An increase in cytosolic Ca^{2+} delays cAMP oscillations in *Dictyostelium* cells

Dieter MALCHOW*, Ralph SCHALOSKE and Christina SCHLATTERER

Faculty of Biology, University of Konstanz, Postfach 55 60, D-78434 Konstanz, Federal Republic of Germany

We have shown that calmidazolium (R24571) causes a transient increase in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in *Dictyostelium discoideum* [Schlatterer and Schaloske (1996) *Biochem. J.* **313**, 661–667]. Here we have used R24571 to artificially increase $[\text{Ca}^{2+}]_i$ during light-scattering oscillations and have found that, depending on the time of addition during the oscillatory cycle, R24571 suppressed cAMP synthesis and delayed

the next spike for several minutes. Addition of Ca^{2+} to the medium, which also elevates $[\text{Ca}^{2+}]_i$, induced phase delays and resulted in a similar phase response curve as R24571. The magnitude of the phase delay was correlated with the point during the oscillatory cycle at which Ca^{2+} was added, indicating that an artificial increase in $[\text{Ca}^{2+}]_i$ also resets the phase of the intrinsic oscillator.

INTRODUCTION

Aggregation of starving *Dictyostelium* cells depends on the pulsatile synthesis and release of cAMP (for reviews see [1–3]). cAMP is synthesized as a result of receptor activation and signal transduction involving the $\beta\gamma$ subunits of the heterotrimeric $\text{G}\alpha 2$ -protein and a novel protein known as cytosolic regulator of adenylate cyclase (CRAC) [4]. Adenylate cyclase activation seems to depend on the function of the mitogen-activated protein kinase ERK 2 (extracellular-signal-regulated kinase 2), since cells lacking this kinase failed to relay cAMP but were normal with regard to CRAC and cGMP synthesis [5]. In addition, Ca^{2+} may participate in the regulation of adenylate cyclase activation. Sudden elevations of the extracellular Ca^{2+} concentration led to a marked decrease in cAMP production [6,7]. However, it remained an open question as to whether Ca^{2+} was acting extracellularly or intracellularly.

During periodic signal relay, other messengers oscillate besides cAMP, i.e. cGMP and the extracellular concentrations of K^+ , H^+ and Ca^{2+} (for reviews see [8,9]). In fact, there seems to exist a basal oscillation of Ca^{2+} of sinusoidal character, on which is superimposed a spike-like pattern of Ca^{2+} fluxes at the time of pulsatile cAMP signalling [10]. Cytosolic Ca^{2+} oscillations in the pre-aggregation state have not yet been measured, probably because the changes in the concentration of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) are too small or are intimately localized [11,12]. Furthermore it is not known whether there is an oscillatory link between cAMP and Ca^{2+} , although both are tightly coupled in time [10].

Recently we have shown that calmidazolium (also known as compound R24571) can be used to elevate $[\text{Ca}^{2+}]_i$ transiently in *Dictyostelium* [13]. We therefore applied R24571 to cell suspensions during different phases of oscillation and found that, depending on the time of addition during the oscillatory cycle, R24571 caused phase delays and suppressed cAMP synthesis, suggesting that endogenous $[\text{Ca}^{2+}]_i$ increases regulate cAMP production in each cycle.

MATERIALS AND METHODS

Materials

Fura-2 was obtained from Molecular Probes, calmidazolium

(R24571) was from Sigma (Munich, Germany) and the cAMP enzyme immunoassay Biotrak was from Amersham (Braunschweig, Germany). $[2,8\text{-}^3\text{H}]\text{cAMP}$ was purchased from ICN (Eschwege, Germany).

Culture of cells

The axenic strain Ax2 was grown in liquid medium as described [14] supplemented with vitamin B12 ($5\text{ }\mu\text{g/l}$) and folic acid ($200\text{ }\mu\text{g/l}$). Differentiation was induced by washing exponentially growing amoebae twice with ice-cold Sørensen phosphate buffer [$17\text{ mM } (\text{KH}_2/\text{Na}_2\text{H})\text{PO}_4$], pH 6.0. The cell density was adjusted to 2×10^7 cells/ml and the cells were shaken until use at $23\text{ }^\circ\text{C}$.

Light-scattering measurements and $[\text{Ca}^{2+}]_i$ determination

The changes in light scattering of suspensions of amoebae were recorded with a Zeiss spectrophotometer (PM6) according to the method of Gerisch and Hess [15], modified as in [10], at a cell density of 2×10^7 cells/ml in Sørensen phosphate buffer starting at 4 h after induction of development.

$[\text{Ca}^{2+}]_i$ measurements were performed as described [13].

