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Cytoplasmic free-Ca\textsuperscript{2+} level rises with repellents and falls with attractants in Escherichia coli chemotaxis

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Contributed by Julius Adler, August 10, 1995

ABSTRACT Cytoplasmic free-Ca\textsuperscript{2+} levels in Escherichia coli were measured by use of the fluorescent Ca\textsuperscript{2+}-indicator dye fura-2. Chemotactically wild-type E. coli regulated cytoplasmic free Ca\textsuperscript{2+} at ~100 nM when no stimuli were encountered, but changes in bacterial behavior correlated with changes in cytoplasmic free-Ca\textsuperscript{2+} concentration. For chemotactically wild-type E. coli, addition of a repellent resulted in cells tumbling and a transient increase in cytoplasmic free-Ca\textsuperscript{2+} levels. Conversely, addition of an attractant to wild-type cells caused running and produced a transient decrease in cytoplasmic free-Ca\textsuperscript{2+} levels. Studies with mutant strains showed that the chemoreceptors were required for the observed changes in cytoplasmic free-Ca\textsuperscript{2+} levels in response to chemical stimuli.

In eukaryotic cells Ca\textsuperscript{2+} plays a role as a second messenger for many events such as signal transduction, behavior, and differentiation (1–3). There has been growing support for a function of Ca\textsuperscript{2+} in the behavior of bacteria (4–13, 37). Some of the most recent of this support comes from our own re-investigation of the role of Ca\textsuperscript{2+} in Escherichia coli chemotaxis (12, 13).

Certain mutants defective in calcium transport (5) have elevated levels of cytoplasmic Ca\textsuperscript{2+}, tumble continuously, and are defective in chemotaxis (31). On the basis of our studies, we have proposed a working model (12). In the absence of stimuli, cells maintain a steady-state level of cytoplasmic free Ca\textsuperscript{2+} and swim in an unbiased random-walk pattern consisting of runs and tumbles. Upon the addition of a repellent, cells tumble; our model predicts that repellents cause the cytoplasmic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) to rise transiently and then return to the steady-state level. On the other hand, the addition of an attractant causes running, and our model predicts that attractants cause [Ca\textsuperscript{2+}] to drop transiently and then return to steady-state level.

We wanted to test this model by directly measuring [Ca\textsuperscript{2+}]. Gangola and Rosen (14) first measured the [Ca\textsuperscript{2+}] in E. coli by use of the fluorescent Ca\textsuperscript{2+}-indicator dye fura-2 (15); the bacteria tightly regulate [Ca\textsuperscript{2+}] at ~90 ± 10 nM, similar to the level seen in eukaryotic cells (14). In that work Gangola and Rosen loaded uncoupled, Tris/EDTA-permeabilized E. coli cells not with fura-2 but with the membrane-permeable pentaacetoxymethyl ester of fura-2. Once this ester was inside, the cells slowly hydrolyzed it to form fura-2, the compound needed for measuring free-Ca\textsuperscript{2+} levels. To circumvent this requirement for hydrolysis, we directly loaded the cells with fura-2 itself by electroporation of that membrane-impermeable Ca\textsuperscript{2+} indicator. Then changes in bacterial behavior resulting from the addition of repellents or attractants were correlated with corresponding changes in [Ca\textsuperscript{2+}], in wild-type and mutant strains. The results of this study are described here. Preliminary reports of this have already been presented.† Some similar results were published recently by Watkins et al. (16).

MATERIALS AND METHODS

Bacterial Strains. The bacteria used were derivatives of E. coli K-12. Chemotactically wild-type strains are AW405 (17), AW574 (18), and AW607 (18). RP5882 is D\textit{tsr} (J. S. Parkinson, University of Utah). AW539 (19) and AW633 (20) are \textit{tar} mutants. CP362 is D\textit{tsr} D\textit{tg} D(tar-tap) (21). RP3098 is D\textit{fhCD} (ref. 22; renamed in ref. 23).

Growth Conditions. Cells were grown in Vogel–Bonner medium (24) containing the required amino acids at 1 mM and 50 mM glycerol (minimal glycerol medium) or in tryptone broth consisting of 1% Bactotryptone (Difco)/0.5% NaCl.

