CHARACTERIZATION OF TUBULIN IN THE BOVINE ADRENAL CORTEX CYTOSOL

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1. Introduction

Microtubular structures appear to exist in all eukaryotic cells [1]. Tubulin has been characterized as their major constituant and the colchicine-binding properties of this protein have been widely used for this purpose [2].

A possible role of microtubules in the regulation of steroidogenesis in the adrenal cortex has recently been discussed [3,4]. However, only very limited knowledge of the colchicine-binding properties of this tissue is available [5]. Characterization of this interaction should be of interest for further evaluation of microtubular functions in the adrenal gland.

In the present work, the characterization of bovine adrenal cortex tubulin was carried out by the study of colchicine-binding properties of the adrenal high speed supernatant (cytosol). The specificity and the kinetic properties of the colchicine binding, as well as several molecular parameters of the colchicine-binding moiety allowed its identification as adrenal cortex tubulin. This protein was evaluated to represent about 3% of the total adrenal cortex cytosolic proteins.

2. Materials and methods

2.1. *Preparation of cytosol*

Fresh bovine adrenal glands were collected on ice before processing (0–4°C) within the next hour. Scraped adrenal cortex tissue was homogenized in 2 vol. (P/V) cold 20 mM sodium phosphate 10 mM sodium glutamate, pH 6.8 buffer (PG buffer). The high speed supernatant (cytosol) was obtained after centrifugation at 100 000 × *g* for 60 min. Determination of protein concentration of cytosol by the biuret method with bovine serum albumin as standard [6] yielded routinely an average of 20 mg/ml.

2.2. *Chemicals*

[*ring C-methoxy*-^{3}H] Colchicine (16.05 Ci/mmol) from New England Nuclear was used as tracer and diluted to lower specific activity by addition of purified [7] unlabelled colchicine (Boehringer). Preparation of lumicolchicine was performed according to Wilson and Friedkin [7]. Podophyllotoxin was purchased from Aldrich, cytochalasin B from Serva, vinblastine sulfate from Eli Lilly and melatonin from Sigma. Griseofulvin was a gift from Clin-Comar-Byla, prostaglandins A_1 (PG A_1), E_2 (PG E_2), $F_{2\alpha}$ (PG $F_{2\alpha}$) from Upjohn (Dr J. Pike).

2.3. *Methods*

2.3.1. Colchicine-binding assay

Standard conditions were used with a charcoal procedure for separation of free and bound colchicine after incubation with cytosol [5]. Each tube contained 200 μl undiluted cytosol and 100 μl PG buffer containing colchicine and/or other compounds. The capped tubes were incubated in darkness at 37°C for 2 h and thereafter processed in the cold (4°C). After 15 min, 100 μl of a stirred 20 mg/ml charcoal (acid washed, neutralized and activated Norit A) suspension in PG-buffer was added, mixed immediately and allowed to sit for 30 min before centrifugation at 5000 × *g* for 10 min. A 150 μl supernatant aliquot was counted; calculations for bound radioactivity were made according to Sherline et al. [5]. Unless indicated

each result is the mean of triplicate corrected for loss of binding activity using appropriate values obtained from the decay rate of the binding complex.

2.3.2. Gel-filtration

Gel-filtration was made with Sephadex G-200 (Pharmacia) in small columns (0.7 cm i.d. X 9 cm) equilibrated at 37°C with PG-buffer; gel-filtration procedure and calibration were those described by Cochet et al. [8].

2.3.3. Sucrose-gradient centrifugation

Linear sucrose-gradients (0–20%) were prepared with PG-buffer and spun at 4°C and 100 000 X *g* for 14 h; 6 drop fractions were collected and counted or read at 280 nm for protein profile.

2.3.4. Thin-layer chromatography

Thin-layer chromatography of colchicine was performed according to Schönharting et al. [9].

3. Results and discussion

3.1. *Evidence for a saturable high affinity colchicine-binding in adrenal cortex cytosol*

3.1.1. Differential dissociation

The differential dissociation technique of Milgrom et al. [10] was used at 4°C and showed a typical biphasic curve (fig.1) : a fast decrease of bound radioactivity during the first 30 min then a much slower decrease indicating the presence of a high affinity binding. In the same conditions the radioactivity contained in PG buffer fell to background level within the first 3 min.