Determination of cAMP

The amount of cAMP (intracellular and extracellular) present during spike-shaped oscillations and before and after stimulation with 100 nM cAMP was measured using an enzyme immunoassay (Biotrak; Amersham) according to the manufacturer's instructions. Samples were prepared as described [16].

Measurement of cAMP binding to cell surface receptors

High- and low-affinity binding of $[^3\text{H}]\text{cAMP}$ was determined in Sørensen phosphate buffer at 10 nM and 100 nM cAMP respectively, as described previously [17]. Binding was measured before and 1 and 2 min after addition of $20\text{ }\mu\text{M}$ R24571 to the cell suspension. Binding in the presence of R24571 was $114 \pm 14\%$ ($n = 4$) after 1 min and $120 \pm 15\%$ ($n = 4$) after 2 min of pre-stimulation levels of high-affinity binding; the corresponding values were $112 \pm 14\%$ ($n = 3$) after 1 min and $110 \pm 9\%$ ($n = 2$) after 2 min for low-affinity binding.

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; CRAC, cytosolic regulator of adenylate cyclase.

* To whom correspondence should be addressed.

Control cAMP binding was 62–166 fmol/ 10^7 cells and 0.24–1.1 pmol/ 10^7 cells for high- and low-affinity binding respectively. The slight increase in binding probably results from an increase in the number of single cells, which can also be observed as an increase in light scattering.

RESULTS

The oscillatory activity of *Dictyostelium* cells can be conveniently analysed by the light-scattering technique introduced by Gerisch and Hess [15]. Cells undergo rhythmic changes of light scattering beginning after about 4 h of starvation in suspension (Figure 1). Subsequent analysis has shown that, simultaneously with these optical changes, cAMP concentrations also oscillate. The peak of cAMP virtually coincides with the peak of light scattering ([18], and see Figure 3).

Free-running light-scattering oscillations can be altered by the addition of R24571. Figure 1 shows that R24571 suppressed the occurrence of the next spike and delayed its appearance for

Figure 1 R24571 induces a phase shift of free-running light-scattering oscillations in *Dictyostelium*

Light-scattering oscillations were recorded at 500 nm as described in the Materials and methods section. Addition of 20 μ M R24571 at 0.6 of the oscillatory cycle (the time between peaks of the spikes is normalized to 1.0) induced a phase delay of 0.85 of the cycle. The broken line indicates the theoretical position of the next spike in an undisturbed oscillation. One out of seven independent experiments is shown.

Figure 2 Phase diagram depicting the magnitude of the phase shifts induced by R24571

Phase shifts (either advances or delays) are plotted against the time point of addition of 10–20 μ M R24571 within the phase. The duration of a phase (the time between the peaks of the spikes) was normalized to 1.0. Each point represents one experiment.

Figure 3 R24571 induces a phase delay of free-running cAMP oscillations

At 0.71 of the phase (where the time between peaks of the spikes is normalized to 1.0), 20 μ M R24571 induced a phase delay of 0.80 of the oscillatory cycle. cAMP was determined as described in the Materials and methods section. The concentration was expressed in μ mol/l of cell suspension. The broken line indicates the theoretical position of the next spike in an undisturbed oscillation. The increase in cAMP is suppressed, and was delayed until the occurrence of the next light-scattering spike. One out of two independent experiments is shown.

several minutes. After recovery, the oscillatory activity continued at about the same frequency as before. The cellular response depended on the point during the oscillatory cycle at which R24571 was added. If added shortly after the occurrence of a spike, no change or very small phase advances were observed (Figure 2). However, application in the 'trough' period and shortly before the next spike caused increasingly marked phase delays.

We next investigated whether the phase shift was restricted to the suppression of the light-scattering spike or whether cAMP synthesis was also suppressed. cAMP concentrations were determined during free-running oscillations before and after addition of R24571. Figure 3 demonstrates that cAMP synthesis was inhibited during the time at which the next spike would have occurred, and resumption of synthesis was delayed until the appearance of the next light-scattering spike.

After the occurrence of a spike, the cells become refractory to further stimulation due to receptor adaptation [19]. Receptors regain their ability to respond to external stimulation by 5–10 nM cAMP after about one-quarter of the cycle. These additions entrain the cells. If stimulation occurs before one-half of the cycle has occurred, phase delays are induced; if later than this, then phase advances occur [15,20]. We tested whether calmidazolium can interfere with the cAMP-induced cAMP relay. Figure 4 shows that, in the presence of R24571, the usual peak of cAMP synthesis at 90 s after application of 100 nM cAMP did not occur, indicating that the drug inhibited both spontaneous cAMP production and the cAMP-induced relay.

The effect of R24571 might be due to inhibition of cAMP binding to cell surface receptors. However, this was not the case.

