]

Ion	Motility				Concn in seawater (M)[c]
	In presence of 10 mM Ca^{2+}		In absence of Ca^{2+}		
	10 μM[b]	1 mM	2 mM	10 mM	
Ca^{2+}	Motile[d]	Motile	Motile	Motile	1.0×10^{-2}
Mg^{2+}	Motile	Motile	Nonmotile	Nonmotile	5.3×10^{-2}
Sr^{2+}	Motile	Motile	Nonmotile	Nonmotile	9.0×10^{-5}
Fe^{2+}	Motile	Nonmotile[e]	Nonmotile	Nonmotile	3.5×10^{-8}
Zn^{2+}	Motile	Nonmotile[e]	Nonmotile	Nonmotile	7.5×10^{-8}
Mn^{2+}	Motile	Motile	Nonmotile	Nonmotile	4.0×10^{-9}
Cu^{2+}	Motile	Nonmotile[e]	Nonmotile	Nonmotile	4.0×10^{-9}
Co^{2+}	Motile	Nonmotile[e]	Nonmotile	Nonmotile	4.2×10^{-9}
Ni^{2+}	Motile	Nonmotile	Nonmotile	Nonmotile	9.0×10^{-9}
Co^{2+}	Motile	Nonmotile[e]	Nonmotile	Nonmotile	9.7×10^{-10}

[a] All measurements were made in modified AN medium minus Ca^{2+} (250 mM NaCl, 30 mM $MgSO_4$, 20 mM $MgCl_2$, 10 mM KCl, 3 mM $NaHCO_3$); 1% BSA was added to prevent cells from sticking to the coverglass.

[b] Ion concentration. The counterion in all cases was Cl^-.

[c] From the *Handbook of Chemistry and Physics* (19).

[d] For all conditions in this table, motile cells swam at the same speed as untreated cells and nonmotile cells were at full stop. Swimming was not affected by additional Ca^{2+}, tested up to 50 mM.

[e] Cells clumped together; free cells were nonmotile.

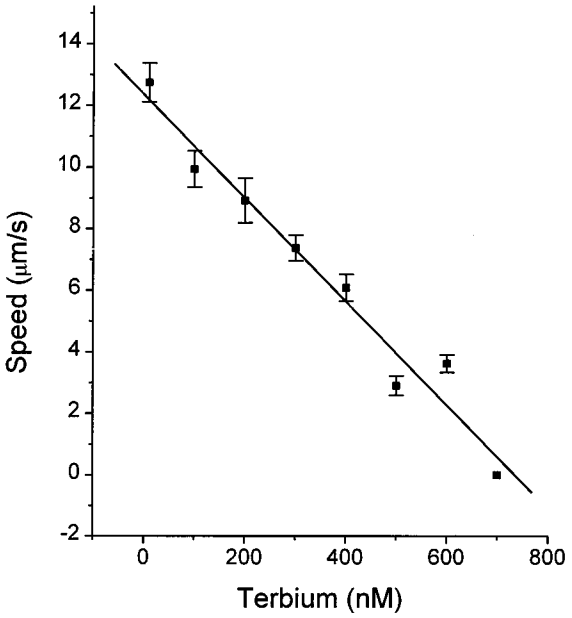

FIG. 3. Effect of terbium on swimming speed in the presence of 10 mM calcium. Cells were aliquoted, pelleted by centrifugation, and resuspended in a mixture of 250 mM NaCl, 10 mM KCl, and 10 mM $CaCl_2$. Terbium was then added to each tube to give the desired concentration, and the cells were assayed for swimming speed. The maximum speed was lower than that shown in Fig. 2 because of differences in the media.