3.1.2. Binding study

A study of the binding of 5×10^{-6} M [^{3}H]-colchicine to cytosol as a function of time was made at 37°C; the association curve corrected for half-time decay of binding activity showed that a plateau was practically attained at two hours.

3.1.3. Gel-filtration

Gel-filtration on Sephadex G-200 of a cytosol aliquot incubated at 37°C with 10^{-6} M colchicine showed two well-separated peaks. The first one was totally suppressed after incubation of the cytosol

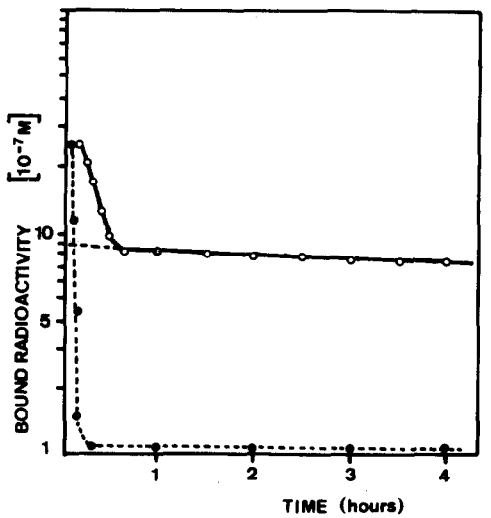

Fig.1. Differential dissociation curve: 2 ml cytosol was incubated at 37°C with 10^{-4} M [^{3}H]colchicine for 2 h, chilled on ice 15 min and then added to 1 ml stirred charcoal suspension equilibrated at 4°C. At regular intervals thereafter, 150 µl aliquots were removed from the mixture and centrifuged as described in the methods; 100 µl supernatant aliquot was counted. Control was made with PG-buffer in the same conditions. (o——o) Open circles, cytosol. (•- - -•) Closed circles, PG-buffer (control).

with a 100-fold excess (10^{-4} M) of unlabelled colchicine and thus could be attributed to colchicine bound to a saturable system. The second peak eluted after one column volume and was represented by free colchicine.

3.2. *Specificity of colchicine-binding activity*

Several compounds were tested for possible interference with colchicine-binding to cytosol (table 1). In the experimental conditions used no effect upon colchicine-binding activity was observed with lumicolchicine (a biologically inactive derivative of colchicine), cytochalasin B (a compound interacting with microfilaments) and griseofulvin (a biologically active compound on cell-division) [11]. Melatonin, which has been shown to antagonize colchicine, induced mitotic arrest in cultures of Hela and Kb cells [12], did not alter [^{3}H]colchicine binding. PG A_1, PG E_2 and PG $F_{2\alpha}$ were also ineffective. On the contrary podophyllotoxin inhibited [^{3}H]colchicine binding to cytosol; this inhibition was of a com-

Table 1
Specificity of [^{3}H]colchicine binding

Compounds (5.0×10^{-6} M)	No added compound	With added compound	% Bound radioactivity
	(dpm/μg protein)		
Colchicine	22.7	5.9	26
Lumicolchicine	22.7	22.8	100
Podophyllotoxin	22.7	5.2	23
Griseofulvin	25.4	24.7	97.4
Cytochalasin B	25.4	24.2	95.5
Melatonin	25.4	25.5	100
PG A_1	22.7	22.6	100
PG E_2	22.7	22.6	100
PG $F_{2\alpha}$	22.7	22.8	100

A 5.0×10^{-6} M concentration of the indicated compound was preincubated 1 h at 37°C with 200 μl cytosol, thereafter incubated as described in the methods with a tracer amount of [^{3}H]colchicine. Griseofulvin, cytochalasin B and melatonin were dissolved with 1% (final concentration in PG-buffer) dimethylsulfoxide (DMSO). The corresponding controls contained the same amount of DMSO.

