CYCLIC ADENOSINE MONOPHOSPHATE AND
THE DEVELOPMENT OF POLYSPHONDYLIUM

M. E. JONES AND A. ROBERTSON
Department of Biophysics and Theoretical Biology, University of Chicago, Chicago 60637, U.S.A.

SUMMARY

Centre formation in Polysphondylium violaceum is delayed for 2 h on buffered agar containing 10^-3 M c-AMP, and for up to 22 h on unbuffered agar with the same c-AMP concentration. With ambient c-AMP concentrations as low as 10^-6 M, P. pallidum forms numerous, small, atypical aggregates which do not fruit. This effect is independent of whether the agar is buffered.

P. violaceum amoebae are weakly attracted to the tip of a microelectrode containing 10^-3 or 10^-4 M c-AMP, but the electrode cannot compete when natural centres form nearby. P. pallidum amoebae are not attracted.

Aggregates of P. violaceum and of P. pallidum are strongly attracted to a microelectrode releasing c-AMP. The observation of Shaffer that Polysphondylium grex switch over from secreting an acrasin that attracts homologous amoebae to one that attracts the larger Dictyostelium species suggests that the second acrasin might be c-AMP. The above results strengthen this conjecture.

As c-AMP inhibits centre formation, the secretion of c-AMP by older aggregates may explain an inhibition of centre formation in the 'overlay' experiments of Shaffer.

INTRODUCTION

Amoebae of the cellular slime moulds Polysphondylium pallidum and Polysphondylium violaceum enjoy a unicellular existence until their food supply is exhausted. The unicellular vegetative stage then ceases, and after a variable 'interphase' of several hours the cells come together at aggregation centres to form multicellular pseudoplasmodia which lead to the formation of a fruiting body. The aggregation process involves the secretion of, and chemotactic response to, a diffusible chemotactic agent or 'acrasin'.

The closely related genus Dictyostelium has a similar life cycle. For the species D. discoideum, D. purpureum and D. mucoroides, the acrasin appears to be 3'5'-cyclic adenosine monophosphate (c-AMP) (Konijn, Barkley, Chang & Bonner, 1968; Bonner et al. 1972). Although c-AMP does affect the development of Polysphondylium (Konijn, 1972), and although P. violaceum is known to produce an extracellular phosphodiesterase (Gerisch et al. 1972), evidence suggesting different acrasins for Dictyostelium and Polysphondylium has been accumulating since the co-aggregation experiments of Raper & Thom (1941).

During the aggregation phase of the cellular slime moulds, interspecific and intergeneric interactions are complex. Broadly speaking, we expect species to co-aggregate if they use the same chemotactic agent or 'acrasin' in the aggregation
process. The species, *D. discoideum*, *D. purpureum*, and *D. mucoroides* are known to co-aggregate, but *Polysphondylium violaceum* does not co-aggregate with any of these three (Raper & Thom, 1941).

There is, however, considerable evidence for some attraction between the genera *Dictyostelium* and *Polysphondylium*. Sussman (1956) reported 'Leachings of *Dictyostelium discoideum* and *Polysphondylium violaceum* were tested on homologous and heterologous myxamoebae. Young pseudoplasmodia of *Polysphondylium* were specific, but at later stages could attract both groups of myxamoebae.' Shaffer (1957a) found that 'Cells of *Dictyostelium discoideum*, *D. mucoroides*, and *D. purpureum* were attracted to the older centres of *Polysphondylium violaceum*, both primary and secondary ones, and to its developing fruiting bodies.' Later, Shaffer (1962) reports that 'in *violaceum* the grex has switched over almost entirely from secreting an acrasin that attracts homologous amoebae to one that attracts those of the large *Dictyostelium* species', and his article demonstrates that the tip of the *P. pallidum* grex not only attracts cells of *D. mucoroides*, but also induces them to relay.

