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Calcium ions are involved in *Escherichia coli* chemotaxis

(bacterial behavior/"caged" calcium/nitr-5/DM-nitrophen/diazo-2)

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ABSTRACT **_Escherichia coli_ regulates intracellular free Ca^{2+} at about 90 nM [Gangola, P. & Rosen, B. P. (1987) *J. Biol. Chem.* 262, 12570–12574]. To increase intracellular free Ca^{2+}, nitr-5/Ca^{2+}, a "caged" Ca^{2+} compound, was electroporated into cells and then its affinity for Ca^{2+} was reduced by exposure to 370-nm light. Upon release of the Ca^{2+} ions, the cells tumbled. Studies on mutant strains showed that the receptor proteins (methyl-accepting chemotaxis proteins, MCPs) were not required for the Ca^{2+}-induced tumbling but that CheA, CheW, and CheY proteins were required. Similar results were obtained with DM-nitrophen/Ca^{2+}, another caged calcium compound that releases Ca^{2+} upon illumination at 340 nm. Diazo-2, a caged Ca^{2+} chelator that takes up Ca^{2+} upon illumination at 340 nm, was used to decrease intracellular free Ca^{2+}, and this caused smooth swimming.**

Calcium ions play a role in eukaryotic cells as a second messenger for many events such as signal transduction, behavior, and differentiation (1–3). The role of Ca^{2+} in prokaryotes is not as well defined (for reviews, see refs. 4 and 5).

Ca^{2+} participates in chemotaxis (the ability of motile cells to be attracted or repelled by chemicals) by *Bacillus subtilis* (6, 7) and in both chemotaxis and phototaxis (the ability of motile cells to be attracted or repelled by light) by *Halobacterium halobium* (8–10). There is also evidence indicating Ca^{2+} involvement in gliding motility by myxobacteria (11) and cyanobacteria (12).

With *Escherichia coli* two lines of evidence have suggested that Ca^{2+} does not play a role in chemotaxis. First, cells that were repeatedly treated with the Ca^{2+} chelator EGTA exhibited a >10-fold reduction in their Ca^{2+} content but were still chemotactic (13, 14). Second, increases in external Ca^{2+} did not affect chemotaxis (15).

Gangola and Rosen (16) measured the intracellular concentration of free Ca^{2+} in *E. coli* by use of the fluorescent indicator dye fura-2 (16). These bacteria tightly regulate intracellular free Ca^{2+} at about 90 nM, similar to the level observed in eukaryotic cells. Treating cells with EGTA reduced the total cellular content of Ca^{2+} but had no affect on the intracellular free-Ca^{2+} concentration. Elevating the level of extracellular Ca^{2+} increased total cellular Ca^{2+} content but also had no affect on intracellular free-Ca^{2+} level.

We were interested in the behavioral effects of changing the level of intracellular Ca^{2+} in *E. coli*. Since changes in external Ca^{2+} levels have no effect on intracellular free-Ca^{2+} levels (16), we developed a different approach. "Caged" Ca^{2+} compounds are photolabile Ca^{2+} chelators which change their affinity for Ca^{2+} after exposure to certain wavelengths of light (for reviews, see refs. 17–19). Nitr-5 and DM-nitrophen are two caged Ca^{2+} compounds which release Ca^{2+} upon illumination (20, 21), while diazo-2 is a caged Ca^{2+} compound which takes up Ca^{2+} upon illumination (22). To test the effects of transient changes in intracellular free-Ca^{2+} levels on bacterial behavior, these caged compounds were introduced into *E. coli* cells, and then the cells were illuminated.

MATERIALS AND METHODS

Bacteria. All strains used in this study are derivatives of *Escherichia coli* K-12 and are listed in Table 1. Cells were grown in tryptone broth consisting of 1% tryptone (Difco) and 0.5% NaCl.

Electroporation Conditions. Cells were grown in tryptone broth with shaking at 35°C until they reached an OD_{590} of 0.4–0.6. Then they were harvested by centrifugation at 6000 × *g* for 3 min. The supernatant was discarded, the pellet was resuspended, and 5 ml of electroporation buffer [1 mM Hepes, pH 7.2/10% (vol/vol) glycerol] was added. This was followed by two more such washes in electroporation buffer, and finally the cells were suspended in 1 ml of electroporation buffer to an OD_{590} of 2–6 ($1.5–4.5 \times 10^9$ bacteria per ml). Cells were stored on ice until electroporation.

