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Inhibition of *Escherichia coli* Chemotaxis by ω-Conotoxin, a Calcium Ion Channel Blocker

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***Escherichia coli* chemotaxis was inhibited by ω-conotoxin, a calcium ion channel blocker. With Tris-EDTA-permeabilized cells, nanomolar levels of ω-conotoxin inhibited chemotaxis without loss of motility. Cells treated with ω-conotoxin swam with a smooth bias, i.e., tumbling was inhibited.**

Motile bacteria such as *Escherichia coli* perform chemotaxis, i.e., they move toward chemical attractants (1) and away from chemical repellents (30). Each bacterium possesses (i) a sensory apparatus to recognize the chemical signal, (ii) a signal transduction mechanism, (iii) a pathway to conduct the message to the flagella, and (iv) a response mechanism to modify the direction of rotation of the flagella so as to achieve running in the case of increasing concentration of attractants and tumbling in the case of increasing concentration of repellents (10).

Bacteria sense a variety of chemical gradients by use of transmembrane receptors called methyl-accepting chemotaxis proteins (MCPs). There are four known MCPs in *E. coli*: MCP I (Tsr or serine receptor), MCP II (Tar or aspartate receptor), MCP III (Trg or ribose and galactose receptor), and MCP IV (Tap or dipeptide receptor). The cytoplasmic proteins CheW, CheA, CheY, and CheZ transmit the excitatory signal from the receptors to the flagellar motor by a phosphorylation-dephosphorylation cascade (6, 27). To attenuate the signal during the continued presence of the stimulus, the cytoplasmic CheR and CheB proteins are involved in the adaptation process by methylation and demethylation, respectively, of the MCPs.

Calcium ions have been implicated to play a role in chemotaxis by *Bacillus subtilis* (20) and *Halobacterium halobium* (5, 24). With *E. coli*, two lines of evidence have suggested that calcium ions do not play a role in chemotaxis. First, cells repeatedly treated with the calcium ion chelator EGTA exhibited a 20-fold reduction in their calcium content, but they were still chemotactic (21, 26). Second, increases in external calcium ion concentration did not affect chemotaxis (11).

Gangola and Rosen (12) measured the intracellular free calcium ion concentration in *E. coli* by use of the fluorescent indicator dye fura-2. These bacteria tightly regulate and maintain a low level (90 nM) of intracellular free calcium ions similar to the level observed in eukaryotic cells. Treating cells with EGTA reduced the total cellular content of calcium ions, but had no effect on the free intracellular calcium ion concentration. Elevating extracellular calcium ions increased total cellular calcium ion content, but also had no affect on intracellular free calcium ion level.

The ω-conotoxins, a class of peptide neurotoxins from fish-hunting cone snails (19), specifically block calcium ion channels (7, 16, 22). The affinity (K_d) of ω-conotoxin for its target proteins in eukaryotes is in the picomolar to subpicomolar range (4, 8). This neurotoxin has been shown to inhibit *B. subtilis* chemotaxis to L-alanine without affecting growth or motility (14). Voltage-dependent Ca^{2+} uptake was also inhibited by ω-conotoxin, which supports a role for calcium ions in *Bacillus* chemotaxis (15).

With these new insights, we decided to examine the effect of ω-conotoxin on *E. coli* chemotaxis.

(A preliminary report of this work was presented at the 91st General Meeting of the American Society for Microbiology [29].)

MATERIALS AND METHODS

Strains. AW405 was used as a chemotactically wild-type strain of *E. coli* (17).

Growth conditions. Cells were grown in Vogel-Bonner medium (31) containing 1 mM L-leucine, 1 mM L-threonine, and 1 mM L-histidine (required for growth by AW405) and 50 mM DL-lactate (minimal lactate medium) or in tryptone broth consisting of 1% tryptone (Difco Laboratories) and 0.5% NaCl.

Chemotaxis assay. Cells were grown in this Vogel-Bonner lactate medium by shaking them at 35°C until they reached an optical density of 0.4 to 0.6 at 590 nm. Then they were harvested by centrifuging at 6,000 × *g* for 3 min. The supernatant fluid was discarded, the pellet was resuspended, and chemotaxis medium (10 mM K^+ phosphate, [pH 7.0], 0.1 mM K^+ EDTA) was added. This was followed by two more such washes in chemotaxis medium, and finally the cells were resuspended in chemotaxis medium to an optical density of 0.01 at 590 nm (about 7×10^6 bacteria per ml). Chemotaxis was assayed by the capillary method (2).

Chemical-in-plug assay. Repellent responses were measured by the chemical-in-plug procedure (30).

Osmotaxis assay. Osmotaxis was measured by the chemical-in-plug method (13).

