The signal from fruiting body and conus tips of Dictyostelium discoideum

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SUMMARY

Tips from fruiting bodies and conuses were transplanted into interphase fields of Dictyostelium discoideum amoebae. Progressively increasing concentrations of beef-heart phosphodiesterase added to the fields significantly decreased the chemotactic range of the responding amoebae. The findings suggest that the tip secretes c-AMP. We also find that the chemotactic range is independent of the size of the tip implying that the tip may produce a regulating gradient.

INTRODUCTION

During the final stages of aggregation of the cellular slime mold Dictyostelium discoideum, a nipple-shaped tip forms on top of the hemispherical aggregate (Raper, 1940; Bonner, 1967; Cohen & Robertson, 1972). The tip retains its integrity throughout the subsequent multicellular (pseudoplasmodial) stages of the life cycle which include slug migration and fruiting body culmination (Raper, 1940; Krivanek & Krivanek, 1958; Muller and Hohl, 1973). It has been suggested that the tip is necessary for definition of the developmental axis, acting as the organizing center of the pseudoplasmodium and showing some of the properties of a classical developmental organizer (Robertson & Cohen, 1972; Farnsworth, 1973; Rubin & Robertson, 1975).

The tip from any pseudoplasmodial stage of D. discoideum will organize (Raper, 1940; Rubin & Robertson, 1975) in the following sense. When a tip from one stage is transplanted into the side of a pseudoplasmodium at any other stage, the transplanted tip induces the formation of a secondary developmental axis in the body of the host leading eventually to a second pseudoplasmodium. The unique feature of the tip’s signal is that it is a continuous secretion of the acrasin or chemotactic agent (Robertson et al. 1972; Durston, 1974; Rubin & Robertson, 1975). This is an important property as aggregation competent amoebae are initially attracted toward individuals or groups of cells that secrete periodic pulses of c-AMP (Konijn, Van de Meene, Bonner & Barkley, 1967; Gerisch, 1968; Robertson, Drage & Cohen, 1972). The cells in the field

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are controlled by the pacemaker with the highest frequency of pulsation (Cohen & Robertson, 1971; Robertson, Drage and Cohen, 1972; Durston, 1973). However, each cell's response is followed by a refractory period during which time it cannot respond by relaying a c-AMP signal (Gerisch, 1968; Durston, 1973; Robertson & Drage, 1975). The tip's continuous signal represents the fastest possible pacemaker, allowing the tip to drive individual cells in an aggregating field at their own intrinsic refractory periods (Durston, 1974; Rubin & Robertson, 1975). This allows the tip to entrain all other autonomous pacemakers and leads to a steady increase in the frequency of the propagated signal as the refractory period decreases with cell age (Robertson & Drage, 1975). The signal from the tip appears to be independent of the origin of the tip in that the structure formed by the grafted tip and the cells it pulls in from the host is similar to the host. Thus a slug tip transplanted into a fruiting body induces a second fruiting body, while a fruiting-body tip placed into a slug induces the formation of a second slug (Rubin & Robertson, 1975).

The tip appears to secrete more of the acrasin than the rest of the pseudoplasmodium (Bonner, 1949), and the acrasin for aggregation of *D. discoideum* is c-AMP (Konijn *et al.* 1967; Konijn, Barkley, Chang & Bonner, 1968). Thus it is suspected that the tip also secretes c-AMP as its chemotactic agent (Rubin and Robertson, 1975) although further evidence is needed to support this hypothesis.

Before they can aggregate the amoebae develop several competences. They first develop the competence to chemotax toward sources of acrasin (Robertson, Drage and Cohen, 1972; Robertson & Cohen, 1972), then acquire the ability to relay a c-AMP signal (Gingle & Robertson, 1976), and finally certain cells begin to secrete c-AMP autonomously (Raman, Hashimoto, Cohen & Robertson, 1976). Fortunately, the acquisition of each competence is well separated temporally from the other two, so that it is possible to obtain fields of cells that have attained the first, the first and second, or all three competences. Because of this separation of competences, *D. discoideum* amoebae in interphase can provide an assay for the tip's signal. If tip transplants are made into fields of cells that are only chemotactically sensitive to the tip's signal, the range of the chemotactic response will only be a function of the tip's signal, and one can therefore obtain a quantitative measure of the magnitude of the tip's signal. Secondly, by transplanting tips into fields containing an added c-AMP phosphodiesterase, one can determine if the tip's signal is indeed c-AMP. The experiments in this paper are presented with these two ideas in mind.