Compounds were introduced into 200 μl of cells by electroporation with a single pulse at a capacity of 25 μF with a field intensity of 5 kV/cm at 200 Ω for 4–6 msec in a Bio-Rad Gene Pulser. Immediately after electroporation, the cells were diluted with 1 ml of filtered used growth medium and incubated at room temperature for 15 min. The cells were collected by centrifugation at 6000 × *g* for 3 min. The supernatant was discarded, the pellet was resuspended, and 5 ml of filtered used growth medium or chemotaxis medium (10 mM potassium phosphate, pH 7.0/0.1 mM potassium EDTA/1 mM L-methionine) was added. This was followed by an additional wash in either medium, and finally the cells were suspended in either medium to an OD_{590} of 0.1 (about 7×10^7 bacteria per ml).

"Caged"-Ca^{2+} Experiments. Nitr-5 (Calbiochem) (18, 20) and Ca^{2+}, each at 300 μM unless otherwise stated, were electroporated into cells as described above. The swimming behavior of these nitr-5-loaded cells was observed at 30°C by phase-contrast microscopy at a magnification of ×400 with infrared illumination (>700 nm). The cells were exposed to 370-nm light to release Ca^{2+} from nitr-5. Control cells without nitr-5 or Ca^{2+} were exposed to otherwise identical conditions. Microscopic observations were videotaped and analyzed (28). In the case of DM-nitrophen (Calbiochem) and diazo-2 and diazo-3 (Molecular Probes), cells were loaded as described above for nitr-5 and then were exposed to 340-nm light instead of 370-nm light.

RESULTS

Effect of Increased Intracellular Ca^{2+} in Chemotactically Wild-Type Bacteria. Nitr-5, a "caged" Ca^{2+} chelator, was introduced with Ca^{2+} into *E. coli* cells. Photocleavage of nitr-5 by 370-nm light results in two photoproducts: a ni-

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Abbreviation: MCP, methyl-accepting chemotaxis protein.

Table 1. Bacterial strains

Strain	Relevant phenotype	Ref. or source
AW405	Chemotactically wild type	23
AW574	Chemotactically wild type	24
CP362	Δ*tsr* Δ*trg* Δ(*tar–tap*)	25
HCB429	Δ*tsr* Δ*trg zbd*::Tn*5* Δ(*tar–tap*)	26
HCB326	Δ*tsr trg*::Tn*10* Δ(*cheA–cheZ*)	26
HCB437	Δ*tsr* Δ*trg zbd*::Tn*5* Δ(*cheA–cheZ*)	26
RP1788	Δ*cheA*	27
RP1078	Δ*cheW*	27
RP5232	Δ*cheY*	*
RP1616	Δ*cheZ*	*
RP4971	Δ*cheB*	*
RP1254	Δ*cheR*	*
HCB482	Δ*tsr* Δ*trg zbd*::Tn*5* Δ(*cheA–cheZ*) *fliM* (*scyA2*)	26
HCB483	Δ*tsr* Δ*trg zbd*::Tn*5* Δ(*cheA–cheZ*) *fliM* (*scyA3*)	26
HCB484	Δ*tsr* Δ*trg zbd*::Tn*5* Δ(*cheA–cheZ*) *fliG* (*scyB10*)	26
AJW378	Δ*tsr* Δ*trg zbd*::Tn*5* Δ(*cheA–cheZ*) P_{lac}-*cheY*	†

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troketone with a lowered Ca^{2+} affinity and water (18, 20). Nitr-5 is selective for Ca^{2+} (K_d = 145 nM before light, 6 μM after) over Mg^{2+} (K_d = 8.5 mM before light, 8 mM after) (18, 20).

The results in Table 2 show the effect of photolysis-induced release of intracellular Ca^{2+} on the swimming behavior of wild-type *E. coli*. Under unstimulated conditions, cells typically swam in a pattern of runs (also called smooth swimming) and tumbles. Cells not electroporated, or cells electroporated in the absence of nitr-5, exhibited typical swimming behavior. Exposure to 370-nm light caused no change in their swimming behavior. Cells loaded with nitr-5/Ca^{2+} tumbled after exposure to 370-nm light. This light resulted in photocleavage of the nitr-5, lowering its affinity for Ca^{2+} and thus releasing bound Ca^{2+}. The released, free Ca^{2+} is presumed to cause the tumble response. The intensity of the tumble response was correlated to the amount of Ca^{2+} bound to nitr-5. Cells loaded with nitr-5/Ca^{2+} in a 1:1 ratio produced the strongest tumble response. This tumble response was specific for Ca^{2+}. Replacing Ca^{2+} with Mg^{2+} or Ba^{2+} did not induce the tumble response. When nitr-5 was introduced into the cells without bound Ca^{2+}, the cells swam with a smooth bias. This result suggests that nitr-5 reduced the level of intracellular free Ca^{2+} and thus caused smooth swimming.

