THE PRESENCE OF PHOSPHOLIPIDS AND DIGLYCERIDE KINASE ACTIVITY IN MICROTUBULES FROM DIFFERENT TISSUES

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SUMMARY: Microtubular preparations obtained by different procedures from chick embryonic muscles and brains and from HeLa cells have associated, in addition to the protein kinase which can phosphorylate tubulin, an enzymatic activity which has been identified as that of a diglyceride kinase. They have also consistently associated various phospholipids, lecithin being the principal one. The phosphatidic acid formation is greatly stimulated by exogenous diglycerides or pretreatment with phospholipase C, and it is not present in partially purified preparations of cytoplasmic protein kinases of chick muscle. The significance of these findings is discussed.

Phosphorylation of brain tubulin has been described by several authors (1-4) and we have reported (5) that chick embryonic muscle and HeLa cell tubulins can also be phosphorylated, suggesting that this is a general phenomenom. In the course of these studies we had observed that part of the ^{32}P-radioactivity of a TCA[1] precipitated tubulin that has been labelled *in vitro* or *in vivo* could be removed by organic solvents. These observations prompted an investigation on whether microtubules had normally associated phospholipids and an enzymatic activity capable of synthetizing *in vitro* a phosphate containing lipid. The results of this investigation are the subject of the present communication.

METHODS. The standard reaction mixture for ^{32}P-incorporation studies "in vitro" has the following composition: 10-20 µg of microtubular protein, 10 mM sodium α-glycerophosphate, pH 6.8, 25 mM NaF, 40 mM Mg acetate and 0.15 mM [γ-^{32}P]-ATP (0.5-2 Ci/mmol) in a final volume of 0.1 ml. After incubation at 30° for 10 min the reaction was stopped by addition of 25 µl of 50 mg/ml albumin - 0.1 M EDTA and 2 ml of ice cold 5% TCA.

^{32}P-lipid and ^{32}P-tubulin isolation. TCA-precipitated microtubules were pelleted at 1200 x g for 10 min, washed twice with TCA, dissolved with 0.3 ml

[1]Abbreviations used: TCA, trichloroacetic acid; PA, phosphatidic acid; PMS buffer, a solution of 10 mM potassium phosphate - 10 mM $MgCl_2$-0.22 M sucrose, pH 6.5; TLC, thin layer chromatography.

of 1 M NH_4NaHPO_4, and reprecipitated with TCA. Upon repeating the NH_4NaHPO_4-TCA step once more the pellet was resuspended in 0.15 ml of n-butanol saturated with water, cooled and centrifuged. Butanolic extraction was repeated twice with 0.1 and 0.05 ml. The pooled extracts were washed with 0.05 ml of water saturated with butanol, and the radioactivity present in the organic phase ("butanol extract") and in the extracted pellet ("tubulin") was determined.

Analytical procedures. Paper chromatography was carried out in the following systems: (I), isopropanol-acetic acid-water (27:4:9); (II), butanol-pyridine-water (6:4:3); (III), isopropanol-ammonia-water (7:2:1); (IV), phenol saturated with water containing 0,12% V/V concentrated ammonia. Paper electrophoresis was carried out with pyridine-acetic acid buffer, pH 6.5 for 2 hr at 20 V/cm. TLC was performed on silica gel H (Merck) in 1% $K_2(COO)_2$ (V), or 1 mM Na_2CO_3 (IV). The solvent systems used were: (V), chloroform-methanol-4N ammonia (9:7:2); (VI), chloroform-methanol-ammonia-water (24:12:1:1). Phosphate was detected with the Burrow's reagent (8) and lipids with I_2 vapors.

Deacylation and alkaline phosphatase treatment. Deacylation of butanolic extracts was carried out under mild alkaline conditions (0.1 N LiOH in chloroform-methanol (4:1),30°-30min, followed by partition into a chloroformic and aqueous phase. The latter was treated with 2 μg of E.Coli alkaline phosphatase in 5% methanol-0.1 M Tris-HCl, pH 8.2 for 4 hours at 37°.