It was not until about 6 years after Shaffer’s article that the acrasin for *D. discoideum*, *D. mucoroides* and *D. purpureum* was identified as c-AMP. Following this identification, subsequent work on *D. discoideum* showed that the 'acrasin' (c-AMP) of its aggregation phase was also responsible for the organization of the migrating grex (Bonner, 1949; Robertson & Grutsch, 1974; Rubin & Robertson, 1975). It therefore appears that in *D. discoideum* the process of aggregation and of organization of the grex are regulated by gradients of the same intercellular transmitter, and that the different structures which result are not a response to different transmitters, but the response to the same transmitter of cells at different stages of development.

The work of Sussman, and of Shaffer, suggests that a different situation may obtain in *Polysphondylium*; intercellular transmission in the grex stage may use a chemotactic agent different from the ‘acrasin’ of aggregation. That this transmitter might be c-AMP is suggested by the attraction of c-AMP-sensitive *Dictyostelium* myxamoebae to the grex.

It was the purpose of the following experiments to investigate the effect of c-AMP on the development and organization of *Polysphondylium*.

**MATERIALS AND METHODS**

Vegetative amoebae were grown and harvested as described elsewhere (Jones, 1976). Agar plates containing c-AMP were prepared by adding equal volumes of 2% agar at 80°C and c-AMP solution to give the required final concentration of c-AMP in 1% agar. The solutions were made up in either deionized water or KK2 buffer (Bonner, 1967).

Microelectrode plates were prepared by a modification of the method of Robertson, Drage & Cohen (1972). The method there outlined involves introducing the micropipette from above the agar, the tip being several microns below the surface. Four disadvantages of this method are: (1) for low impedance electrodes, the tip diameter is sufficiently large to make gravitational emptying of the electrode significant; (2) a meniscus forms around the tip of the agar surface, and refraction from this precludes seeing an area of about 200 μm diameter around the tip; (3) small movements often bring the tip off the agar surface; and (4) small air-bubbles forming at the end of the microelectrode are invisible because of refraction from the glass–air interface of the microelectrode.
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To overcome these problems, we introduced the microelectrode from below the agar. A 1-cm-square hole is removed from the centre of a 100 x 15 mm plastic Petri dish (Optilux 1001), and a plain microscope slide placed inside the dish, covering the hole. A 45° angle is made 1 cm from the end of a glass microelectrode, a sheath of aluminium foil protecting the tip from heat while the angle is made. This electrode is filled with buffered c-AMP, which has been boiled to drive off dissolved air. A 1.5-mm hole drilled in the side of the Petri dish admits the microelectrode which is glued in place with its tip above the slide over the base hole, and inclined up at a 45° angle. A further hole admits the chlorided silver electrode. The technique of pouring agar on to this arrangement is critical, for the level of the tip cannot now be adjusted. The following method was found to be satisfactory. One percent agar in KK2 buffer is introduced in 3 stages. First, a small amount is introduced around the microscope slide, the microelectrode and the chlorided silver electrode. This is allowed to set to seal off all potential holes. A second pouring of agar brings the surface to within 1 mm of the microelectrode tip, and is left to cool to room temperature so that there will be no further contraction from cooling in this layer. The final pouring, done at 40 °C, must bring the surface of the agar sufficiently above the tip to allow for contraction on cooling. In a 10-cm-diameter Petri dish, about 0.5 ml of agar, in addition to that required to cover the tip, is satisfactory.

The reverse bias and pulsing of the microelectrode has been described elsewhere (Robertson et al. 1972).

RESULTS

Aggregation of P. violaceum on agar containing c-AMP

c-AMP at $10^{-3}$ M delayed development of centres of P. violaceum by 2 h on 1 % buffered agar (containing magnesium) and by 22 h on 1 % agar made up in deionized water. Concentrations of c-AMP from $10^{-4}$ to $10^{-10}$ M caused no significant deviation from development on c-AMP-free controls.

Aggregation of P. pallidum on agar containing c-AMP

The effect of c-AMP on P. pallidum is independent of whether the agar is buffered. At concentrations of $10^{-7}$ M and less, there was no difference between the experimental plates and the c-AMP-free controls. With $10^{-6}$ M c-AMP, numerous small aggregates form, many of which fail to develop further. Those which do proceed form diminutive fruiting bodies. With higher concentrations of c-AMP, small, flattened aggregates form, but none proceed to the formation of fruiting bodies. Konijn (1972) reports a similar finding with the periodic application of $10^{-2}$ M c-AMP near Polysphondylium in the 'Konijn test' for chemotaxis, but does not distinguish the different responses of the 2 species.