**MATERIALS AND METHODS**

*D. discoideum* (NC-4 haploid) amoebae stock originally obtained from Professor K. B. Raper were used. The amoebae were grown in association with either *Aerobacter aerogenes* or *Enterobacter cloacae*. Culturing, harvesting, and plating techniques were as previously described (Rubin & Robertson, 1975).
The amoebae were either allowed to enter interphase and eventually complete the life-cycle, or they were harvested in the vegetative phase as described below. Harvesting vegetative cells synchronizes them to the beginning of interphase (Bonner, 1967; Gingle, 1976).

Harvested amoebae suspended in buffer were adjusted to a final concentration using a haemocytometer so that the amoeba density on the agar surface was $3.5 \times 10^4$ cells/cm$^2$. Two per cent purified DIFCO agar made up in phosphate (KK$_2$) buffer served as the filming surface. The cells were allowed to progress for 6 h into interphase, at which time tips were transplanted into the field of cells. A 6-h field is fully competent to respond chemotactically to a c-AMP signal (Robertson et al. 1972), but, at the densities used, cannot relay the signal (Gingle & Robertson, 1976).

In some experiments beef-heart phosphodiesterase (PDE) was used (Sigma Chemical Co., 0.18 units/mg protein). Dilutions of the PDE were made beforehand and added to the washed amoebae to give known activities. For the highest PDE concentrations used, 500 $\mu$g/cm$^2$ or $10^3$ $\mu$g/cm$^2$, the amoebae were added directly to the lyophilized enzyme. When the enzyme had dissolved, cell-enzyme suspensions were added directly to the agar surface.

Tips were removed from fruiting bodies or conuses as previously described (Rubin & Robertson, 1975). Two tips were generally placed in the field to be filmed in each experiment. The distance between the tips was at least 1.5 mm, which ensured that they did not influence each other. At the field density and age used, only chemotaxis could be observed and its maximum range was about 600 $\mu$m.

Films

The films were taken in a constant temperature room ($21 \pm 1 ^\circ C$) with a Bolex 16 mm movie camera and a Nikon CFMA camera drive. The filming microscope was either a Nikon Apophot or a Nikon 'SKE' using continuous transmitted light with a green filter to enhance contrast on the panchromatic film. Exposure was set automatically on the SKE or manually using a photoelectric cell with the Apophot. Frame rates for filming were 4, 7.5 or 8 frames/min. All magnifications were set at 2.5 (ocular = 5 $\times$, objective = 2 $\times$, and $\frac{1}{2} \times$ camera relay lens). The experiments were recorded on Kodak 16 mm 4 X reversal film.

Measurements

Measurements were taken from films projected onto a work bench, using an overhead front-surfaced mirror, by a Traid Selecta-frame projector, Model 16N/LS. The projector was equipped with an automatic frame counter. If the image of the transplanted tip could be well approximated by a circle, then a circle was drawn around the margins of the tip and the center of the circle was defined. Concentric circles were drawn around the tip, and successive approxi-
mations were made of the chemotactic range of the tip. At each distance, the number of cells crossing the circle toward the tip and away from the tip were counted. The \( \chi^2 \) was taken comparing the numbers obtained to those that would be expected if there were no chemotaxis and only a random amoeboid motion. A \( P < 0.025 \) was used as the criterion for rejecting the null hypothesis that there was no chemotaxis. Steps of approximately 50 \( \mu \text{m} \) were taken between successive circles until the null hypothesis was rejected. If the tip's border could not be approximated by a circle, then its boundary was traced and parallel outlines of the tip were drawn at various distances to obtain the successive approximations.

All of the chemotactic measurements were made during the first hour to hour and a half of real time after the transplant. The \( \chi^2 \) analysis was performed to compare the number of crossings during the periods: 0–30 min, 0–60 min and sometimes 0–90 min after each transplant was made. We had noted previously that for conus tips nearly all the transplants had begun to form a pseudoplasmodium within the first 90 min and that a large minority of fruiting body tips had regulated by this time (Rubin & Robertson, 1975). It was also observed that the maximum chemotaxis toward tips occurred during the first 90 min; after the multicellular pseudo-plasmodium began to form on tip regulation, chemotaxis and thus acrasin secretion decreased markedly.

**Tips transplanted onto plain agar**

Tips were transplanted onto buffered agar as previously described. About half of the transplants were filmed at 4 frames/min and the remainder were observed at various intervals to see whether the pseudoplasmodium that formed after regulation was a migratory slug or whether no migration occurred and a fruiting body formed directly. The occurrence of migration could be checked by observing whether or not there was a slime sheath leading from the residual base of the transplant to the subsequent fruiting body.

