Attractant-induced Changes and Oscillations of the Extracellular Ca\(^{++}\) Concentration in Suspensions of Differentiating Dictyostelium Cells

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ABSTRACT We used a Ca\(^{++}\)-sensitive electrode to measure changes in extracellular Ca\(^{++}\) concentration in cell suspensions of Dictyostelium discoideum during differentiation and attractant stimulation. The cells maintained an external level of 3-8 \(\mu\)M Ca\(^{++}\) until the beginning of aggregation and then started to take up Ca\(^{++}\). The attractants, folic acid, cyclic AMP, and cyclic GMP, induced a transient uptake of Ca\(^{++}\) by the cells. The response was detectable within 6 s and peaked at 30 s. Half-maximal uptake occurred at 5 nM cyclic AMP or 0.2 \(\mu\)M folic acid, respectively. The apparent rate of uptake amounted to \(2 \times 10^7\) Ca\(^{++}\) per cell per min. Following uptake, Ca\(^{++}\) was released by the cells with a rate of \(5 \times 10^6\) ions per cell per min. Specificity studies indicated that the induced uptake of Ca\(^{++}\) was mediated by cell surface receptors. The amount of accumulated Ca\(^{++}\) remained constant as long as a constant stimulus was provided. No apparent adaptation occurred. The cyclic AMP-induced uptake of Ca\(^{++}\) increased during differentiation and was dependent on the external Ca\(^{++}\) concentration.

During development the extracellular Ca\(^{++}\) level oscillated with a period of 6-11 min. The change of the extracellular Ca\(^{++}\) concentration during one cycle would correspond to a 30-fold change of the cellular free Ca\(^{++}\) concentration.

The development of the cellular slime mold, Dictyostelium discoideum, to a multicellular organism is mediated by extracellular cyclic AMP signals (1-3). It is assumed that cyclic AMP is secreted periodically by center cells. Neighboring cells react chemotactically and relay the signal (4-6). Signal transduction is initiated by binding of cyclic AMP to cell surface receptors (7-10). Fast cellular responses are an increase of the intracellular cyclic GMP level (11, 12), which occurs due to the activation of guanylate cyclase (13), a transient uptake of \(^{45}\)Ca\(^{++}\) (14), a decrease of the extracellular pH (15), and a dephosphorylation of myosin heavy chain (16, 17). Adenylate cyclase is activated (18) and cyclic AMP is synthesized and secreted in the extracellular medium (19, 20).

Calcium ions in the extracellular medium are reported to enhance center formation (21) and the speed of development (22). Also, Ca\(^{++}\) is apparently involved in the process of contraction and assembly of actin filaments (23-25) and in the regulation of myosin heavy chain kinase (17, 26) and adenylate cyclase (27, 28). Here we extend our studies on the role of extracellular Ca\(^{++}\) during attractant-induced excitation and differentiation using a Ca\(^{++}\)-sensitive electrode (29).

MATERIALS AND METHODS

Chemicals: Cyclic AMP, cyclic GMP, 5\(^{i}\) AMP, ADP, ATP, adenosine were purchased from Boehringer, Mannheim Biochemicals (Federal Republic of Germany). Folic acid and analytical grade EGTA were obtained from SERVA (Heidelberg, Federal Republic of Germany). Cyclic AMP \(^{32}\) tracer and cyclic AMP antiserum were brought from NEN (Dreieich, Federal Republic of Germany). Adenosine 3',5'-cyclic phosphorothioate (cyclic AMP) was a gift of Dr. F. Eckstein, Max-Planck-Institut für Experimentelle Medizin (Göttingen, Federal Republic of Germany); nitrotetrazolium acid was obtained analytical grade from Fluka (Buchs, Switzerland), and dithiothreitol was from Sigma Chemical Co. (Munich, Federal Republic of Germany).

Culture Conditions: D. discoideum strain Ax-2 and a morphogenetic mutant of Ax-2, agi53, were used. Agi53 was the gift of P. Brachet, Pasteur Institute (Paris, France).