Table 2. Effect of intracellular Ca^{2+} release from nitr-5/Ca^{2+} in wild-type *E. coli* (strain AW574)

Condition*	Swimming behavior: Before photolysis	Swimming behavior: After photolysis
Control cells (no electroporation)	Smooth–tumbly	Smooth–tumbly
Electroporated cells		
No additions	Smooth–tumbly	Smooth–tumbly
$CaCl_2$	Smooth–tumbly	Smooth–tumbly
Nitr-5	Very smooth	Weak tumbly
Nitr-5/$CaCl_2$ (1:0.25)	Smooth	Tumbly
Nitr-5/$CaCl_2$ (1:1)	Smooth–tumbly	Strong tumbly
Nitr-5/$BaCl_2$ (1:1)	Smooth–tumbly	Smooth–tumbly
Nitr-5/$MgCl_2$ (1:1)	Smooth–tumbly	Smooth–tumbly

Photolysis was achieved with 370-nm light. "Smooth–tumbly" means smooth swimming (running) and tumbling. Results were confirmed by motion analysis (data not shown).
*Molar ratios are given in parentheses.

Fig. 1 illustrates the effect of nitr-5/Ca^{2+} on the swimming behavior of wild-type *E. coli*. To provide objectivity, our observations were subjected to computer analysis. Free-swimming bacteria were observed and videotaped, and the data were digitized and analyzed as described (28). 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 proportional, respectively, to the tumbling frequency: an increase in the angular speed reflects an increase in the time spent tumbling, whereas a decrease corresponds to a reduction in tumbling.

Upon illumination, cells loaded with nitr-5/Ca^{2+} exhibited an increase in their angular speed and a decrease in linear speed; this reflects an increase in tumbling frequency. This increase in angular speed and decrease in linear speed continued for >100 sec after illumination and then the bacteria returned to the original speeds observed before illumination. The control cells lacking nitr-5 did not show any change in angular speed or linear speed during and after illumination. These data indicate that a transient increase in intracellular free Ca^{2+} causes tumbling.

Mutant Studies. To determine the site of action for Ca^{2+} ions in the chemotactic pathway, we tested the effect of Ca^{2+} release from nitr-5 on chemotactic mutants (Table 3). Mutant strains lacking all four MCPs were unable to respond to most chemical stimuli and swam with a smooth bias. When nitr-5/Ca^{2+} was loaded into such cells, and before the release of Ca^{2+}, the cells still swam with a smooth bias. Upon illumination and intracellular Ca^{2+} release, the cells tumbled. This result indicates that the Ca^{2+} effect occurs downstream of the sensory receptors (the MCPs). However, "gutted" strains, lacking all four MCPs and all six Che proteins, failed to tumble after illumination and intracellular Ca^{2+} release. This result indicates that some or all of the Che proteins are required for the Ca^{2+}-induced tumbling. Tests on strains deleted for individual Che proteins showed that CheA, CheW, and CheY were required for the effect (Table 3). It is difficult to judge the requirement for CheZ, since CheZ-negative cells were already tumbly before the release of Ca^{2+}. To find out whether CheB was required, attractant

Table 3. Effect of intracellular Ca^{2+} release from nitr-5/Ca^{2+} in mutants of *E. coli*

Phenotype*	Strain	Swimming behavior: Before photolysis	Swimming behavior: After photolysis
Wild type	AW405	Smooth–tumbly	Tumbly
	AW574	Smooth–tumbly	Tumbly
No MCPs	CP362	Smooth	Tumbly
	HCB429	Smooth	Tumbly
No MCPs or Che proteins	HCB326	Smooth	Smooth
	HCB437	Smooth	Smooth
Δ*cheA*	RP1788	Smooth	Smooth
Δ*cheW*	RP1078	Smooth	Smooth
Δ*cheY*	RP5232	Smooth	Smooth
Δ*cheZ*	RP1616	Tumbly	Tumbly
Δ*cheB*	RP4971	Tumbly	Tumbly
Δ*cheR*	RP1254	Smooth	Tumbly

Cells were loaded with nitr-5 and Ca^{2+} (1:1 molar ratio) and photolysis was achieved with 370-nm light. Results were confirmed by motion analysis (data not shown).
*MCP, methyl-accepting chemotaxis protein; no MCPs, MCP $I^-II^-III^-IV^-$; no Che proteins, Δ*cheA* Δ*cheW* Δ*cheY* Δ*cheZ* Δ*cheB* Δ*cheR*.