Figure 4 Inhibition of the cAMP relay by R24571

R24571 (20 μ M) was added 1 min before the application of 100 nM cAMP (at time point zero) to an oscillating cell suspension (at 0.56 of the phase, where the time between peaks of the spikes was normalized to 1.0), and 30 μ l samples were withdrawn at the indicated times to determine total concentrations. Two independent experiments are shown. Note that the added cAMP is hydrolysed within 60 s by extracellular and cell-surface-bound cAMP phosphodiesterases, and that no peak of cAMP synthesized by the cells appears at 90 s after cAMP stimulation.

Figure 5 [Ca²⁺]_i increase induced by R24571

The experiment was performed in nominally Ca²⁺-free buffer. The ratio R340/380, as a measure of the [Ca²⁺]_i of a single fura-2-loaded cell, was plotted as a function of time. The arrow indicates the time point of addition of 10 μ l of 100 μ M R24571 to 100 μ l of medium. The increase in [Ca²⁺]_i amounted to 27 ± 5 nM ($n = 4$). One out of four independent experiments is shown.

Calmidazolium did not decrease high- or low-affinity binding of cAMP as specified in the Materials and methods section.

Although cAMP causes Ca²⁺ influx by binding to cell surface receptors, no [11] or only very small [12] global increases in [Ca²⁺]_i have been reported to occur in response to cAMP. The situation is different with R24571, which induces both Ca²⁺ release from intracellular stores and Ca²⁺ influx across the plasma membrane, resulting in global [Ca²⁺]_i increases [13], possibly due to inhibition of Ca²⁺ uptake into intracellular Ca²⁺ stores [21]. Since light-scattering oscillations were measured in the absence of added Ca²⁺, we confirmed that an increase in [Ca²⁺]_i occurred following addition of R24571 to the cells in nominally Ca²⁺-free buffer (Figure 5). The [Ca²⁺]_i transient amounted to about 30 nM and lasted 60 s. This global increase is fairly small; however, if it were to originate locally, i.e. close to the plasma membrane, substantial local changes in [Ca²⁺]_i could be attained. In fact, cAMP stimulation causes an influx of Ca²⁺ resulting in a calculated change of 10–20 μ M Ca²⁺ (final cellular concentration) that cannot be measured as a global

Figure 6 Ca²⁺ induces a phase delay of free-running light-scattering oscillations

CaCl₂ (1 mM) was added at 0.60 of the phase (where the time between peaks of the spikes was normalized to 1.0) and induced a phase delay of 0.42 in the oscillatory cycle. One out of eight independent experiments is shown.

Figure 7 Phase response curve depicting the magnitude of the phase shift induced by Ca²⁺

The experiment was performed as described in the legend to Figure 2, except that Ca²⁺ was added at the indicated time of the phase instead of R24571. The onset of the phase delays induced by Ca²⁺ occurred earlier during the phase than those induced by R24571. Each point represents one experiment.

increase in [Ca²⁺]_i [11,14]. Local [Ca²⁺]_i increases that oscillate are known to occur in defined cytoplasmic areas, e.g. in chromaffin cells [22], but have not been detected in *Dictyostelium* due to the small size of the cells.

We next asked whether an increase in [Ca²⁺]_i is sufficient to induce a phase delay in light-scattering oscillations. Recently we found that an increase in the extracellular Ca²⁺ concentration causes a transient elevation of [Ca²⁺]_i [13]. Application of 1 mM Ca²⁺ to an oscillating cell suspension elicited a phase delay (Figure 6). As with R24571, the cellular response depended on the time during the oscillatory cycle at which Ca²⁺ was added. If it was added shortly after the appearance of a spike, no changes were seen (Figure 7). However, Ca²⁺ addition thereafter caused increasingly marked phase delays that were correlated with the time of addition: the later the addition, the greater the delay.

DISCUSSION

Several models for periodic cAMP control and synthesis in *Dictyostelium* have been published [23–26]. One of these, by Monk and Othmer [26], incorporated changes in [Ca²⁺]_i due to

receptor-mediated Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ regulation by internal Ca^{2+} stores as part of the oscillatory cycle, assuming that adenylate cyclase activity is inhibited by elevated $[\text{Ca}^{2+}]_i$.