RESULTS. When chick embryonic muscle cells are incubated in a phosphate-deficient medium containing $^{32}P_i$ (5) and the microtubules isolated by the vinblastine procedure they are radioactive. Approximately 50% of this radioactivity is soluble in 5% TCA, most of it being accounted for by GTP. Eighty percent of TCA-insoluble radioactivity is extractable by organic solvents (chloroform-methanol (2:1) or n-butanol saturated with water), the residue (20%) representing the ^{32}P-covalently bound to tubulin. In order to determine if the presence of the lipophilic ^{32}P-radioactivity was an artifact due to the vinblastine procedure used on embryonic muscles, other tissues were employed and their microtubules isolated by different procedures (Table I). It was found that, even though to a different extent, in all instances the microtubular fractions contained radioactivity that could be extracted with organic solvents. This radioactivity migrates on paper chromatography with an R_f value of 0.95 in solvents I (e.g. Fig. 1E), II, and IV, confirming its lipophilic nature.

Under slightly different conditions than those previously reported (5) for in vitro phosphorylation of tubulin it was now found that a part of the radioactivity incorporated into the TCA-precipitate is extractable with organic solvents, suggesting a phosphorylation not only of tubulin but also of a lipophilic endogenous acceptor. The association of this enzymatic activity to

TABLE I - In vivo and in vitro ^{32}P-incorporation into protein and butanol extracts of microtubules from different tissues[a].

Tissue of Origin	Cell Disruption	Microtubule Isolation	In Vivo Labelling[b]		In Vitro Labelling[c]	
			Tubulin	Organic Phase	Tubulin	Organic Phase
			cpm/mg tubulin		pmol/min/mg tubulin	
HeLa	Sonication	Vinblastine	105 000	32 500	30	15
Muscle	Homogenization	Vinblastine[d]	36 300	51 000	15	12
Muscle	Homogenization + Sonication	Vinblastine[d]	37 500	52 700	17	15
Brain	Homogenization	Vinblastine	8 200	15 300	28	26
Brain	Homogenization + Sonication	Vinblastine	7 700	27 700	30	28
Brain	Homogenization	Polymerization	6 900	4 400	25	10
Brain	Homogenization	DEAE-cellulose	[e]	[e]	27	8

[a]HeLa cells or chick embryonic muscles and brains were homogenized (5) and/or sonicated three times for 15 sec each, and the microtubules isolated either by vinblastine precipitation (5) or by the temperature-dependent polymerization-depolymerization procedure in the presence of glycerol-GTP (6), or by slight modifications of existing DEAE-cellulose (Whatman DE52) chromatographic procedures (1,7).

[b]Labelling of HeLa cells and of chick embryonic muscle and brain slices with $^{32}P_i$ was carried out under conditions essentially similar to those described in Table 7 of reference 5. The isolated microtubules were precipitated with carrier albumin and TCA, and extracted with butanol as described under Methods.

[c]Microtubules isolated in parallel from unlabelled cells were incubated *in vitro* with $[\gamma\text{-}^{32}P]$-ATP under the standard conditions, and the radioactivity incorporated into tubulin and the organic phase determined as indicated under Methods.

[d]The isolation used in these experiments included, over the standard procedure (5), an additional washing with PMS buffer of the microtubular pellet.

[e]Not determined. However, Eipper (2) has reported that tubulin isolated by DEAE-chromatography from rat brains incubated with $^{32}P_i$ contains associated phospholipids.

different microtubules was explored, and it was found that - as with the phospholipids - it is present in microtubules of different origin and prepared by independent procedures (Table I last column). In all instances the lipophilic product of the *in vitro* reaction migrates with the front upon paper chromatogra