Table 2
Effect of various agents on colchicine binding

Agent	Concentration	No added agent	With added agent	% Bound radioactivity
		(dpm/μg protein)		
Temperature	0°C	23.2	0.1	0
	100°C	23.2	0.4	1.7
Ribonuclease	0.005%	25.2	25.4	100
Deoxyribonuclease	0.027%	24.6	24.8	100
Phospholipase A	0.005%	24.8	25.0	100
Trypsin[a]	0.0013%	23.2	1.8	7.7
Trypsin + Trypsin inhibitor[a]	0.0013% 0.0014%	23.2	23.4	100

[a] Cytosol pH adjusted to 7.4

The tests were made using the standard binding assay (see methods) with tracer amount of [^{3}H]colchicine. Effect of temperature was studied by incubating cytosol at 0°C (instead of 37°C for control) or by boiling it (2 min) before the assay. Trypsin, RNAase, DNAase, phospholipase A were added after a standard incubation for an additional 1 h at 37°C.

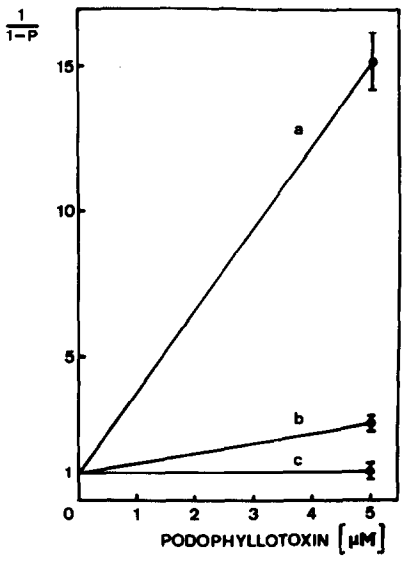

Fig.2. Competitive inhibition of colchicine binding to cytosol by podophyllotoxin: different concentrations of [^{3}H]-colchicine (respectively, a, tracer amount, b, 10^{-6} M and c, 5.0×10^{-6} M) were incubated under standard conditions without (control value) or with 5×10^{-6} M podophyllotoxin. *P* represents the percentage of binding inhibition in the samples containing podophyllotoxin as compared to the control value. Bars represent ± 1 SD.

petitive type (fig.2) [13]. From this experiment the inhibition constant K_i was calculated for podophyllotoxin with the following formula:

$$\frac{B}{Bi} = 1 + \frac{(K_d\, x) \times [i]}{(K_i) \times (K_d\, x + [x])}$$

were x is the ligand (colchicine), i the competitive inhibitor (podophyllotoxin), B the bound ligand in absence of competitive inhibitor and Bi the bound ligand in its presence. The calculated value $K_i = 3.5 \times 10^{-7}$ M is close to that reported for tubulin in chick embryo brain cytosol [14].

3.3. *Effect of various agents*

Trypsin was the only tested enzyme able to abolish the cytosol colchicine-binding property, (table 2) as previously reported by Wilson and Friedkin [15]. Trypsin inhibitor abolished this effect. Ribonuclease (RNAase), deoxyribonuclease (DNAase) and phospholipase A were without effect. Extreme conditions of temperature (0°C and 100°C) prevented the binding reaction.

3.4. *Decay-rate of the binding activity*

The stability of the binding activity of the cytosol was tested according to the time-decay procedure proposed by Wilson [16]. A loss of binding activity following a first-order kinetic was observed at 37°C within a range of colchicine concentration from 10^{-9}–10^{-5} M (table 3). When measured with 10^{-6} M [^{3}H]colchicine, the decay-rate was influenced by temperature. The inactivation rate was decreased at

Table 3
Half-time (min) for the inactivation of colchicine-binding activity at 37°C

Colchicine concentration (M)	Without added GTP	With added GTP (0.1 mM)
10^{-9}	272	337
10^{-8}	279	325
10^{-7}	288	310
10^{-6}	275	296
10^{-5}	276	358
	278 ± 6 (mean ± SD)	325 ± 24 (mean ± SD)

Adrenal cortex cytosol was preincubated at 37°C for 0, 2, 4, 6 or 8 h, after which 200 μl aliquots were processed for determination of bound colchicine using the standard assay described in the methods. For each experimental condition, half time was calculated from the slope of the straight line representing the first-order decay rate.

low temperature (4.6, 9.5, 10.2 and 21 h at 37, 20, 10 and 4°C respectively). It was also significantly decreased ($p < 0.001$) by 0.1 mM guanosine triphosphate (GTP) (table 3) and 10^{-4} M vinblastine sulfate which stabilized the binding activity over the 8 h period studied (data not shown).