Phosphotransferase system chemotaxis assay. Cells used in phosphotransferase system chemotaxis assays were grown in Vogel-Bonner medium with D-fructose replacing lactate as a carbon and energy source (3). Chemotaxis was determined by the capillary assay described above, except that 10 mM D-fructose was used as an attractant.

Tris-EDTA permeabilization. Cells grown in Vogel-Bonner lactate (or better, glycerol) medium were made permeable by Tris-EDTA treatment (28).

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ω-Conotoxin. ω-Conotoxin GIVA was purified as described by Olivera et al. (19) or purchased from Research Biochemical Inc. Subsequently it will be called ω-conotoxin.

Inactivation of ω-conotoxin. Native ω-conotoxin was inactivated by reduction with dithiothreitol and then alkylated with iodoacetamide to stabilize the reduced form (19).

Analysis of free-swimming behavior. Bacterial swimming behavior was observed at 30°C by phase-contrast microscopy at a magnification of ×400. The cells were suspended in chemotaxis medium or in filtered used growth medium to an optical density at 590 nm of 0.1. The microscopic behavior was videotaped and analyzed by motion analysis (23).

RESULTS

The effect of ω-conotoxin on *E. coli* chemotaxis was tested by the capillary assay (Fig. 1). ω-Conotoxin inhibited chemotaxis toward both L-serine (sensed by Tsr) and L-aspartate (sensed by Tar). Chemotaxis was inhibited 90% by 1 μM ω-conotoxin (Fig. 1A). Cell motility, as measured by the capillary assay, was not affected by ω-conotoxin up to 1 μM, although at 2 μM there was inhibition (data not shown). Cell viability was not affected by 1 μM ω-conotoxin (data not shown).

The outer membrane of *E. coli* acts as a diffusion barrier to the inner cytoplasmic membrane; it allows passage of small molecules (<600 molecular weight) while blocking large molecules (18). ω-Conotoxin is a peptide composed of 27 amino acids with a molecular weight of 3,043 (19). The large size of this peptide would prevent its effective translocation to the cytoplasmic membrane. Cells were made permeable to ω-conotoxin by Tris-EDTA treatment, which has little or no effect on chemotaxis or motility (28). After this treatment, lower levels of ω-conotoxin inhibited serine chemotaxis (Fig. 1B). Motility as measured by the capillary assay was not affected at 0.25 μM, but there was partial inhibition at 0.5 μM (data not shown). L-Aspartate or D-fructose chemotaxis was also inhibited by ω-conotoxin for Tris-EDTA-treated cells more severely than for untreated cells (data not shown). These results demonstrate that ω-conotoxin was more effective in blocking bacterial chemotaxis after the cytoplasmic membrane had been exposed.

When measured by the chemical-in-plug assay, ω-conotoxin inhibited the repellent response to 5 mM $NiSO_4$, 1 mM $CoCl_2$, 0.5 M $NaNO_3$, or 1 M ribitol (data not shown). This indicates that ω-conotoxin inhibits both negative chemotaxis and osmotaxis.

ω-Conotoxin itself did not act as an attractant or a repellent when included with chemotaxis medium in the capillary but not in the bacterial suspension, and it did not act as a repellent in the chemical-in-plug assay (data not shown).

Disulfide reduction of ω-conotoxin eliminates its biological activity (19). The effect of inactivated ω-conotoxin on chemotaxis was tested by the capillary assay (Fig. 2). While native ω-conotoxin inhibited chemotaxis, inactivated ω-conotoxin had no effect on chemotaxis. This shows that ω-conotoxin must be in its biologically active form for inhibition of chemotaxis.

The effect of Ca^{2+} and other cations on recovery of inhibition of chemotaxis by ω-conotoxin was tested by the capillary assay (Table 1). The free concentration of each cation required to restore 50% recovery from inhibition of chemotaxis by 0.2 μM ω-conotoxin was determined from these experiments. The results are shown in Table 1. Cation effectiveness, from strongest to weakest, was $Ca^{2+} > Mg^{2+} > Ba^{2+} > Na^{+} = K^{+}$.

