BIOCHEMICAL SOCIETY TRANSACTIONS

Preferential binding of a chick brain tau isoform to microtubules assembled in the presence of aluminium fluoride

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Introduction

The tau proteins are microtubule-associated proteins (MAPs). They are the product of differential splicing of a single gene [1–3], have molecular masses of ∼36–40 kDa [2], and are found almost exclusively in neuronal cells [4]. Tau is also a structural component of the paired helical filaments (PHFs) characteristic of the hippocampal cells of patients suffering from senile dementia of the Alzheimer’s type (SDAT) [e.g.5].

Tau stimulates microtubule assembly and suppresses dynamic instability [6]. This instability is due to two interconvertible populations [7], represented by microtubules with or without a terminal GTP-capped [8], with the assembly-dependent GTP hydrolysis inducing a change in the conformation of the tubulin dimer as measured by the dimer dissociation rate constant (k\text{GTP}\to\text{GDP}) [10].

Aluminium fluoride (AlF\text{3}) stabilizes microtubules by lowering K\text{GDP} [9]. Aluminium has also been implicated in the ontogeny of SDAT [10]. I demonstrate that microtubules assembled in the presence of AlF\text{3} contain an enhanced amount of a tau isoform; this observation may link the indirect evidence that aluminium is involved in SDAT and the well-characterized localization of tau in the PHFs.

Materials and methods

Microtubule protein was prepared from day-old chick brains [11, 12], including (Fig. 1b) or omitting (Figs. 1a and 1c) 25% glycerol during the assembly step. Immediately before use, the protein was eluted through a 0.9 cm × 28 cm Sephadex G-50 column equilibrated with the assembly buffer consisting of 100 mm-Mes, 62 mm-NaCl, 5 mm-NaF, 1 mm-dithiothreitol and 0.5 mm-MgCl\text{2} (pH 6.4 with KOH). The salt is included to dissociate the MAP:tubulin oligomers, so simplifying the elongation kinetics [13], and to suppress the MAP-inhibition of dynamic instability [14].

The eluted protein was assembled with increasing concentrations of AICl\text{3}, as previously described [15]. The formation during homogenization, did not alter the B\text{max} ratio of the two ligands (control, 1.7; treated, 1.9).

Fourthly, we then used a salt/acid washing procedure, as described in [8], to check the possibility that one of the ligands could bind to extra- and intra-vesicular βAR because of the difference in their lipophilicity. No such internalization was observed.

Finally, we postulated that [\text{[H]}\text{H}]DHA may bind to different sites, as was suggested by a very poor definition of the non-specific binding. Non-specific binding was respectively 29 ± 2% and 9 ± 1% of the total for [\text{[H]}\text{H}]DHA and [\text{[H]}\text{H}]CGP 12177. The [\text{[H]}\text{H}]DHA/proprenolol displacement curve was best fitted by a two-site model (R\text{max} = 92 ± 1%, K\text{d} = 4 ± 2 nM; R\text{max} = 8.7 ± 1%, K\text{d} = 3.2 ± 2 μM; n = 3, P<0.0025; Fig. 1). These results are in agreement with recent work on a different membrane preparation [9].

Our results show that [\text{[H]}\text{H}]CGP 12177 appears to be the ligand of choice for studying rat brain βAR regulation.

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Figure 1. Competition curve of (-)-alprenolol for [\text{[H]}\text{H}]DHA binding in rat cerebral cortex synaptoneurosomes

The concentration of [\text{[H]}\text{H}]DHA was 1.0 nM. Non-specific binding was defined by the curve-fitting program and it was determined as 29% of total [\text{[H]}\text{H}]DHA binding. The competition curve fit best to a two-site model (P<0.0025), with K\text{d} = 4 ± 2 nM and K\text{d} = 3 ± 2 μM (means ± SEM of three separate competition experiments which were pooled). These values were obtained by setting the affinity of [\text{[H]}\text{H}]DHA for the two sites at 1.8 nM.

or the binding assay by an endogenous agonist was also excluded as the saturation isotherm after 15 min preincubation in the presence of 1 μM (-)-isoprenaline gave the same B\text{max} and K\text{d} values for the ligands [\text{[H]}\text{H}]DHA (B\text{max} = 84 ± 5 fmol/mg of protein, K\text{d} = 1.81 ± 0.24 nM, n = 2) and [\text{[H]}\text{H}]CGP 12177 (B\text{max} = 43 ± 3 fmol/mg of protein, K\text{d} = 0.20 ± 0.04 nM, n = 2). Pretreatment in vivo with the βAR antagonist (±)-propranolol, which can block a possible βAR desensitization during homogenization, did not alter the B\text{max} ratio of the two ligands (control, 1.7; treated, 1.9).


