NON PHOSPHORYLATION *IN VITRO* OF THE 6 S TUBULIN FROM BRAIN AND THYROID TISSUE

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1. Introduction

Tubulin, a protein subunit of microtubules, binds colchicine with a high affinity [1–3]. It is generally assumed that the biological processes which are inhibited by colchicine involve the participation of microtubules. In the thyroid gland, for instance, Williams and Wolff [4] and Neve et al. [5] have shown that colchicine blocks the TSH-dependent pinocytosis of thyroglobulin and therefore the release and secretion of thyroxine. The first aim of this work was to purify thyroid tubulin and to compare its properties to those of brain neurotubulin. On another hand pinocytosis is activated in the thyroid gland by TSH; *c*AMP mimics this action [6,7]. The only known enzymes which are activated by the cyclic nucleotide in various tissues and in the thyroid gland [8] are the *c*AMP-dependent protein-kinases. It has been assumed by Goodman et al. [9] and Rasmussen [10] that neurotubulin is phosphorylated *in vitro* by an intrinsic, *c*AMP dependent, protein-kinase. In contrast with this conclusion our results show that both thyroid and brain 6 S tubulin are not phosphorylated *in vitro*.

2. Materials and methods

2.1. *Products*

[^{3}H] colchicine (Amersham); [γ-^{32}P] ATP (CEA); colchicine (Sigma); GTP (Boehringer); Sephadex G-50 and DEAE-Sephadex A-50 (Pharmacia); DEAE-cellulose (Bio-Rad).

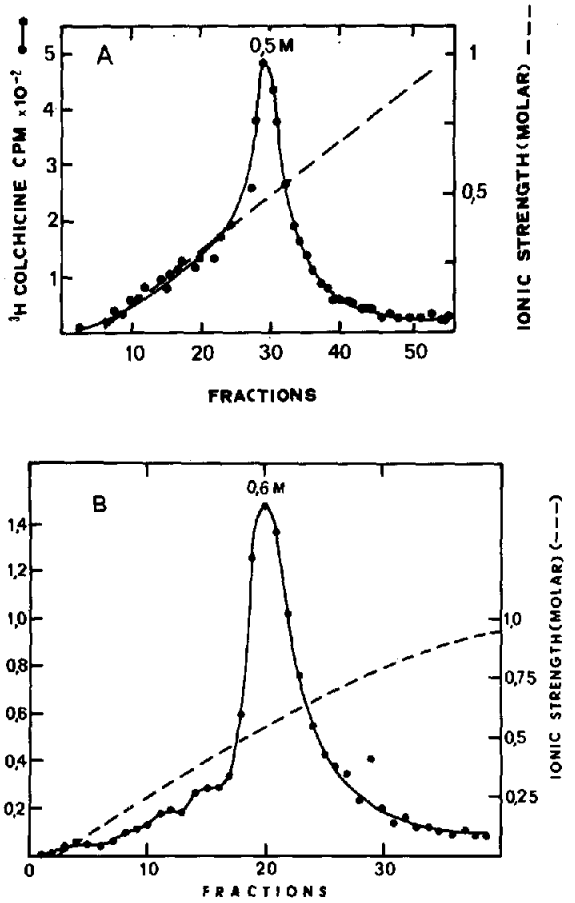

Fig. 1. DEAE-column chromatography of thyroid (fig. 1A) and brain (fig. 1B) tubulin labeled by [^{3}H] colchicine as described in Materials and methods.

2.2. *Thyroid and brain tubulin purification*

Brain neurotubulin was purified by the procedure of Weisenberg et al. [3,11].