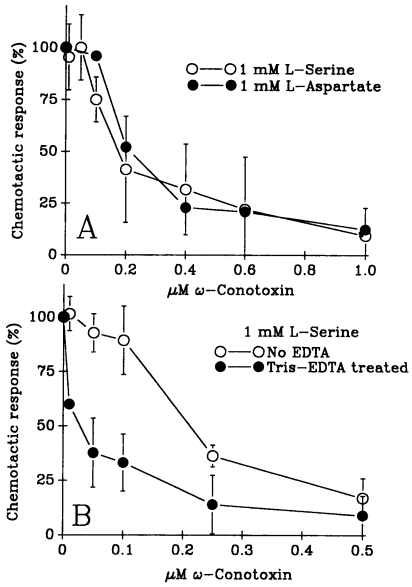

FIG. 1. (A) Effect of ω-conotoxin on chemotaxis and motility by wild-type *E. coli* (strain AW405). Bacteria were incubated for 45 min at 30°C in chemotaxis medium with different concentrations of ω-conotoxin and were then presented with capillaries containing 1 mM L-serine, 1 mM L-aspartate, or no attractant, all in chemotaxis medium. In each case, the concentration of ω-conotoxin in the capillary was the same as in the pond of bacteria. This experiment was repeated a number of times with similar results. In each experiment, all points were determined in triplicate. The averages of three to five experiments are plotted, and the range of results is indicated by the error bars. The chemotactic responses without ω-conotoxin (100%) were 87,100 ± 17,900 and 119,000 ± 17,900 bacteria in the capillary for L-serine and L-aspartate, respectively. (B) Effect of Tris-EDTA permeabilization of bacteria on ω-conotoxin inhibition of chemotaxis. Wild-type *E. coli* (strain AW405) cells were permeabilized by treatment with Tris-EDTA as described in Materials and Methods. Then bacteria were incubated for 30 min at 30°C in chemotaxis medium with different concentrations of ω-conotoxin and were presented with capillaries containing 1 mM L-serine in chemotaxis medium. In each case, the concentration of ω-conotoxin in the capillary was the same as in the pond of bacteria. This experiment was repeated a number of times with similar results. In each experiment, all points were determined in triplicate. The averages of three to seven experiments are plotted, and the range of results is indicated by the error bars. The chemotactic responses without ω-conotoxin (100%) were 68,000 ± 7,500 and 64,300 ± 14,400 bacteria in the capillary for Tris- and Tris-EDTA-treated cells, respectively.

Cells were observed microscopically to determine the effect of ω-conotoxin on *E. coli* swimming behavior. Wild-type chemotactic cells swim under unstimulated conditions with alternating runs and tumbles. Cells treated with ω-conotoxin (0.2 to 1.0 μM) swim with a running bias, i.e., tumbling is blocked. Tris-EDTA permeabilizing of *E. coli* allowed ω-conotoxin to produce this running bias at lower concentrations (0.01 to 0.25 μM).

To provide an objective analysis of the effect of ω-conotoxin, we subjected these observations to computer analysis. The behavior of free-swimming bacteria was videotaped, digitized, and computer analyzed (23). This technique measures both the average angular speed (the rate of change in direction) and the average linear speed (the rate of movement in a straight line) of a population of motile bacteria. The angular and linear speeds are directly and inversely propor-

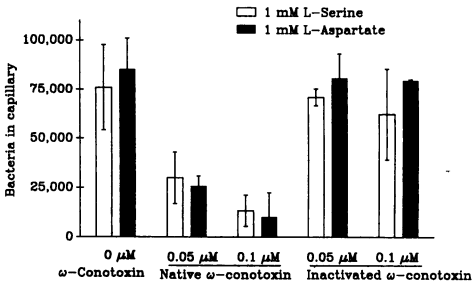

FIG. 2. Effect of inactive ω-conotoxin on chemotaxis by wild-type *E. coli* (strain AW405). Wild-type *E. coli* cells were permeabilized by treatment with Tris-EDTA as described in Materials and Methods. Then bacteria were incubated for 30 min at 30°C in chemotaxis medium with no ω-conotoxin, native ω-conotoxin, or inactivated ω-conotoxin and were presented with capillaries containing 1 mM L-serine or 1 mM L-aspartate, both in chemotaxis medium. In each case, the quality and the concentration of ω-conotoxin in the capillaries were the same as in the ponds of bacteria. This experiment was repeated a number of times with similar results. In each experiment, all points were determined in triplicate. The averages of three to five experiments are plotted, and the range of the results is indicated by the error bars.

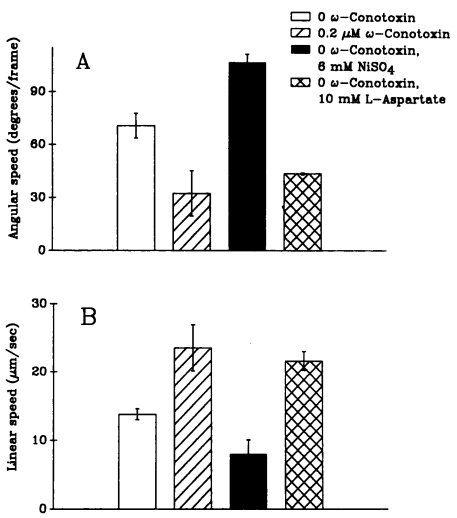

FIG. 3. Effect of ω-conotoxin on the average angular and linear speeds of chemotactically wild-type *E. coli* (strain AW405) in minimal lactate medium. The free-swimming behavior was videotaped, digitized, and analyzed as described in Materials and Methods. This experiment was repeated a number of times with similar results. Averages for three to six measurements of different fields are plotted, and the range of the results is indicated by the error bars. (A) Average angular speeds; (B) average linear speeds.

tional, respectively, to the tumbling frequency. An increase in the angular speed reflects an increase in the time spent tumbling, while a decrease corresponds to a reduction in tumbling.