Electroporation Conditions. Bacteria were grown in tryptone broth by shaking them at 35°C until they reached an OD\textsubscript{590} value of 0.4–0.6. Then they were harvested by centrifugation at 6000 × g for 3 min. The supernatant fluid was discarded, the pellet was resuspended, and 5 ml of electroporation buffer (1 mM Hepes, pH 7.2/10% glycerol) was added. This procedure was followed by two more such washes in electroporation buffer, and finally the cells were suspended in 1 ml of electroporation buffer at an OD\textsubscript{590} value of 2–6. The washed cells were stored on ice until electroporation. In some experiments, cells were grown in minimal glycerol medium instead of tryptone broth, and then in all subsequent steps minimal glycerol medium replaced tryptone broth.

Fura-2 (25–100 μM) was introduced into cells (200-μl suspension) with electroporation by the use of a single pulse of electricity at a capacity of 25 μF with a field intensity of 5 kV/cm at 200 Ω for 4–6 ms in a Bio-Rad gene pulser. The resultant cytoplasmic concentration of fura-2 was about the same as the concentration of fura-2 added to the cells. Immediately after electroporation, cells were diluted with 1 ml of filtered used tryptone broth (in which E. coli had grown as described above) and incubated at room temperature for 15 min. Under these conditions 90% or more of the cells were still viable, they ran and tumbled, and they responded to repellents and attractants as judged microscopically. Cells were collected by centrifugation at 6000 × g for 3 min. The supernatant fluid was discarded, the pellet was resuspended, and 5 ml of HKM buffer (50 mM Hepes, pH 7.5/100 mM KC1/1 mM MgCl\textsubscript{2}) was added. This was followed by an additional wash in the same medium, and finally the cells were resuspended in HKM buffer at an OD\textsubscript{590} of 0.1 (~7 × 10\textsuperscript{7} bacteria per ml), and these cells were used for fluorescence measurements. In some experiments, HKM buffer was replaced by chemotaxis medium (10 mM potassium phosphate, pH 7.0/0.1 mM potassium EDTA).

Abbreviations: [Ca\textsuperscript{2+}], cytoplasmic free Ca\textsuperscript{2+} concentration; MCP, methyl-accepting chemotaxis protein.

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‡Tisa, L. S. & Adler, J., Annual Meeting of the American Society for Microbiology, May 28, 1992, New Orleans (abstr.); Second Meeting on Bacterial Locomotion and Signal Transduction, January 14–18, 1993, Austin, TX (abstr.); Third Meeting on Bacterial Locomotion and Signal Transduction, January 12–16, 1995, Austin, TX (abstr.).
0.1 mM l-methionine), and the results were similar to those obtained by the above procedure. We have evidence that electroporation loads fura-2 into the cytoplasm rather than primarily into the periplasm (31).

Alternatively, in some experiments, cells grown overnight in tryptone broth or minimal glycerol medium were used. Immediately after electroporation these cells were diluted into 10 ml of fresh growth medium of the same type and incubated at 35°C from an OD_{590} level of 0.05–0.1 up to 0.4–0.6. This procedure allows restoration of any small molecules lost during electroporation. Cells were collected by centrifugation at 6000 x g for 3 min. The supernatant fluid was discarded, the pellet was resuspended, and the same filtered used-growth medium or chemotaxis medium was added. This step was followed by an additional wash in either medium, and finally cells were resuspended in either medium at an OD_{590} value of 0.1.

**Fluorescence Measurements.** Fluorescence was measured at 30°C with an SLM Aminco spectrophotofluorimeter interfaced with a computer. During the measurements the cell suspension was continuously stirred. The standard monochromator settings were at wavelength 340 nm for excitation and 510 nm for emission; excitation slit widths were 8 nm and 4 nm, respectively. Data were collected in 200-ms intervals. Reagents were added (10–100 μl into a total volume of 2 ml) with a microliter syringe through a light-protected port.

**Measurement of [Ca^{2+}].** [Ca^{2+}] was calculated by use of the following equation (25):

\[
[Ca^{2+}] = K_d (F - F_{min})/(F_{max} - F),
\]

where \( F \) is the fluorescence intensity of dye in the cells, \( F_{max} \) and \( F_{min} \) are the intensities at saturating and zero Ca^{2+} concentrations, respectively, and \( K_d \) is the dissociation constant for Ca^{2+}. We determined that at 30°C fura-2 had a \( K_d \) for Ca^{2+} of 192 nM. After the measurement of fluorescence intensity, cells were disrupted by sonication. \( F_{max} \) was measured by addition of 1 mM CaCl₂, and to determine \( F_{min} \) 10 mM EGTA was added.

