INTERACTIONS OF *MYO*-INOSITOL WITH BRAIN MICROTUBULES

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1. Introduction

Labelling experiments in vivo with $^{32}P_i$ have shown that a significant proportion of the radioactivity incorporated into brain microtubules was present in a phospholipid fraction purified from chloroform/methanol extracts of the isolated protein [1]. Further, in short-term (2 h) labelling experiments, between 25% and 40% of the ^{32}P radioactivity present in purified microtubule-associated phospholipids was recovered in the phosphoinositide fraction which consitutes, at most, 5–10% of the total phospholipid P recovered [2–4]. This observed enrichment of label in the phosphoinositide fraction is similar to that seen when synaptosomal membrane phospholipids are labelled with ^{32}P, under similar conditions [5] and could therefore simply reflect the labelling pattern of membrane phospholipids that are preferentially associated with microtubular components (see ref. [4]). It was postulated [1] on the basis of these biochemical observations that phospholipids associated with isolated microtubules may reflect the occurrence, in situ, of functional associations between microtubules and neuronal membranes, for which there now exists some morphological evidence [6,7]. More specifically, it was suggested [3,4] that microtubules may generally participate in the regulation of membrane-bound phosphoinositide metabolism that is associated with physiological responses in stimulated tissues (see ref. [8]).

In the course of further experiments, to test these possibilities, it has now been observed that *myo*-inositol itself interacts with brain microtubular protein. This report describes evidence that inositol markedly affects the temperature-dependent assembly and disassembly of microtubules in vitro, and that microtubular proteins bind up to 1 mol inositol/mol tubulin. It is proposed in the light of these and other observations that inositol may play a physiological role through regulation of the functional state of microtubules in various cell types.

2. Materials and methods

Microtubular protein was isolated by one cycle of assembly/disassembly from 4–6 day-old chick brain or from 4–6 week-old rat brain by the procedure of Borisy et al. [9], except that PIPES (piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)) was replaced throughout by MES (2-(*N*-morpholino)ethanesulphonic acid) in the buffer solution which will be referred to as MT-reassembly buffer. This contained 100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$ and 1 mM GTP (freshly added), adjusted to pH 6.9 with 2 N NaOH at 4°C.

Microtubule polymerisation was assayed by a turbidimetric procedure [10] in a split-beam Unicam SP1800 UV spectrometer, fitted with a 4 sample automatic sample changer thermostated to 4°C or 37°C as required. Readings were taken at 350 nm at 1 min intervals and recorded on a Unicam AR25 Linear Recorder. Samples of the purified protein (ca. 2.5 mg protein/ml) were diluted to give ca. 1 mg/ml protein in MT-reassembly buffer containing freshly added GTP (1 mM, final concn.) and incubated in the presence or absence of inositol added in the cold at zero time.

