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Immunofluorescence Evidence for the Distribution of Cyclic AMP in Cells and Cell Masses of the Cellular Slime Molds

(histochemistry/fluorescent antibody staining/cyclic nucleotide localization)

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ABSTRACT With immunofluorescent techniques it has been possible to show that bound cyclic AMP is uniformly distributed in the nucleus and cytoplasm of a number of species of cellular slime molds. One species (which does not respond to cyclic AMP as an acrasin) is an exception and has its cyclic AMP concentrated in the nucleus during the feeding and aggregation stage. In cell masses of *Dictyostelium discoideum* that show early signs of differentiation the anterior, prestalk cells contain more cyclic AMP than the posterior, prespore cells.

There is evidence that cyclic AMP plays at least two important roles in the development of the cellular slime molds: for some species it is the acrasin, or chemotactic agent which attracts the amoebae to central collection points to produce a multicellular organism (1), and in some species it is known to induce stalk cell formation, at least when added to isolated cells (2). For obvious reasons it would be interesting to know the distribution of bound cyclic AMP in cells and cell masses of a number of species, with special emphasis on *Dictyostelium discoideum*, which responds to cyclic AMP in both of the two ways cited above.

MATERIALS AND METHODS

Preparation of Cells. The following species of cellular slime molds were used: *Dictyostelium discoideum* (no. NC-4H), *D. rosarium* (no. CC-7), *D. mucoroides* (no. 11), *D. purpureum* (no. 12), *Polysphondylium violaceum* (no. 1), and *P. pallidum* (no. 2). The amoebae were grown on a two-membered culture with *Escherichia coli* on a suitable medium (3) for 40 hr at 21°. They were then washed from the plate and centrifuged at 25 × *g* for three 7-min periods in a 1% physiological salt solution (4). The final suspension was brought to a concentration of 1×10^6 cells per ml. Single drops were placed on acid-washed cover slips and spread with another. Air-dried specimens of *D. discoideum* and *P. violaceum* were prepared by placing droplets of cells (1×10^7 cells per ml.) on cover slips resting on the surface of 2% agar plates. When aggregation streams appeared, the cover slips were carefully washed with phosphate-buffered saline (5) and air dried before staining. To obtain migrating cell masses the washed amoebae were put directly on 2% agar plates.

Preparation of Immunoreagent. Antisera to cyclic AMP were obtained from randomly bred rabbits which had been previously injected with repeated doses of 2′-*o*-succinyl cyclic AMP conjugated to human serum albumin or polylysine (6). Conjugate (0.25 mg/ml) suspended in complete Freund's adjuvant was injected into each footpad. Booster injections of 0.6 mg of conjugate were administered into the rear thighs, upper back region, and rear footpads after 6 weeks. Then 10–14 days following booster injections, the rabbits were bled from the ear. The serum immunoglobulin (IgG) fraction was obtained by ammonium sulfate fractionation (the precipitate at 40% saturation) and subsequent DEAE-cellulose column purification with 0.02 M pH 7.0 phosphate buffer. By means of radioimmunoassay using [I^{125}]succinyl cyclic AMP tyrosine methyl ester antigen (Collaborative Research, Waltham, Mass.) (7) the various IgG fractions from the different antisera were shown to have relatively high specific titers. The I^{125} isotope was counted in an Intertechnique SL30 scintillation counter, with antibody–antigen pellet first solubilized in 0.2 ml of 0.1% sodium dodecyl sulfate. Maximal binding (35–50%) of antibody was obtained. These fractions of IgG were then used for staining the slime mold cells and sections.

Sectioning and Staining of Cells and Cell Masses. Migration stages of *D. discoideum* were gently lifted by their slime trail end with a fine needle and placed in tiny grooves made on the surface of 0.75-cm^3 2% agar blocks. The agar was then mounted on a metal specimen holder with Ames O.C.T. Compound (Ames Co., Division of Miles Laboratories, Inc., Ind.) and the upper surface of the agar block was uniformly covered with Cryoform (Damon, IEC Division). Rapid freezing of the agar and specimens was then accomplished by packing the metal chuck into powdered dry ice. With material thus frozen, cryostat sections (5–10 μm) of the unfixed samples were made, thawed on glass slides and briefly dried in a gentle stream of filtered air.

