Involvement of acetylated tubulin in the regulation of Na^+,K^+-ATPase activity in cultured astrocytes

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Abstract The results presented support the view that the modulation of Na^+,K^+-ATPase activity in living cells involves the association/dissociation of acetylated tubulin with the enzyme. We found that the stimulation of Na^+,K^+-ATPase activity by L-glutamate correlates with decreased acetylated tubulin quantity associated with the enzyme. The effect of L-glutamate was abolished by the glutamate transporter inhibitor DL-*threo*-β-hydroxyaspartate but was not affected by either specific agonists or antagonists. The effect of L-glutamate seems to be mediated by Na^+ entry resulting from glutamate transport, since the Na^+ ionophore monensin produced stimulation of Na^+,K^+-ATPase activity with concomitant decrease of acetylated tubulin quantity associated with the enzyme.

Key words: Tubulin; Acetylated tubulin; ATPase; Astrocyte

1. Introduction

Tubulin, the main protein of microtubules, is present in various types of membranes [1–7]. In membranes isolated from neural tissue, we found that a fraction of the tubulin pool is associated with the Na^+,K^+-ATPase α-subunit [8] and that the associated tubulin consists mainly, if not exclusively, of the acetylated isotype [9]. Tubulin associated with Na^+,K^+-ATPase behaves as a hydrophobic compound, whereas tubulin separated from Na^+,K^+-ATPase shows hydrophilic behavior. The terms 'hydrophilic' and 'hydrophobic' refer to tubulins that partition into the aqueous phase and the detergent phase, respectively. When brain membranes are extracted with a solution containing 1% Triton X-114, after partitioning, the acetylated tubulin/Na^+,K^+-ATPase complex partitions into the detergent phase, whereas free tubulin partitions into the aqueous phase. In contrast, when the associated tubulin is separated from Na^+,K^+-ATPase, it no longer partitions into the detergent-rich phase but into the aqueous phase [9,10]. These properties allowed us to easily determine the quantity of tubulin associated with Na^+,K^+-ATPase, which we refer to in this paper as hydrophobic acetylated tubulin (HAT).

We found recently that the association of tubulin with Na^+,K^+-ATPase inhibits the enzyme activity in vitro [11]. In the present study, to ascertain whether tubulin participates in the regulation of Na^+,K^+-ATPase activity in vivo, we pharmacologically modified activity of the enzyme in cultured astrocytes and determined the quantity of HAT resulting from the modified activity. We found that increased Na^+,K^+-ATPase activity consistently correlated with decreased quantity of HAT, and vice versa. Our results indicate that the association/dissociation of acetylated tubulin with Na^+,K^+-ATPase is involved in the regulation of the enzyme activity in living cells.

2. Materials and methods

2.1. Materials

Triton X-114, ATP, L-glutamate, L-aspartate, D-aspartate, mouse monoclonal antibody 6-11B-1, anti-mouse IgG conjugated with peroxidase, and Dulbecco's modified Eagle's medium containing 25 mM glucose and 44 mM $NaHCO_3$ were from Sigma Chemical Co. [γ-^{32}P]ATP and ^{86}RbCl were from NEN Life Science. Fetal calf serum and fetal bovine serum were from Invitrogen.

2.2. Cell culture

Primary cultures of cerebral cortical astrocytes were prepared from 1–2-day-old Swiss Albino mice and cultivated at 37°C in a water-saturated atmosphere containing air/CO_2 (19:1) as described previously [12].

2.3. Na^+,K^+-ATPase activity assay

The enzyme activity was determined by two different methods.

2.3.1. ^{86}Rb uptake method. We followed essentially the procedure described by Pellerin and Magistretti [13]. Confluent cells, after removal of culture medium, were washed twice with 1 ml HEPES buffer (25 mM HEPES, pH 7.4, containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 125 mM NaCl, and 6 mM KCl) and then incubated at 37°C with 1 ml HEPES buffer containing L-glutamate at the concentrations indicated for the respective experiments. Next, 1 ml HEPES buffer containing ^{86}RbCI (2.6 μCi/ml) and 0 or 1 mM ouabain was added, and incubation continued for 20 min. The medium was removed, and cells were rinsed three times with 3 ml cold (0–4°C) HEPES buffer and then lysed by adding 2 ml Tris-buffered saline (TBS)–Triton (50 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl and 1% Triton X-100). Appropriate aliquots were used to determine radioactivity, protein, and HAT. Na^+,K^+-ATPase-mediated ^{86}Rb uptake was calculated as the difference in ^{86}Rb uptake between samples incubated in the presence and in the absence of 1 mM ouabain.

