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COLCHICINE BINDING ACTIVITY IN THE RAT PANCREAS CYTOSOL[1]

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SUMMARY

Colchicine binding activity was tested in rat pancreas cytosol. This binding is time and temperature dependent ; it is stabilized by vinca alkaloids, GTP[3] and inhibited competitively by podophyllotoxin. The integrity of the colchicine binding moiety is necessary. These findings describe the kinetic properties of colchicine binding activity and demonstrate the existence of a tubulin-like protein in the rat pancreas cytosol.

INTRODUCTION

Microtubules are found in all eukaryotic cells (1) and have been involved in many biological processes, including cell motility, cell division, morphogenesis, axonal transport and secretion (1,2). Most of these works are based on the use of antimitotic drugs known to exert an effect on microtubules, especially colchicine and the vinca alkaloids. In the exocrine pancreas, these agents inhibit the release of enzymes induced by bethanechol, caerulein, carbamylcholine or ionophore A 23187 (3-5). Our recent findings (6) showed that this antisecretagogue effect could be ascribed to the inhibition of the intracellular transport of exportable proteins. These results suggested that the microtubular system of the pancreas may be of great physiological importance, and could be a site at which regulation of these various secretory processes occur. It is well known that colchicine binds to tubulin or a tubulin-like protein ; thus the biochemical characterization of these binding properties may be a prerequisite for the understanding of microtubular function in the rat pancreas.

In the present work, we report, for the first time, the existence of a colchicine binding activity in a high speed supernatant (cytosol) of rat pancreas. The specificity and the kinetic properties of

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[3] GTP = Guanosine-5'-triphosphate

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the colchicine binding, as well as several parameters of the colchicine binding moiety, allowed its identification as a tubulin-like protein.

METHODS

Rat pancreas was minced and homogenized in 5 vol (W/V) of cold 10 mM sodium phosphate, 10 mM $MgCl_2$, pH 6.8 (PM Buffer). The homogenate was kept for 30 min on ice, then centrifuged at 100 000 x g for 60 min at 4°C in a MSE High Speed 75 centrifuge to obtain the high-speed supernatant (cytosol). The protein concentration of the cytosol was determined by the LOWRY method (7) with bovine serum albumin as a standard. Colchicine binding assays were carried out by adding 100 µl of [^{3}H]colchicine (5 µCi/ml) to 200 µl of undiluted cytosol (containing at least 2 mg of protein) and 700 µl of PM Buffer supplemented or not with other compounds. Samples were incubated for 60 min at 37°C in a water bath before the reaction was terminated by transferring the tubes to an ice bath. After 5 min, each sample was collected onto stacks of four Whatman DE81 filter papers in a Millipore filter apparatus. The filters already contained 5 ml of PM Buffer, additional buffer was added to complete the volume to 10 ml. Suction was applied with a water pump and the filters were washed 4 times more with 10 ml portions of PM Buffer at room temperature and allowed dry for 10 sec after the last wash. The filter stack was placed in a counting vial containing 10 ml of Picofluor 15 (Packard) and counted after digestion for 18h at 20°C in a Packard Tri-Carb (Model 3375) scintillation spectrometer. Quench correction was by the external standards/channels ratio method. Samples were set up in triplicate for all time points. Blank runs contained 200 µl of PM Buffer in place of the cytosol.

Ring-[A-4-^{3}H]colchicine (7.7 Ci/mmol) was from Radiochemical centre (Amersham, England) ; it was diluted to lower specific activity by addition of unlabelled colchicine from Boehringer (Mannheim, Germany) Lumicolchicine was prepared by irradiating a solution of colchicine in 95 % ethanol with long wave (350 nm) ultra-violet light (8). The conversion of colchicine to lumicolchicine was followed by measuring the decrease in absorbancy at 350 nm. Podophyllotoxin was purchased by Aldrich Chemical Company (Milwaukee, USA), cytochalasin B by EGA-chemie (Steinheim Albuch, W. Germany), melatonin by Sigma (St Louis, USA), Vinblastine sulfate was a generous gift from Eli Lilly (St-Cloud, France).

RESULTS

The binding of colchicine to pancreas cytosol was determined in function of time at different temperatures (0°C, 25°C, 37°C). As shown in figure 1, the colchicine binding activity of the cytosol was clearly temperature and time dependent. The amount of colchicine bound after incubation of pancreas cytosol with 10^{-6}M[^{3}H] colchicine at 0°C was less than 1 % of the value obtained at 37°C. Optimal binding appeared at 37°C, this temperature was employed for all subsequent experiments. The association curve showed that a plateau was practically attained at 60 min. A 60 min incubation period was therefore adopted for all experiments. This colchicine binding activity was linearly related to the protein concentration of the cytosol (Fig. 2).

