appears (at least in the short term experiments described here) that extracellular Ca^{++} is not required for gregarine motility.

A stock solution of 1 mM TFP (Smith, Klinde & French, Welwyn Garden City, Herts.) in Hedon-Fleig medium was prepared and appropriate dilutions made. The potential inhibitory effect of TFP was tested using the experimental protocol described by King and Lee[6], i.e. 0.1 ml of gregarine preparation (20 → 60 individuals) was placed in a single compartment (depth ~ 1 cm, diameter = 0.6 cm) of a flat bottomed plastic tissue culture tray, and the number of motile gregarines present was recorded; 0.1 ml of TFP solution was added and the number of motile gregarines present at 20-min intervals was recorded. The original number of motile cells counted just prior to the addition of test solution was normalized to a value of 100%. Suitable controls were carried out using Hedon-Fleig solution in place of the TFP solutions under test. The table shows that TFP markedly inhibited the motility of gregarines in a dose dependent manner and within the range of concentration ($10^{-5} \rightarrow 10^{-4}$ M) previously found to be effective in other systems[12,13].

At the light microscope level no change in gregarine shape was observed in the presence of TFP in contrast to the marked change in shape (discoidal → spherical) of human blood platelets when transfered to solutions containing TFP[12]. External Ca^{++} is not required for gregarine motility but the inhibition of motility by TFP indicates that Ca^{++}-calmodulin plays an important role. One would assume that sequestered Ca^{++} stores occur within the gregarine having a role similar to the sacroplasmic reticulum of muscle[11] and the Ca^{++} storage sites in the dense tubular system of platelets[12]. One presumes that under conditions suitable for motility to occur, Ca^{++} is released from these stores, complexes with calmodulin leading to the activation of enzyme system(s) required for motility. One prime candidate would be myosin kinase, however there are a large number of Ca^{++}-calmodulin activated enzyme systems[8,9]. Gregarine motility would seem to be worthy of further study particularly with regard to Ca^{++} stores and possible role of calmodulin. Since gregarines are large cells (volume $10^4 \rightarrow 10^6$ μm^3) they can be conveniently microinjected[14] and might offer an interesting model system for the study of calcium intracellular reactions.

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Induction of cell contact sites by Ca^{2+}-EDTA pulses in *Dictyostelium discoideum*

S. Miyamoto[1]

Department of Biology, Tsukuba University, Sakura-mura, Ibaraki (Japan), 31 October 1980

Summary. The effect of extracellular Ca^{2+} on the differentiation of the cellular slime mold *D. discoideum* was examined. In the case of sustained application of calcium, cells required at least 60 min for the presence of calcium to show the induction of EDTA resistant cell contact sites (csA). However, the application of 12 pulses of calcium, followed after 30 sec by EDTA, giving a total time of Ca^{2+}-exposure of 6 min, could induce csA on the cell surface.

Cellular slime mold *Dictyostelium discoideum* ameobae enter a phase of aggregation when they are starved. Cells aggregate by chemotaxis towards cAMP producing cells. Cyclic AMP pulses, either spontaneously generated or artificially applied, were reported to induce some proteins specific for the development phase[2]. Expression of contact sites (csA)[3], that mediate tight intercellular cohesion, is also developmentally regulated and is induced by cAMP pulses. The mechanism of the induction of csA by cAMP pulses was not defined. But calcium ions are believed to play some role in the inducing mechanism because calcium influx was observed within 10 sec after stimulation of cells with cAMP[4]. It was reported that cells could not aggregate in the calcium free medium[5]. Most work concerning the effects of calcium was performed using a sustained application of calcium[6,7]. But in this report, it was added in a pulsatile manner. Calcium pulses instead of cAMP pulses also induced csA.