2.3.2. [γ-^{32}P]ATP hydrolysis method. Na^+,K^+-ATPase activity was determined by measuring [γ-^{32}P]ATP hydrolysis and expressed

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazol-4-propionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-AP5, D-(−)-2-amino-5-phosphono-valeric acid; HAT, hydrophobic acetylated tubulin; L-AP3, L(+)-2-amino-3-phosphono-propionic acid; NMDA, *N*-methyl-D-aspartic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; THA, DL-*threo*-β-hydroxyaspartate

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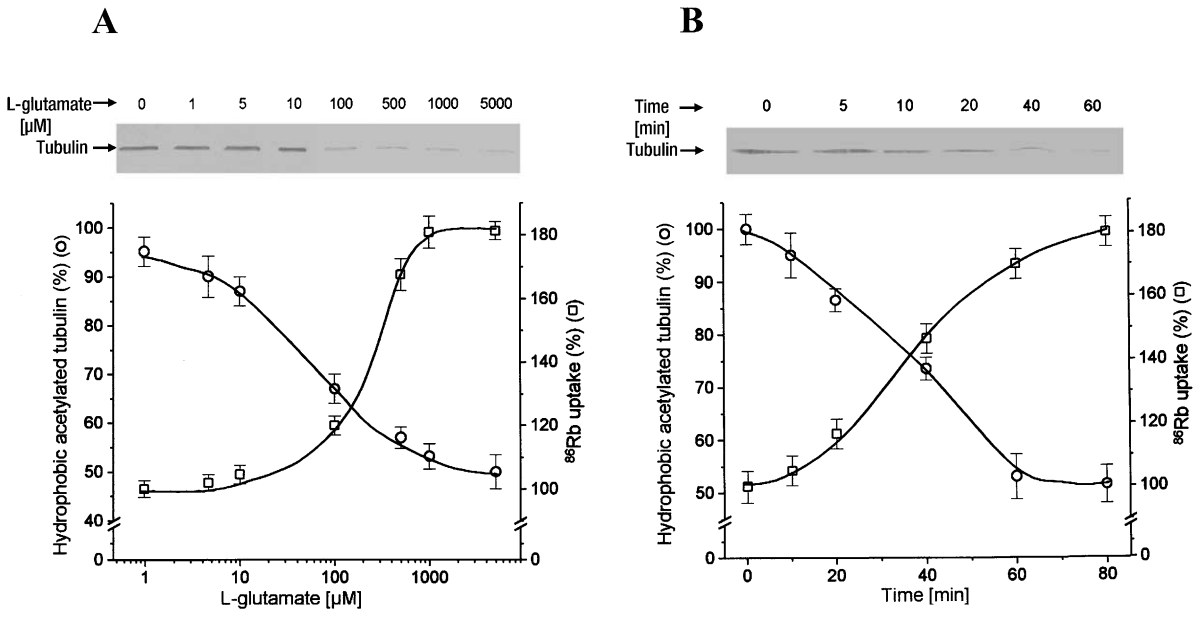

Fig. 1. Effect of L-glutamate on HAT quantity and ^{86}Rb uptake. Astrocytes were incubated for 60 min at 37°C in the presence of L-glutamate at the indicated concentrations (A) or at 37°C in the presence of 1 mM L-glutamate at the indicated times (B). At the end of incubation, HAT was obtained in the detergent-rich phase and immunoblotted with antibody 6-11B-1 as described in Section 2. Tubulin bands (upper panels) were quantified, and the values are shown in the lower panels. Data are expressed as percentages of control incubated in the absence of L-glutamate (A) or at $t=0$ (B). Data are mean ± S.D. from three independent experiments. The tubulin bands shown in the upper panels are from a representative experiment.

as ouabain-sensitive ATPase activity. It was determined essentially as described by Goldin [14]. Confluent cells were permeabilized by incubating with HEPES buffer containing 3 μg/ml digitonin for 5 min at room temperature, and rinsed twice with HEPES buffer. The enzymatic assay was started by adding 1 ml HEPES buffer containing 2 mM [γ-^{32}P]ATP (450 dpm/nmol) and 0 or 1 mM ouabain. After 20 min at 37°C, the reaction was stopped by adding 50 μl of 66% (w/v) trichloroacetic acid. Released $^{32}P_i$ was quantified as described by Goldin [14]. Na^+,K^+-ATPase activity was estimated from the difference between samples incubated in the absence and in the presence of ouabain.