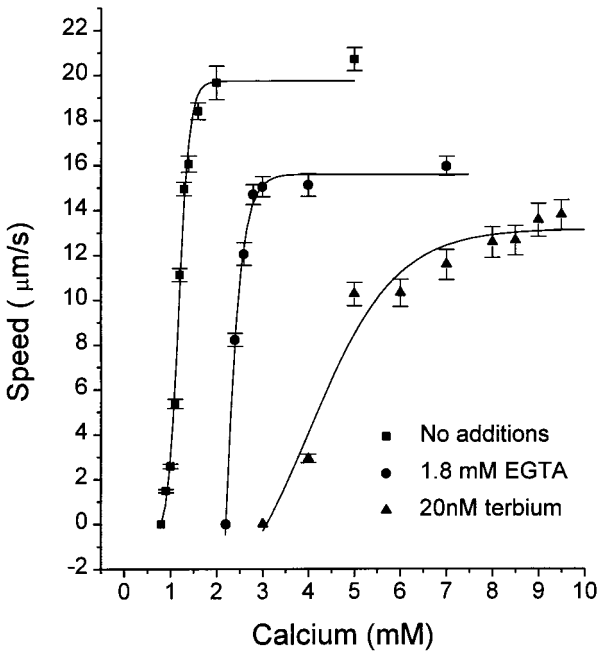

FIG. 4. Effect of subinhibitory concentrations of EGTA or terbium on speed as a function of calcium concentration. Cells were aliquoted, pelleted by centrifugation, and resuspended in half the original volume of AN medium plus 1% BSA (for the EGTA curve) or in 250 mM NaCl–10 mM KCl (for the terbium curve). An equal volume of $CaCl_2$ plus inhibitor was then added to each tube to give the desired final concentration. The cells were then assayed for swimming speed. Data represented by solid squares are the same as those shown in Fig. 2. Again, differences in maximum speeds reflect differences in media.

a control experiment, the pH of a suspension of cells in AN medium was adjusted from 8.3 to 6.0 with HCl with no reduction in swimming speed. Therefore, the changes in speed shown in Fig. 5 were not due to changes in pH. The effect of both EGTA and EDTA was reversible, indicating that the cell integrity was not compromised at concentrations required to inhibit motility (Fig. 6). Higher concentrations of chelators did permanently damage the cells, EDTA more so than EGTA (Fig. 6). Subinhibitory concentrations of EGTA shifted the calcium speed curve to the right approximately by the amount of EGTA added and limited the maximum speed of the cells (Fig. 4). These data indicate two roles for EGTA in this system. First, the calcium concentration must exceed the EGTA concentration for enough free calcium to be available for motility (about 1 mM). Second, EGTA, acting as a weak calcium ionophore, limits the top speed of the cells, probably by lowering the calcium potential. This explains the limiting of speed in Fig. 4, the sharp drop in speed in Fig. 5, and the reversible nature of the EGTA effect (Fig. 6). The fact that an extremely high calcium concentration (50 mM) cannot outcompete the action of 2 mM EGTA suggests that the cell cannot pump calcium out at a rate faster than the EGTA can bring it in. The separation of the curves in Fig. 5 and 6 are due either to the differential competition for magnesium between the two chelators (logarithms of stability constants [17] are, for EGTA, 11.0 $[Ca^{2+}]$ and 5.2 $[Mg^{2+}]$ and, for EDTA, 10.7 $[Ca^{2+}]$ and 8.7 $[Mg^{2+}]$) or to a differential ability of these chelators to transport calcium across the cell membrane.

Organic calcium channel blockers inhibit motility. Verapamil and nitrendipine, two inhibitors of voltage-gated calcium channels in eukaryotic cells, inhibit motility of *Synechococcus*. These inhibitors do not function at the ion-binding site (8, 18, 33) but, rather, hold the channel in an inactive conformation by an allosteric interaction with the gating mechanism (2, 8). The water-soluble calcium channel blockers conotoxin and diltiazem did not inhibit motility nor did orthovanadate, which inhibits the P-type calcium ATPase (3, 9) (Table 2). The calcium ionophore 4-bromo A23187 was not an effective inhibitor of motility at 10 μM, but at 100 μM, it caused the cells to clump. However, a few free cells were able to swim slowly under these conditions.

Photosynthesis is not required for motility. Cells continued to swim for over an hour in the absence of photosynthesis, indicating that loss of motility in calcium-free media was not due to an inhibition of photosystem II and that respiration can power motility. Cells also continued to swim in the presence of cyanide (10 mM; data not shown) provided that they were continuously illuminated, indicating that photosynthesis can power motility in the absence of respiration.

DISCUSSION

We have demonstrated that calcium is required for motility of *Synechococcus* strain WH8113. Swimming speed increased dramatically as the calcium concentration was increased from 0.9 to 2 mM (Fig. 2). Motility was inhibited by low concentrations of terbium ion, a competitive inhibitor of calcium (Fig. 3 and 4), and also by the calcium chelators EGTA and EDTA, which appeared to act as calcium ionophores (Fig. 4 to 6). In addition, motility was inhibited by nitrendipine and verapamil (Table 1), suggesting that one site of action might be a voltage-gated calcium channel similar to those found in cardiac tissue (33). The data suggest that a calcium gradient in *Synechococcus*, probably across the cytoplasmic membrane, is required for motility. The linear decrease in speed in response to terbium ion (Fig. 3) is consistent with the notion that a large number of calcium channels function in parallel and act independently.

