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Enhancement of tubulin polymerization by Cl^--induced blockade of intrinsic GTPase

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ABSTRACT

In growing neurite of neuronal cells, it is suggested that α/β-tubulin heterodimers assemble to form microtubule, and assembly of microtubule promotes neurite elongation. On the other hand, recent studies reveal importance of intracellular Cl^- in regulation of various cellular functions such as cell cycle progression, differentiation, cell migration, and elongation of neurite in neuronal cells. In this study, we investigated effects of Cl^- on *in vitro* tubulin polymerization. We found that efficiency of *in vitro* tubulin polymerization (the number of microtubule) was higher (3 to 5-fold) in Cl^--containing solutions than that in Cl^--free solutions containing Br^- or NO_3^-. On the other hand, GTPase activity of tubulin was lower (2/3-fold) in Cl^--containing solutions than that in Cl^--free solutions containing Br^- or NO_3^-. Efficiency of *in vitro* tubulin polymerization in solutions containing a non-hydrolyzable analogue of GTP (GpCpp) instead of GTP was much higher than that in the presence of GTP. Effects of replacement of GTP with GpCpp on *in vitro* tubulin polymerization was weaker in Cl^- solutions (10-fold increases) than that in Br^- or NO_3^- solutions (20-fold increases), although the efficiency of *in vitro* tubulin polymerization in Cl^- solutions containing GpCpp was still higher than that in Br^- or NO_3^- solutions containing GpCpp. Our results suggest that a part of stimulatory effects of Cl^- on *in vitro* tubulin polymerization is mediated via an inhibitory effect on GTPase activity of tubulin, although Cl^- would also regulate *in vitro* tubulin polymerization by factors other than an inhibitory effect on GTPase activity.

1. Introduction

Microtubule formed by reversible association of tubulin proteins is one of major cytoskeletal systems in eukaryotic cells, and is involved in various basic and essential cellular functions such as chromosome segregation, intracellular vesicle transport, establishment of cellular polarity, cell motility and elongation of neurite in neuronal cells [1–4]. Tubulin consists of highly conserved α/β heterodimeric proteins forming microtubule via self-assembly. Both subunits can bind guanine nucleotides; one exchangeable (β-subunit binding) and the other unexchangeable (α-subunit binding). Only a GTP-bound form of tubulin can be assembled to tubulin forming microtubule, while tubulin has an intrinsic GTPase activity contributing to disassembly of polymerized tubulin via hydrolysis of GTP bound to tubulin [5,6]. Therefore, GTPase activity of tubulin is one of key factors regulating polymerization of tubulin.

Recent studies have revealed that intracellular Cl^- is an important factor regulating various cellular functions such as cell cycle progression [7–16], proliferation [17–20], and adhesion/migration of cells [21–24]. We have recently reported that reduction of intracellular Cl^- concentration ($[Cl^-]_i$) enhances gene expression of the α subunit of epithelial Na^+ channel (α-ENaC) [25,26]. In addition, Menegazzi et al. have reported that changes in $[Cl^-]_i$ are associated with physiological functions of leukocyte such as migration, adhesion and production of reactive oxygen species [22,23]. We have recently reported that in rat pheochromocytoma PC12 cells and their subclonal PC12D cells NGF-induced neurite outgrowth requires uptake of Cl^- into the intracellular space via Na^+–K^+–$2Cl^-$ cotransporter 1 (NKCC1) [27–29], and activation of NKCC1 by dietary flavonoids, quercetin and genistein, enhances NGF-induced neurite outgrowth [30–32]. In addition, NGF-induced neurite outgrowth is enhanced by inhibition of K^+–Cl^- cotransporter 1 (KCC1) [33,34], which is another type of Cl^- cotransporter mediating excretion of K^+ and Cl^- from intracellular space. These observations suggest that intracellular Cl^- would be an important factor regulating the neurite outgrowth. As mentioned above, polymerization of tubulin from free tubulin in the tip of growing neurite is essential for the elongation of neurite [35]. Therefore, we speculated if intracellular Cl^- would contribute to elongation of neurite by regulating tubulin polymerization.

In the present study, we investigated if Cl^- affects polymerization of tubulin, and demonstrated that Cl^- affected intrinsic GTPase

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activity of tubulin and the action of Cl^- on intrinsic GTPase activity of tubulin would be one of important factors regulating tubulin polymerization (formation of microtubule).

2. Materials and methods

2.1. Materials

We purchased purified porcine brain tubulin, TRITC-labeled tubulin, and PhosFree Phosphate Assay Biochem Kit from Cytoskeleton Inc. (Denver, CO, USA), guanosine-5′-triphosphate (GTP) from Sigma–Aldrich (St Louis, MO, USA), non-hydrolyzed guanosine-5′-[(α,β)-methyleno]triphosphate (GpCpp) from Jena Bioscience GmbH (Jena, Germany), and any other chemicals not listed above from Sigma–Aldrich or Wako Pure Chemical (Osaka, Japan).