Abbreviations used: MAP, microtubule-associated protein; PHF, paired helical filaments; SDAT, senile dementia of the Alzheimer’s type.
The assembled microtubules were pelleted, dissolved in SDS/PAGE sample buffer, and fractionated on a 10% (w/v) acrylamide/6 M-urea gel (Fig. 1b) or a 4–15% (w/v) acrylamide/4-8% urea gel (Fig. 1c) slab gel. After staining and clearing [17], individual bands were eluted [18], and the Coomassie stain was measured at 550 nm.

**Results**

AIF₂ (500 μM) reduces $k_{GTP}$, as measured from the kinetics of microtubule shortening on dilution as visualized by immunofluorescence, from $\simeq 200$ s$^{-1}$ to $\simeq 50$ s$^{-1}$ [19] (M. Symmons & R. G. Burns, unpublished work). It therefore affects the conformation of the assembled tubulin dimer. AIF₂ also affects the incorporation of [3H]GTP into pre-assembled microtubules (Fig. 1a), with an apparent $K_0$ of $\approx 20$ μM-AIF₂, consistent with its observed inhibition of dynamic instability.

Analysis of such assembled microtubules by SDS/PAGE reveals a remarkable enhancement of a single co-purifying component (band 2, Fig. 1b). Western blotting using a specific anti-tau monoclonal antibody (tau-1, generously donated by Dr. L. I. Binder, University of Alabama), solubility in 2.5% (v/v) perchloric acid [20], and electrophoretic mobility all confirm that band 2 is a tau component (data not shown). The recovery of other tau components, one with a slightly slower mobility (band 1, Fig. 1b) and two which co-migrate with β-tubulin, are not affected. Quantitative analysis shows that the band-2 enhancement exhibits a $K_0$ of $\approx 100$ μM-AIF₂ (Fig. 1c), whereas the recovery of other components, including band 1 and a protein [band 3] migrating ahead of β-tubulin, is independent of the AIF₂ concentration (Figs. 1b and 1c).

**Discussion**

The increased recovery of a single tau component by assembling microtubule protein with 500 μM-AIF₂ (Fig. 1b) clearly indicates that the conformation of the tubulin lattice affects the binding of this specific component. Tau heterogeneity is due to differential gene splicing (e.g. [2]) and the level of phosphorylation [21]. AIF₂ is unlikely to selectively induce the dephosphorylation of just one isoform; the selectively bound isoform is therefore probably a specific splicing product. Splicing yields products with three or four tubulin-binding sites, with the four-site form being uniquely expressed late in development [2, 22]. This additional site may be exquisitely sensitive to the AIF₂-induced change in the conformation of the tubulin lattice.

The apparent $K_0$ for the enhanced tau recovery is significantly higher than that for inhibiting subunit addition to steady-state microtubules ($\approx 100$ vs $\approx 20$ μM-AIF₂, Figs. 1a and 1c). This implies that the enhanced recovery of tau requires a high fraction of the assembled tubulin dimers to be in the altered conformation, and that tau binding is a cooperative process. Significantly, the assembly-promoting activity of synthetic peptides of the tubulin-binding sites is much less than that of the native protein [23].

The observed enhanced binding induced by AIF₂ is of considerable interest in view of the reported role of aluminium in the ontogeny of SDAT and the presence of tau in the core of the characteristic PHFs of patients suffering from this debilitating disease.

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Characterization of cysteine sulphinate transport by intact rat brain cerebrocortical synaptosome fractions

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A number of endogenous sulphur-containing amino acids have been shown in electrophysiological studies to possess excitatory properties similar to those exhibited by L-glutamate and L-aspartate [1]. The physiological significance of these neuroexcitatory actions is still unclear, although it is speculated that certain of the acidic sulphur-containing amino acids may function as putative excitatory neurotransmitters. The evidence is particularly strong for t-cysteine sulphinate.

