Characterization of Colchicine-Binding Activity in *Dictyostelium discoideum*

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(Received 11 July 1977)

A colchicine-binding component was detected in vegetative amoebae of *Dictyostelium discoideum* by using a Millipore-filter assay. The colchicine-binding activity is temperature- and time-dependent, maximum binding occurring at 22–35°C after 60 min incubation. Further increases in temperature are without effect on the extent of binding, but bound colchicine is released with increased time of incubation. Furthermore, colchicine-binding activity itself decreased in the high-speed supernatant from *D. discoideum*, with half the activity being lost in approx. 2.5 h. Several lines of evidence, including the saturation kinetics of colchicine binding, enhancement of colchicine binding by tartrate, insensitivity to lumicolchicine, precipitation of the binding protein by vinblastine and behaviour of the binding protein on DEAE-cellulose and Sephadex resins, suggest that the colchicine-binding protein may be tubulin.

Microtubules have been found in all eukaryotes. Tubulin, the subunit protein of microtubules, has the property of binding alkaloids such as colchicine (Wilson *et al.*, 1974), and this binding usually interferes with microtubule function. However, in certain simple eukaryotes, for example *Saccharomyces cerevisiae* (Haber *et al.*, 1972) and *Physarum* (Jockusch, 1973), although microtubules have been observed, no colchicine-binding protein has been detected. In other simple eukaryotes, for example *Allomyces neomoniliformis* (Olson, 1973), although a colchicine-binding protein has been detected, it has a mol. wt. of only 30 000, compared with a mol. wt. of 115 000–120 000 for tubulin from higher eukaryotes.

Moens (1976) has demonstrated the existence of microtubules in the mitotic apparatus of *Dictyostelium discoideum*, and it has also been shown that colchicine will inhibit not only mitosis (Zada-Hames, 1977) but also development (Cappuccinelli & Ashworth, 1976) in this organism, although very high concentrations of the drug are necessary. The present study was initiated to determine whether a colchicine-binding protein could be detected in *D. discoideum* and, if so, to characterize its properties.

Experimental

Materials

Colchicine was obtained from BDH Chemicals,

Abbreviation used: Mes, 4-morpholine-ethanesulphonic acid.

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Poole, Dorset, U.K.; Vinblastine was a gift from Eli-Lilly and Co., Basingstoke, Hants., U.K.; DEAE-cellulose (DE-52) and Sephadex resins were purchased from Sigma (London) Chemical Co., London W.5, U.K. Lumicolchicine was prepared by exposure of 1 mM-colchicine in 95% (v/v) ethanol to u.v. light for 14 h. Actual conversion of colchicine into lumicolchicine was monitored by measuring the shift in the absorbance spectrum.

Methods

Growth of myxamoebae and preparation of cell extracts. Myxamoebae of strain Ax-2 (A.T.C.C. 24397) of *D. discoideum* were grown axenically in HL5 medium containing 86 mM-glucose and harvested in the exponential phase of growth (Hames & Ashworth, 1974). The cells were washed twice with cold water and suspended in ice-cold binding buffer (0.1 M-Mes/0.5 mM-MgCl₂/1 mM-EGTA/1 mM-GTP, pH 6.5) to give a final concentration of 1.5×10^8 cells/ml. Cell suspensions were disrupted by sonication with a 100 W MSE ultrasonic disintegrator (peak-to-peak amplitude 10 μ m) for four periods of 15 s with continuous cooling in an ice/salt bath. Sonicated preparations were stirred in an ice bath for 30 min and then centrifuged at 100 000 g for 60 min at 4°C. The high-speed supernatant was assayed for protein by the method of Lowry *et al.* (1951), and samples were diluted with binding buffer to the desired protein concentration, and immediately used for the colchicine-binding assay.

Colchicine-binding assay. A portion (0.2 ml) of the high-speed supernatant was incubated at 35°C in the dark with an appropriate concentration of [³H]-colchicine (usually 2.5–3.0 μ M-[ring C-methoxy-³H]-colchicine, sp. radioactivity 5.9 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.).

After incubation for 30–120 min with gentle stirring, the reaction was stopped by the addition of 1 ml of ice-cold binding buffer containing unlabelled 2.5–3.0 μM -colchicine. Bound colchicine was separated from the unbound material by using a modification of the filter assay technique of Weisenberg *et al.* (1968). Thus 0.2 ml of the sample was filtered under gravity through a Millipore filter (25 mm diam., 0.45 μm pore diam.) previously equilibrated in binding buffer containing unlabelled colchicine. Unbound radioactivity was removed by washing with 20 ml of this buffer and the filters were dried, dissolved in a toluene-based scintillation fluid and counted for radioactivity. The efficiency of the system was determined by the internal-standard method.