Indeed, we found that by elevating $[\text{Ca}^{2+}]_i$ using R24571, oscillatory or cAMP-induced cAMP synthesis could be transiently suppressed. Since an increase in $[\text{Ca}^{2+}]_i$ may not be the only consequence of R24571 application, we also increased $[\text{Ca}^{2+}]_i$ by addition of Ca^{2+} to the medium. Both treatments resulted in similar phase response curves, and in both cases oscillations were not affected when cells were treated early in the cycle. Therefore only a transient event can explain the results. A transient consequence of both applications is the elevation of $[\text{Ca}^{2+}]_i$. What, then, are the cellular consequences of an increase in $[\text{Ca}^{2+}]_i$? Ca^{2+} activates phospholipase C and inhibits guanylate cyclase activity [2,9]. There is no known link between $\text{Ins}(1,4,5)\text{P}_3$ or cGMP and cAMP synthesis. In fact, mutants defective in either cGMP (KI-8) or InsP_3 (KI-7) production were almost normal with regard to cAMP synthesis [27]. The latter is blocked by caffeine, and these authors assumed that caffeine acts via an increase in $[\text{Ca}^{2+}]_i$ [28]. Klein [29] and Schaap et al. [30] reported inhibition of adenylate cyclase activity by Ca^{2+} *in vitro*.

Although a direct effect of Ca^{2+} on adenylate cyclase activity thus seems possible, indirect effects on enzyme regulation via phosphorylation or dephosphorylation, or on CRAC activity, are also likely. However, a puzzle still remains. Ca^{2+} does not seem to be a general terminator of adenylate cyclase activity. In the relay experiments, where cAMP binds to cell surface receptors and elicits the synthesis of cAMP which in turn is secreted [31], there is an influx of Ca^{2+} before cAMP synthesis starts, an efflux of Ca^{2+} during the early stages of cAMP synthesis and no measurable change in extracellular Ca^{2+} thereafter [14,31]. Indeed, it is not known why cAMP synthesis is delayed for 90 s after receptor stimulation, compared with cGMP synthesis peaking at 10 s, even if CRAC needs to be recruited. In contrast, during oscillations Ca^{2+} influx peaks shortly after cAMP production, and Ca^{2+} efflux continues after resting levels of cAMP have been attained [10].

The artificial increase in $[\text{Ca}^{2+}]_i$ may have two consequences: (i) a direct or indirect inhibition of adenylate cyclase activity, and (ii) to reset the clock of the basic Ca^{2+} oscillator [8,9]. Figure 8 shows possible mechanisms for the effect of Ca^{2+} . A precocious increase in $[\text{Ca}^{2+}]_i$ counteracts the onset of cAMP synthesis until the cytoplasmic Ca^{2+} is sequestered and/or released to the external medium. In this case the next spike could be either omitted (Figure 8a) or delayed until the increase in $[\text{Ca}^{2+}]_i$ is removed (Figure 8b). Even if we allow both for a local increase in $[\text{Ca}^{2+}]_i$ which may last longer than the 1 min measured for the global $[\text{Ca}^{2+}]_i$ increase (Figure 5) and for secondary enzyme reactions, inspection of the data in Figure 7 reveals that the next spike always appeared much later, about one unit length (0.96 ± 0.09 ; $n = 12$) of the cycle after the pulse of Ca^{2+} was administered (Figure 8c). This result indicates that Ca^{2+} initiates a new cycle, i.e. it is resetting the clock.

We have shown previously that there is a basic Ca^{2+} oscillation in *Dictyostelium* and that spike-like cAMP production is coupled to it [9,10]. It is conceivable that a sudden increase in $[\text{Ca}^{2+}]_i$ disturbs this cycle in such a way that Ca^{2+} transport into the stores is activated. This latter event is also induced by cAMP stimulation [32], and a new oscillatory cycle starts. Finally, we found that additions of $2.5 \mu\text{M}$ Ca^{2+} induced small phase delays (1.18 ± 0.11 ; $n = 3$) of extracellular Ca^{2+} oscillations in *Dictyostelium*, indicating that the intrinsic oscillator reacts to small perturbations of Ca^{2+} . Future experiments will aim to show the extent to which endogenous changes in $[\text{Ca}^{2+}]_i$ contribute to the regulation of adenylate cyclase activity during oscillation.

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Figure 8 Hypothetical scheme showing the possible mechanisms by which $[\text{Ca}^{2+}]_i$ might alter periodic spike generation

Free-running spikes are shown, with an artificial increase in $[\text{Ca}^{2+}]_i$ shortly before generation of the next spike (at 0.7 of the phase; lasting for 1 min as in Figure 5). (a) cAMP synthesis is suppressed and the next spike is omitted (dotted spike). The internal oscillator is not affected. The residual cAMP concentration in the medium triggers a new spike in phase with the old one. (b) cAMP synthesis is delayed until the increase in $[\text{Ca}^{2+}]_i$ is removed. The relief from suppression of adenylate cyclase activity initiates a new cycle. (c) $[\text{Ca}^{2+}]_i$ initiates a new cycle and spike formation is delayed for one unit length of the cycle. The results of our experiments support possibility (c). LS, start of light-scattering period.

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