Cells were grown on nutrient medium supplemented with 1.8% maltose.
(30). Streptomycin (20 μg/ml) was added to cultures of agip 53. Growing cells (3–8 \times 10^6 cells/ml) were induced to differentiate by washing three times with 5 mM tricine (NTris(hydroxymethyl)-methyl)glycine) pH 7.0, 5 mM KCl, resuspending at 2 \times 10^9 cell/ml in the same buffer, and shaking at 150 rpm, 23°C, until use. The time, in hours, following induction of differentiation was designated \( t_0 \). Beginning at \( t_0 \), Ax-2 cells started to aggregate and formed EDTA-resistant cell contacts (31). To test for EDTA-resistant cell contacts, cells were examined microscopically following a 30-min incubation in buffer containing 10 mM EDTA. During incubation in Sorensen phosphate buffer pH 6.0, agip 53 cells do not develop any characteristics of aggregation competence (2). When agip 53 cells were treated with 20-nM pulses of cyclic AMP at intervals of 6 min from \( t_0 \) to \( t_5 \), however, they acquired the capacity to form EDTA-stable cell contacts (2). At \( t_5 \) the cells were washed and resuspended in 5 mM tricine buffer pH 7.0, 5 mM KCl.

**Ca** \(^{2+} \) Determination: Ca** \(^{2+} \) was measured using a calcium-ion-sensitive electrode (29) obtained from W. Möller (Zurich, Switzerland). Electrode potentials were recorded using a Metrohm pH-meter E 510 (Herisau, Switzerland). The electrode was of the following type: Ag-AgCl, 10 mM CaCl\(_2\), membrane sample, 3 M KCl, Ag-AgCl. The membrane consisted of a neutral carrier (N,N'-Di[1-ethylcarboxy]lundecylen,N,N',4,5-tetramethyl-3,6-dioxoacetamide) in o-nitrophenyl-o-octylether as membrane component in a polyvinyl chloride (PVC) matrix (29). The electrode was specific for Ca** \(^{2+} \) (32).

The selectivity of Ca** over K\(^+\), Na\(^+\), H\(^+\), and Mg** in cell suspension was 9,000, 4,500, 1,000 and 40, respectively. The tricine/KCl buffer contained 0.2–0.8 mM Ca**. Cyclic AMP addition to the buffer did not cause a significant millivolt change. The response time of the electrode was tested. When the Ca** concentration was raised from 10 to 100 μM the electrode indicated 95% of the total change within 2 s. Ca** sensitive membranes were usually used for \~ 1 ms. Calibration curves in water and in 5 mM tricine buffer pH 7.0, 5 mM KCl, were linear from 0.2 to 10,000 μM Ca** with a slope of 28–30 mV per one decade of Ca** concentration. In control experiments at ionic strength of 0.1 nitrotriacetic acid was used to prepare calibration buffers with 0.1–100 mM Ca**. The following constants were used to calculate free Ca** concentrations: log K\(_{1}\) = 9.73, log K\(_{2}\) = 2.49, log K\(_{3}\) = 1.89, log K\(_{4}\) = 6.46 (33, 34). EGTA was used to make buffers with 0.1–11 mM Ca**; log K\(_{5}\) = 6.68 (pH 7.0, 35). Measurements of Ca** uptake and exit were performed in a thermostated glass vessel at 23°C. 5–20 \times 10^6 cells/ml were stirred by means of a magnetic stirrer and were aerated with 20 ml oxygen per min. When a known amount of Ca** was added to a cell suspension the increase in free Ca** was less than expected. This Ca** increase was less than expected.

**Cyclic AMP Determination:** Extracellular cyclic AMP in cell suspensions was measured by radioimmunoassay as described (12). Samples of the cell suspension were made 10 mM of dithiothreitol, centrifuged for 15 s (Eppendorf, microfuge; Hamburg, Federal Republic of Germany), and supernatants inactivated by addition of equal volumes of 2 N perchloric acid. 10 mM dithiothreitol efficiently inhibited hydrolysis of 4 \times 10^{-6} [H]cyclic AMP by culture supernatant of agip 53 (19 nmol \times min^{-1} \times ml^{-1}); after incubation for 50 s at 23°C, 94.5% of [H]cyclic AMP was left.

**RESULTS**

**Changes of Extracellular Ca** \(^{2+} \) Concentration During Differentiation to Aggregation Competence**

After initiation of differentiation amoebae released Ca** to a concentration of 3–8 μM within the time of first measurement (Fig. 1). This level was maintained until EDTA-stable cell contacts began forming at about \( t_5 \). Subsequently, the extracellular Ca** concentration decreased by about two-thirds within 4 h, possibly indicating that Ca** was taken up by the cells. During these 4 h, amoebae became fully competent for aggregation.