3. Results and discussion

3.1. *Effects of inositol on microtubule assembly*

The results of experiments using protein from rat

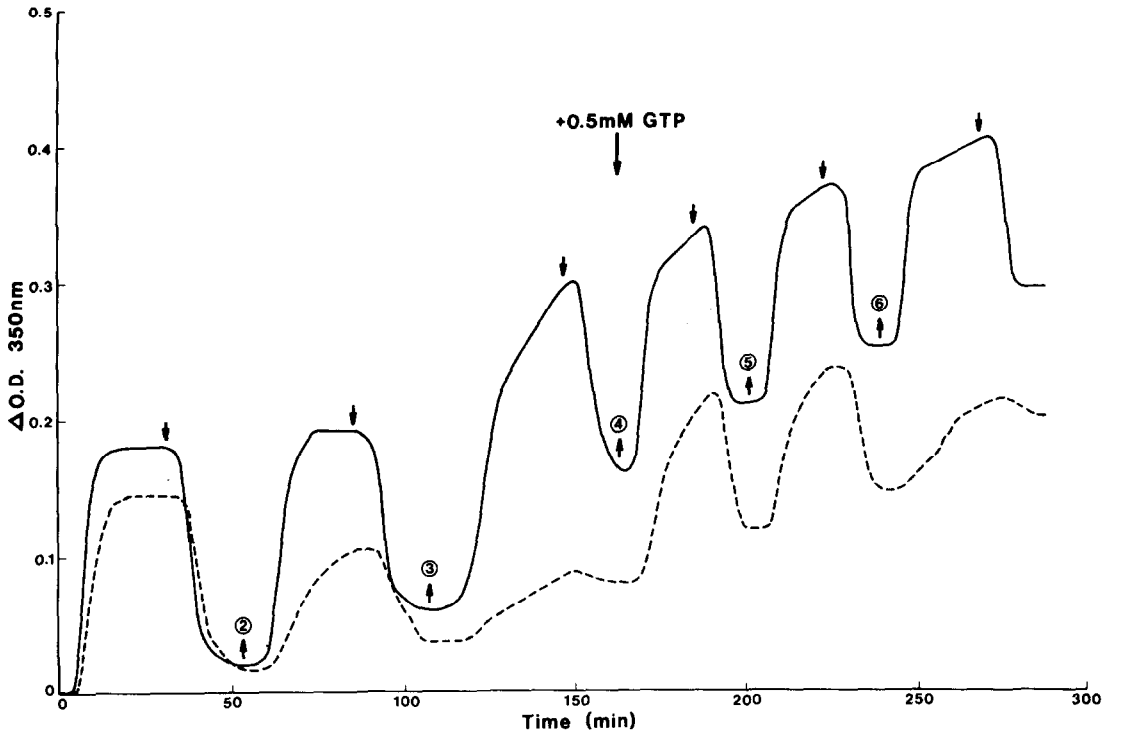

Fig.1. Turbidity changes during successive cycles of assembly/disassembly of rat brain S_3 fraction in the presence (– – –) and absence (——) of *myo*-inositol (added at zero time, 250 mM final concn.). Turbidity changes were monitored simultaneously for control and inositol-containing samples. Arrows indicate temperature increase to 37°C (↑) or decrease to 2°C (↓) and circled numbers indicate the start of each cycle. For details see text.

brain show that inositol decreased the rates of both assembly and disassembly by more than 30% and the maximum turbidity developed (plateau values), by about 25%, during the first cycle of assembly/disassembly (fig.1 and 2). Inositol acted in a concentration-dependent manner, maximum effects occurring at about 100 mM (fig.2). Presumably, the effects seen during the first cycle resulted from an immediate interaction of inositol with the precursor pool of polymerisable protein, occurring at 4°C or during the period (< 2 min) required for re-equilibration of the system to 37°C.

These effects were considerably amplified during successive cycling of the protein (cycles 2–6, fig.1). It was also evident that whereas the rapid initial increases in turbidity seen during the first cycle resulted in the establishment of a well-defined plateau. However, in subsequent cycles the initial phase of turbidity increase was followed by a characteristically slow phase that was maintained at a constant rate during incubation at 37°C. In samples incubated with inositol the development of this secondary slow phase was already apparent earlier in the history of the protein than in control samples (cf. cycles 2 and 3, fig.1), thus accounting, presumably, for the amplification of the effects of inositol observed during successive cycles of assembly/disassembly.

The secondary phase of slow increase in turbidity probably reflects the gradual formation and accumulation of a pool of 'inactive' microtubular protein aggregates that do not readily depolymerise in the cold, as can be inferred from the progressive increase, during successive cycling of the proteins in the basal level of turbidity attained on equilibration of the samples at 4°C (fig.1). Such protein aggregates could be related to the cold-stable microtubular protein fraction that is obtained (and normally discarded)