Fresh hog thyroid glands (200 g) were homogenized (Ultra-turrax) in 2 vol of 0.25 M sucrose in a solution (A) containing: phosphate buffer 10 mM, pH 6.5; $MgCl_2$ 10 mM; 10^{-4} M GTP. The homogenate was centrifuged 40 min at 33 000 *g*. The supernatant was precipitated with $(NH_4)_2SO_4$ (final conc. 32%); the supernatant was brought to a final concentration of $(NH_4)_2SO_4$ of 43%. The precipitate was redissolved in 200 ml of solution (A) and then adjusted to 0.4 M by addition of KCl. Thyroglobulin which is present in huge excess in the thyroid supernatant is not absorbed in these conditions of molarity, during the subsequent batch purification using DEAE-Sephadex A-50 (200 ml of packed anionic exchanger). After stirring during 30 min the resin was collected by centrifugation (12 000 *g*, 10 min) and washed twice (2 × 150 ml) with solution (A) containing 0.4 M KCl. Tubulin was then eluted by 2 × 100 ml of 0.8 M KCl in solution (A) and precipitated by $(NH_4)_2SO_4$ (final conc. 43%). The precipitate redissolved in solution (A) was freed from $(NH_4)_2SO_4$ on a Sephadex G-50 column. Tubulin was then precipitated by 0.5 M $MgCl_2$ (final concentration 50 mM). The precipitate was redissolved in solution (A) and dialysed 18 hr against the same buffer.

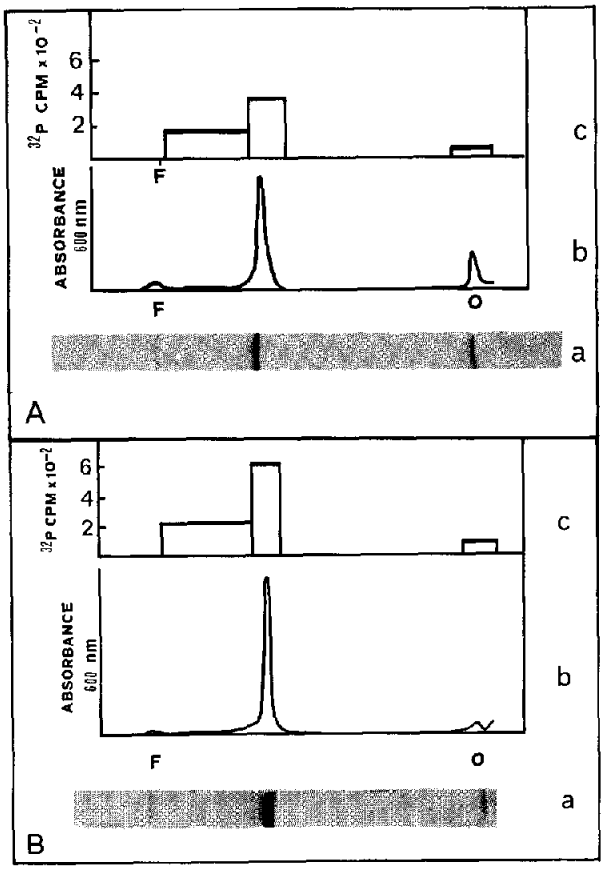

Fig. 2. Disc gel electrophoresis of thyroid and brain purified tubulins. Fig. 2a represents photographs of both preparations after amidoschwartz staining; Fig. 2b densitometer scanning at 600 nm; Fig. 2c ^{32}P-radioactivity measured in different portions of the gels.

2.3. *[^{3}H] Colchicine binding assay*

It was performed according to Weisenberg et al. [3]. The excess of this drug was removed according to Williams and Wolff [4]; an aliquot of the [^{3}H] colchicine labeled fraction was analyzed by sucrose gradient (5–20% in buffer A) ultracentrifugation (36 000 rpm; 18 hr; Rotor SW 50 L; Spinco L_2 50) or by DEAE--Sephadex column chromatography (elution by a linear gradient 0–1.0 M NaCl in buffer A; pH 6.8).