**Attractants Induce a Transient Uptake of Calcium Ions**

Folic acid attracts cells of the early preaggregation phase only (36). As shown in Fig. 2a, folic acid elicited a rapid uptake of external Ca** in \( t_5 \) cells. The extracellular Ca**

**FIGURE 1** Extracellular Ca** \(^{2+} \) concentration during development to aggregation competence.

The cell density was 5 \times 10^6 cells per ml.
FIGURE 3 Cyclic AMP-induced change of the extracellular Ca** concentration during development. (upper panel) 0.1 μM cyclic AMP was added to the cell suspension at various developmental times at time zero. Recorder tracings of 2 x 10⁶ cells/ml are shown. (lower panel) The induced change in the extracellular Ca** concentration at various developmental times was plotted as a function of developmental time. This change is the difference between the extracellular Ca** level before and the minimum extracellular level after stimulus.

Specificity of Cyclic AMP-induced Uptake of Ca**

The amount of Ca** taken up by the cells depended on the concentration of cyclic AMP added. This is shown for aggregation-competent cells in Fig. 4. The half-maximal response was obtained at about 5 nM cyclic AMP. Uptake began to saturate at 0.1 μM cyclic AMP and amounted to 1.6 nmol per 5 x 10⁶ cells, or 1.9 x 10⁻⁶ ions per cell. A small further increase in Ca** uptake occurred up to 10 μM cyclic AMP.

Table 1 shows that 100 μM cyclic GMP elicited the same change of the extracellular Ca** concentration as 0.1 μM cyclic AMP. Cyclic AMPS and cyclic GMP are agonists of cyclic AMP binding to the cell surface. Their affinities are, however, 10-fold and 1,000-fold lower, respectively, than that for cyclic AMP (37, 8). 5' AMP, adenosine, ADP, and ATP did not cause a measurable uptake of Ca**. These results indicate that the specificity of the cyclic AMP-induced uptake of Ca** apparently reflects the specificity of cyclic AMP binding sites (7–9, 37).

A dose response curve for the folic acid-induced Ca** response of t₁ cells is also shown in Fig. 4. Half-maximal stimulation appears to occur at 0.2 μM folic acid.

Significance of External Ca** Concentration

Fig. 5 shows that the cyclic AMP-induced uptake of external Ca** increases with the extracellular Ca** concentration until ~10 μM and then remains approximately constant. Addition of EGTA inhibited the induced uptake of Ca** by lowering free external Ca** concentrations. At 0.3 μM external Ca**, no uptake could be detected (data not shown).

Ca** Uptake in Response to Constant Cyclic AMP Stimuli

In the preceding experiments changes in the Ca** concentration were measured in response to one-step additions of attractants. Such additions result in an instantaneous increase of the attractant concentration in the suspension followed by a concentration decrease as the attractant is inactivated (38). To characterize a chemoreception system one would like to know how it responds to constant attractant concentrations. To answer this question experiments were performed with mutant agp 53, which does not synthesize cyclic AMP in

![Diagram](image-url)

FIGURE 4 Dose response curves for cyclic AMP and folic acid-induced uptake of Ca**, 5 x 10⁶ cells per ml were assayed for cyclic AMP-induced Ca** changes (x, o) at t₀ and 2 x 10⁸ cells per ml were tested for cyclic acid-induced Ca** changes at t₁. During one experiment (x) the extracellular Ca** concentration declined from 1.8 to 1 μM. All values were adjusted to 1.8 μM, assuming a linear relationship (see Fig. 5). In the other experiments (o, g) the extracellular Ca** concentration was 4 μM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Change of the external Ca** concentration</th>
<th>No. of experiments</th>
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<tr>
<td></td>
<td>μM</td>
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<tr>
<td>Cyclic AMP</td>
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<td></td>
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In each experiment the uptake of Ca** elicited by 0.1 μM cyclic AMP served as 100% control value. 2 x 10⁶ cells were tested at t₀. ADP and ATP caused a small, immediate reduction of the external Ca** concentration when added to the buffer, probably due to complexation of Ca**. The same change, but no additional change, was observed when ADP or ATP was added to cell suspensions. nd, not detectable.

