Biochemical and Biophysical Research Communications 425 (2012) 225–229

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 13 July 2012
Available online 22 July 2012

Keywords:
GTPase
Non-hydrolyzable GTP
Microtubule
Cytoskeleton
Anions

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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http://dx.doi.org/10.1016/j.bbrc.2012.07.072

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^-

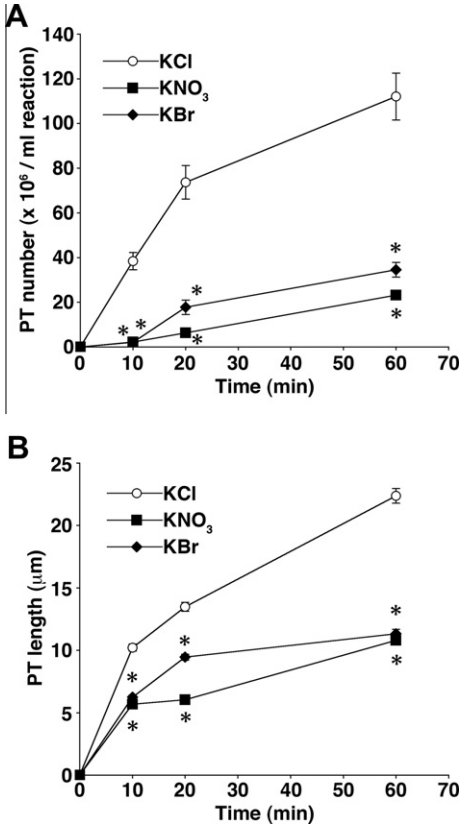

Fig. 1. Effects of Cl^- on *in vitro* polymerization of tubulin. Purified porcine tubulin was incubated in solutions containing 100 mM KCl (open circles), KNO_3 (closed squares) or KBr (closed diamonds) with 1 mM GTP 10 min, 20 min, and 60 min at 37 °C after starting the reaction. Numbers (A) and lengths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