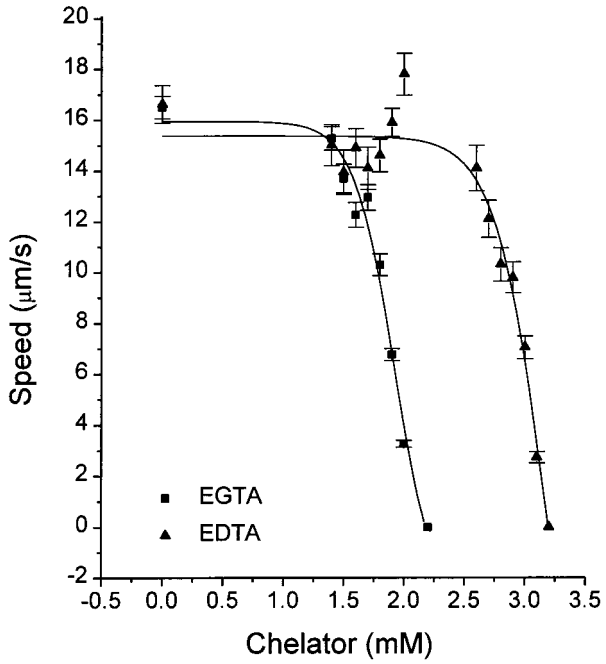

FIG. 5. Effect of EGTA or EDTA on swimming speed in the presence of 10 mM calcium. Cells were aliquoted, pelleted by centrifugation, and then resuspended in AN medium plus 1% BSA with 10 mM calcium. Chelators were then added to give the desired concentration, and the cells were assayed for swimming speed.

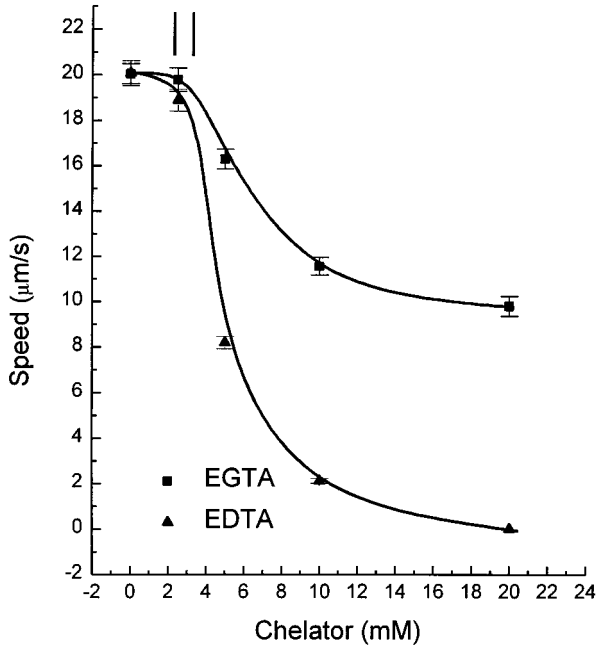

FIG. 6. Recovery of cells after brief exposure to chelators. Cells were aliquoted, pelleted by centrifugation, and then resuspended in AN medium plus 1% BSA with 10 mM calcium. Chelators were added to give the desired concentration, and the suspensions were incubated for 5 min at room temperature. The cells were then washed three times by centrifugation with fresh AN medium containing no chelator and assayed for swimming speed. The figure shows the final speeds. Vertical lines indicate the concentration of chelator which caused total inhibition of motility (left line, EGTA; right line, EDTA) (see Fig. 5).

The sharp increase in speed with addition of calcium (in the absence of terbium) (Fig. 2) is consistent with the idea that a calcium potential must be at or above a threshold level before the channels will operate. If EGTA and EDTA act as calcium ionophores, they should decrease that potential and thereby abruptly inhibit motility when the potential drops below a critical level, as observed (Fig. 5).

