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Excitation, Adaptation, and Deadaptation of the cAMP-Mediated cGMP Response in Dictyostelium discoideum

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ABSTRACT Extracellular cAMP induces chemotaxis and cell aggregation in Dictyostelium discoideum cells. cAMP added to a cell suspension is rapidly hydrolyzed (half-life of 10 s) and induces a rapid increase of intracellular cGMP levels, which reach a peak at 10 s and recover prestimulated levels at about 30 s. This recovery is not due to removal of the stimulus because the nonhydrolyzable analogue adenosine 3',5'-monophosphorothioate-Sp-stereoisomer (cAMPS) induced a comparable cGMP response, which peaked at 10 s, even at subsaturating cAMPS concentrations.

When cells were stimulated twice with the same cAMP concentration at a 30-s interval, only the first stimulus produced a cGMP response. Cells did respond to the second stimulus when the concentration of the second stimulus was higher than that of the first stimulus. By increasing the interval between two identical stimuli, the response to the second stimulus gradually increased. Recovery from the first stimulus showed first-order kinetics with a half-life of 1-2 min.

The stimulation period was shortened by adding phosphodiesterase to the cell suspension. The cGMP response was unaltered if the half-life of cAMP was reduced to 2 s. The peak of the transient cGMP accumulation still appeared at 10 s even when the half-life of cAMP was 0.4 s; however, the height of the cGMP peak was reduced. The cGMP response at 10 s after stimulation was diminished by 50% when the half-life of 10^{-7} M cAMP was 0.5 s or when the half-life of 10^{-8} M cAMP was 3.0 s.

These results show that the cAMP signal is transduced to two opposing processes: excitation and adaptation. Within 10 s after addition of cAMP to a cell suspension the level of adaptation reaches the level of excitation, which causes the extinction of the transduction of the signal. Deadaptation starts as soon as the signal is removed, and it has first-order kinetics with a half-life of 1-2 min.

The cellular slime mold Dictyostelium discoideum lives in the soil where it feeds on bacteria. After exhaustion of the food supply the amoebae pass an interphase, followed by cell aggregation and the formation of a fruiting body consisting of dead stalk cells, and spores embedded in a slime droplet, on top of them. Cell aggregation is mediated by chemotaxis to cAMP (8). Aggregating cells probably detect cAMP by cell surface receptors (9).

Addition of cAMP to aggregative cells induces several responses such as the entrance of calcium, an increase of cGMP levels, the methylation of proteins and phospholipids, the accumulation of dephosphorylated myosin heavy chains, the excretion of protons, and the production and excretion of cAMP (for a review, see reference 26).

The detection and analysis of chemotactic signals by the slime molds is poorly understood. Investigations of the cAMP-mediated production and excretion of cAMP revealed that this relay response is controlled by an adaptation process (4-6); cells react to an increase of the cAMP concentration, but accommodate to constant concentrations by extinguishing the relay response. Adaptation of the relay response is essential during cell aggregation in D. discoideum (23). However, the
properties of the relay response cannot be simply transferred to the detection of chemotactic signals since (a) many species do not have a relay mechanism although they react chemotactically (e.g., *D. lacteum* and *D. minutum*) and (b) adaptation of the relay response is a relatively slow process (several minutes), whereas directed pseudopod formation is very fast (about 5 s [7]).

Chemotactic stimulation induces an increase of cGMP levels within 2 s; cGMP levels reach a peak at 10 s and prestimulated levels are recovered at about 30 s after stimulation [12]. Several other chemotactants such as folic acid (19), pterin (20), and partially purified active extracts that attract specifically *Dictyostelium lacteum* (15), or *Polysphondylium violaceum* (28), induce similar transient elevations of cGMP levels in sensitive cells [10, 16, 27]. The involvement of cGMP during chemosensory transduction is further suggested by mutants that have altered cGMP metabolism and altered chemotactic behavior [11, 21].