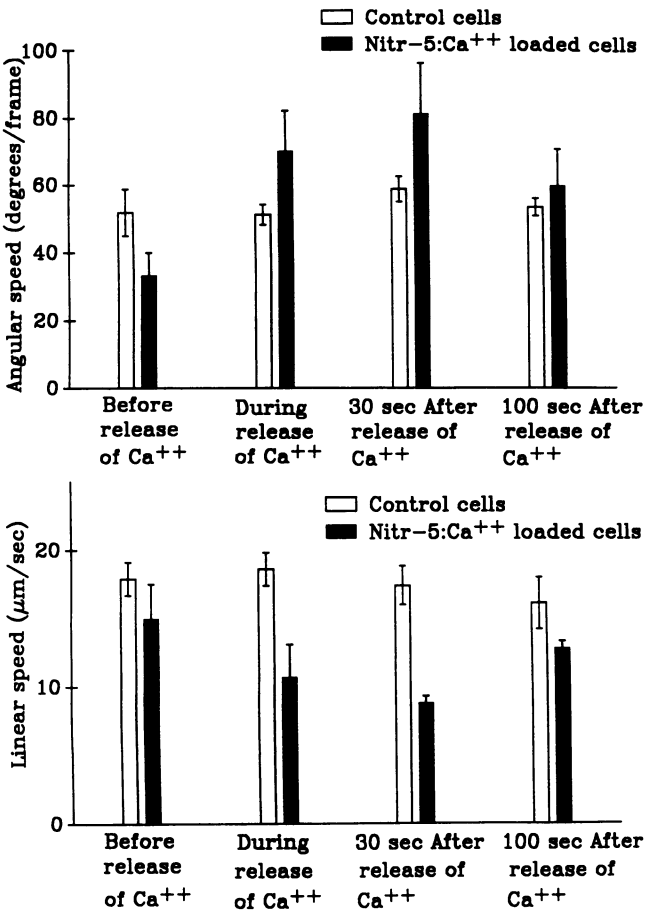

FIG. 1. Effect of intracellular Ca^{2+} release from nitr-5/Ca^{2+} on average angular and linear speeds of wild-type *E. coli* (strain AW405) in chemotaxis medium. Cells were loaded with nitr-5/Ca^{2+} (1:0.75 molar ratio) by electroporation. Bacteria were exposed to 370-nm light to release Ca^{2+} from nitr-5. The swimming behavior was observed by phase-contrast microscopy at 30°C, videotaped, and subjected to computer analysis. The average angular speed (rate of change in direction) is an objective measure of the tumbliness for a population of cells. The average linear speed is a measure of the movement in a straight line. Filled bars, nitr-5/Ca^{2+}-loaded cells; open bars, control cells electroporated without nitr-5 and without Ca^{2+}. Shown are the average of 4–10 measurements, with the error bars indicating the range of the measurements.

(20 mM L-aspartate) was added to Δ*cheB* cells to promote smooth swimming. Upon illumination and intracellular Ca^{2+} release from nitr-5/Ca^{2+}, cells tumbled, indicating that CheB was not required. Control Δ*cheB* cells with aspartate, electroporated in the absence of nitr-5/Ca^{2+}, maintained smooth swimming (data not shown). CheR was not required (Table 3).

It was possible that these mutants ("gutted" strains, Δ*cheA*, Δ*cheW*, and Δ*cheY*) exhibited too strong a smooth swimming bias to show a tumble response to increased intracellular Ca^{2+}. To test this possibility, gutted strains containing switch mutations that caused smooth–tumbly swimming (*fliG* and *fliM*) were used (Table 1). Upon illumination and release of Ca^{2+}, none of these mutant strains showed an increase in tumbling frequency (data not shown). Another gutted strain (AJW378) containing the *cheY* gene under the control of a *lac* promoter was tested. The amount of CheY protein synthesized was controlled by the amount of inducer (isopropyl β-D-thiogalactopyranoside) added; the more CheY was made, the more the cells swam with a smooth–tumbly bias. Such cells are able to tumble in the presence of acetate, though not in the presence of benzoate or other weak acids (29). Loaded with nitr-5/Ca^{2+}, these induced cells failed to increase their tumbling frequency upon illumination. These data indicate that the site of action of Ca^{2+} is not downstream of CheY or at the flagellar switch. These experiments also imply that the failure of the *cheY* mutant to respond to Ca^{2+} was not due to a too-strong smooth bias.