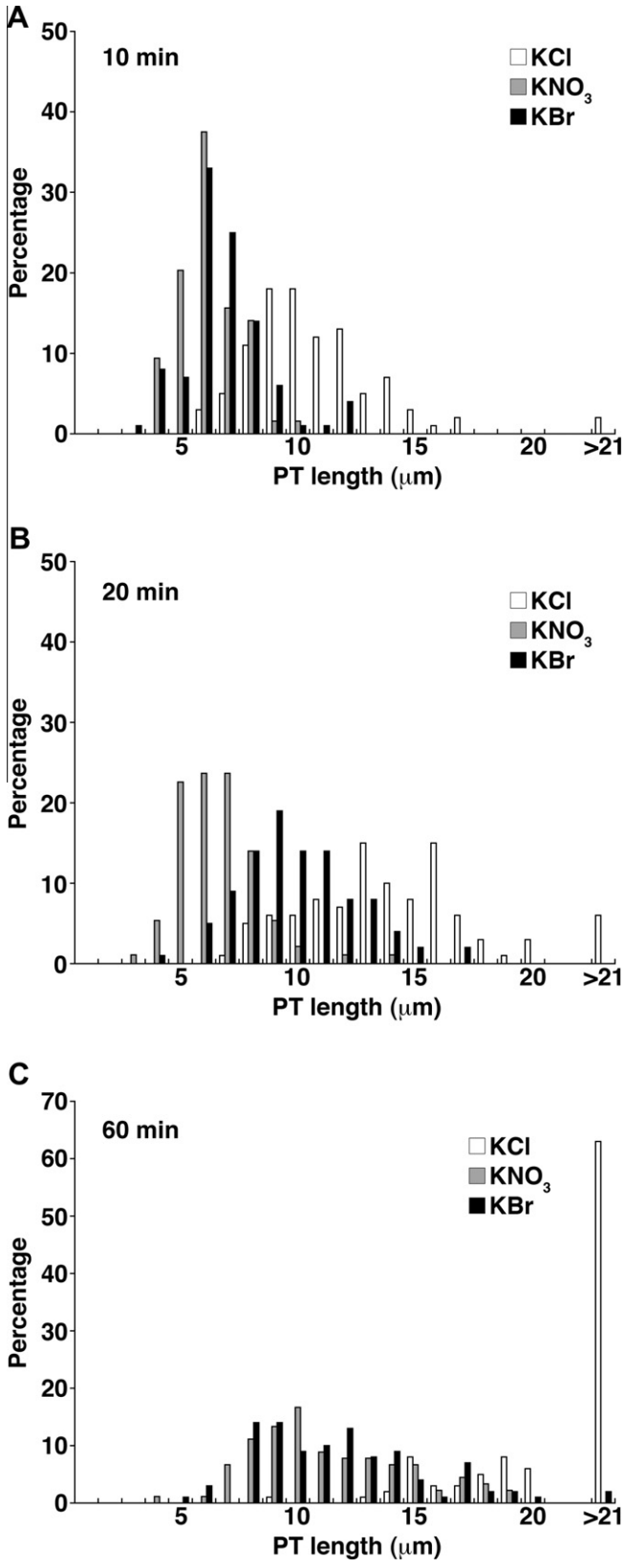

Fig. 2. Histograms of lengths of polymerized tubulin (PT) in solutions containing 100 mM KCl (open columns), KNO_3 (gray columns), or KBr (closed columns) with 1 mM GTP 10 min (A), 20 min (B), and 60 min (C) at 37 °C after starting the reaction.

(47–49 μM Pi for 150 min) than that in solutions containing Cl^- (about 31 μM Pi for 150 min).

Table 1
Effects of Cl^- on GTPase activity.

	GTPase activity (released Pi; μM)			
	0 min	20 min	60 min	150 min
KCl	8.93 ± 1.62	19.88 ± 2.67	22.06 ± 2.99	31.11 ± 2.73
KNO_3	10.81 ± 1.84	22.57 ± 3.10	32.32 ± 2.74*	47.06 ± 3.83*
KBr	9.85 ± 1.97	16.10 ± 2.80	34.83 ± 3.05*	48.82 ± 3.08*

Purified porcine tubulin was incubated in 100 mM KCl, KNO_3, or KBr with 1 mM GTP for 20 min, 60 min, and 150 min at 37 °C. Released inorganic phosphate (Pi) was measured by using PhosFree Phosphate Assay Biochem Kit. Results were presented as mean ± SEM.
* Indicates $p < 0.05$ vs KCl.

3.3. Effects of non-hydrolyzable analogue of GTP (GpCpp) on in vitro polymerization of tubulin

Next, we assessed if lower efficiency of tubulin polymerization is caused by higher GTPase activity in solutions containing NO_3^- or Br^-. We tested the effect of a non-hydrolyzable analogue of GTP, GpCpp [36–38], on the *in vitro* polymerization of tubulin in solutions containing Cl^-, NO_3^- or Br^-. We observed approximately 1,000 × 10^6/ml polymerized tubulin (microtubule) 60 min after starting the reaction in solutions containing Cl^-, and approximately 500–600 × 10^6/ml polymerized tubulin (microtubule) in solutions containing NO_3^- or Br^- (Fig. 3A). Although the number of polymerized tubulin was larger in solutions containing Cl^- than that in solutions containing NO_3^- or Br^-, the difference of numbers of polymerized tubulin (microtubule) in Cl^-- and NO_3^- or Br^--containing solutions

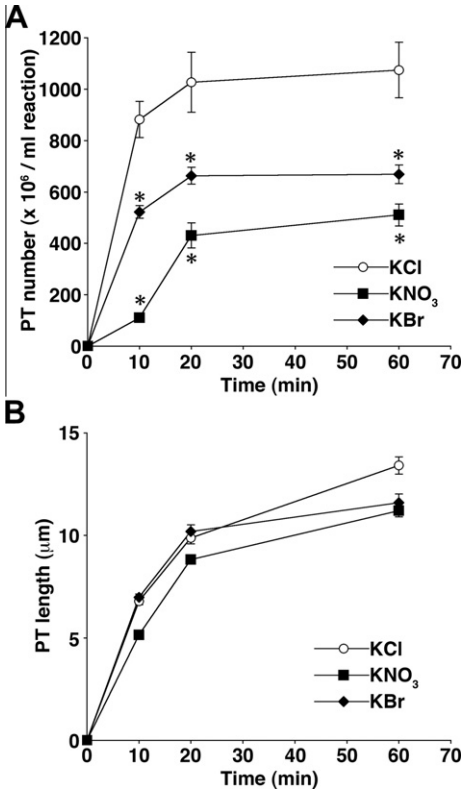

Fig. 3. Effects of non-hydrolyzable analogue of GTP (GpCpp) on *in vitro* polymerization of tubulin. Purified porcine tubulin was incubated in solutions containing 100 mM KCl (open circles), KNO_3 (closed squares), or KBr (closed diamonds) with 0.1 mM GpCpp 10 min, 20 min, and 60 min at 37 °C after starting 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.