Materials and methods. Dictyostelium discoideum NC-4 cells were cultivated on nutrient agar in association with *Enterobacter aerogenes* at 22 °C. Growth phase cells were collected, washed twice with 17 mM phospate buffer and resuspended in the same buffer. Aliquots of 6 ml of the cell suspension in 20 ml flasks were incubated in the shaker (T_0). After 2 h of incubation (T_2), 30 µl of $CaCl_2$ and/or EDTA solution was added manually with a micropipette. Protocols for pulsatile application and sustained application are shown in figure 1. In the case of Ca^{2+}-EDTA pulses, EDTA was added equimolar to the $CaCl_2$ solution 30 sec after the $CaCl_2$ addition. This alternate addition, which represented 1 pulse, was repeated at 5-min intervals. During the 5-min interval, calcium ions existed in the suspension for the 1st 30 sec, then for at least 4.5 min, the calcium was absorbed by EDTA. In all cases, either the pulse or sustained application, EDTA solution that was equivalent to the Ca^{2+} was added after the $CaCl_2$ solution was applied in order to avoid the effect of Ca^{2+} remaining in the suspension. Because, at the end of incubation, the cell contact sites A was measured as the amount of cell aggregates that were resistant to EDTA.

Samples of cells with or without stimulation were taken after 7 h of incubation (T_7), and shaken for 30 min in 10 mM EDTA solution. EDTA resistant cell contact were examined microscopically. Non-aggregated cells that remained single or double at the end of incubation were counted and expressed as the percentage of total cell number.

Results and discussion. Slime mold cells were starved in 17 mM phosphate buffer. After 7 h of incubation (T_7), EDTA resistant cell contacts were counted. Singlet and doublet cells were about 90% of the total population. Aggregation did not begin until T_8.

1 drop (30 µl) of $CaCl_2$ solution was added at T_2 at the final concentration of 50 µM. 2 h later (T_4), 1 drop of EDTA that was equimolar to the $CaCl_2$ was added. In this case, cells were sustained in Ca^{2+} for 120 min. Singlet and doublet cells were reduced to about 60% when cell aggregation was counted at T_7. Their aggregation was stimulated by calcium.

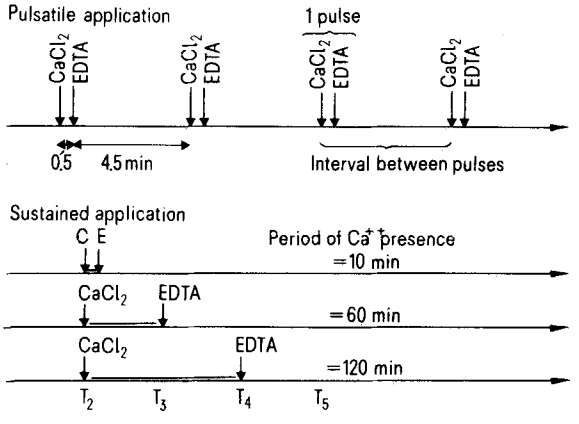

Figure 1. Experimental procedures for pulsatile application and sustained application of calcium. The time-scales of the 3 are different. A drop (30 µl) of $CaCl_2$ or EDTA solution at the final concentration of 50 µM was added with a micropipette. The 1st pulse was added at T_2, and when the interval was 5 min, the 12th pulse was at $T_{2:55}$. In sustained application, a drop of $CaCl_2$ solution was added at T_2, and following drops of EDTA were added at various times as shown in figure 2. The interval between $CaCl_2$ addition (T_2) and EDTA addition was assumed to be the period of Ca^{2+} presence.

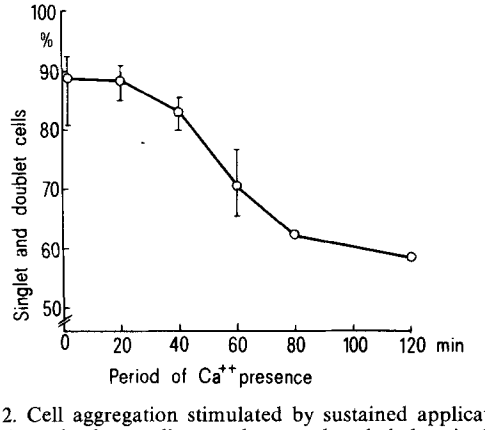

Figure 2. Cell aggregation stimulated by sustained application of $CaCl_2$. Growth phase cells were harvested and shaken in 17 mM phosphate buffer. Procedure for the application of $CaCl_2$ and EDTA solution is shown in figure 1. $CaCl_2$ was added at T_2 in every case. EDTA drops were added at 30 sec, 20, 40, 60, 80, and 120 min, after the $CaCl_2$ addition. Cell aggregation was observed at T_7.