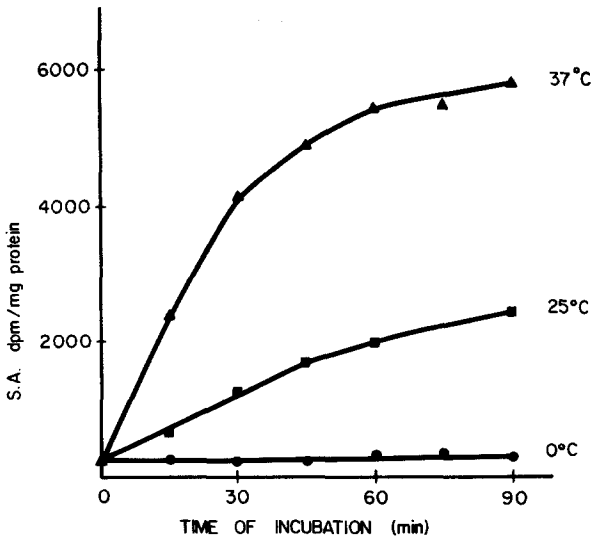

Fig. 1. Time and temperature dependency of the colchicine binding capacity of rat pancreas high-speed supernatants. 200 µl of supernatant containing 2.5 mg of protein were incubated with 10^{-6}M [^{3}H] colchicine at 0°C, 25°C and 37°C.

Several compounds were tested for their possible interference with colchicine binding activity (Table 1). Lumicolchicine, a compound which is structurally similar to colchicine but lacks antimitotic activity and which apparently does not bind to tubulin (9), can be used in ascertaining the specificity of colchicine binding. Lumicolchicine

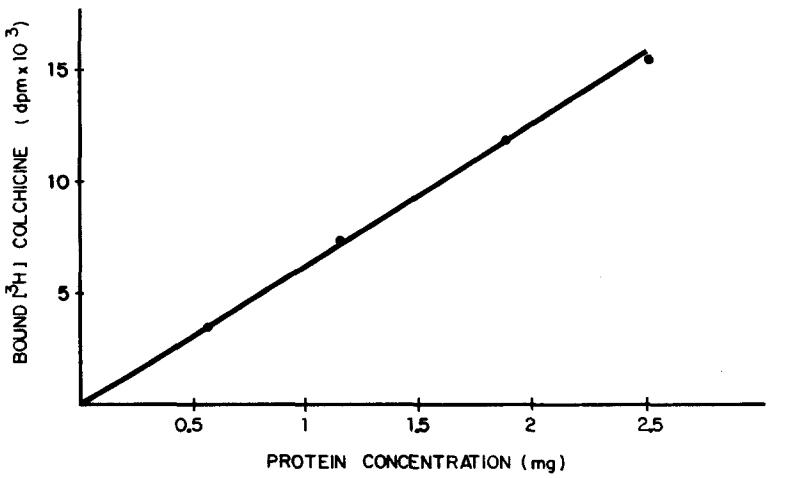

Fig. 2. Protein dependency of the colchicine binding activity. Samples of pancreas high-speed supernatants containing various amounts of protein were incubated for 60 min at 37°C in presence of 10^{-6}M [^{3}H] colchicine.

Table 1. Specificity of [^{3}H] colchicine binding. 200 µl of pancreas high speed supernatants were incubated with 10^{-6}M [^{3}H] colchicine and a 10^{-6}M concentration of the indicated compound. Melatonin, cytochalasin B, podophyllotoxin were dissolved in 95 % ethanol, final concentration in the PM - buffer being 1 %. The corresponding controls contained the same amount of ethanol.

COMPOUNDS (10^{-6}M)	% BOUND RADIOACTIVITY
CONTROL	100
MELATONIN	91.4
CYTOCHALASIN B	94.3
LUMICOLCHICINE	92.9
PODOPHYLLOTOXIN	32.1

(10^{-6}M) had no effect upon colchicine binding activity at 37°C. On the contrary, podophyllotoxin inhibited colchicine binding to rat pancreas cytosol (table 1).

Drugs which induce mitotic arrest (melatonin) on cultures of Hela and Kb cells (10) or which disrupt microfilaments (cytochalasin B) were ineffective.

The inhibition of colchicine binding by podophyllotoxin was studied with different concentrations of colchicine ($0.25.10^{-6}$M - $0.75.10^{-5}$M) in the absence or the presence of 10^{-6}M podophyllotoxin. Podophyllotoxin appeared to inhibit competitively the binding of colchicine to rat pancreas cytosol (Fig. 3). From this experiment the inhibition constant K_i was calculated for podophyllotoxin ($K_i = 3.3.10^{-7}$M). The affinity constant (Km) for colchicine gave a value of $2.5.10^{-6}$M).