**Chemicals.** Fura-2 was purchased from Molecular Probes.

**RESULTS**

Cells were loaded with the fluorescent Ca^{2+}-indicator dye fura-2 by electroporation, and then fluorescence was monitored to reveal [Ca^{2+}] while different chemical repellents or chemical attractants were added to the cells.

**Measurements of [Ca^{2+}] in Chemotactically Wild-Type E. coli Responding to Repellents and Attractants.** Fig. 1 shows results for chemotactically wild-type cells (AW574). Repellents such as acetate, indole, or L-leucine (18), sensed by methyl-accepting chemotaxis protein I (MCP-I), also called Tsr (26, 27), increased [Ca^{2+}] in the wild type for a time that was brief, lasting only seconds, and then the level returned to near the steady-state level (Fig. 1 A–C). Addition of the attractants L-serine or L-aspartate (19), sensed by MCP-I and MCP-II, also called Tar, respectively (26, 27), transiently decreased [Ca^{2+}], which also returned to near steady-state level (Fig. 1 D and E). For L-serine, the complete return tended to be immediate (Figs. 1D and 4A). For L-aspartate, two degrees of return were seen. The first shows a rapid partial return (Fig. 1E); the second required several minutes for any return (see Fig. 5A). The addition of β-alanine, which is neither an attractant nor repellent (19), had no effect on [Ca^{2+}], (Fig. 1F).

The size of the response to any particular stimulus varied; for example, compare the response to indole in Figs. 1B and 2A or the response to L-leucine in Figs. 1C and 3A. This result may

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**FIG. 1.** Changes in cytoplasmic Ca^{2+} concentration in chemotactically wild-type (tar^{+} tar^{+} trg^{+} tap^{+}) E. coli (AW574) in response to repellents or attractants. Bacteria were loaded with fura-2 by electroporation as described. Cells were suspended in chemotaxis medium and with stirring continuously monitored for changes in fluorescence at excitation wavelength 510 nm and excitation wavelength 340 nm. At zero time (arrow), repellent or attractant was added to the cell suspension. (A) Sodium acetate (17 mM; repellent). (B) Indole (0.3 mM; repellent). (C) L-Leucine (10 mM; repellent). (D) L-Serine (1 mM; attractant). (E) L-Aspartate (1 mM; attractant). (F) β-Alanine (1 mM; neither attractant nor repellent).
be explained, in part, by the short duration of the response, which makes its full capture difficult.

Because the MCP-II repellents Ca\(^{2+}\), Mn\(^{2+}\), and Zn\(^{2+}\) quench fura-2 fluorescence (15), they were not tested. Repellents and attractants for MCP-III, also called Trg, or MCP-IV, also called Tap, were not tested.

The results of these experiments are summarized in column 1 of Table 1. Similar results were obtained with other chemotactically wild-type strains such as AW405 and AW607 (data not shown).

**Studies with Mutants Lacking One Particular Sensory Receptor.** A mutant lacking any one particular receptor was presented with a stimulus for that missing receptor, and it showed no change in [Ca\(^{2+}\)] (Figs. 2D, 3D, 4D, and 5D and the last column of Table 1).

**Chemotactically wild-type was AW574; in some experiments AW405 and AW607 were also used.**†, Transient increase in cytoplasmic Ca\(^{2+}\); †, transient decrease in cytoplasmic Ca\(^{2+}\); †, no change in cytoplasmic Ca\(^{2+}\).

**Studies with Mutant Lacking Four Sensory Receptors.** Strain CP362 lacks four sensory receptor proteins (MCP-I, -II, -III, and -IV), and consequently does not respond to chemical stimuli sensed by them (21). The effect of stimuli on the [Ca\(^{2+}\)] in strain CP362 was tested. In the absence of these four sensory receptors, attractants or repellent sensed by them caused no change in [Ca\(^{2+}\)].