2.4. *Phosphorylation of purified tubulins*

1 ml of tubulin preparation (approx. 4 mg of protein) was incubated 30 min at 30° in presence of [γ-^{32}P] ATP (20 μCi/20 μM final conc.) after addition of the medium (final vol. 2 ml) described by Miyamoto et al. [12]. Excess ATP was eliminated by Sephadex G-50 column chromatography. The phosphorylated proteins were analysed by sucrose gradient (5–20% in solution A) ultracentrifugation (36 000 rpm; 18 hr; Rotor SW 50 L; Spinco L_2 50) and by urea–polyacrylamide gel electrophoresis according to Weisenberg [3].

3. Results

3.1. *Molecular properties of thyroid and brain tubulins*

Both preparations when labeled with [^{3}H] colchicine exhibit sedimentation coefficient of 6.4 S. Thyroid tubulin is eluted from a DEAE-cellulose column (fig. 1A) at 0.5 M KCl when the brain protein is eluted at 0.6 M (fig. 1B) as described by Weisenberg et al. [3]. The electrophoretic mobility (fig. 2) of both the urea treated proteins is similar (R_f 0.64–0.68).

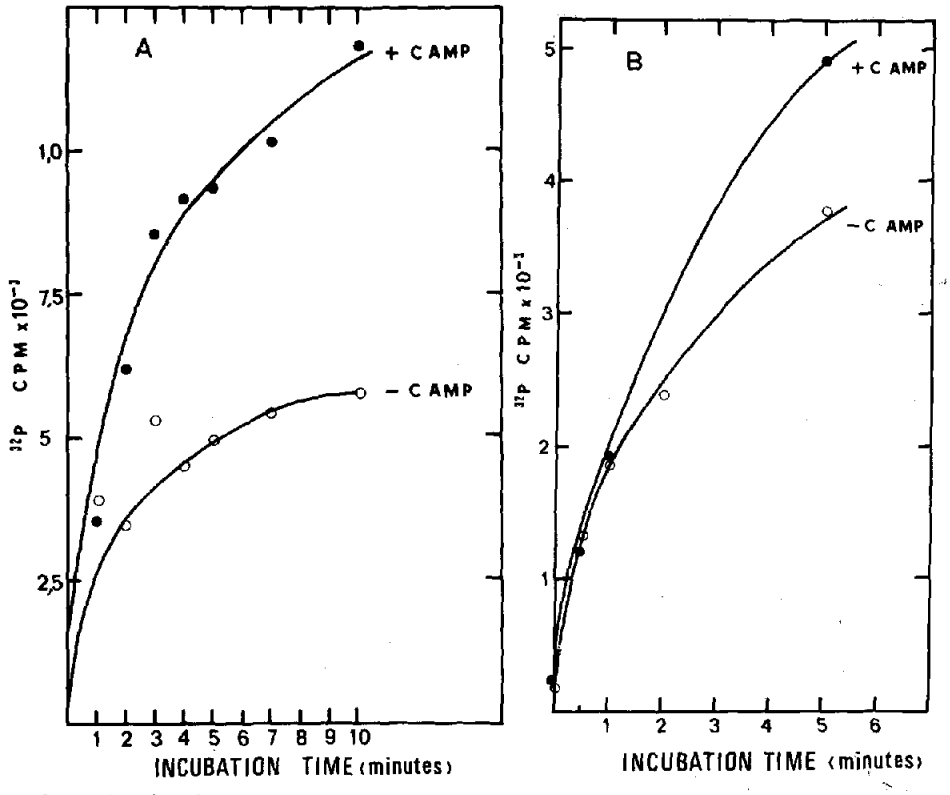

Fig. 3. Kinetics of autophosphorylation of purified thyroid (A) and brain (B) tubulins in presence and absence of 10^{-5} M *c*AMP.

3.2. *Phosphorylation of thyroid and brain purified tubulins*

Fig. 2 shows the results of polyacrylamide gel analysis of thyroid (A) and brain (B) purified tubulins. A major band of tubulin is present in both cases (fig. 2a). Minor faster contaminants are detected either by increasing the amount of protein analysed or by densitometric scanning (fig. 2b). The major tubulin band contains 3/4 of ^{32}P-radioactivity. A faster radioactive band contains approx. 1/4 of ^{32}P-radioactivity (fig. 2c).