To investigate the way cells detect chemotactic signals we measured the cGMP response of aggregative *D. discoideum* cells to cAMP under a variety of dynamic conditions of the cAMP stimulus. The results show that the transduction of the signal is rapidly (within 10 s) terminated by an adaptation process. Cells remain adapted as long as the stimulus is present, and they immediately start to deadapt after removal of the stimulus. Deadaptation is relatively slow and shows first-order kinetics with a half-life of 1–2 min.

**MATERIALS AND METHODS**

**Chemicals:** cAMP was purchased from Boehringer Mannheim Biochemicals (Mannheim, W. Germany); (8-<sup>H</sup>)cAMP (0.9 TBq/m mole) and the cGMP radio-immunoassay were obtained from Radiochemical Centre (Amersham, England). Snake venom (Ophiophagus hannah) was obtained from Sigma Chemical Co. (St. Louis, MO). Adenosine 3′,5′-monophosphorothioate-8′-stereoisomer (cAMPS) (2) was a generous gift of Drs. Baraniak and Stec.

**Culture Conditions:** *D. discoideum* NC-4 (H) was grown in association with Escherichia coli B/r (13) on a solid medium containing 3.3 g peptone, 3.3 g glucose, 4.5 g KH<sub>2</sub>PO<sub>4</sub>, 1.5 g NaH<sub>2</sub>PO<sub>4</sub>-H<sub>2</sub>O, and 15 g agar per liter. Late log phase cells were harvested in 10 mM NaH<sub>2</sub>PO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6.0 (PB) and freed from bacteria by centrifuging three times at 150 g for 4 min. Cells were stored on nonnutrient agar (15% agar in PB) at a density of 1.5 × 10<sup>7</sup> cells/cm<sup>3</sup>. After 4.5 h, cells were collected, washed twice with PB and, unless mentioned otherwise, suspended in PB at a density of 10<sup>7</sup> cells/ml. Air was bubbled through the suspension at a rate of 75 ml/h/l. After 20 h the cells were separated from the suspension by centrifugation for 4 min at 100 × g and washed twice with PB to remove contaminating lipids. Late log phase cells were harvested in 10 mM NaH<sub>2</sub>PO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6.0 (PB) and freed from bacteria by centrifuging three times at 150 g for 4 min. Cells were starved on nonnutrient agar (15% agar in PB) at a density of 1.5 × 10<sup>7</sup> cells/cm<sup>3</sup>. After 4.5 h, cells were collected, washed twice with PB and, unless mentioned otherwise, suspended in PB at a density of 10<sup>7</sup> cells/ml. Air was bubbled through the suspension (150 ml/min) for at least 10 min.

**Cell Stimulation and cGMP Assay:** Cells were stimulated under a variety of conditions; the exact protocols are given in the legends of the figures. Log phase cells were harvested in 10 mM NaH<sub>2</sub>PO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6.0 (PB) and lysed while vigorously shaken. Isolation of Cyclic Nucleotide Phosphodiesterase: Phosphodiesterase was isolated from starved *D. discoideum* cells as described previously [18]. About 2 × 10<sup>9</sup> vegetative cells were suspended in 200 ml PB and incubated at 22°C on a rotary shaker at 150 rpm. cAMP was added to this suspension at a rate of 75 µmol/h/l. After 20 h the cells were separated from the supernatant by centrifuging for 4 min at 250 g. The subsequent experiments were carried out at 4°C. The supernatant was centrifuged for 15 min at 30,000 g. Ammonium sulfate (440 g/l) was slowly added to the newly obtained supernatant. After 3 h the precipitate was collected by centrifugation for 30 min at 30,000 g. The pellets were combined and resuspended in 40 ml of 10 mM phosphate buffer, pH 7.0 (PB7), containing 2.5 M NaH<sub>2</sub>PO<sub>4</sub> and centrifuged again. The new pellet was dissolved in 5 ml of PB7 and concentrated to 0.5 ml by Minicon B15 (Amicon, Oosterhout, the Netherlands). The concentrate was washed twice with 4.5 ml of PB7 on Minicon B15. The final concentration was diluted with PB7 to 1.3 ml. The preparation contained 2.0 mg protein/ml and it hydrolyzed about 1,500 nmoI cAMP/min/mg protein at 10<sup>-3</sup> M substrate concentration. The activity is reduced about 50% after storage at −20°C for two months.