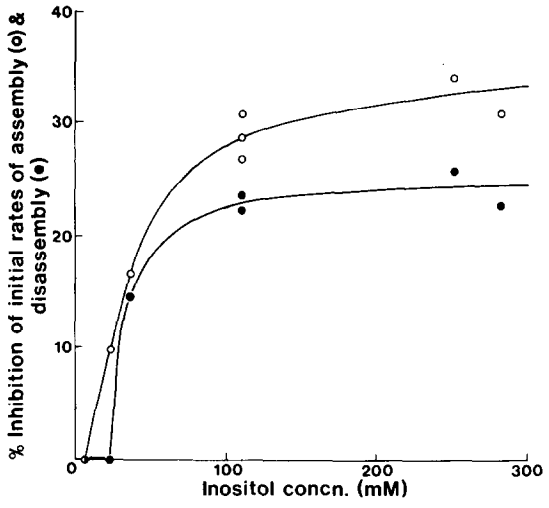

Fig.2. Effect of inositol on initial rates of assembly and disassembly of microtubular protein prepared by one cycle of polymerisation/depolymerisation. Initial rates are expressed in arbitrary units calculated from maximum slope of the polymerisation curves. Protein concn.: 1 mg/ml incubation mixture. For details, see text.

during purification of the protein (see ref. [3,12]).

Preliminary experiments indicate that inositol can also stabilise brain microtubules against the rapid depolymerisation induced by calcium ions. Thus, the addition of 3 mM $CaCl_2$ to a twice-polymerised preparation of rat brain microtubules decreased turbidity within 2 min to 45% of the maximal plateau levels attained in control samples, whereas a fall of only 30% in turbidity was produced in samples containing 250 mM (final concn.) inositol (data not shown).

Finally, it can be seen from the data shown in fig.1 and 3 that the addition of GTP (0.5 mM, final concn.) to the test system after 3 cycles of polymerisation caused a significant increase in the initial rates of assembly and of disassembly, in both control and inositol-containing samples (fig.3A) and a partial alleviation of the 'inhibitory' effects of inositol on these processes during the first cycle following addition of the nucleotide (cycle 4, fig.1 and 3B). However, the addition of GTP did not eliminate the secondary slow phase of turbidity development (see fig.1 and 3, cycles 4–6). GTP also induced an apparent stabilisation of the initial rates of assembly and of disassembly in control samples, though not in the presence of inositol (fig.3A), indicating that the effects of inositol and GTP might be related. It is suggested that the observed effects of GTP are most likely mediated through its protection of microtubular protein against 'ageing' (see ref. [11,13,14]), thus increasing the pool of 'active' intermediates available either for microtubule assembly, in the control preparations, or for the generation of non-equilibrating 'stabilised' species of the protein, in the presence of inositol (see below and fig.5).

A plausible interpretation of these findings is that

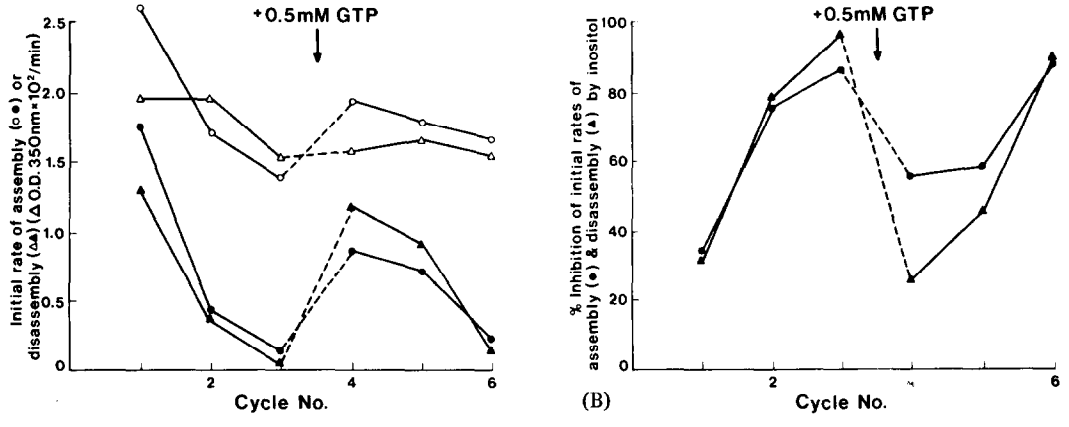

Fig.3. The effects of added GTP on initial rates of assembly and disassembly of microtubules in the presence (filled symbols) or absence (open symbols) of inositol (A) and on the percentage inhibition of the initial rates by inositol (B).