Characterization of the colchicine binding fraction. A portion (3 ml) of the high-speed supernatant (10–20 mg of protein/ml) in 0.5 mM- MgCl_2 /0.1 M-sodium phosphate buffer (pH 6.8) was incubated for 60 min with 5 μM - ^3H colchicine and then applied to a column (1 cm $\times$ 7 cm) of DEAE-cellulose previously equilibrated in this buffer, and the column was developed by increasing the NaCl concentration from 0 to 0.8 M. Fractions were collected and assayed for radioactivity and A_{280} . Colchicine-binding activity was eluted in a peak corresponding to 0.21 M-NaCl concentration. These peak fractions were pooled, re-incubated with 5 μM - ^3H colchicine for 60 min at 35°C, and portions immediately applied to a column (13 cm $\times$ 1 cm) of Sephadex G-25 or a column (30 cm $\times$ 1 cm) of Sephadex G-75 equilibrated with 0.5 mM- MgCl_2 /0.1 M-sodium phosphate buffer (pH 6.8).

Fig. 1. Linearity of colchicine binding with protein concentration

Various quantities of a 100000g supernatant of *D. discoideum* were incubated for 60 min with 3 μM - ^3H -colchicine and bound radioactivity was measured by using a Millipore-filter assay.

Results

Characterization of the colchicine-binding assay

Linearity of the assay. To check the linearity of the assay, samples (0.2 ml) containing different amounts of protein were incubated with 3 μM - ^3H colchicine at 35°C, the reaction was stopped after 60 min and the colchicine binding determined as described under 'Methods'. The colchicine-binding assay was linear up to a concentration of 7.5 mg of protein/ml (Fig. 1). The lack of linearity above this protein concentration was shown by appropriate control experiments to be due to saturation of the protein-binding capacity of the Millipore filter rather than any non-linearity of colchicine binding itself. However all colchicine-binding assays were routinely carried out with a protein concentration below 6 mg of protein/ml of sample.

Effect of temperature and time of incubation. As shown in Fig. 2, far more binding occurred at 22°C than at 2°C, but further increases of temperature did not alter the maximum binding observed. Furthermore, the binding reaction is time-dependent, reaching a maximum after about 60 min. After this time the amount of colchicine bound decreases, slowly at first but then at a faster rate, such that after 3 h incubation, only 20% of the maximum binding remains. This is due to a release of previously bound colchicine. The ability of the binding component to retain colchicine is therefore unstable.

Effect of tartrate. Since it is known that the rate of colchicine binding to tubulin is enhanced by certain anions, especially tartrate (Bhattacharyya & Wolff, 1976), we have determined the effect of different concentrations of tartrate on the colchicine-binding activity in *D. discoideum*. As shown in Fig. 3, tartrate does indeed increase the rate of colchicine binding,

Fig. 2. Effect of temperature and time of incubation on colchicine binding

High-speed supernatant of *D. discoideum* was incubated with 3 μM - ^3H colchicine at 2°C (Δ), 12°C ($\triangle$), 22°C ($\bullet$) and 35°C ($\blacksquare$), and bound ^3H colchicine was determined as a function of time.

Fig. 3. Effect of L-tartrate on colchicine binding High-speed supernatant of *D. discoideum* was incubated with 3 μ M- $[^3\text{H}]$ colchicine in the absence (Δ) or in the presence of 0.3 mM- ($\triangle$), 1.5 mM- ($\bullet$), or 3 mM-tartrate ($\blacksquare$), and at intervals bound $[^3\text{H}]$ -colchicine was determined.

Fig. 4. Colchine-binding activity as a function of colchicine concentration High-speed supernatant of *D. discoideum* was incubated for 60min with various concentrations of $[^3\text{H}]$ colchicine, and protein-bound $[^3\text{H}]$ colchicine was determined.

with 3 mM-tartrate being the most effective concentration, resulting in maximum binding after incubation for 30 min, compared with 60 min in the absence of tartrate. Further increases in tartrate concentration are without effect.