2.4. Determination of HAT

The acetylated tubulin associated with Na^+,K^+-ATPase (HAT) was found in the Triton X-114 reach phase as described previously [11]. The detergent-rich phase, which contained HAT, was washed once with TBS (50 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl). To determine the HAT quantity, aliquots were subjected to electrophoresis and immunoblotting.

2.5. Electrophoresis and immunoblotting

Proteins were separated by SDS–PAGE by the procedure of Laemmli [15] on 10% polyacrylamide slab gels. After electrophoresis, proteins were transferred to nitrocellulose and the blots were probed with mouse monoclonal antibody 6-11B-1 (dilution 1/1000) to determine HAT [16]. The nitrocellulose sheet was incubated with anti-mouse IgG conjugated with peroxidase, and intensities of tubulin bands were quantified by Scion imaging software.

2.6. Protein determination

Protein concentration was determined by the method of Bradford [17].

3. Results

3.1. Correlation between quantity of HAT and Na^+,K^+-ATPase activity

Tubulin isolated from brain membranes as a hydrophobic compound is associated with Na^+,K^+-ATPase, and this association inhibits Na^+,K^+-ATPase activity in vitro [8,11]. In view of the observation that exogenous L-glutamate stimulates Na^+,K^+-ATPase activity in cultured astrocytes in a concentration-dependent manner [13], we investigated whether this activation implies dissociation of the acetylated tubulin/Na^+,K^+-ATPase complex. If dissociation occurs, the quantity of HAT should be reduced. To test this possibility, we exposed cultured astrocytes to various concentrations of L-glu-

Table 1
Effect of L-glutamate on HAT quantity and Na^+,K^+-ATPase activity

Experiment	1 h incubation	Medium renewed and 1 h further incubation	Medium renewed and 1 h further incubation	HAT (% of control)	^{86}Rb uptake (nmol/mg of protein/min)
1	Control			100 ± 4.2	26.5 ± 5.3
	+Glutamate			48 ± 2.2	46.5 ± 4.7
2	Control	Yes		100 ± 7.3	25.0 ± 5.2
	+Glutamate	Yes		53 ± 7.8	49.3 ± 7.3
	+Glutamate	Yes+glucose		105 ± 6.9	25.5 ± 6.0
3	Control	Yes	Yes	100 ± 4.3	25.2 ± 4.5
	+Glutamate	Yes+glucose	Yes+glutamate	52 ± 4.2	40.5 ± 6.7

Astrocytes were incubated at 37°C in HEPES buffer under the conditions indicated, for 1 h (experiment 1), 2 h (experiment 2), or 3 h (experiment 3). Glutamate and glucose were added at 1 mM final concentration. Data are mean ± S.D. from three independent experiments.

Table 2
Influence of glutamate agonists and antagonists on glutamate-induced effects

	Addition	HAT (% of control)	^{86}Rb uptake (nmol/mg of protein/min)
Control	None	100 ± 6.8	29.5 ± 6.7
	Glutamate	45 ± 6.7	49.1 ± 5.3
Agonist	NMDA	101 ± 9.4	31.3 ± 4.5
	AMPA	107 ± 6.7	28.8 ± 5.3
Antagonist	L-AP3+glutamate	58 ± 8.8	45.5 ± 7.4
	D-AP5+glutamate	53 ± 7.3	49.3 ± 8.2
	CNQX+glutamate	59 ± 6.2	44.0 ± 4.2

Astrocytes were incubated at 37°C for 60 min in HEPES buffer containing, where indicated, 0.5 mM glutamate, 1 mM agonist and 1 mM antagonist. For antagonists, a 20 min period of incubation preceded the 1 h incubation with L-glutamate. Data are mean ± S.D. from three independent experiments.

tamate during 60 min and determined HAT quantity and Na^+,K^+-ATPase activity as reflected by ^{86}Rb uptake. The former parameter decreased, whereas the latter increased (Fig. 1A). It can be seen that maximal stimulation of Na^+,K^+-ATPase activity was reached at 1 mM L-glutamate. However, at this L-glutamate concentration, HAT decreased only about 50%. An explanation for this apparent discrepancy is given in the Section 4. Fig. 1B shows the decrease of HAT quantity and the stimulation of Na^+,K^+-ATPase activity as a function of the incubation time in the presence of 1 mM L-glutamate.