Effect of Other Caged Calcium Compounds. Nitr-5 is only one of several caged Ca^{2+} chelators available. DM-nitrophen is another compound which releases Ca^{2+} upon illumination (21). While nitr-5 binds Ca^{2+} almost exclusively, DM-nitrophen is less selective, binding both Ca^{2+} (K_d = 5 nM) and Mg^{2+} (K_d = 2.5 μM). The photoproducts of this compound are iminodiacetic acid and nitrosoacetophenone-substituted iminodiacetic acid, both with a lowered affinity for both Ca^{2+} (K_d = 3 mM) and Mg^{2+} (K_d = 3 mM) (21). Light-induced (340 nm) release of Ca^{2+} from DM-nitrophen/Ca^{2+} induced a tumble response (data not shown). Upon illumination, both an increase in angular speed and a decrease in linear speed were observed.

Diazo-2 is a caged Ca^{2+} chelator which *takes up* Ca^{2+} upon illumination instead of releasing it (the opposite of nitr-5 and DM-nitrophen) (22). Photochemical rearrangement of a diazoacetyl group to a carboxymethyl group results in the conversion of a chelator with a low affinity for Ca^{2+} (K_d = 2 μM) to one with a higher affinity for Ca^{2+} (K_d = 73 nM) (22). The effect of decreasing intracellular free Ca^{2+} in chemotactically wild-type cells was tested. Cells loaded with diazo-2 exhibited a reduced angular speed and an increased linear speed after photolysis (data not shown); this indicates that the cells swam with a smooth bias after photolysis, which was confirmed by microscopic observations. Cells loaded with diazo-3, which does not change its affinity for Ca^{2+} upon photolysis (K_d > 1 mM before and after treatment) (22), did not exhibit a smooth bias. They showed an increase in angular speed and a decrease in linear speed (data not shown); this indicates an increase in the tumbling frequency, which was confirmed by microscopic observations. This increase in tumbling was most likely the result of proton release from photolysis of diazo-3 (22). Protons are also a photoproduct of diazo-2 (22), but their effect was not observed. This would indicate that a decrease in intracellular free Ca^{2+}, which causes smooth swimming, has a greater effect than an increase in intracellular H^+, which causes tumbling.

DISCUSSION

We have developed a method to manipulate levels of intracellular free Ca^{2+} in *E. coli* which should be generally adaptable to other cells. Caged Ca^{2+} chelators were electroporated into *E. coli*. Nitr-5/Ca^{2+} and DM-nitrophen/Ca^{2+}, which upon illumination lower their affinity for Ca^{2+} and thereby release bound Ca^{2+}, were used to increase intracellular free Ca^{2+}. Electroporating free nitr-5 without bound Ca^{2+} lowered the free-Ca^{2+} levels without illumination. Diazo-2, which upon illumination increases its affinity for Ca^{2+}, was also used to decrease intracellular free Ca^{2+}.

Elevating intracellular free-Ca^{2+} levels by releasing Ca^{2+} from either nitr-5 (Fig. 1, Table 2) or DM-nitrophen (data not shown) resulted in an increase in tumbling by chemotactically wild-type *E. coli*. Decreasing intracellular free-Ca^{2+} levels by binding to free nitr-5 (Table 2) or to diazo-2 (data not shown) inhibited tumbling and promoted smooth swimming by chemotactically wild-type *E. coli*.

Since DM-nitrophen and diazo-2 release weak acids and protons, respectively, most of the work in this study focused on using nitr-5 as a caged Ca^{2+} chelator because in that case no other molecules (except H_2O) are involved. Both weak acids and protons are known repellents sensed by the MCPs

(30, 31). In spite of this complication, these two compounds were used to confirm and support the results obtained with nitr-5. Although diazo-2 releases protons upon illumination, cells containing diazo-2 still showed increased smooth swimming (decreased tumbling frequency) after the reduction in intracellular free Ca^{2+} which resulted from binding of Ca^{2+} to diazo-2 upon illumination. Control cells containing diazo-3, which upon illumination does not bind Ca^{2+} but does release protons, increased their tumbling frequency upon illumination.