Results from computer analysis (23) of the effect of ω-conotoxin on the behavior of chemotactically wild-type *E. coli* in minimal lactate medium are shown in Fig. 3. The addition of ω-conotoxin caused both a reduction in angular speed and an increase in linear speed (Fig. 3), and this was found to the same degree for cells in tryptone broth (data not shown). As expected, addition of repellent ($NiSO_4$) in the absence of ω-conotoxin caused an increase in angular speed and a decrease in linear speed, while addition of attractant (L-aspartate) did the opposite (Fig. 3).

DISCUSSION

ω-Conotoxin, a calcium ion channel blocker, inhibits chemotaxis of *E. coli*, and once the outer membrane is removed, the toxin inhibits chemotaxis at an even lower dosage. This suggests that ω-conotoxin acts on the cytoplasmic membrane or possibly in the cytoplasm. The low dosage required to block chemotaxis (10 nM for 50% inhibition in permeabilized cells) was similar to the doses required to block calcium channels in eukaryotic systems (10 nM for 50% inhibition of $^{45}Ca^{2+}$ uptake by chick brain synaptosomes [22]). ω-Conotoxin blocks bacterial chemotaxis by stopping tumbles; it does not stop runs at these concentrations.

One possible explanation for ω-conotoxin inhibition of chemotaxis could be that ω-conotoxin causes a reduction of the proton motive force in *E. coli*. This would eliminate tumbling, and it would cause slower swimming. Although ω-conotoxin did eliminate tumbling, it did not reduce the swimming speed at concentrations that blocked chemotaxis. Therefore, we do not believe that ω-conotoxin is causing a reduction in the proton motive force.

The discovery that a Ca^{2+} ion channel blocker affects *E. coli* chemotaxis provides a new tool for the study of *E. coli* behavior. We are presently attempting to learn how ω-conotoxin inhibits the tumbling mechanism of the chemotaxis system. Presumably, ω-conotoxin acts by blocking a calcium ion channel which is needed for tumbling and consequently for chemotaxis.

TABLE 1. Cation concentration required for 50% recovery of 0.2 μM ω-conotoxin inhibition of L-serine chemotaxis by wild-type *E. coli* (strain AW405)[a]

Cation	Concn (M) of free cation required for 50% recovery
Ca^{2+}	4.0×10^{-8}
Mg^{2+}	6.1×10^{-6}
Ba^{2+}	9.7×10^{-5}
Na^{+}	1.7×10^{-4}
K^{+}	2.0×10^{-4}

[a] The effect of cations on inhibition of chemotaxis by ω-conotoxin was measured by the capillary assay as described in the legend to Fig. 1A. ω-Conotoxin (0.2 μM) and various concentrations of each cation were present in both the capillary and the bacterial suspension. Because of the presence of EDTA in the chemotaxis medium, the levels of free cation had to be calculated for each cation based on the cation-EDTA stability constants (9, 25). The concentration of free cation required for 50% recovery was determined for each cation tested. In each case, Cl^- salts were used. The average response without ω-conotoxin was 155,300 ± 14,600 bacteria in the capillary, and the average response with 0.2 μM ω-conotoxin was 34,600 ± 14,800 bacteria in the capillary.

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ADDENDUM IN PROOF

Two other studies have implicated that Ca^{2+} participates in chemotaxis by *E. coli* Omirbekova et al. observed constant tumbling of *E. coli* in the presence of Ca^{2+} and an ionophore (N. G. Omirbekova, V. L. Gabai, M. Y. Sherman, N. V. Vorobyeva, and A. N. Glagolev. FEMS Microbiol. Lett. **28:**259–263, 1985). See also Discussion by R. N. Brey and B. P. Rosen (J. Bacteriol. **139:**824–834, 1979). Recently, we have described the use of "caged" Ca^{2+} compounds to modulate intracellular Ca^{2+} levels in *E. coli*: increasing intracellular Ca^{2+} caused tumbling, while decreasing intracellular Ca^{2+} caused smooth swimming (L. S. Tisa and J. Adler, Proc. Natl. Acad. Sci. USA **89:**11804–11808, 1992).

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