Specificity of colchicine binding. When the amount of the high-speed supernatant is constant and the colchicine concentration is increased, binding is saturated at approx. 2.5 μ M-colchicine (Fig. 4). Further increases in colchicine concentrations are without effect until very high concentrations are present (greater than 15 μ M), when increased binding is observed, probably of a non-specific nature. Therefore below 10 μ M-colchicine, binding is satur-

Table 1. Specificity of $[^3\text{H}]$ colchicine binding in *D. discoideum* high-speed supernatants

High-speed supernatants from *D. discoideum* were preincubated for 30 min at 35°C with and without various concentrations of vinblastine, colchicine and lumicolchicine to determine the specificity of colchicine binding. After centrifugation (10000 g for 20 min), the binding of $[^3\text{H}]$ colchicine was measured. Results are given as means $\pm$ s.d for four experiments.

Preincubation conditions	Incubation conditions	Bound $[^3\text{H}]$ -colchicine (c.p.m./mg of protein)
—	—	48 $\pm$ 7
—	$[^3\text{H}]$ Colchicine (3 μ M)	5610 $\pm$ 520
Vinblastine (10 μ M)	$[^3\text{H}]$ Colchicine (3 μ M)	6070 $\pm$ 635
Vinblastine (2 mM)	$[^3\text{H}]$ Colchicine (3 μ M)	240 $\pm$ 85
Colchicine (3 μ M)	$[^3\text{H}]$ Colchicine (3 μ M)	297 $\pm$ 155
Lumicolchicine (3 μ M)	$[^3\text{H}]$ Colchicine (3 μ M)	5475 $\pm$ 830

able, suggesting specificity. Scatchard-plot analysis (Scatchard, 1949) of the results suggest a colchicine-binding constant of approx. 2×10^5 litres/mol, in comparison with the value of 6×10^5 litres/mol found for rat liver tubulin (Rapaport *et al.*, 1975).

Evidence for the specificity of colchicine binding to tubulin was gained by the use of vinblastine, which is known to precipitate tubulin at high vinblastine concentration (2 mM) but stabilizes colchicine-binding activity at low concentration (10 μ M) (Patzelt *et al.*, 1975) and lumicolchicine, a stereoisomer of colchicine, which binds to most proteins that bind colchicine non-specifically, but fails to bind to tubulin (Wilson & Meza, 1973). When high-speed supernatant is preincubated with 10 μ M-vinblastine and the supernatant centrifuged (10000 g for 20 min) before the colchicine-binding assay, no decrease of colchicine binding is observed, but at 2 mM-vinblastine, colchicine binding is decreased to background values, as would be expected if the colchicine was binding specifically and solely to tubulin (Table 1). Furthermore, preincubation with lumicolchicine is without effect on colchicine binding, as would be expected if colchicine is binding only to tubulin. Therefore we conclude that high-speed supernatant of *D. discoideum* contains a protein that binds colchicine specifically and that is probably tubulin.

Decay of colchicine-binding activity

To determine the stability of the colchicine-binding component, *D. discoideum* supernatant was

Fig. 5. Time-dependence of colchicine-binding activity. A high-speed supernatant fraction of *D. discoideum* was incubated at 37°C. At the time indicated, samples were removed, incubated with 3 μ M-[³H]colchicine for 60 min at 35°C, and bound colchicine was assayed. Extrapolation of the line to include the time of [³H]colchicine incubation (60 min) yields the initial binding capacity. The half-time of the colchicine-binding activity is calculated from the slope of the line.

Fig. 6. Fractionation of colchicine-binding on DEAE-cellulose

Elution profiles of protein (—) and radioactivity (●) from a DEAE-cellulose (DE-52) column loaded with 3 ml of a high-speed supernatant fraction of *D. discoideum* amoebae (10–20 mg of protein/ml) incubated for 60 min with 5 μ M-[³H]colchicine. Protein was eluted with a gradient of 0–0.8 M NaCl (----) at 4°C.

incubated at 35°C and samples were removed at intervals and assayed for colchicine-binding activity. The binding activity was found to decrease to 50% of the initial activity in approx. 2.5 h (Fig. 5). This represents a decrease in the ability to bind colchicine rather than a release of bound colchicine.

Fractionation of colchicine-binding activity

The colchicine-binding component of the high-speed supernatant of *D. discoideum* binds to DEAE-cellulose and is eluted by a total salt concentration of

Fig. 7. Chromatography of colchicine-binding protein on Sephadex resins

Elution profiles of protein (—) and radioactivity (●) from (a) a Sephadex G-25 and (b) a Sephadex G-75 column loaded with colchicine-binding protein of *D. discoideum* prepared by the DEAE-cellulose technique (see under 'Methods') and incubated with 5 μ M-[³H]colchicine for 60 min. DEX and BSA indicate elution positions of Blue Dextran 2000 and bovine serum albumin respectively. Chromatography was performed at 4°C.