**Table 1.** Effect of repellents or attractants on cytoplasmic Ca\(^{2+}\) of chemotactically wild-type and mutant strains of *E. coli*

<table>
<thead>
<tr>
<th>[Ca(^{2+})]</th>
<th>Chemotactically wild type</th>
<th>tsr(^{-})</th>
<th>tar(^{+})</th>
<th>tsr(^{-})tar(^{+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate</td>
<td>(17 mM)</td>
<td>†</td>
<td>—</td>
<td>†</td>
</tr>
<tr>
<td>L-Leucine (10 mM)</td>
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<tr>
<td>L-Indole (0.3 mM)</td>
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<tr>
<td>L-Serine (1 mM)</td>
<td>†</td>
<td>—</td>
<td>—</td>
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</tr>
<tr>
<td>L-Aspartate (1 mM)</td>
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<td>—</td>
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<tr>
<td>β-Alanine (1 mM)</td>
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</tbody>
</table>

Chemotactically wild-type was AW574; in some experiments AW405 and AW607 were also used.†, Transient increase in cytoplasmic Ca\(^{2+}\); †, transient decrease in cytoplasmic Ca\(^{2+}\); †, no change in cytoplasmic Ca\(^{2+}\).
DISCUSSION

[Ca^{2+}], values were measured directly by using electroporated fluorescent Ca^{2+}-indicator dye fura-2. The addition of a repellent, which promotes tumbling, transiently increased [Ca^{2+}]. The addition of an attractant, which promotes running, transiently decreased [Ca^{2+}]. These results are compatible with others presented recently, which were obtained with the Ca^{2+}-activated photoprotein aequorin (16).

In our report, chemotactically wild-type cells showed these changes in Ca^{2+} levels for all repellents and attractants tested. Experiments with mutant strains lacking particular receptors (MCPs) illustrate that these changes in [Ca^{2+}] depend on the presence of the appropriate receptor (Table 1).

The changes in [Ca^{2+}] in response to chemoeffectors typically lasted only a few seconds (except for L-aspartate where it was sometimes longer for reasons not understood). However, the behavioral responses to these chemoeffectors at these concentrations are much more prolonged, lasting from 30 s to several minutes.

How an increase in cytoplasmic Ca^{2+} is associated with tumbling and a decrease is associated with running remains to be determined. Elevation of Ca^{2+} could result in maintaining CheY in its phosphorylated state and thereby stimulate tumbling, whereas decreased Ca^{2+} could result in keeping CheY unphosphorylated to stimulate running. Some published results support this hypothesis (28).

Aside from the question of the site of action of Ca^{2+}, another question is the following: Where does the Ca^{2+} come from and go to? There are two possibilities. (i) Ca^{2+} comes from and goes to a site outside the cytoplasm and thereby enters or exits the cytoplasm. Accordingly, a Ca^{2+} entry/exit mechanism must be coupled to some interaction with the receptors because the receptors are required for a change in [Ca^{2+}]. (ii) A Ca^{2+}-binding mechanism exists inside the cytoplasm and is the immediate source or sink of Ca^{2+}; this binding site is coupled to some interaction with the receptors. In the latter case, the Ca^{2+} entry and exit mechanisms are of secondary importance, functioning to keep the necessary [Ca^{2+}] available.

The changes in cytoplasmic Ca^{2+} brought about by repellents and attractants in bacteria have their analogs in eukaryotic microorganisms (32-36) and plants: "It is now clear that many physical and hormonal signals (touch, wind, gravity, light, cold, auxin, gibberellic acid, abscisic acid, salt, and fungal elicitors) induce rapid changes in cytosolic Ca^{2+} levels and these changes precede the physiological responses. Although the signals induce an increase in cytosolic Ca^{2+} in a majority of cases, there are instances where the cytosolic Ca^{2+} level is decreased by signals" (29).

Also in animals Ca^{2+}-mediated signal transduction is a common occurrence (for review, see ref. 30).

We thank G. L. Hazelbauer and J. S. Parkinson for bacterial strains. This investigation was supported by National Science Foundation Grant BNS-8804849 and by a grant from the Lucille P. Markey Charitable Trust. The work was helped also by a postdoctoral fellowship to L.S.T. from the U.S. Public Health Service, National Institutes of Health (F32-GM12187).