In sustained application, periods of Ca^{2+} presence were varied from 30 sec to 120 min (fig. 2). $CaCl_2$ droplets were added at T_2 in all cases. Stimulation of cell aggregation was observed when cells were kept in $CaCl_2$ for more than 60 min. Short period application had no effect. This suggested that added calcium was mostly absorbed by the following addition of EDTA.

Ca^{2+}-EDTA pulses were added from T_2 at 5-min intervals. 12 pulses were added and at T_7, aggregation was observed. Singlet and doublet cells were reduced to 40% (fig. 3). The effect of Ca^{2+}-EDTA pulses was dependent on the number of pulses.

In the case of pulsatile application, Ca^{2+} was present for 30 sec for 1 pulse. When 12 pulses were added, the period of calcium presence summed up to 6 min. This was contrasted with the 60 min which was required in the sustained incubation. In pulse application, with a period of Ca^{2+} presence only $^1/_{10}$ as long, cell aggregation could be stimulated.

Intervals between a pulse and the next pulse were varied. In contrast to cAMP pulses, which showed a clear dependence on the interval between pulses, Ca^{2+}-EDTA pulses induced csA even at the interval period of 1 min (fig. 4). Intervals shorter than 1 min were not tested. 1 pulse of Ca^{2+}-EDTA of 360 µl (12-fold more than normal pulse in volume: a model for a zero interval) showed slight acceleration of csA expression.

Concentration dependence was determined for 12 pulses of Ca^{2+}-EDTA application. Final concentrations of 5 or

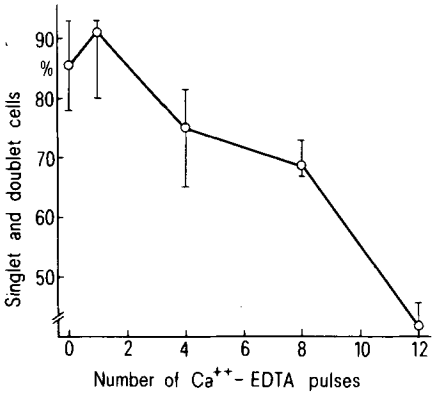

Figure 3. Dependence of cell aggregation on the number of pulses. Cell were stimulated by 1, 4, 8 and 12 pulses of Ca^{2+}-EDTA. Experimental procedure is shown in figure 1. Aggregation was assayed at T_7.

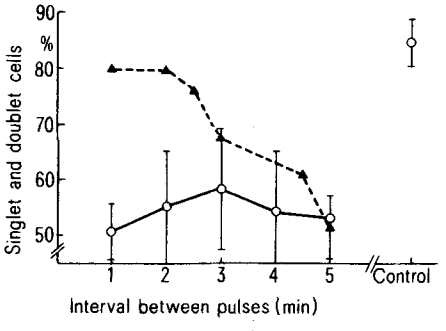

Figure 4. Effects of cAMP and Ca^{2+}-EDTA pulses, varying the interval between pulses. The 1st pulse was always added at T_2 at the final concentration of 50 µM. 12 pulses of cAMP (▲) and Ca^{2+}-EDTA (○) were added at the intervals indicated.

50 μM $CaCl_2$ stimulated aggregation. A higher concentration of $CaCl_2$ seemed to be inhibitory.
Specificity of calcium was tested. Mg^{2+}-EDTA pulses and sustained $MgCl_2$ application had no effect. When EGTA was used instead of EDTA, it induced csA similarly.
This study shows that Ca^{2+}-EDTA pulses stimulated the formation of EDTA resistant cell contacts as well as cAMP pulses did. As in the case of cAMP, pulse application of calcium could induce cell contacts in a shorter period than sustained application. It has been reported that 3 pulses of $CaCl_2$ induced spontaneous oscillation of light scattering in cell suspension. The induction of cell contact by Ca^{2+}-EDTA pulses may be due to triggered cellular oscillations. Calmodulin[9] may take part in this process.