P. violaceum grex on agar containing c-AMP

Older P. violaceum centres and grex transferred to buffered agar recover from the insult and develop normally. If the agar contains $10^{-3}$ M c-AMP, however, some small clumps of cells can be seen migrating centrifugally from the main cell mass.

Microelectrode plates

With concentrations of $10^{-3}$ and $10^{-4}$ M c-AMP in the microelectrode, vegetative P. violaceum amoebae are attracted to the microelectrode tip. This attraction does not
result in the formation of a morphologically distinct 'centre', nor is there any stream formation. When autonomous centres of aggregation form, the attraction of amoebae to these is far greater than the attraction toward the microelectrode. When these naturally forming centres are about 2 h old, however, they migrate to the c-AMP microelectrode. The appearance of a centre thus attracted to the microelectrode tip is indistinguishable from the appearance of one which, in normal development, is 'taken over' by, and attracted into, a neighbouring aggregate.

The same process appears to take place with *P. pallidum* aggregates. We have not filmed the process, but examination of the terminal state shows abnormally large *P. pallidum* aggregates over the microelectrode tip, where previously there were no aggregates.

**DISCUSSION**

On agar containing c-AMP, the development of *P. pallidum* is inhibited far more than that of *P. violaceum*. This is perhaps to be expected; it has been known for some time that under conditions of aggregation *P. violaceum* secretes a phosphodiesterase whereas *P. pallidum* does not (Bonner et al. 1972; Gerisch et al. 1972). That *P. violaceum* on plain agar is inhibited longer by 10^{-3} M c-AMP than it is by the same concentration on buffered agar containing magnesium may reflect different phosphodiesterase activity under these different circumstances. *P. pallidum*, lacking a phosphodiesterase, is equally affected on plain and buffered agar.

*P. pallidum* forms aggregates in the presence of c-AMP, but does not proceed to the erection of a fruiting body. If c-AMP were the acrasin it would be surprising for aggregation to proceed at such high ambient levels. Conversely, the interruption of development at this stage suggests that c-AMP might be involved in the development of the migrating grex. This evidence adds to that already in the literature suggesting a major reorganization of the control of development at this stage in *Polysphondylium* (Shaffer, 1957a, b, 1962). If c-AMP were to function chemotactically in the migrating grex of *Polysphondylium*, as it does in *Dictyostelium*, high c-AMP levels would interrupt development at this point.

The chemotaxis of individual *P. violaceum* cells toward a c-AMP electrode in the microelectrode plates can be interpreted in 2 ways. It may represent a nonspecific chemotaxis, such as vegetative amoebae might use in seeking food. Certainly it is of a much lower magnitude than chemotaxis toward a centre of aggregation, and is insufficient to cause amoebae to chemotact beyond the confines of a 'drop' in the Konijn drop test (Konijn et al. 1968). No chemotaxis is apparent using c-CMP.

An alternative explanation is that this represents a chemotactic potential which is not yet fully developed, but which is involved at the stage of the migrating grex. The secretion from the *Polysphondylium* grex of a substance attracting *Dictyostelium*, the interruption of development by high c-AMP levels, and the attraction of *Polysphondylium* pseudoplasmodia up a c-AMP gradient, suggest that c-AMP or a c-AMP-like substance can control development of the *Polysphondylium* grex much as it does in the *Dictyostelium* grex. Bonner has suggested that the c-AMP produced by *P. violaceum*
and *P. pallidum* may be involved in the differentiation of stalk cells, and this conjecture now seems even more likely. Both the ability to attract *Dictyostelium* and the chemosensitivity to c-AMP are limited to older *Polysphondylium* grex; young pseudoplasmodia do not attract the larger *Dictyostelium* species (Raper & Thom, 1941), nor are they attracted to the c-AMP microelectrode. Migration of clumps of cells away from a mass of *P. violaceum* aggregates deposited on agar containing c-AMP probably reflects the same phenomenon, the c-AMP gradient being caused by the phosphodiesterase which *P. violaceum* is known to produce.