An inverse correlation between HAT quantity and Na^+K^+-ATPase activity was also found for L- and D-aspartate which were previously described as Na^+,K^+-ATPase stimulators [13] (results not shown).

3.2. The effect of L-glutamate on HAT quantity and Na^+,K^+-ATPase activity is reversible

The changes produced by L-glutamate on HAT quantity and Na^+,K^+-ATPase activity were completely reversed by a subsequent 1 h incubation of astrocytes in L-glutamate-free medium containing D-glucose (Table 1, compare Exp. 1 with Exp. 2). Such reversal did not occur when D-glucose was omitted from the medium. Because stimulation of Na^+,K^+-ATPase activity by L-glutamate is accompanied by glucose consumption [12,13], we hypothesized that replenishment with D-glucose is necessary for the reversal, and this was confirmed by our experiment. Following the D-glucose treatment, incubation with L-glutamate led again to reduction of HAT quantity and increased Na^+,K^+-ATPase activity (Table 1, Exp. 3).

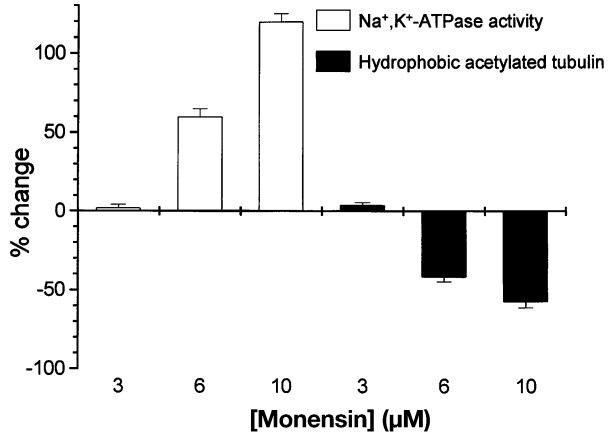

Fig. 2. Effect of monensin on HAT quantity and Na^+,K^+-ATPase activity. Astrocytes were incubated in HEPES buffer in the presence of monensin at the indicated concentrations. After 60 min at 37°C, HAT quantity and [γ-^{32}P]ATP hydrolysis were determined. Results are expressed as percentages of control which was incubated in the absence of monensin. Data are mean ± S.D. from three independent experiments.

3.3. Mechanism of the L-glutamate effect

We investigated if the effect of L-glutamate on HAT quantity involved interaction of the amino acid with membrane receptors. We tested the effect of several well-known L-glutamate agonists and antagonists on HAT quantity and Na^+,K^+-ATPase activity. The agonists *N*-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methylisoxazol-4-propionic acid (AMPA) and the antagonists L(+)-2-amino-3-phosphono-propionic acid (L-AP3), D-(−)-2-amino-5-phosphono-valeric acid (D-AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) practically did not affect those parameters, indicating that the effect produced by L-glutamate was not mediated by specific L-glutamate receptors (Table 2). Pellerin and Magistretti [13] considered the stimulation of Na^+,K^+-ATPase activity by L-glutamate to be due to Na^+ entry resulting from glutamate transport. A similar mechanism apparently underlies the effect of L-glutamate on HAT quantity, since this effect is abolished by glutamate transporter inhibitor DL-*threo*-β-hydroxyaspartate (THA) (Table 3). Since glutamate uptake is accompanied by Na^+ entry, the glutamate effect on HAT quantity may be mediated by Na^+ ions. The addition of monensin, a Na^+ ionophore [18], to cultured astrocytes resulted in a lower HAT quantity similar to that obtained with L-glutamate (Fig. 2).