**RESULTS**

**Tips into fields**

Tips from early fruiting bodies (Farnsworth, stage 17 or 18) or conus tips (Farnsworth, stage 12 or 13) were placed into 6 h interphase fields of amoebae and the chemotactic ranges were measured (see Figs. 1 and 2). The data show that fruiting body tips transplanted into fields of amoebae mixed with increasing concentrations of c-AMP phosphodiesterase experienced up to a five-fold decrease in their measured chemotactic range. The range for fruiting body tips decreased from a mean of 506 \( \mu \text{m} \) in fields without any added PDE to 106 \( \mu \text{m} \) for fields with \( 10^3 \mu \text{g/cm}^2 \) of agar surface. The same type of response was seen with conus tips where a greater than an order of magnitude decrease in the chemotactic range, 447–34 \( \mu \text{m} \), occurred over the same PDE concentration ranges.
Fig. 1. Plot of the chemotactic range of fruiting body tips transplanted into interphase fields of ameobae vs. increasing concentrations of beef-heart PDE added to the fields.

Fig. 2. Plot of the chemotactic range of conus tips transplanted into interphase fields of ameobae vs. increasing concentrations of beef-heart PDE added to the fields.
The differences between the measurements of tips in fields without PDE and those with $10^3 \mu g/cm^2$ of agar surface was significant ($P < 0.001$ for fruiting bodies, $P < 0.01$ for conuses using a two-tailed $t$ test). Although the range of fruiting body tips tended to be slightly greater at all PDE concentrations than the range of conus tips, the difference between individual points was not significant ($P > 0.05$).

Effect of size of tip

Although the number of amoebae in the field could be well controlled, the size of the tip depended very much on the size of the conus or fruiting body from which it was taken. To see if the tip's size altered its capacity to attract amoebae in the field, plots of range vs. tip radius were made for tips placed into fields without PDE (see Figs. 3 and 4). The sample regression coefficient was not significant, $P > 0.05$, for either conus or fruiting body tips.

Tip transplants into high-density fields

We showed previously that both fruiting body and conus tips transplanted into fields of densely packed amoebae, $1 \times 10^6$ cells/cm$^2$, generally produce a migratory slug before culmination (Rubin & Robertson, 1975). However, tips

transplanted onto plain agar surfaces tend to culminate without migration (Gregg, 1968). To compare this result with ours, conus and fruiting body tips were transplanted onto plain agar. The product of the tip's regulation was checked for either immediate culmination or migration before culmination. As the results show (Table 1), fruiting body and conus tips form migratory slugs significantly more often on high density fields of amoebae than on plain agar surfaces.

Table 1. Comparison of the results of conus tips and fruiting-body tips transplanted into high density fields of interphase amoebae and tips transplanted onto plain agar

<table>
<thead>
<tr>
<th>Field</th>
<th>No. of tips fruiting immediately</th>
<th>No. of tips forming a migratory phase before fruiting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conus tip transplants</td>
<td></td>
</tr>
<tr>
<td>High density*</td>
<td>20</td>
<td>58</td>
</tr>
<tr>
<td>No cells</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>$P &lt; 0.005$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F.B. tip transplants</td>
<td></td>
</tr>
<tr>
<td>High density</td>
<td>2</td>
<td>46</td>
</tr>
<tr>
<td>No cells</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>$P &lt; &lt; 0.005$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The density used was $1 \times 10^6$ cells/cm$^2$, which is almost close packing. In each case the difference between the results was significant by the $\chi^2$ test.
Fig. 3. Histogram showing the relationship of the effective radius or radius of fruiting body tips to the chemotactic range. The effective radius is the radius of the circle whose area is equivalent to the area of the tip measured. A regression analysis of the chemotactic range onto the tip radius demonstrated no significant difference from the null hypothesis ($P > 0.05$).

Fig. 4. Histogram showing the relationship of the radius of conus tips to the chemotactic range. A regression analysis of the chemotactic range onto the tip radius demonstrated no significant difference from the null hypothesis ($P > 0.05$).
Regulation of the tip's signal

A very interesting point has arisen from these results. At least for the range of tip sizes used, there is no dependence of the chemotactic range on the tip diameter (or volume). Thus the gradient of acrasin produced by the tip appears to be regulated. If this is so, then the slime mold organizing system provides a simple example of a regulating gradient.

This may be important because the idea of a regulating gradient is frequently invoked to account for regulative development. Recent models for regulating gradients have been proposed by Wolpert, Hicklin & Hornbruch (1971), Cohen (1971) and Lawrence, Crick & Munro (1973). All suffer from two defects: ad hoc assumptions about kinetics and lack of knowledge of the chemical nature of the postulated morphogen. It might seem that the concept was too tenuous to retain did it not arise naturally from the well-established idea of a morphogenetic field controlled by a graded parameter. Much of the difficulty might be circumvented if a specific regulating gradient were known.