**Phosphodiesterase Assay:** 100-µl aliquots of aggregative *D. discoi-
after the addition of the stimulus. The mechanism by which signal transduction terminates was investigated by cell stimulation at 0 s and 30 s with different cAMP concentrations and detection of the cGMP levels during 60 s. In those cases in which cGMP levels changed, the peak was always reached at about 10 s after stimulation. A summary of the results has been shown in Fig. 2.

Cell stimulation with different cAMP concentrations at 0 s and 30 s (A and O) was corrected for 16% recovery of responsiveness (see Fig. 4C). The results shown are the mean of three experiments. The subsaturating concentration had no effect on the response to the saturating concentration, which makes the existence of a short absolute refractory period unlikely (data not shown).

Although the previous experiments point to an adaptation process, they do not exclude the possibility of a short absolute refractory period that lasts less than 30 s. This is important to investigate, because cells extrude a pseudopod to a capillary filled with cAMP within 5 s (7). Therefore, cells were stimulated with a subsaturating cAMP concentration (10^{-9} M) at 0 s, followed by a saturating cAMP concentration (10^{-7} M) at 5 s. The subsaturating concentration had no effect on the response to the saturating concentration, which makes the existence of a short absolute refractory period unlikely (data not shown).

Cell aggregation in vivo is a dynamic oscillating process with periodicity of about 5 min (23), and with a fast detection of the cAMP concentration (7). Therefore, the kinetics of signal detection and recovery from stimulation were measured.

The Kinetics of Signal Transduction to Excitation and Adaptation

The rate of signal transduction was investigated by adding various amounts of phosphodiesterase activity to the cell suspension, followed by stimulation with 10^{-7} M cAMP. 30 s after the addition of cAMP, the slowly hydrolyzable analogue cAMPS was added. The cGMP responses to these two stimuli were measured. The rationale behind this experiment is that the first stimulus (cAMP) has different half-life periods; the cGMP response reveals the rate of entrance of the signal to excitation. The second stimulus (cAMPS) is slowly hydrolyzable and saturating. The response to this stimulus reveals the...
level of adaptation caused by the first stimulus and thus shows the rate of entrance of the signal to adaptation.

The half-life periods of the cAMP stimuli (10^{-7} M) were
determined from the hydrolysis of 10^{-7} and 10^{-9} M 3H-cAMP
at 3 s after their addition (Fig. 3A); for calculations, we assume
Michaelis-Menten kinetics with a Km equal to 1 μM (24). Fig.
3B shows that addition of phosphodiesterase to the cell sus-
pension reduces the cGMP response to 10^{-7} M cAMP. Two
controls show that this is due to the increased hydrolysis
velocity of cAMP, and not to other components of the phos-
phodiesterase preparation; boiled phosphodiesterase does not
reduce the response to cAMP, and active phosphodiesterase
does not reduce the response to the slowly hydrolyzable ana-
logue cAMPS. Fig. 3B also reveals that the response to the
second slowly hydrolyzable stimulus (cAMPS) increases if the
first stimulus (cAMP) is hydrolyzed faster. Apparently, by the
addition of phosphodiesterase, cAMP is not present for suffi-
cient time to achieve complete transduction of the signal to
excitation and to adaptation. In the experiment of Fig. 3B the
cGMP levels were determined only at 10 s after stimulation.
The results of Fig. 3C show that this is justified; cGMP levels
still peak at 10 s after stimulation even if 50% of the first
stimulus is hydrolyzed after 0.4 s.