Table 3
Effect of THA on HAT quantity and Na^+,K^+-ATPase activity

Condition	HAT (% of control)	Na^+,K^+-ATPase activity (μmol P_i/mg of protein/min)
Control	100 ± 3.5	1.28 ± 0.3
Glutamate	49 ± 8.5	3.53 ± 0.4
THA	98 ± 9.0	1.37 ± 0.2
THA+glutamate	96 ± 9.2	1.56 ± 0.3

Astrocytes were incubated at 37°C for 1 h in HEPES buffer containing, where indicated, 0.5 mM glutamate and 1 mM THA. In the case of THA+glutamate, astrocytes were incubated with THA for 20 min at 37°C, followed by addition of glutamate and 1 h further incubation. Next, HAT quantity and [γ-^{32}P]ATP hydrolysis were determined. Data are mean ± S.D. from three independent experiments.

4. Discussion

We found that the increase of Na^+,K^+-ATPase activity is correlated with decreased HAT quantity. This result, and the fact that this tubulin form originates from acetylated tubulin/Na^+,K^+-ATPase complex [9,11], indicate that the stimulation of the enzyme activity involves dissociation of the complex. The effect was reversible, i.e. if astrocytes after L-glutamate treatment were maintained in L-glutamate-free medium containing 1 mM D-glucose, the enzyme activity decreased and HAT quantity increased (Table 1). Such a reversal did not occur in the absence of glucose. These results indicate that glucose is necessary to restore acetylated tubulin/enzyme complex after dissociation by L-glutamate.

Glutamate mediates most of the excitatory synaptic transmissions in the brain by interacting with specific receptors [19]. In nerve terminals it is stored in synaptic vesicles with an estimated concentration of 60–210 mM [20–22], and on depolarization of the nerve terminal it is released by a calcium-dependent exocytotic process. The exocytosis of a single synaptic vesicle can raise the concentration of the amino acid in the synaptic cleft to about 1 mM [23]. In our experiments, this concentration of glutamate was sufficient to decrease HAT quantity and to maximally stimulate Na^+,K^+-ATPase activity (Fig. 1). The action of glutamate in the synaptic cleft is terminated by uptake systems localized in the plasma membrane of neurons and astrocytes. A major role of glutamate transporters is to prevent excessive stimulation of glutamate receptors by limiting the concentration of free glutamate in the extracellular space [24]. As in the case of stimulation of Na^+,K^+-ATPase activity [13], the effect of L-glutamate on HAT quantity appears to be mediated through glutamate transporters, not by L-glutamate-specific receptors, i.e. the effect of L-glutamate was abolished by the glutamate transporter inhibitor THA (Table 3), but was not affected by L-glutamate-specific agonists and antagonists (Table 2).

In astrocytes, three Na^+ ions (or two Na^+ and one H^+) accompany glutamate entry, while one K^+ is transported out accompanied by either one OH^- or one HCO_3^- [25]. Therefore, glutamate uptake by transporters increases Na^+ concentration within the cell. The effect of glutamate on HAT quantity appears to be mediated by Na^+ entry resulting from glutamate transport, in a similar manner to the stimulation of Na^+K^+-ATPase activity [13]. This is supported by the finding that the addition of Na^+ ionophore monensin produced a decrease in the amount of HAT (Fig. 2). The regulation of HAT quantity by L-glutamate through a mechanism similar to that for stimulation of Na^+,K^+-ATPase reflects the close relationship between acetylated tubulin and Na^+,K^+-ATPase.

At maximal stimulation of Na^+,K^+-ATPase activity, only about 50% of hydrophobic tubulin was converted to the hydrophilic form. Considering that there are several isotypes of tubulin [26,27] and Na^+K^+-ATPase [28–30], there may be some forms of acetylated tubulin–enzyme complex that are not dissociated by an L-glutamate-induced mechanism. Another possibility is that part of the acetylated tubulin pool was associated with membrane components other than Na^+,K^+-ATPase. In any case, our data support the hypothesis that the regulation of Na^+,K^+-ATPase activity in living cells involves the association/dissociation of the enzyme with acetylated tubulin. According to previously published results [10], the acetylated tubulin/Na^+,K^+-ATPase complex can be obtained from membranes or from native microtubules depending on the conditions of tissue homogenization. To determine the possible involvement of native microtubules in the effect of glutamate described herein, experiments are in progress.

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