inositol in some way 'stabilises' the main species of microtubular protein involved in the subunit–polymer equilibrium, thereby reducing their availability for assembly or for disassembly of microtubules. In so doing, inositol would effectively promote the formation of a pool of 'stabilised' microtubular protein aggregates that do not readily equilibrate with 'active' forms of the protein which participate in the polymerisation or depolymerisation processes.

The effects of inositol could be exerted through its binding to species of microtubular protein participating in the subunit–polymer equilibrium. Indeed, preliminary experiments show that incubation of once-cycled microtubular protein in the presence of [^{3}H]inositol resulted in the binding of up to 1 mol [^{3}H]inositol/mol of tubulin dimer as determined by gel filtration on Sephadex G-100 (data not shown). Further, when such preparations are chromatographed on Sepharose 6B (see ref. [1]) to separate the oligomeric (30–36 S) and dimer (6 S) species of tubulin, about 60% of the bound ^{3}H cpm were co-eluted with the first protein peak (I), the remainder appearing with the dimer fraction (peak II), as shown in fig.4.

The preferential binding of inositol to assembly-competent tubulin oligomers (peak I, fig.4), and the consistently observed protection by inositol of microtubules against cold and calcium-induced depolymerisation, support the view that inositol preferentially stabilises both microtubules and the intermediate aggregate species of tubulin with which they are in dynamic equilibrium. This interpretation of our results can be readily incorporated in a general scheme for microtubule assembly (see ref. [11]), as illustrated in fig.5, in which no assumption is made regarding the nature of the components present in the pool of 'active intermediates', though this would presumably include tubulin oligomers giving rise to the various disc- and spiral-shaped structures described in the literature (see refs. [11,13]), some of which can be

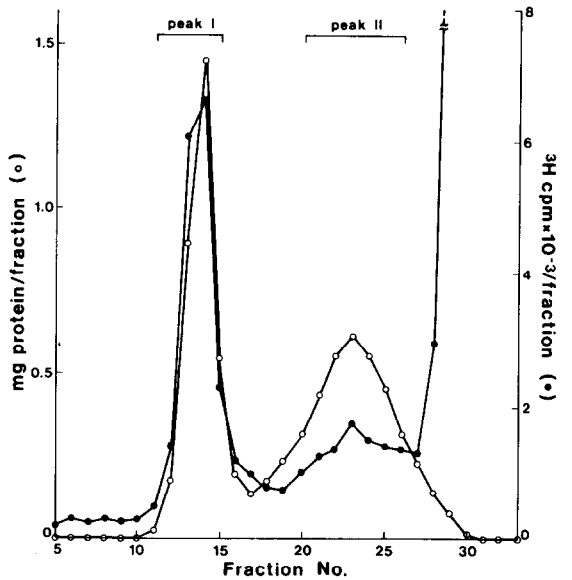

Fig.4. Protein and [^{3}H]inositol elution profiles for chick brain MTP, prepared by one cycle of assembly/disassembly, during chromatography on Sepharose 6B. Freshly prepared MTP was incubated for 30 min at 37°C in the presence of [^{3}H]inositol (25 nmol/ml, 30 nCi/ml reaction mixture). After depolymerisation on ice for 30 min samples containing 10–15 mg protein (in 5 ml) were chromatographed at 4°C on 1.8 × 30 cm columns of Sepharose 6B preequilibrated and then eluted with MT-reassembly buffer minus GTP. Flow rate: 10 ml/h. Usually 89% of the loaded protein was recovered in the eluate. Counts per min in peak I and II correspond to 6.0 nmol and 3.5 nmol bound [^{3}H]inositol/mg protein, respectively.