Sections or whole cells were then immediately stained by the indirect immunofluorescent method (8) in a moist chamber using fluorescein isothiocyanate-conjugated goat immunoglobulin (antibodies to rabbit IgG—Miles Laboratories, 64-173). Following staining, slides of cells were mounted in 90% glycerol, 10% phosphate-buffered saline and then scanned by dark field fluorescence microscopy with transmitted or incident illumination (Zeiss microscopes with BG-12 excitation filter and 470- to 500-nm barrier filters).

Various controls were run to show that the stain was specific for cyclic AMP. No staining was observed if: (*1*) IgG fractions from unimmunized rabbits were used as stain and (*2*) anti-cyclic AMP serum was incubated for 5 hr with cyclic AMP prior to use in staining. However, if other cyclic compounds, IMP, GMP, UMP, ATP, and 5′AMP (mM) were incubated with the anti-cyclic AMP for 5 hr at 4°, only slight

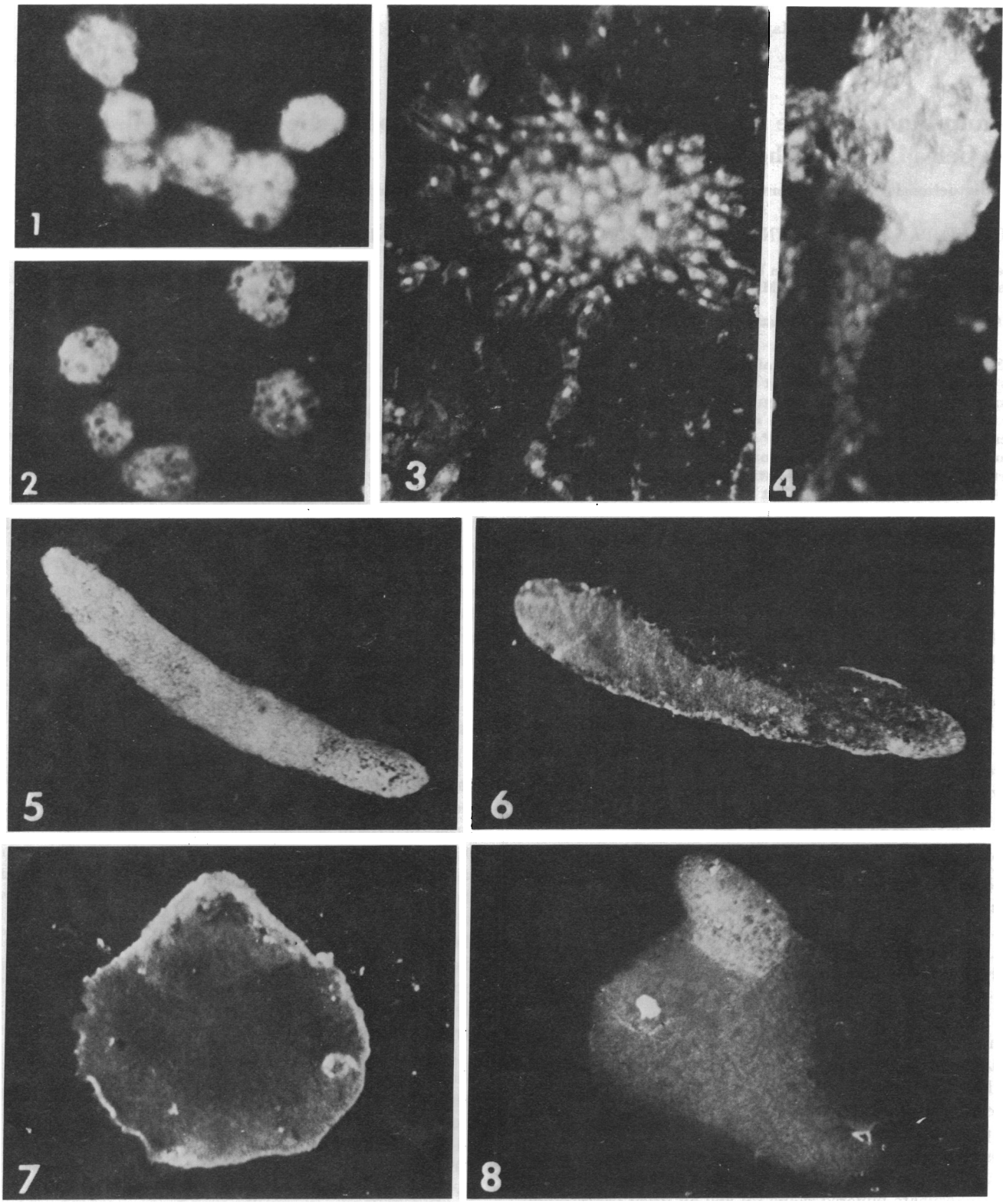

FIG. 1. The vegetative amoebae of *Dictyostelium discoideum* showing an even distribution of cyclic AMP. (×1500)