The results of the experiments as shown in Figs. 3A and 3B
are combined in Fig. 3D. This reveals that cAMP has to be
present for only a few seconds to achieve complete transduction
of the signal. The cGMP response is still half-maximal when
the half-life of cAMP is reduced to 0.5 s. Interestingly, the level
of adaptation and the magnitude of the response depend on
the half-life of cAMP in a similar way. Apparently, the entrance
of the signal to excitation and to adaptation has the same
velocity. Detection of the cGMP response to 10^{-7} M cAMP
with different half-lives is also shown in this figure. A cell
detects 10^{-7} M cAMP more slowly than 10^{-5} M cAMP; half-
maximal transduction requires a half-life of ~3 s.

Because we detect the result of excitation at 10 s after
addition of cAMP, and we measure the level of adaptation at
30 s after addition of cAMP, Fig. 4D does not inform us of the
kinetics of excitation and adaptation themselves, but only of
the kinetics of the entrance of the signal to excitation and
adaptation.

The Kinetics of Deadaptation

The rate of recovery from adaptation (deadaptation) was
investigated by variation of the time interval between two
cAMP stimuli and detection of the cGMP response to the
second stimulus (Fig. 4). The first stimulus (10^{-8} M or 10^{-7} M)
cells, phosphodiesterase was added after lysis of the cells; (■)
cGMP levels at 10 s, phosphodiesterase was added at 10 s; (△)
cGMP levels at 10 s, boiled phosphodiesterase was added at 10 s.
At point A a determination. Data from the same experiment as shown
in A. (■) Phosphodiesterase (20 μl, amount indicated on the
abscissa) was added, followed by the addition of 20 μl
3H-cAMP at 0 s (10^{-7} or 10^{-5} M, final concentrations, both containing about 5 kBq).
The degradation was terminated at 3 s by adding
100 μl perchloric acid. Hydrolysis of 3H-cAMP was
determined in the neutralized lysates. (○) Hydro-
dalysis of 10^{-7} M cAMP; (□) hydrolysis of 10^{-8}
M cAMP. From these data the half-life periods of
10^{-7} M cAMP (x) were calculated. The means of
duplicate determinations of a single experiment
are shown. (B) At about -10 s, 20 μl phosphodi-
esterase was added to 100-μl cell suspensions,
and these cells were lysed at 40 s with 20 μl cAMP
(10^{-7} M, final concentrations). At 10 s, perchloric
acid (100 μl) was added to half of the suspensions.
The other suspensions were stimulated again at
30 s with 20 μl cAMPS (3 × 10^{-5} M, final concentra-
tion), and these cells were lysed at 40 s with
100 μl perchloric acid. cGMP was determined in
the neutralized lysates. (○) cGMP levels at 10 s;
(●) cGMP levels at 40 s. The following controls
were taken: (□) cGMP levels of nonstimulated
aggregative cells at 10 s; (■) cGMP levels at 10 s.
Phosphodiesterase was added after lysis of the cells,
and phosphodiesterase was added to the cell sus-
pension varied from 7 to 14 s. The response was calculated from the increase
of cGMP levels at 10 s; the response to 10^{-7} M cAMP without adding phosphodiesterase (x) was set at 100%. The level of adaptation
was calculated from the increase of cGMP levels at 40 s (y) using the equation:

\[
\text{Adaptation} = \frac{(1 - y/x)}{0.84}
\]

The factor 0.84 is derived from a 16% deadaptation during a 30-s period (see Fig. 4C). By a procedure similar to that in A and B, the
response to 10^{-8} M cAMP with different half-lives was determined. (○) Response to 10^{-7} M cAMP; (+) adaptation to 10^{-7} M cAMP;
(□) response to 10^{-8} M cAMP.