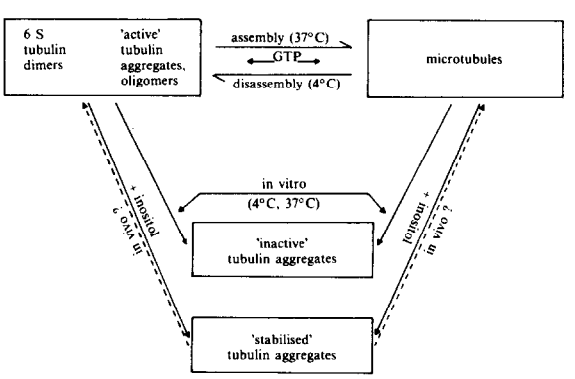

Fig.5. A generalised scheme showing possible sites of interaction of inositol with various pools of microtubular protein involved in assembly and disassembly in vitro. The diagrammatic representation of the microtubule–subunit equilibrium (top) does not distinguish between in vitro and in vivo models proposed (see ref. [11,19]) for microtubule assembly. Dashed lines indicate processes that are postulated to occur in vivo.

readily differentiated from the 6 S tubulin dimer fraction by gel-permeation chromatography.

3.2. *General implications*

The results described above clearly show that *myo*-inositol interacts with polymerisable forms of microtubular protein and influences the extent to which it participated in the temperature-dependent subunit–polymer equilibrium at concentrations above 20 mM, maximum effects occurring at about 100 mM (see fig.2). Such effects could be of physiological significance, if one considers that these levels of inositol are of the same order as those which are found in vivo (10–25 μmol free inositol/g fresh tissue, see ref. [15,16]), when they are related, on a molar basis (mol inositol/mol tubulin), to the estimated content of polymerisable tubulin (ca. 10% of total soluble protein) present in brain extracts (see ref. [11]). The unusual behaviour of *myo*-inositol as a water-structuring compound [17] suggests that some of the effects observed could result from an alteration in the state of hydration of microtubular protein, since this factor is apparently of great importance in entropically-driven polymerisation processes such as microtubule assembly [13,18,19].

In attempting to assess the possible physiological significance of the present results, it seems noteworthy that stimulation of surface receptors in various cell types, where an increased turnover of phosphatidyl inositol ('PI effect') is observed (see ref. [8]), generally results in processes in which microtubules are apparently involved; for example, the redistribution of surface receptors in plasma membranes (see, eg ref. [20,21]) and diverse secretory phenomena (eg. [22,23]). It has been suggested [8] that the primary significance of the 'PI effect' is related to the production of a metabolite (ie., inositol or its phosphorylated precursors) mediating the effects of extracellular stimuli (ref. [24]), although the cellular targets for the inositol metabolite have not been identified.

On the basis of the above considerations, it is tempting to speculate that the relatively high intracellular levels of free inositol found in nervous and secretory tissues [15,16], which are richly endowed in microtubules, may reflect a unique and novel role for this compound in directly controlling the functional states of microtubules and hence, their participation in the regulation of various cellular activities. Thus, in this view, microtubules could act as a target for the inositol 'messenger' released as a consequence of the increased breakdown of phosphatidylinositol during cell stimulation ('PI effect').

In addition, it can be inferred from the binding data presented that microtubules may also contribute to the intracellular compartmentation of inositol. Such a role could be of great importance in the light of recent evidence showing:

(a) That brain microtubule-associated phospholipids, which are presumably derived from membranes, are enriched in a pool of metabolically-active phosphoinositides [2–4].

(b) That two of the key enzymes involved in the recycling of membrane phosphoinositides [8] are closely associated with microtubular protein [25,26].

In this way, microtubules could also function in compartmentalising enzymes and substrates involved in the breakdown and re-synthesis of phosphatidylinositides (see ref. [3,4,27]).

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