Although I cannot say, at this stage, how the tip's signal is regulated, a few possibilities may be suggested. Approximately the anterior \( \frac{1}{4} \) of the pseudoplasmodium was removed in my experiments and considered to be the tip. It is possible that the actual cells that control slime mold development represent only a fraction of the morphologically distinct tip, and the transplants presented here might include other cells along with the effective ‘tip’. Other possibilities which cannot be excluded are non-linearities in the enzyme kinetics or some sort of active-transport mechanism (see Cohen, 1971, for a review).

2. Chemical nature of the tip’s signal

One problem with the models in other systems is that their morphogens are still unknown. A large part of the ambiguity generated in the explanation of multi-cellular development in other systems is due to the myriad of postulated inducing substances without the isolation of any specific chemical (Deuchar, 1975). However, the results from experiments in which tips were transplanted into fields mixed with beef-heart phosphodiesterase show that the tip’s morphogen is c-AMP. The results show clearly that beef-heart phosphodiesterase significantly decreases the chemotactic range of the tip’s signal. Konijn et al. (1967, 1968) first suggested that c-AMP was the natural acrasin for the slime mold D. discoideum. It was noted that concentrations of c-AMP would attract interphase amoebae, and interphase amoebae would chemotax toward an exogenously produced signal of c-AMP (Robertson et al. 1972). D. discoideum also produces a cyclic nucleotide phosphodiesterase (Chang, 1968). The phosphodiesterase will hydrolyze any of the cyclic nucleotides; however, besides c-AMP, the amoebae will only chemotax to c-GMP, to which they are three orders of magnitude less sensitive (Konijn, 1972). Thus given Konijn’s result, it
is safe to conclude that the tip does secrete c-AMP. It is also known that the tip secretes the highest concentration of acrasin (Bonner, 1949), and that the tip is necessary for the completion of the multi-cellular stages of the life-cycle (Raper, 1940; Farnsworth, 1973; Rubin & Robertson, 1975). It therefore is likely that an extracellular c-AMP signal is necessary for development through the life-cycle (Bonner, 1970).

3. **Quantity of the tip's signal**

In a previous paper (Rubin & Robertson, 1975) it was suggested that fruiting-body tips might produce a stronger signal than conus tips. The results in this paper show that although the fruiting-body tips seem to produce a slightly stronger signal on the average, there is no significant difference between the amounts of c-AMP secreted by the two different types of tips. The difference in the probabilities for the induction of relaying and wave propagation by the two types of tips as noted in the earlier paper may have been due to the shorter regulation time observed for conus tips, or to the fact that conus tips produce a slime sheath whereas fruiting-body tips do not (Bonner, 1967).

To approximate the amount of c-AMP secreted by the tip, I used equation (7) of Cohen, Drage & Robertson (1975). This equation assumes no PDE activity and a point source. To calculate the amount of c-AMP secreted per cell in the tip, I approximated the tip’s volume by a hemisphere of radius 100 \( \mu m \) and took the average cell radius to be 7 \( \mu m \). The value obtained is \( 7 \times 10^3 \) molecules of c-AMP secreted/cell/second. This value is a lower bound since no PDE activity is assumed, and it is well within the plausible range since the maximum possible secretion rate is about \( 10^6 \) molecules/cell/sec (Robertson & Drage, 1975).

4. **The tip as an organizing centre**

From the results of Raper (1940), Farnsworth (1973) and Rubin & Robertson (1975) it is apparent that the tip does act as an organizing region during the pseudoplasmodial stages of *D. discoideum* development. Rubin and Robertson also showed that all tips were at least qualitatively similar in the properties, particularly with respect to their signal. In this paper I have shown that the signal is a regulated gradient of c-AMP. Thus, in analogy with the organizer of Spemann (1938), the tip behaves autonomously in grafting experiments and releases a signal which does not vary, but whose interpretation by responding cells is varied. While the tip of *D. discoideum* may represent a unique case it is at least possible that other organizing regions behave in a similar fashion, releasing a morphogen of small molecular weight.
5. Response of interphase cells to the tip's signal

As mentioned in the previous paper (Rubin & Robertson, 1975), the tip's signal is stage independent and is interpreted by the field according to the responding cell's ability. The tip transplants into high density fields further illustrate this point. Tips transplanted onto plain agar surfaces by themselves do not usually migrate, but in high-density fields the tip and the cells that it pulls in form a migratory slug. Interphase cells normally produce a migratory slug after aggregation, and if the tip's signal were non-specific, a pseudoplasmodium incorporating cells that were recently in interphase would also be expected to have a migratory phase before culminating into a fruiting body. This is indeed the case.

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REFERENCES


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