Colchicine binding activity of animal tubulin can be influenced by the presence of GTP, vinblastine, sucrose and glycerol (11) and therefore we investigated the effects of these substances on colchicine binding in pancreas cytosol. Only GTP or vinblastine increased the level of binding, the maximal value was obtained with vinblastine 10^{-4}M (table 2) these compounds added together had no additive effect. GILLESPIE (12) has reported that DTT[1] partially stabilizes colchicine binding activity from rat brain tubulin. Addition of a similar compound such as DTE[2]

[1] DTT = dithiothreitol

[2] DTE = dithioerythritol

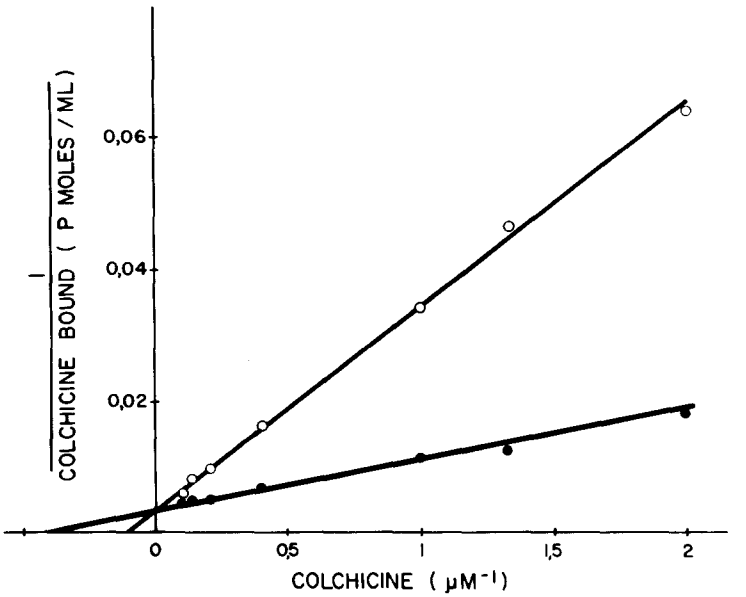

Fig. 3. Effect of podophyllotoxin on the binding of colchicine. Samples of pancreas high-speed supernatants were incubated with different concentrations of [^{3}H] colchicine for 60 min at 37°C in the absence (•) or in the presence (o) of 10^{-6}M podophyllotoxin.

Table 2. Effect of stabilizing agents on colchicine binding. The tests were made using the standard binding assay with 10^{-6}M [^{3}H]colchicine.

AGENT	CONCENTRATION	% BOUND RADIOACTIVITY
SACCHAROSE	0.25 M	99.7
	1.00 M	92.2
GLYCEROL	0.25 M	98.3
	1.00 M	88.7
DTE	10^{-3} M	98.0
	10^{-4} M	87.4
GTP	10^{-3} M	128.8
	10^{-4} M	120.9
VINBLASTINE	10^{-3} M	122.6
	10^{-4} M	135.3
VINBLASTINE + GTP	10^{-4} M 10^{-3} M	135.3

Table 3. Effect of proteases on colchicine binding. The tests were made using the standard binding assay with 10^{-6}M [^{3}H]colchicine.

AGENT	CONCENTRATION	% BOUND RADIOACTIVITY
TRYPSIN	0.013 ‰	3.0
STI	0.014 ‰	100.0
TRYPSIN + STI	0.013 ‰ + 0.014 ‰	90.5
CHYMOTRYPSIN	0.140 ‰	1.3
PMSF	0.170 ‰	100.0
CHYMOTRYPSIN + PMSF	0.140 ‰ + 0.170 ‰	95.9

(10^{-3} or 10^{-4}M) did not influence markedly the binding activity in the cytosol of the rat pancreas.

Trypsin and chymotrypsin abolished the cytosol colchicine binding property (Table 3), their respective inhibitors STI[3] and PMSF[4] counter-acted this effect.

DISCUSSION

In this paper, we report the existence of colchicine binding in the rat pancreas cytosol. The kinetic properties of this binding activity are temperature and time dependent as it has been mentioned in the brain (9) or in the liver (13). The competitive inhibition by podophyllotoxin has been described in isolated vinblastine-induced microtubule crystals (14) or in the bovine adrenal cortex (15), the K_i value is close to that reported for tubulin in chick embryo brain (16). Colchicine binding capacity extrapolated from Fig. 3 is 1.4 nmole of bound colchicine per gram of gland, similar value has been obtained for the microtubule protein of rat lacrymal glands (17). The effects of stabilizing agents vary considerably in function of animal sources of tubulin. In our experimental conditions, these agents are without any effects on colchicine binding,

[3] STI = Soybean trypsin inhibitor

[4] PMSF = phenyl-methyl sulfonyle fluoride

only GTP and vinblastine enhance the binding, this observation has been made in chick embryo brain (9). As shown in table 3, colchicine binding is sensitive to proteases, these results suggest that the colchicine binding moiety is certainly a protein. The parameters described in this study agree well with those of WILSON and BRYAN (18) and allowed us to demonstrate the existence of a protein-colchicine complex in the rat pancreas cytosol. However, the nature of this protein has not been yet established. Therefore, further work is in progress to purify this protein and to compare its biochemical characteristics with tubulin from different animal sources.

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