Fig. 3 shows the kinetics of autophosphorylation of brain (A) and thyroid (B) purified tubulin incubated with [γ-^{32}P] ATP; *c*AMP 10^{-5} M, stimulates about two times the autophosphorylation of thyroid tubulin. The stimulation is lower with the brain preparation.

Fig. 4A, B, shows the ^{32}P- radioactivity profile after sucrose gradient ultracentrifugation of autophosphorylated thyroid (A) and brain (B) tubulins. Radioactivity is always present only at the top and the bottom of the tube, never at the level of the tubulin as defined by the [^{3}H] colchicine binding assay.

4. Discussion

A simple procedure has been described which permits the purification of thyroid tubulin and the elimination of thyroglobulin which is present in huge amounts in the thyroid postmicrosomal supernatant. Thyroid tubulin is very similar to neurotubulin. Both preparations seem to be phosphorylated when incubated with [γ-^{32}P] ATP in presence or absence of *c*AMP. When analysed by polyacrylamide gel electrophoresis thyroid tubulin and neurotubulin contain most of the ^{32}P label. These results agree with those reported by Goodman et al. [9]. However, analysis of the same phosphorylated preparations by sucrose gradient ultracentrifugation have showed repeatedly that ^{32}P-radioactivity does not sediment with the 6 S tubulin whereas a [^{3}H] colchicine binding peak can

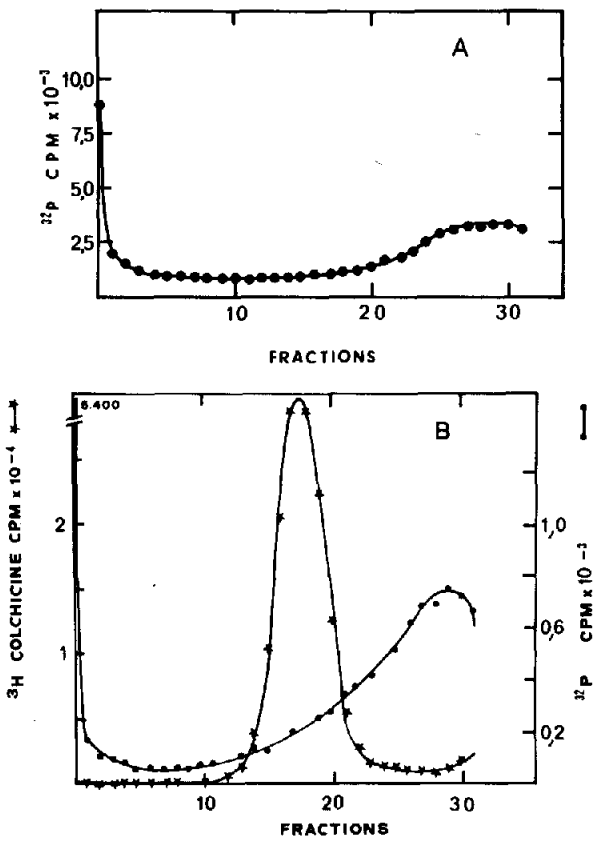

Fig. 4. Sucrose gradient ultracentrifugation of ^{32}P-labeled purified tubulins from thyroid (A) and brain (B) obtained by autophosphorylation with [γ-^{32}P] ATP and purified as described in Materials and methods. In (B) neurotubulin preparation was first labeled with [^{3}H] colchicine, then phosphorylated and finally analysed by ultracentrifugation.

be very easily demonstrated in this region. On the contrary, ^{32}P-labeled proteins are constantly present at the bottom and the top of the ultracentrifugation profile. It seems therefore that both thyroid and brain 6 S tubulins are, *in vitro* as well as *in vivo* [13], a very poor substrate, if not at all, for protein kinases.

Acknowledgement

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