in the presence of non-hydrolyzable analogue of GTP (GpCpp; Fig. 3A) was smaller than that in the presence of hydrolysable GTP (Fig. 1A). Further, the effect of replacement of GTP with non-hydrolyzable GpCpp on the number of polymerized tubulin (microtubule) was higher in NO_3^- or Br^--containing solutions (20-fold increases) than that in Cl^--containing solutions (10-fold increases) (compare Fig. 3A with Fig. 1A). In other words, replacement of GTP with a non-hydrolyzable GTP (GpCpp) diminished the enhancing action of Cl^- on tubulin polymerization compared with NO_3^- or Br^-. These observations suggest that the enhancing action of Cl^- on polymerization of tubulin would be partially mediated by low activity of GTPase in Cl^--containing solutions. In the presence of GpCpp as a guanine nucleotide, lengths of polymerized tubulin (microtubule) were identical in Cl^-, NO_3^- and Br^--containing solutions (Figs. 3B and 4). Lengths of polymerized tubulin (microtubule) became shorter by replacement of GTP with a non-hydrolyzable GTP (GpCpp) in Cl^--containing solutions (Figs. 1B and 3B), while lengths of polymerized tubulin (microtubule) were not affected by replacement of GTP with a non-hydrolyzable GTP (GpCpp) in NO_3^- or Br^--containing solutions (Figs. 1B and 3B). Namely, replacement of GTP with a non-hydrolyzable GTP (GpCpp) shortened the length of polymerized tubulin (microtubule) in Cl^--containing solutions, but not in NO_3^- or Br^--containing solutions. These observations suggest that a non-GTP-hydrolysis condition caused by replacement of GTP with a non-hydrolyzable GTP (GpCpp) would efficiently trigger tubulin polymerization (formation of microtubules), increasing the number of polymerized tubulin (Figs. 1A and 3A) but rather prohibiting incorporation of GTP-bound tubulin into long polymerized tubulin (Figs. 1B and 3B).

4. Discussion

Gokarn et al. have reported that anions modulate oligomerization of protein [39]. Cl^- is one of major anions existing in our body. Recent studies have revealed that intracellular Cl^- regulates various cellular functions. We have recently reported that intracellular Cl^- plays an important role in regulation of cell cycle progression and cell proliferation via activation of stress activated protein kinases [13,14,18]. Moreover, Nakajima et al. have reported that Cl^- inhibits GTPase activity of heterotrimeric G protein (G_k) [40]. In the present study, we indicate that Cl^- modulated GTPase activity of tubulin (Table 1). The present study indicates that Cl^- showed an inhibitory effect on GTPase activity (Table 1) and a stimulatory effect on formation of tubulin polymerization (Fig. 1A) compared with NO_3^- or Br^-. We considered two possibilities to explain how Cl^- shows high rates of tubulin polymerization (Figs. 1 and 2) based on Cl^--induced inhibition of GTPase activity (Table 1). One possibility is that: (1) NO_3^- and Br^- causes diminution (or depletion) of GTP-bound form of β-subunit of tubulin required for tubulin polymerization by stimulating the intrinsic GTPase activity of monomer tubulin compared with Cl^-, and 2) diminution (or depletion) of GTP-bound form of β-subunit of tubulin induces low rates of tubulin polymerization. The other possibility is that even if tubulin polymerization occurs in solutions containing NO_3^- or Br^- similar to Cl^-, polymerized tubulin would be rapidly depolymerized in solutions containing NO_3^- or Br^- compared with Cl^-, resulting in small numbers of stable polymerized tubulin. Roychowdhury et al. [5,6] have reported that α-subunit of heterotrimeric G protein stimulates the GTPase activity of tubulin and modulates microtubule dynamics (polymerization/deplolymerization of tubulin). Therefore, NO_3^- or Br^- would stimulate the GTPase activity of tubulin directly or indirectly via other factors such as G protein after polymerization of tubulin leading to depolymerization of tubulin, although further studies are necessary to clarify the mechanism how NO_3^- or Br^- stimulates intrinsic GTPase activity of tubulin. As mentioned above, Cl^- enhanced formation of tubulin polymerization (the number of microtubules) compared