1 Present address: Department of Anatomy, Dokkyo University School of Medicine, Mibu, Tochigi (Japan).
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Antifeedant activity of precocenes and analogs on *Rhodnius prolixus*[1]

P.D. Azambuja, W.S. Bowers[2], J.M.C. Ribeiro and E.S. Garcia

Department of Physiology, Universidade Federal Fluminense, C.P. 183, Niteroi, R.J., 24000 (Brazil), and Department of Entomology, New York State Agricultural Experiment Station, Cornell University, Geneva (New York 14456, USA), 27 April 1982

Summary. Precocene and analogs added to the meal of 4th instar larvae of *Rhodnius prolixus* were tested as antifeedants. While precocene II had a strong antifeedant effect (ED_{50} = 48 μg/ml), the other compounds showed no drastic inhibition of feeding (ED_{50} > 140 μg/ml). ATP, a phagostimulant, did not reverse the antifeedant action of precocene II. The mechanism of feeding inhibition is discussed.

Precocenes are plant derived substances that induce precocious metamorphosis in immature hemipterans and prevent ovarian development in some adult insects[3-6]. Precocenes are selectively cytotoxic to the secretory cells of the corpus allatum of the insect, thereby eliminating the production of juvenile hormone[7-12]. However, Slama[13] concluded that premature metamorphosis and ecdysis prevention induced by precocene in certain insects were due to its antifeedant action, as prothetelic insects always appeared among the larvae with the most disturbed growth, close to lethality. In fact, Slama found that precocene profoundly inhibited feeding and growth as well as oviposition in *Pyrrhocoris* and *Dysdercus*. He concluded that the latter effect was identical to that obtained with females treated by the antifeedants myristicin and elemicin. In contrast, although Bowers and Ferugia, and Bowers and Aldrich[14,15] had noted a disinclination by adult female milkweed bugs to feed following treatment with just subtoxic dosages of precocene II, at effective sterilizing concentrations substantial feeding and weight gain occurred. In similar experiments with the milkweed bug it was demonstrated that the main difference in weight between normal and precocene-treated insects was due to the inhibition of ovarian development in the latter[16]. These results emphasize that the antifeedant and anti-hormonal actions of precocene are distinct and especially that the antihormonal activities are not the result of a reduction or elimination of food intake. Considering antifeedants as substances which when tasted or initially consumed can result in cessation of feeding either temporarily or permanently depending on potency, we reported recently that precocene II, added to the diet, acted as a feeding deterrent for *Rhodnius prolixus*, reducing the amount of blood consumed by this insect[17]. We now describe the inhibition of feeding induced by precocene and its analogs in the bloodsucking bug *Rhodnius prolixus*.

Materials and methods. Precocene II, ethoxy precocene (i.e., 7-ethoxy-6-methoxy-2,2-dimethyl chromene[18]) and the chromane of precocene II were synthesized by one of us (W.S.B.); precocene I was purchased from Sigma Chemical Co. All other reagents were of analytical grade. Following ecdysis, the 4th-instar larvae of *R. prolixus* were starved for 20-30 days and then fed on blood or artificial food containing precocene through a special feeding apparatus[19]. Precocenes dissolved in ethanol were added to the citrated blood or artificial diet (1 μl of Tween 80, 20 μl of ethanol plus sample, 10 ml of 30 μM ATP in 0.15 M NaCl) at doses ranging from 10 to 100 μg/ml. Control experiments showed

Table 1. Inhibition of feeding of *Rhodnius prolixus* by the precocenes and analogs

Substances	Effective dose (ED_{50}) (μg/ml)
Chromane of precocene II*	> 300
Precocene I*	260
Precocene II*	48
Precocene II**	43
Ethoxy precocene*	140

* Drugs diluted in blood; ** drugs diluted in artificial diet.

Table 2. Effect of ATP on the inhibition of feeding induced by precocene II in *Rhodnius prolixus*

ATP (μm)	Precocene II (μg/ml)*	Meal ingested (mg)
30	-	94.5 ± 5.5
30	50	29.0 ± 6.4
300	50	36.5 ± 7.0
3000	50	34.8 ± 5.6

* Artificial meal.