The notion that EDTA and EGTA are active as ionophores has not been well documented, and most researchers assume that they simply buffer calcium. However, Kovac has shown that EDTA, and to a lesser extent calcium-bound EDTA, is transported into *Saccharomyces* spp., and he suggests that the same is true for EGTA (15). Our data indicate that these chelators have an effect on motility that involves more than calcium sequestration. The high ionic strength of seawater should favor passage of neutralized EGTA through the cell envelope. The relatively poor inhibition by 4-bromo A23187 is difficult to interpret without actually measuring the intracellular concentration of calcium. It is possible that the ionophore simply does not work in *Synechococcus*. Currently, we are trying to measure the intracellular calcium concentration in *Synechococcus.*

A wave of depolarization has been shown to occur in the filamentous gliding cyanobacterium *Phormidium uncinatum* (10, 11, 23) in response to a light-dark transition or addition of a chemorepellent (24). This organism responds to a step down in light intensity by reversing the direction of motion, a photophobic response. The light stimulus can be localized to the first third of the trichome, but the wave of depolarization propagates its entire length. This action potential is produced only in the presence of calcium, as is the photophobic response (22, 23). Häder and Poff (12) showed a transient increase of $^{45}Ca^{++}$ uptake into *P. uncinatum* after a light-to-dark stimulus, further implicating calcium as the depolarizing ion. Studies using the calcium ionophore A23187 demonstrated that increasing the internal calcium concentration increased the frequency of reversals (1, 24). Ruthenium red, an inhibitor of electrogenic calcium transport, and the calcium analog lanthanum both inhibited reversals in intact trichomes without affecting cell speed (13). It is thought that this action potential is used to communicate the reversal signal to all the cells in the trichome (23). Womack et al. showed that calcium is required both for gliding (0.1 to 0.3 mM) and induction (1 mM) of the gliding machinery in myxobacteria (38).

Calcium has also been shown to affect tumbling in bacterial chemotaxis. Ordal showed that an increase in intracellular calcium levels in *Bacillus* spp. causes cells to undergo incessant

TABLE 2. Effect of calcium channel inhibitors and ionophores on motility

Material	Concn (μM)[a]	Motility	Mode of action (references)
Verapamil[b]	100	Nonmotile	Ca^{2+} channel blocker (8, 18, 33)
Nitrendipine[b]	100	Nonmotile	Ca^{2+} channel blocker (2, 8, 18, 33)
Conotoxin	1	Motile	Ca^{2+} channel blocker (21, 31)
Diltiazem	100	Motile	Ca^{2+} channel blocker (8, 18, 33)
Orthovanadate	100	Motile	P-type Ca^{2+} ATPase inhibitor (3, 9)
4-Bromo A23187	10	Motile	Calcium ionophore (5, 27)
4-Bromo A23187	100	Nonmotile[c]	

[a] The concentration chosen was typical of that used in other prokaryotic systems (about 10 times the concentration used in the heart), as referenced under mode of action.

[b] Drugs were dissolved in ethanol (10 mM). Solvent-only controls had no effect on motility.

[c] Cells clumped together; free cells swam slowly.

tumbling, thus inhibiting chemotaxis (26). Kusaka and Matsushita demonstrated that *Bacillus* cytoplasmic membrane contains voltage-activated calcium uniporters which are inhibited by nitrendipine, verapamil, lanthanum chloride, and conotoxin (16). Later, these authors showed that these drugs also inhibit chemotaxis (21). Tisa et al. used the calcium channel blocker conotoxin to show that blocking calcium entry into cells caused a cessation of tumbling, inhibiting chemotaxis in *Escherichia coli* (31). Later, Tisa and Adler demonstrated that an increase of intracellular calcium caused tumbling in *E. coli* (32), as it does in *Bacillus* spp.

The water-soluble inhibitors diltiazem and conotoxin did not inhibit *Synechococcus* motility, but this could have been due to a lack of access to the cytoplasmic membrane. Conotoxin was a more effective inhibitor when the outer membrane of *E. coli* was permeabilized by EDTA (31). Such a treatment of *Synechococcus*, however, would interfere with swimming, and the results would be uninterpretable.

Our data are consistent with a mechanism of motility in which longitudinal or transverse waves are propagated down the length of the cell (7, 30). The prediction is that a cell could swim at 25 μm/s by propagating a wave of 0.02-μm amplitude, 0.2-μm length, and 160-μm/s speed. Such a wave would be undetectable by standard phase-contrast microscopy. Calcium depolarization might serve as either a timing mechanism for a yet-unknown motor (12) or, as envisioned by Koch (14), as a means for generating a local swelling of the cell envelope which might propagate down the cell body. We are in the process of looking for such displacements (see Ehlers et al. [7]).

ACKNOWLEDGMENTS

We thank Linda Turner and Angelica Seitz for helpful discussions and comments on the manuscript, John Waterbury for providing strains, and Bianca Brahamsha for many helpful discussions.

This work was supported by the Rowland Institute for Science.

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