0.31 M; free colchicine passed through the column unretarded (Fig. 6). Attempts to determine accurately the molecular weight of the eluted colchicine-binding protein by gel filtration on Sephadex G-200 failed, since colchicine was released from the binding protein before the Sephadex resin had resolved the protein components of the sample. However, by using the faster-running Sephadex G-25 and G-75 resins it was possible to show that the binding protein was eluted in the void volume of each (Fig. 7), indicating that the active complex has mol.wt. greater than 70000. Free colchicine was eluted in the inclusion volume of each column.

Discussion

Evidence has been given in the present paper that vegetative cells of *D. discoideum* contain a colchicine-binding protein that can be assayed in high-speed supernatant of the organism. In this respect *D. discoideum* seems to be similar to most eukaryotic cells and unlike some of the simple eukaryotes such as *S. cerevisiae* (Haber *et al.*, 1972) and *Physarum* (Jockusch, 1973), for which no colchicine-binding protein has been detected. Thus our finding is in contrast with the general suggestion of the absence of colchicine-binding proteins from lower organisms (Burns, 1973).

In *D. discoideum* both the ability to bind colchicine and the ability of the complex to retain bound colchicine are unstable, decreasing to 50% of maximum in approx. 2–2.5 h. Instability of colchicine-binding protein has also been reported for *Saprolegnia ferax* (Brent-Heath, 1975), where two binding components are observed, one losing the ability to bind colchicine after 1–2 h at 37°C, and the other stable for 20 h at this temperature. We have not examined *D. discoideum* for the existence of a similar stable component.

Evidence in the present paper suggests that the colchicine-binding activity of *D. discoideum* supernatant is probably due to tubulin. Thus the binding of colchicine is clearly saturable at reasonable concentrations of colchicine, suggesting that it is specific rather than being due to a general affinity of supernatant proteins for colchicine. An extremely similar curve is observed for the binding of colchicine by tubulin in mammalian liver (Rapaport *et al.*, 1975), where saturation is reached at concentrations of 2.5–5.0 μM (compared with approx. 2.5 μM for *D. discoideum*), and non-specific colchicine binding does not occur until the colchicine concentration exceeds 50 μM (compared with approx. 15 μM for *D. discoideum*). Furthermore, the rate of colchicine binding to tubulin is characteristically slow, usually requiring 30–90 min, shows a high temperature coefficient and is enhanced by certain anions, particularly tartrate (Bhattacharyya & Wolff, 1976). The colchicine-binding activity of *D. discoideum* displays all these properties. Also lumicolchicine, which would be expected to prevent non-specific binding because of its structural similarity to colchicine but is unable to prevent specific colchicine binding to tubulin (Wilson & Meza, 1973), is without effect on the observed binding, suggesting that this is due to tubulin. Furthermore, preincubation with high concentrations of vinblastine precipitates the colchicine-binding activity from *D. discoideum* supernatant, as would be expected if tubulin were the colchicine-binding protein (Patzelt *et al.*, 1975).

In another fungus, *A. neomoniliformis*, tubulin has been shown to have a mol.wt. of 30000 (Olson, 1973),

whereas the molecular weight of most tubulins, including those from plant sources (Hart & Sabnis, 1975), is approx. 115000–120000 in the normally isolated dimer form (Bryan, 1972; Haber *et al.*, 1972; Owellen *et al.*, 1972; Wilson & Meza, 1973). The exclusion of *D. discoideum* binding protein from Sephadex G-25 and G-75 indicates a mol.wt. greater than 70000, which is at least compatible with the concept of a dalton unit of 120000. The binding with tubulin of mol.wt. 30000 observed in *Allomyces* would have been well separated on the Sephadex G-75 column but there was no evidence for such a peak. Thus on the basis of retention by DEAE-cellulose and molecular weight, *D. discoideum* tubulin is comparable with most other reported tubulins. However, one should be cautious in using the cellular content of colchicine-binding protein to derive an estimate of the cellular content of tubulin, since some tubulin is known to be bound to membranes (Feit & Barondes, 1970; Lagnado *et al.*, 1971; Stadler & Franke, 1974) and may be poorly solubilized by the methods used by us. Furthermore, we have no results on the proportion of *D. discoideum* tubulin that will bind colchicine; for example, it is known that incompletely depolymerized tubulin fails to bind colchicine (Wilson & Meza, 1973). Quantification of the cellular content of tubulin will therefore require methods other than the colchicine-binding assay described in the present paper.

We thank Professor J. M. Ashworth for helpful discussion and the Science Research Council for financial assistance. P. C. was supported by an E.M.B.O. fellowship.

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