2.2. in vitro polymerization of tubulin

Purified porcine tubulin mixed with TRITC-labeled tubulin (non-lebeled:labeled = 10:1) was dissolved in stock buffers (10 mM Hepes-KOH, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, and 1 mM GTP with pH 7.4) at a concentration of 10 mg protein/ml, and was stored in aliquots at −80 °C. Polymerization of tubulin was started by addition of a tubulin stock solution into the reaction buffer consisting of 10 mM Hepes-KOH, 2 mM $MgCl_2$, 0.5 mM EGTA, 5% glycerol, 1 mM GTP or 0.1 mM GpCpp, and 100 mM various KCl, KNO_3, or KBr (pH 7.4) at a final tubulin concentration of 1 mg protein/ml. The reaction was conducted for 10, 20, and 60 min at 37 °C. At each time point, the reaction was stopped by adding a fixative solution (1% glutaraldehyde in stock buffer) of 10-fold volume to the reaction buffer. After incubation for 5 min at room temperature, the stock buffer was added to the fixed samples for further 1/10 to 1/1000 fold dilution, and an aliquot of 4 μl was mounted onto glass coverslips. Polymerized tubulin was observed with a confocal microscope FV1000 (Olympus, Tokyo, Japan). We captured images of polymerized tubulin were from randomly chosen field (approximately 7–15 fields), and measured numbers and lengths of polymerized tubulin.

2.3. Measurement of GTPase activity of tubulin

Purified porcine tubulin (without labeled tubulins) was dissolved in the stock buffer at a concentration of 10 mg protein/ml. The enzymatic reaction was started by addition of the tubulin stock solution into the reaction buffer containing 1 mM GTP and 100 mM KCl, KNO_3 or KBr. The reaction was conducted for 20, 60, and 150 min at 37 °C. At each time point, inorganic phosphate (Pi) released from GTP via hydrolysis of GTP was measured by using PhosFree Phosphate Assay Biochem Kit according to the manufacture's protocol (Cytoskeleton Inc.).

2.4. Statistics

All data are presented as means ± SEM. Where error bars are not visible, they are smaller than the symbol. The difference between groups was evaluated with ANOVA. If ANOVA indicated a significant difference, Tukey's HSD was performed to determine the significance between the mean. A p value less than 0.05 was considered as statistically significant.

3. Results

3.1. Effects of Cl^- on in vitro polymerization of tubulin

To assess the effect of Cl^- on *in vitro* polymerization of tubulin, we first measured *in vitro* microtubule polymerization in solutions containing Cl^-, NO_3^-, or Br^-. Fig. 1 shows numbers (Fig. 1A) and lengths of (Fig. 1B) of polymerized tubulin in solutions containing Cl^-, NO_3^-, or Br^-. In Cl^--containing solutions, we observed efficient polymerization of tubulin (formation of microtubule); e.g., polymerized tubulin of approximately 120×10^6/ml with 23 μm in length was observed 60 min after starting the reaction. On the other hand, in NO_3^-- or Br^--containing solutions, polymerized tubulin of approximately 20×10^6/ml with 10 μm in length was observed. We tested the effect of K^+ on the tubulin polymerization by replacing K^+ with Na^+. The replacement of K^+ with Na^+ had no significant effect on polymerization of tubulin (data not shown). Fig. 2 shows histograms of lengths of polymerized tubulin (microtubule) 10 min (Fig. 2A), 20 min (Fig. 2B), and 60 min (Fig. 2C) after starting the reaction. In Cl^--containing solutions we observed many long polymerized tubulin (microtubule) (Fig. 2B and C), but not in NO_3^-- or Br^--containing solutions (Fig. 2B and C).

3.2. Effects of Cl^- on GTPase activity

The GTP/GDP cycle of β-subunit of tubulin is considered to be crucial for tubulin polymerization. The GTP-bound form of β-subunit of tubulin is essentially required for polymerization. GTP of β-subunit of tubulin is hydrolyzable to GDP by GTPase contained in tubulin itself. Therefore, we studied effects of Cl^- on GTPase activity of tubulin [1,2,4] by measuring inorganic phosphate (Pi) released from GTP via GTP hydrolysis using a colorimetric phosphate assay method in solutions containing Cl^-, NO_3^- or Br^-. As shown in Table 1, rates of GTP hydrolysis were higher in solutions containing NO_3^-, or Br^-

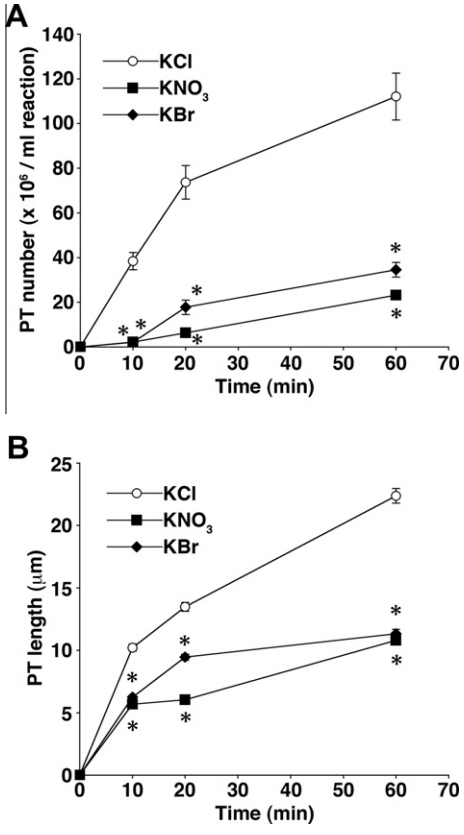

Fig. 1. Effects of Cl^- on *in vitro* polymerization of tubulin. Purified porcine tubulin was incubated in solutions containing 100 mM KCl (open circles), KNO_3 (closed squares) or KBr (closed diamonds) with 1 mM GTP 10 min, 20 min, and 60 min at 37 °C after starting the reaction. Numbers (A) and lengths (B) of polymerized tubulin (PT) were measured with a confocal microscope. Results were presented as mean ± SEM.*indicates $p < 0.01$ vs KCl.