To our knowledge, the binding affinities of these caged compounds for metal ions other than Ca^{2+} and Mg^{2+} have not been determined; thus it is possible that Ca^{2+} is mimicking the effect of another metal ion.

We have previously shown that ω-conotoxin, a Ca^{2+}-channel blocker, inhibits *E. coli* chemotaxis (32). Cells treated with ω-conotoxin swim with a smooth bias as a result of inhibited tumbling. Several other Ca^{2+} antagonists also inhibit chemotaxis and cause a smooth swimming bias (L.S.T., J.J. Sekelsky, and J.A., unpublished work). Mutant strains defective in Ca^{2+} transport were found to be defective in chemotaxis (unpublished work). Based on those observations and the present study, we propose a working model (Table 4). In the absence of stimuli, cells maintain a steady-state level of intracellular free Ca^{2+} and swim in an unbiased random-walk pattern consisting of runs (smooth swimming) and tumbles. Upon the addition of a repellent, cells tumble; our model predicts that repellents cause the levels of intracellular free Ca^{2+} to rise transiently and then return to the steady-state level. The addition of an attractant causes smooth swimming, and our model predicts that attractants cause the level of intracellular free Ca^{2+} to drop transiently and then return to the steady-state level.

Intracellular free Ca^{2+} levels may be measured directly by the use of the fluorescent Ca^{2+}-indicator dye fura-2. Gangola and Rosen (16) used fura-2 to measure intracellular Ca^{2+}, but they loaded uncoupled, Tris/EDTA-permeabilized cells with the membrane-permeable acetoxymethyl ester of fura-2. Once inside, the cells then had to slowly hydrolyze the ester to form fura-2. To circumvent these requirements, we have loaded cells with fura-2 itself by electroporation of the membrane-impermeable fura-2. Preliminary results show that addition of a repellent produced a transient increase in intracellular free Ca^{2+}, whereas addition of an attractant produced a transient decrease (unpublished work). This is expected from the model (Table 4).

Where does Ca^{2+} act on the signal transduction pathway? It is clear from use of chemotactic mutants (Table 3) that the Ca^{2+} effect does not require chemosensory transducer proteins (MCPs) and instead occurs downstream from them. This observation indicates that Ca^{2+} is not just a stimulus recognized by the MCPs. Two lines of evidence indicate that Ca^{2+} does not act downstream of CheY directly on the flagellar switch. First, gutted strains, lacking receptor proteins (MCPs) and all six Che proteins, did not tumble in response to increased intracellular free Ca^{2+} (Table 3). Second, a gutted strain containing only CheY, which tumbles in response to acetate but not benzoate, did not tumble in response to increased intracellular free Ca^{2+}. The failure of this strain to tumble also eliminates the possibility of cross talk from EnvZ or KdpD, two known Ca^{2+}-stimulated kinases (33, 34).

Table 4. Working model for the effect of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) on *E. coli* chemotaxis

Stimulus	$[Ca^{2+}]_i$	Behavior
None	Steady state	Smooth–tumbly
Add repellent	Increase	Tumbly
Add attractant	Decrease	Smooth

Changes in $[Ca^{2+}]_i$ are transient, and the concentration returns to the steady-state level.

Ca^{2+}-induced tumbling required the presence of CheA, CheW, and CheY proteins (Table 3). These cytoplasmic proteins are involved in generating the tumble signal (35). The most likely sites of action for Ca^{2+} are either in generating the tumble signal or in maintaining the tumble signal. Based on what is presently known biochemically about chemotaxis (36, 37), one possibility is that autophosphorylation of CheA or phosphotransfer from phosphorylated CheA to CheY is stimulated by Ca^{2+}. In the EnvZ–OmpR system, Ca^{2+} stimulates autophosphorylation of EnvZ (33). Another possibility involves phosphorylated CheY, the tumble signal. CheY must be phosphorylated to interact with the flagellar switch to initiate a clockwise rotation. Unphosphorylated CheY will produce counterclockwise instead of clockwise rotation. This phosphate group on CheY is unstable (38, 39). Ca^{2+} may help to stabilize and maintain CheY in the phosphorylated state and in this way prolong the tumble signal by allowing interaction of phosphorylated CheY with the switch complex. Some evidence has been presented that Ca^{2+} inhibits dephosphorylation of CheY (40). In view of those observations, we favor the model that Ca^{2+} acts by maintaining the phosphorylated state of CheY.

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