Demonstration of the presence of a selective, Na+-dependent high-affinity transport carrier for physiological inactivation of neurotransmitters is regarded as one of several criteria required for fulfilling the requirement of transmitter candidacy [2]. A number of factors have contributed to the current re-evaluation of the transport characteristics of t-cysteine sulphinate, namely (a) previous transport kinetics studies were undertaken using radiolabelled L-cysteine sulphinate, with the inherent possibility of underestimation of net uptake due to homo- and hetero-exchange reactions; (b) the substrate specificity of the t-cysteine sulphinate transporter has not been fully defined; and (c) the possible existence of a Cl–-dependent transporter for t-cysteine sulphinate in nerve endings, as recently demonstrated in neuronal and glial plasma membranes for t-glutamate [3, 4] and in synaptic membranes for t-cysteate [5], would have implications with regard to previously published kinetic characterization of t-cysteine sulphinate transport. These factors have been investigated using synaptosome fractions, prepared from the cerebral cortices of 220 male Wistar rats, by discontinuous Ficoll-density-gradient centrifugation as described by Nicholls [6]. The synaptosomes recovered from the gradient were maintained as a pellet at 0°C before resuspension (within 3 h) for use in uptake studies. For transport kinetic studies, synaptosomal pellets were resuspended to 8 mg of protein/ml of incubation medium [125 mM-NaCl, 3.5 mM-KCl, 1.2 mM-MgSO₄, 5 mM-NaHCO₃, 0.4 mM-KH₂PO₄, 1.3 mM-CaCl₂, 20 mM-Tris (sodium salt) and 10 mM-d-glucose, pH 7.4], Aliquots were preincubated for 5 min at 35°C before initiation of assays undertaken in the presence of varying concentrations (5–5000 μM) of t-cysteine sulphinate. For substrate specificity assays, synaptosomal fractions were incubated with a fixed concentration (200 μM) of each of a variety of potential transport inhibitors in the presence of either 2 μM-[3H]aspartate (0.3 μCi/ml of assay mixture), 2 μM-[3H]glutamate (0.5 μCi/ml of assay), 3 μM-[3H]Hippurate acid (GABA) (10 μCi/ml of assay mixture), 11 μM-[3H]taurine (2.5 μCi/ml of assay mixture) or 50 μM-l-cysteine sulphinate (non-radiolabelled) as transport substrates. The extent of inhibition of t-cysteine sulphinate uptake by one inhibitor amino acid was assessed against the extent of inhibition of each of the remaining transport substrates by the same inhibitor. In determining the ion specificity and Cl– dependency of transport, the composition of the incubation medium was altered appropriately but always maintaining equal ionic strength in the individual solutions.

This report describes the use of an h.p.l.c. method [7] to measure the total uptake of t-cysteine sulphinate (see Fig. 1). Uptake of [3H]-labelled neurotransmitters was undertaken as described elsewhere [8]. The net intrasynaptosomal accumulation of t-cysteine sulphinate as a function of the external t-cysteine sulphinate concentration exhibited a monophasic dependence when corrected for non-saturable diffusion. Quantification of the kinetic parameters of transport revealed the presence of a single, high-affinity (Kₐ = 36.7 ± 6.17 μM), relatively high capacity (V₉₉ = 1.16 ± 0.13 nmol/min per mg) transport system. This is in contradiction to the results obtained with other brain preparations (e.g. slices) or crude synaptosomal fractions using radiolabelled l-cysteine sulphinate [9, 10] wherein both high- and low-affinity systems appear to be demonstrable.

The inhibition profile of l-cysteine sulphinate transport by a number of l-enantiomers of acidic excitatory amino acids exhibited a significant positive correlation (r = 0.95) when compared with the inhibition of high-affinity l-aspartate or l-glutamate transport by the same inhibitors, but a negative correlation when assessed against inhibition of either l-GABA or l-taurine transport. These observations indicate that l-cysteine sulphinate may share a common transporter with the dicarboxylic excitatory amino acids. At a fixed concentration of 50 μM-l-cysteine...