The factor 0.84 is derived from a 16% deadaptation during a 30-s period (see Fig. 4C). By a procedure similar to that in A and B, the
response to 10^{-8} M cAMP with different half-lives was determined. (○) Response to 10^{-7} M cAMP; (+) adaptation to 10^{-7} M cAMP;
(□) response to 10^{-8} M cAMP.
will induce adaptation. We define the level of adaptation at time \( t \) by \( A(t) \), and the level of responsiveness by \( R(t) \); \( A(t) + R(t) = 1 \). We assume that the response to the second saturating stimulus (10\(^{-7}\) M) represents the level of responsiveness \( R(t) \). We further assume that in the absence of cAMP the transition \( A \rightarrow R \) takes place (cells deadapt with first-order kinetics), that this transition starts at \( t = \alpha \) s after the first stimulus, and that at that moment the level of adaptation equals \( \alpha \) \( (A(\alpha) = \alpha) \). A mathematical description of these assumptions is

\[
-\ln \left( \frac{1 - R(t)}{\alpha} \right) = k(t - \alpha). \quad (3)
\]

The magnitude of \( \alpha \) is derived from Fig. 2; 10\(^{-7}\) M cAMP induces complete adaptation to 10\(^{-7}\) M \((\alpha = 1)\), whereas 10\(^{-8}\) M cAMP induces only half-maximal adaptation to 10\(^{-7}\) M \((\alpha = 0.5)\). The magnitude of the responsiveness to the second stimulus, \( R(t) \), is calculated from the data of Fig. 4A and B by applying

\[
R(t) = \frac{\Delta[cGMP]_{t0(t)}}{\Delta[cGMP]_{t0(\infty)}}, \quad (4)
\]

where \( \Delta[cGMP]_{t0(t)} \) is the increase of cGMP levels at 10 s after stimulation with the second stimulus; the second stimulus is added at \( t \) seconds after the first stimulus.

Substitution of \( \alpha \) and \( R(t) \) in Eq. 3, and expression of the left part of this equation versus \( t \), yields a straight line (Fig. 4C), which affirms the assumption that deadaptation has first-order kinetics. The slope of this line equals \( k \), the rate constant of deadadaptation. Cells deadapt from 10\(^{-8}\) M or 10\(^{-7}\) M cAMP with comparable rate constants \( (k = 7.5 \times 10^{-3} \text{ s}^{-1}; t_{0.5} = 1.5 \text{ min}) \), which confirms that deadaptation has first-order kinetics. Furthermore, both lines intersect the abscissa close to 0 s. Taking into account that hydrolysis of the first stimulus requires only a few s, we conclude that deadaptation starts immediately after removal of the stimulus. The rate of deadadaptation was also measured in the presence of added phosphodiesterase (half-life of 10\(^{-7}\) M cAMP was 2 s); the same rate constant was observed \( (k = 7 \times 10^{-3} \text{ s}^{-1}; \text{data not shown}) \). Fig. 4A reveals that cells do not completely recover the responsiveness of the first stimulus. This has also been observed for deadadaptation of the relay response in D. discoideum (5).

Although the results of Fig. 4A–C clearly suggest that deadadaptation has first-order kinetics, we have observed repeatedly an oscillatorylike responsiveness as is shown in Fig. 4D. Such cells do not oscillate autonomously before addition of the first stimulus (cAMP levels, cGMP levels, or cGMP response to 10\(^{-7}\) M cAMP). The cGMP or cAMP levels also do not oscillate after addition of the first stimulus; only the cGMP responses to second stimulations with cAMP show oscillatory behavior. These results might be explained by two events: deadadaptation as shown in Fig. 4A and B in combination with an intracellular oscillation of responsiveness to extracellular cAMP. These observations are the subject of further investigations.