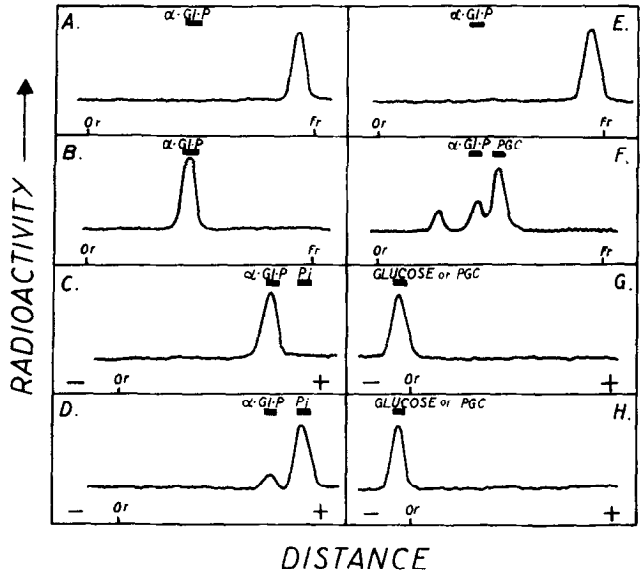

Fig. 1. The effect of deacylation and alkaline phosphatase treatment on the butanol extracts of microtubules. Samples of "in vitro butanol" extracts (left panels, A-D) and of "in vivo butanol" extracts (right panels, E-H) were saponified with LiOH and treated with alkaline phosphatase as indicated under Methods. Chromatography in system I of butanol extracts: (A) and (E); chromatography in system I of the aqueous phase of saponified butanol extracts (APSBE): (B) and (F); electrophoresis of "APSBE": (C) and (G), and of alkaline phosphatase-treated "APSBE" : (D) and (H). Glucose was included as a marker for endosmosis in the electrophoresis. α-Gl-P, GPC, Or, and Fr, stand for α-glycerophosphate, glycerophosphoryl-choline, origin, and front, respectively.

phy on systems I, II, and IV (e.g. Fig. 1A).

Once it was established that the presence of the lipid(s) and of the enzyme in the microtubular fractions does not seem to be an artifact, their identification was undertaken. Of the various conditions shown in Table I the most practical for the preparation of a convenient amount of in vivo and in vitro material was chosen. Thus, "in vivo butanol" and "in vitro butanol" extracts were obtained respectively, from vinblastine isolated microtubules prepared (a) from HeLa cells cultured in the presence of $^{32}P_i$, and (b) from chick embryonic muscles and incubated with $(\gamma\text{-}^{32}P)$-ATP. The ^{32}P-radioactivity of the in vitro and in vivo butanols became upon deacylation 100% and 75-85%, respectively, water soluble. The in vitro water-soluble product gave in all instances a single radioactive peak with a mobility identical to that of α-glycerophosphate by chromatography

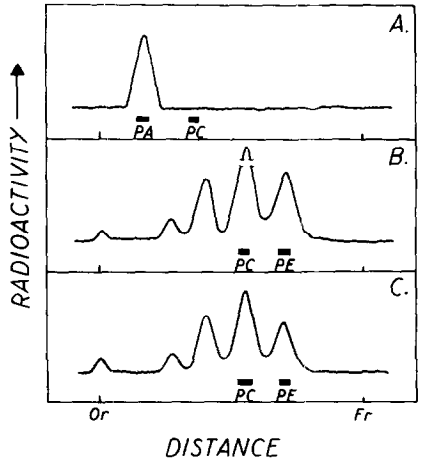

Fig. 2. Thin layer chromatography of butanol extracts. (A), in vitro labelled microtubules, system VI; (B), in vivo labelled microtubules, system V; (C), in vivo labelled 150 000 x g supernatant, system V. PA, PC, and PE, stand for phosphatidic acid, phosphatidyl-choline, and phosphatidyl-ethanolamine, respectively.

(solvent systems I, II, III, and IV) and electrophoresis (e.g. Fig. 1B and C). The in vivo product gave, depending on the solvent system used, three or four peaks, the major one always coinciding with glycerophosphorylcholine (Fig. 1F and G). Alkaline phosphatase treatment of the saponification product of "in vitro butanol" liberates $^{32}P_i$, while the corresponding in vivo product is not modified (Fig. 1 C-D and G-H). All these results suggest that the lipid formed in vitro is phosphatidic acid (PA) and that the in vivo ^{32}P-labelled lipids are a mixture of phospholipids with the predominance of lecithin. TLC in solvent systems V and VI of "in vitro butanol" confirmed this presumption, since in all instances a single peak coinciding with PA was obtained (e.g. Fig. 2A). It should be noted that system V does not separate PA from phosphatidyl-choline, but it does so from phosphatidyl-ethanolamine, -serine, and -inositol. Moreover, the R_f value (0.60) of our radioactive peak differs from that reported for di and tri-phosphoinositides (9). TLC of "in vivo butanol" confirmed that several phospholipids were present, lecithin being the more labelled (Fig. 2B). The phospholipid composition of the microtubules

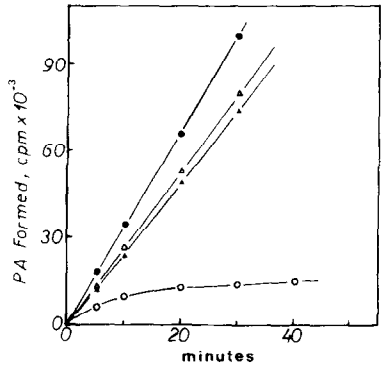

Fig. 3. Time course of phosphatidic acid formation by microtubules incubated in vitro in the presence of diglycerides. Microtubules (40 µg) were incubated under the standard conditions directly (○), in the presence of 5 mM 1,2-dipalmitin (△), or 5 mM 1,2 diolein (▲), or after a pretreatment with phospholipase C (●). The exogenous diglycerides were dispersed by sonication in the phosphorylating reaction mixture, and the phospholipase C (5 µg) pretreatment consisted of an incubation for 5 min at 30° in the presence of 5 mM $CaCl_2$, 50 mM Tris-HCl, pH 7.5, followed by dilution into the phosphorylating buffer. PA was determined by butanol extraction as indicated under Methods.