3.5. *Characterization of the colchicine-binding protein*

3.5.1. Molecular weight

The calibration of the Sephadex G-200 column [8] permitted an evaluation of mol. wt 110 000 for the adrenal cortex colchicine binder, close to reported values for tubulin [17].

3.5.2. Sedimentation coefficient

The determination of sedimentation coefficient was made with a linear sucrose-gradient. ^{14}C-Labelled bovine serum albumin (4.4 S) was used as internal marker; glucose-oxidase (7.9 S) and catalase (11.4 S) were layered both on a separate gradient. The observed peak of radioactivity was lowered by a 100-fold excess (10^{-4} M) of unlabelled colchicine. A sedimentation coefficient of 5.5 S was calculated for this binder, whereas values of 5.8–6.0 S have usually been found in other tissues [18]. Similar sedimentation behavior was observed when the analysis was carried out either in PG-buffer or using the MES-reassembly buffer described by Weisenberg [19], which is thought to stabilize the tubulin moiety [20].

3.5.3. Tubulin content

A saturation curve was realized which showed a plateau at 10^{-6} M colchicine concentration (fig.3). The Scatchard plot of the binding data (fig.3) allowed calculation of the binding capacity of undiluted cytosol (1.0×10^{-6} M) and of the apparent affinity constant of the binder (3.0×10^{6} M^{-1}) this latter result is similar to previously reported values for rat brain [21] and mouse thyroid extracts [22]. The tubulin content of bovine adrenal cortex was evaluated from the binding capacity and found to represent 3% of the cytosolic proteins. This percentage is far lower than that found in brain tissue (20–40%) [23].

3.6. *Nature of the bound ligand*

After a standard binding incubation and charcoal treatment the cytosol was extracted with chloroform and a thin-layer chromatography of the extract was performed [9]. Two radioactive spots were detected: 95% of the total radioactivity (spot I) corresponded to colchicine (R_F 0.59); the remaining 5% radioactivity (spot II) had a R_F of 0.9. The product recovered from spot I had binding properties similar to [^{3}H]-colchicine whereas the product from spot II did not bind to cytosol. This finding is in agreement with the conclusion that chemically unaltered colchicine is the bound ligand to tubulin [15,24].

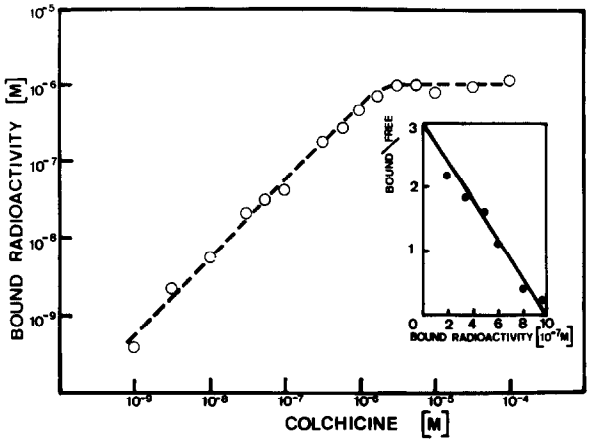

Fig.3. Saturation curve: a 4 h incubation was realized at 37°C in the presence of 10^{-4} M vinblastine with a [^{3}H]-colchicine concentration ranging from 10^{-9}–10^{-4} M. The data were used to draw the corresponding Scatchard plot (inset).

4. Conclusion

According to Wilson and Bryan [25] five parameters permit the characterization of tubulin–colchicine complex. Temperature-dependance, time-dependance, first-order decay and stabilization by Vinca alkaloids, competitive inhibition by podophyllotoxin and lack of effect of lumicolchicine. All these conditions were fulfilled in this study, therefore we conclude that the observed charcoal adsorption resistant binding activity of bovine adrenal cortex high speed supernatant is represented by the association of colchicine with microtubular protein, namely tubulin.

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