### Calculations on the Kinetics of Cell Surface cAMP-Receptors

Aggregative D. discoideum cells contain cell surface receptors for cAMP (9). These receptors might be involved in the transduction of the extracellular cAMP signal to an intracellular accumulation of cGMP. Calculations on the cAMP-receptor interaction under nonequilibrium conditions might be helpful to understand signal destruction, signal transduction, adaptation, and deadaptation. Occupation of the receptor by 10\(^{-7}\) M cAMP is very fast (Fig. 5A). Maximal occupancy is reached.
mediated cGMP response, which appears to be controlled by an adaptation process whenever adaptation is involved in the detection of chemotactic concentration to zero, will facilitate the detection of temporal crease, thus complicating the measurement of still higher concentration differences at different times. As a cell moves up a gradient the background concentration will increase, thus complicating the measurement of still higher concentrations. Again, adaptation, which sets the background concentration to zero, will facilitate the detection of temporal gradients. Cells make a pseudopod in the direction of a capillary filled with cAMP within 5-10 s (7). This suggests that, whenever adaptation is involved in the detection of chemoattractant signals, it will be a fast process. Direct evidence for an adaptation process during chemotaxis has not been presented. Indirect evidence has been derived from observations on the dynamics of chemotaxis and signal relay which reveal that an absolute refractory period, whenever present, does not last longer than 12 s (1), and that cells do not react chemoattractically to a gradient of cAMP that is lower in magnitude and has a polarity opposite that of a previous gradient to which the cells have reacted chemoattractically (23).

All chemoattractants studied so far induce a similar elevation of cGMP levels (10, 12, 16, 27, 29). Besides its general occurrence, the cAMP-response is also a fast reaction. Furthermore, mutants with altered cGMP behavior have altered chemoattractant behavior (11, 21). This may suggest the involvement of cGMP during a chemoattractant reaction; however, direct evidence has not been presented. In this study we investigated the cAMP-mediated cGMP response, which appears to be controlled by an adaptation process.

Adaptation of the cAMP-mediated cGMP response has two characteristics in common with the cAMP-mediated excretion of cAMP (relay) in D. discoideum (5, 6). For both reactions the magnitude of the response and the level of adaptation are additive, and both have similar rate constants of deadaptation. The main difference is the rate of adaptation, which is completed within a few seconds for the cGMP response, and which lasts several minutes for the relay response.

The entrance of the stimulus for a cGMP response is very fast (Fig. 3). The observation that transduction of \(10^{-8}\) M cAMP requires a longer presence of the stimulus than the transduction of \(10^{-7}\) M cAMP suggests that the entrance of the stimulus is a second-order process. Calculations on the occupancy of the cAMP cell-surface receptor under nonequilibrium conditions with different cAMP half-lives reveals that this receptor has the required rate constants to detect these fast changes of the cAMP concentration. The entrance of the stimulus to excitation and to adaptation has the same rate (Fig. 3 D), which may indicate that the signal for excitation and for adaptation enter the cell via the same receptor.

After removal of the signal cells recover from adaptation. The rate of deadaptation remains the same for different magnitudes and duration of the stimulus. This suggests that deadaptation is initiated by dissociation of cAMP from the receptors, but that the rate of deadaptation is cAMP-independent. The cGMP response always reaches a peak at 10 s after stimulation and prestimulated levels are recovered at about 30 s. Also, this is independent of the magnitude and duration of the stimulus. This may indicate that the alterations in the cGMP metabolism are initiated by cAMP, but that the pace of the alterations are stimulus independent.

Mato and Malchow (14) have shown that the cGMP response is produced by activation of the guanylate cyclase, rather than by inhibition of an intracellular phosphodiesterase. We may, therefore, describe the following processes as occurring after addition of cAMP to a cell suspension. In the extracellular space, cAMP is hydrolyzed or is bound to cAMP receptors localized on the cell surface. Occupation of the cAMP receptors activates the guanylate cyclase, producing an increase of cGMP levels. Occupation of the cAMP receptors also activates an adaptation process that rapidly (within 10 s) terminates the increase of cGMP levels, and causes the cells not to respond with an increase of cGMP levels after a second stimulation with the same cAMP concentration. Hydrolysis of cAMP and dissociation of cAMP from the receptors initiates deadaptation. Cells gradually reacquire responsiveness to cAMP in a first-order manner with a half-life of about 1.5 min.

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