FIG. 2. The same for *Polysphondylium pallidum*. (×1500)

FIG. 3. The aggregating streams of *P. violaceum*. Note the relatively bright staining of the nucleus. (×600)

FIG. 4. An advanced center and a stream of *D. discoideum*. (×600)

FIG. 5. A longitudinal section of an early migrating cell mass of *D. discoideum*. While there is a fairly uniform distribution of the fluorescent staining, the anterior end (*left*) appears slightly brighter. (×150)

FIG. 6. A similar section of a late migrating cell mass. Note the uniform intense staining of a large mass of cells at the anterior end (*left*). (×150)

FIGS. 7 and 8. Two sections of cell masses that have righted themselves at the end of aggregation. In both cases the anterior, prestalk region shows more fluorescence than the posterior, prespore region. (×175)

decrease in intensity of fluorescence was observed. Also, anti-cyclic AMP IgG was preincubated with cyclic AMP and this showed no phosphodiesterase or endonuclease activity.

RESULTS

In the vegetative cells of *D. discoideum* there is an even distribution of cyclic AMP in both the cytoplasm and the nucleus, except in the regions of the contractile vacuoles (Fig. 1). This is also true for similar preparations made of vegetative amoebae of *D. purpureum*, *D. mucoroides*, *D. rosarium*, and *Polysphondylium pallidum* (Fig. 2). On the other hand, *P. violaceum* is noteworthy in that only the nucleus shows any significant fluorescence, a condition that is particularly striking during the aggregation of *P. violaceum* (Fig. 3). In *D. discoideum* the cells remain uniformly fluorescent during aggregation, and show intense fluorescence in the central region of the aggregate (Fig. 4) due, no doubt, to the greater concentration of cells.

In the migrating stages of *D. discoideum* there is first a uniform distribution of the cyclic AMP, but later the anterior, prestalk cells show a greater concentration of fluorescence than the posterior prespore region (Figs. 5 and 6). This is especially obvious in the stages just prior to culmination (Figs. 7 and 8). In all, 34 cell masses were sectioned at these stages; of these five showed a gradient highest at the anterior end (Fig. 5), and 15 showed two distinct zones (Figs. 6, 7, and 8). It is presumed that the gradient represents an earlier condition, although there is no evidence to substantiate this.

Finally, in *P. violaceum* the cell masses show a fairly uniform distribution of cyclic AMP, and the cells are no longer merely stained in the nucleus, but in the cytoplasm as well.

DISCUSSION

These results presumably show the distribution of bound cyclic AMP rather than the soluble form of the nucleotide. There are multiple washes during the preparation process which would have removed any soluble cyclic AMP. This is supported by the fact that the vacuoles are empty in the vegetative cells (Figs. 1 and 2).

In considering these results from the point of view of chemotaxis, those species which respond to cyclic AMP as an acrasin have cyclic AMP in their cytoplasm, as does *P. pallidum* which does not respond to cyclic AMP. But *P. violaceum* (which is also chemotactically insensitive to cyclic AMP) shows all of its cyclic AMP in the nucleus during the aggregation stage, although by the migration stage its cytoplasm also has a uniform distribution of cyclic AMP.

From the point of view of the role of cyclic AMP in differentiation, the anterior, prestalk cells show more bound cyclic AMP than the posterior, prespore cells in *D. discoideum*. This is consistent with an earlier observation that the anterior end of the cell mass gives off more acrasin (9) and the recent finding of Maeda and Maeda (10) that before aggregation the prestalk cells of *D. discoideum* secrete more acrasin than the prespore cells. Finally, it is of interest to note that McMahon (11) predicts just such a distribution of cyclic AMP in his model of slime mold development.

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