TABLE II.- Protein kinase and diglyceride kinase activities of various fractions of chick embryonic muscles.

	^{32}P-Incorporated (fentomol/min)			
	Endogenous Acceptor		Exogenous Acceptor	
	Protein	Diglyceride	Casein	Dipalmitin
1.- Total Homogenate	2 480	340	6 290	600
2.- 150 000 x g Supernatant	2 430	290	6 200	850
3.- Microtubules	171	23	470	75
4.- Protein kinase I	-	-	475	0
5.- Protein kinase III	-	-	465	0.3

Total homogenate, 150 000 x g supernatant, and vinblastine precipitated microtubules were prepared from chick embryonic muscles (5). Protein kinase isozyme I and III were obtained by DEAE-cellulose chromatography of the 150 000 x g supernatant as previously described (14). Enzymatic assays were carried out with aliquots of the same equivalent weigth of wet tissue (10 mg) for fractions 1-3, and with an equivalent protein kinase activity (casein as substrate) for fractions 3-5. Assays were performed under standard conditions in the absence of exogenous substrates, as well as in the presence of casein (10 mg/ml) or 1,2-dipalmitin (5 mM).

and of the 150 000 x g supernatant from where they were derived is roughly similar (Fig. 2 B-C).

The identification of PA as the lipophilic product obtained by *in vitro* incubation of microtubules with the sole addition of ATP implies that the enzymatic activity present in these cellular structures is that of a diglyceride kinase and that they have also associated diglyceride(s). PA formation is stimulated by the addition of exogenous dipalmitin or diolein or by preincubation with phospholipase C, which increases *in situ* the diglycerides by hydrolysis of the lechitin present in the microtubules (Fig. 3). Table II shows that the diglyceride kinase activity associated to the microtubular fraction is approximately 9% of that present in the supernatant[2]. It can be seen also that two partially purified isozymes of protein kinase (derived from a supernatant similar to that used to prepare the microtubules) are unable to catalyze the phosphorylation of dipalmitin.

DISCUSSION. The presence of GTP and of protein kinase in tubulins of various origins has been established by several authors (1-5, 15). The presence of an associated ATPase activity (5, 16) and the involvement of a tubulin subunit in a phosphatidylinositol phosphodiesterase activity of rat brain (17) have also been reported. Our finding points out that, in addition, a diglyceride kinase activity, diglycerides, and phospholipids are present in microtubules isolated by independent procedures from different tissues. Even though tubulin is the major component of microtubular preparations, the other constituents that are found consistently associated suggest that they are part of a complex system. Thus, tubulin might be a lipoprotein and the

[2]Diglyceride kinase activity has been reported in several particulated fractions: rat brain microsomes (10), erithrocyte ghosts (11), and E.Coli membranes (12,13). It is also present in our 150 000 x g supernatants of embryonic chick muscles, even though these are free of Na^+, K^+-dependent ATPase, 5'-nucleotidase, and TPNH-dependent cytocrome c oxidase activities, indicating a lack of contamination from plasma membranes and microsomes. Like in the case of protein kinase, the diglyceride kinase activity found in the microtubules is a small fraction of the total. Its properties, which are similar in several aspects to those reported by Weissbach *et al.* (13), differ from those of the chick embryonic muscle supernatant (G. Daleo, M.M. Piras, and R. Piras, unpublished results) suggesting a specific association rather than a contamination.

associated lipids might be important for some of its functional properties. Since diglyceride kinase is a key enzyme in the pathway leading to the synthesis of phospholipids (11), its presence in microtubules is probably not fortuitous, in view that these compounds are also found associated to tubulin. In this regard it will be interesting to know whether the phospholipids of the microtubular fraction constitute a separate pool with different turnover, and whether they play a role in the polymerization-depolymerization process of tubulin, similarly as what has been recently described for the elongation factor I of protein synthesis (18). Experiments along these lines are in progress.

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