* ± SD.
response to external stimulation (39). Because of hydrolysis of cyclic AMP by cell-bound and extracellular phosphodiesterases, continuous addition of cyclic AMP to a cell suspension leads to an increase of the extracellular cyclic AMP concentration up to a constant steady-state level (40). In the experiment shown in Fig. 6, 500 nmol cyclic AMP per min was supplied to a suspension of differentiated agip53 cells. Within 30 s a constant extracellular cyclic AMP concentration of 21 ± 2 nmol per liter cell suspension was attained. The continuous supply of cyclic AMP caused a rapid decrease of the extracellular Ca" concentration to a constant low level. External Ca" remained at this low level as long as the addition of cyclic AMP was continued. As soon as the supply was stopped, the extracellular Ca" concentration increased to a new level, which exceeded the prestimulation level in many experiments.

Oscillation

During differentiation D. discoideum cells acquire the capacity to synthesize and release cyclic AMP in a periodic manner (41). Fig. 7 shows that the external Ca" concentration oscillates as well. Oscillations occurred with a period of 8 min and an amplitude of ~3.5 × 10^6 ions per cell. In other experiments periods of 6–11 min were observed.

DISCUSSION

Saito (42) denies the importance of extracellular Ca" for early differentiation of D. discoideum, whereas other authors report that the speed of aggregation and the formation of aggregates is enhanced by extracellular Ca" (21, 22). We found that amoebae released Ca" at the onset of differentiation, and that a constant level of extracellular Ca" was maintained until the formation of EDTA-stable cell contacts began. During aggregation Ca" was taken up by the cells (Figs. 1 and 3). Addition of 100 µM LaCl₃, an inhibitor of Ca" uptake, at t₀ blocked differentiation (D. Malchow, unpublished results).

It has been shown that cyclic AMP induces uptake and release of Ca" (14). In that study the possibility could not be ruled out that an exchange of Ca" took place. By means of a Ca"-sensitive electrode we found that 0.1 µM cyclic AMP elicits a transient uptake of 1.9 × 10^7 calcium ions per cell of 2 × 10^6 cells per ml at t₀. The amount of Ca" taken up per cell is smaller than in Fig. 4. The induced uptake of Ca" does not increase linearly with cell concentration, presumably because of the formation of aggregates.
cell (Fig. 4). The ions taken up can either be bound to new Ca\textsuperscript{2+} binding sites at the cell surface or cause an increase of the intracellular Ca\textsuperscript{2+} concentration or both. The following arguments taken together tend to favor an influx of calcium ions: (a) Electron microscopic studies revealed an increased formation of calcium deposits inside the plasma membrane during migration (43, 44). (b) The Ca\textsuperscript{2+} ionophore A23 187, applied locally at the cell surface, induced changes of cell shape and orientation (45). (c) The number of calcium ions taken up during stimulation corresponds to a hundred-fold excess of Ca\textsuperscript{2+} over a total of 2 × 10\textsuperscript{5} cyclic AMP binding sites at the cell surface.

Specificity and developmental dependence of the attractant-induced uptake of Ca\textsuperscript{2+} indicate its regulation by attractant receptors. The phase of unresponsiveness to folic acid and cyclic AMP between t\textsubscript{1} and t\textsubscript{2} may reflect an uncoupling of the Ca\textsuperscript{2+} transport system from the receptors. Subsequently, a new coupling to cyclic AMP receptors may occur.

At external Ca\textsuperscript{2+} concentrations above 10 \(\mu\text{M}\) the induced uptake of Ca\textsuperscript{2+} does not increase further with increasing external Ca\textsuperscript{2+} (Fig. 5). A similar situation is observed in other systems, for example in barnacle muscle, where the Ca\textsuperscript{2+} current saturates at large external Ca\textsuperscript{2+} concentrations (46). At external Ca\textsuperscript{2+} concentrations below 10 \(\mu\text{M}\) the induced uptake of Ca\textsuperscript{2+} decreases with declining external Ca\textsuperscript{2+} concentrations (Fig. 5).

A mechanism that can account for these observations consists of (a) a bidirectional Ca\textsuperscript{2+} channel that is opened upon binding of attractants to their receptors, and (b) an outward oriented Ca\textsuperscript{2+} pump. In case of attractant stimulation at external Ca\textsuperscript{2+} greater than internal Ca\textsuperscript{2+} there will be a net influx of Ca\textsuperscript{2+} through the channel and a release of Ca\textsuperscript{2+} by the outward oriented pump. At external Ca\textsuperscript{2+} equal to internal Ca\textsuperscript{2+} no net flux will occur. Such an equilibrium situation may exist at an external Ca\textsuperscript{2+} concentration of ~0.3 \(\mu\text{M}\).