The possibility that the progression from aggregating pseudoplasmodium to migrating grex involves the development of the ability to produce, and to respond to, c-AMP highlights a minor difference in the observed life-cycles of *Dictyostelium* and *Polysphondylium*. In *Dictyostelium*, the migrating pseudoplasmodium is often seen emerging from the aggregation centre at a time when tributary streams are still converging toward the centre. In *Polysphondylium*, there is usually a quiescent period between the formation of pseudoplasmodium and the emergence of the migrating grex. This difference in the observed sequence may reflect the necessity for *Polysphondylium*, but not *Dictyostelium*, to switch from an acrasin controlling aggregation, to one controlling the grex. It is during this quiescent period that sensitivity of *Polysphondylium* to c-AMP develops.

But although such a role might explain the interruption of development by high ambient concentrations of c-AMP, between aggregation and organization of the grex, as seen in *P. pallidum*, it does not suggest a mechanism whereby aggregation itself is inhibited, as in *P. violaceum*. Until we understand more about the mechanism of aggregation in *Polysphondylium* we are unlikely to make precise statements as to how it is interrupted. A recent speculation (Wurster, Pan, Tyan & Bonner, 1976) as to a 'second messenger' role for c-AMP during *Polysphondylium* aggregation, analogous to its role in mammalian hormone systems, offers one mechanism which could be inactivated by high ambient concentrations of c-AMP.

*Polysphondylium violaceum* populations which develop in the presence of added c-AMP are distinguished from those developing normally by the absence of competition between centres. In the normal course of events, many smaller aggregates are 'taken over' by their larger neighbours, but where development begins in high ambient c-AMP concentrations, this phenomenon does not seem to occur. The apparent similarity between the taking over of nearby centres by the c-AMP microelectrode tip on one hand, and the taking over of adjacent aggregates one by the other in the normal sequence of events, on the other hand, suggests that a common mechanism underlies both phenomena. If this is the case, natural competition between adjacent aggregates during the quiescent stage involves attraction of one aggregate up a c-AMP gradient produced by the other. If development takes place on agar containing $10^{-9}$ M c-AMP, the outcome will depend on the ability to secrete sufficient PDE to remove the c-AMP. *P. pallidum*, which lacks a phosphodiesterase is inhibited by c-AMP concentrations exceeding $10^{-7}$ M; *P. violaceum*, which secretes phosphodiesterase, is delayed by $10^{-6}$ M c-AMP on unbuffered agar, and when aggregation does take place, there is no competition between adjacent centres. This lack of competition may reflect the
high phosphodiesterase levels which are necessary to lower the ambient c-AMP concentration.

The variable behaviour of *P. violaceum* in ‘overlay’ experiments is possibly explained by what now appears to be its developmental sequence. When a thin layer of agar carrying chemosensitive *P. violaceum* amoebae is placed over a plate of aggregating amoebae, the response of the upper layer is variable. Often the amoebae of the upper layer are attracted over the streams and centres of the lower layer and there results a clear coincidence of streams and centres of the 2 layers.

But an alternative outcome is that centre formation on the upper layer appears to be actively inhibited in the region of lower layer centres. (For a discussion, see Shaffer, 1962, 1963.)

These two apparently contradictory outcomes might now be interpreted as corresponding to the acrasin-secreting and c-AMP-secreting phase of the lower level *P. violaceum* pseudoplasmodia; c-AMP inhibits centre formation in *P. violaceum* and the inhibition of upper-layer centres by lower-layer centres may represent c-AMP diffusion from the latter.

It is possible that the existence of separate aggregative acrasins represent the step leading to divergence of the 2 *Polysphondylium* species from the *Dictyostelium* species, while the role of c-AMP as a pseudoplasmodial organizer in both *Dictyostelium* and *Polysphondylium* emphasizes its profound importance in intercellular communication during the multicellular phases of development.

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REFERENCES


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