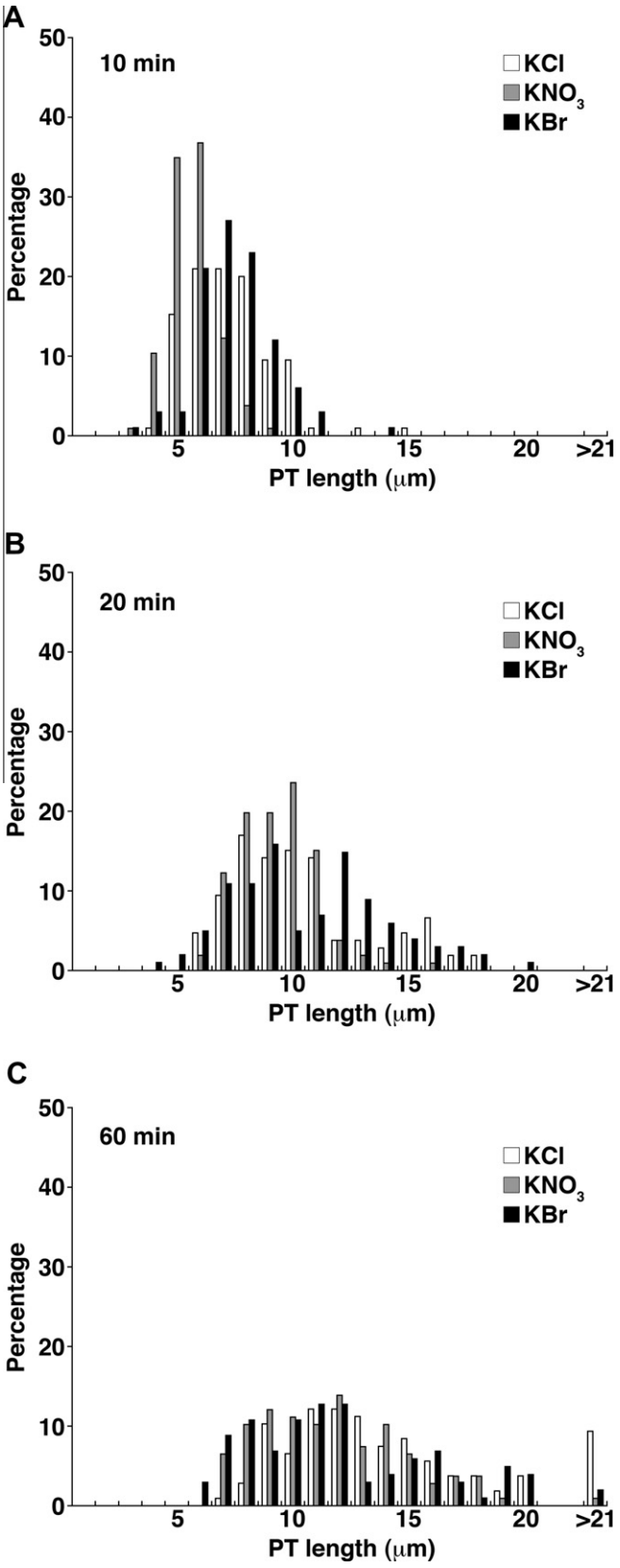

Fig. 4. Histograms of lengths of polymerized tubulin (PT) in solutions containing 100 mM KCl (open columns), KNO_3 (gray columns), or KBr (closed columns) with 0.1 mM GpCpp 10 min (A), 20 min (B), and 60 min (C) at 37 °C after starting the reaction.

with NO_3^- or Br^--containing solutions even in the presence of a non-hydrolyzable GTP (GpCpp). Under this condition, the Cl^- action on formation of tubulin polymerization is not dependent on GTPase activity. Therefore, in addition to the Cl^--modulating action on GTPase activity, Cl^- would have other potentials for triggering of tubulin polymerization; e.g., protein–protein interaction of tubulin subunits. Although further studies are necessary to clarify how Cl^- regulates GTPase activity of tubulin and tubulin polymerization in detail, the present study indicates that the enhancing effect of Cl^- on triggering of tubulin polymerization would be partially mediated by an inhibitory effect of Cl^- on GTPase activity.

Microtubule is crucial for various cellular functions such as chromosome segregation at M phase of the cell cycle, intracellular vesicle transport, and neurite outgrowth in the neuronal differentiation/regeneration. Moreover, microtubule is an important target of cancer chemotherapy [41]. Certain anti-cancer drugs such as Taxol inhibit microtubule functions in cancer cells, leading to apoptosis of cancer cells. Our results show novel regulatory mechanisms forming microtubule cytoskeleton by intracellular Cl^-, and these findings would be useful for searching novel cancer therapies.

Acknowledgment

This work was supported by Grants-in-Aid from Japan Society of the Promotion of Science (18790159, 19590212, 20390060, 20790176, 22790215, 24590282), Research Conference for Cell Function, Fuji Foundation for Protein Research, and The Salt Science Research Foundation (1035, 1235).

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