The cyclic AMP-induced uptake of Ca\textsuperscript{2+} may cause a temporary increase of the cytosolic Ca\textsuperscript{2+} concentration, and this signal may regulate other cellular reactions such as actin reorganization (24, 25), dephosphorylation of myosin heavy chains (16, 17, 26), and cyclic AMP synthesis (27, 28, 41). Contraction of actomyosin in cell-free extracts of \textit{D. discoideum} has been shown to occur in the presence of micromolar Ca\textsuperscript{2+} concentrations (23).

How rapidly does the cellular Ca\textsuperscript{2+} concentration increase to micromolar concentrations? Calculation of the cell volume yields 5.2 × 10\textsuperscript{-10} cm\textsuperscript{3} (\(r = 5 \mu\text{M}\)). Uptake of 1.9 × 10\textsuperscript{7} Ca\textsuperscript{2+} per 30 s (Fig. 4) corresponds to an increase of 1.9 × 10\textsuperscript{7} × 10\textsuperscript{-10} = 3.6 × 10\textsuperscript{-6} calcium ions per cm\textsuperscript{3} or 3.6 × 10\textsuperscript{-6} × 1 M/6 × 10\textsuperscript{-20} = 60 \(\mu\text{M}\) Ca\textsuperscript{2+}. That means that the cellular Ca\textsuperscript{2+} concentration could rise from ~0.3 \(\mu\text{M}\) to 10.3 \(\mu\text{M}\) in 5 s if uptake is linear. A fast contraction of the cells occurs within a few seconds after cyclic AMP stimulation (47) and lasts ~30 s (48), a time consistent with this calculation and duration of Ca\textsuperscript{2+} uptake.

Continuous addition of cyclic AMP induces a decrease of the extracellular Ca\textsuperscript{2+} concentration to a constant low level. The Ca\textsuperscript{2+} concentration remains at this level as long as the cyclic AMP concentration is maintained constant at the steady-state level (Fig. 6). This result indicates that the change of the extracellular Ca\textsuperscript{2+} concentration does not adapt to constant cyclic AMP concentrations. The experiment of Fig. 6 was performed with mutant agp 53. Cells of the parent strain Ax-2 responded to continuous addition of cyclic AMP in a similar manner except that during the first 5 min there was an additional, transient Ca\textsuperscript{2+} uptake. The latter is probably due to cyclic AMP that Ax-2 cells release in response to the added cyclic AMP. In contrast to the apparent lack of adaptation at the level of Ca\textsuperscript{2+} changes, cyclic AMP-stimulated cyclic AMP synthesis completely adapts to constant cyclic AMP concentrations (6, 49). Partial adaptation has been observed for cyclic GMP synthesis in response to constant cyclic AMP stimuli (40).

Adaptation of cyclic AMP synthesis may be a consequence of the nonadapting Ca\textsuperscript{2+} uptake. It has been shown that elevated Ca\textsuperscript{2+} concentrations inhibit cyclic AMP synthesis (27, 28, 41). At present, however, there is still a lack in the understanding of temporal sequences. The uptake of Ca\textsuperscript{2+} is relatively rapid, with a maximum at 30 s, whereas cyclic AMP synthesis peaks 90 s following excitation (38). Since cyclic AMP may be synthesized in vesicles (20), it seems possible that there is a further delay before Ca\textsuperscript{2+} can accumulate in vesicles involved in cyclic AMP synthesis.

Periodic stimulation is important for early differentiation of \textit{D. discoideum}. It has been shown that aggregation of a class of nonaggregating mutants can be induced by pulsatile addition of cyclic AMP (2). Several oscillating parameters of similar period length have been described. These include concentrations of internal cyclic AMP and cyclic GMP, external cyclic AMP, pH (50), carbondioxide (51), and, as shown here, external Ca\textsuperscript{2+}. Although the oscillations in external Ca\textsuperscript{2+} are quite small they may indicate oscillations of the cellular Ca\textsuperscript{2+} concentration of an amplitude of ~10 \(\mu\text{M}\). That means that the cellular Ca\textsuperscript{2+} concentration may rise 30-fold during one oscillatory cycle. Further work has to establish the relationship of the Ca\textsuperscript{2+} oscillations to those of cyclic AMP and cyclic GMP.

We thank V. Nanjundiah and M. Manson for critical reading of the manuscript and helpful comments. We are grateful to B. Schittenhelm for typing the manuscript and to the Deutsche Forschungsgemeinschaft for support.

Received for publication 19 January 1983, and in revised form 2 September 1983.

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