Original Paper

K⁺-Cl⁻ Cotransporter 1 (KCC1) Negatively Regulates NGF-induced Neurite Outgrowth in PC12 Cells

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Key Words

DIOA • NGF • SLC12 family • Chloride ion concentration • siRNA

Abstract

Potassium chloride cotransporters (KCCs) mediate electroneutrally-coupled transport of K⁺ and Cl⁻, and play crucial roles in various cell functions including regulation of cell volume and homeostasis of cellular Cl⁻ content. Four isoforms of KCCs (KCC1, 2, 3, and 4) have been identified. KCC1 is ubiquitously expressed, whereas KCC2 is mainly expressed in neuronal cells of central nervous system. KCC3 is highly expressed in heart, skeletal muscle, kidney, lung and placenta. KCC4 is mainly expressed in epithelial cells. In this study, we investigated roles of KCCs in NGF-induced neurite outgrowth of rat pheochromocytoma PC12 cells. The most abundantly expressed isoform in PC12 cells was KCC1. Inhibition of KCCs using [(dihydrondindenyl)oxy] alkanoic acid (DIOA), an inhibitor of KCCs, enhanced the NGF-induced neurite outgrowth of PC12 cells in a dose-dependent manner. Treatment of PC12 cells with NGF significantly decreased mRNA expression of KCC1, whereas other isoforms, KCC2-4, showed no changes in their mRNA expression in response to NGF treatment. Knockdown of KCC1 using small interfering RNA (siRNA) enhanced the NGF-induced neurite outgrowth. These results suggest that KCC1 negatively regulates the NGF-induced neurite outgrowth of PC12 cells.

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Introduction

Potassium chloride cotransporters (KCCs) are members of cation-chloride cotransporter family (SLC12 family) (see reviews [1, 2]). This family consists of sodium potassium two-chloride cotransporter 1 (NKCC1) and 2 (NKCC2), sodium chloride cotransporter (NCC), and KCCs. All of these transporters mediate electroneutral transport of cation(s) coupled with one or two Cl^- , and contribute to regulation of cell volume and homeostasis of intracellular Cl^- concentration. KCCs extrude one K^+ with one Cl^- from the intracellular space to the extracellular space by using the chemical gradient of K^+ as the driving force for Cl^- extrusion. Four KCC isoforms (KCC1-4) have been identified in mammalian species [3, 4]. KCC1 is a ubiquitously expressed isoform. It was considered that KCC2 would be expressed restrictedly in neuronal cells. However, as it has been reported [5], KCC2 is also expressed in several human cancer cells, such as cervical cancer cells. KCC3 is highly expressed in the heart, skeletal muscles, the kidney, the lung and the placenta. KCC4 is mainly expressed in epithelial cells including the kidney and the lung, but also in the heart and the liver. Recent studies have revealed that KCCs participate in various cellular events such as cancer cell growth and metastasis [6-8], epithelial to mesenchymal transition (EMT) [9], and differentiation of erythroid cells [10].

Cl^- is one of fundamental anions in our body. Recent studies have revealed that Cl^- influences various cellular functions [11-15]. For example, reduction of intracellular Cl^- concentration ($[\text{Cl}^-]_i$) induces the gene expression of the α -subunit of epithelial sodium channel (α -ENaC) in kidney epithelial cells [16]. In addition, it has been reported that physiological functions of leukocytes, such as cell adhesion, spreading, production of reactive oxygen species, and phagocytosis, are associated with changes in $[\text{Cl}^-]_i$ [17-20]. Generally, $[\text{Cl}^-]_i$ is controlled by various Cl^- channels and transporters participating in uptake of Cl^- into cells and release of Cl^- from cells. NKCC1 is involved in the incorporation step of Cl^- into the intracellular space, and KCCs and Cl^- channels are involved in the excretion step of Cl^- from the intracellular space. We have recently reported that in rat pheochromocytoma PC12 cells and its subclonal cell line (PC12D cells), NKCC1 plays an essential role in the nerve growth factor (NGF)-induced neurite outgrowth [21-24]. Moreover, a potent stimulant of NKCC1 (quercetin, a flavonoid) enhances neurite outgrowth, and the stimulatory effect of quercetin on neurite outgrowth is dependent on the extracellular Cl^- concentration [25, 26]. These observations suggest that NKCC1 is responsible for Cl^- influx from the extracellular to the intracellular space, and that Cl^- plays an important role in neurite outgrowth.

The aim of the present study is to clarify roles of KCC(s) in NGF-induced neurite outgrowth of rat pheochromocytoma PC12 cells using siRNA against KCC and DIOA, a KCC inhibitor, although DIOA has inhibitory effects on other Cl^- transporters and channels. We have demonstrated that; 1) inhibition of KCCs with an inhibitor of KCC, DIOA, enhances the NGF-induced neurite outgrowth, 2) the most abundantly expressed isoform in PC12 cells is KCC1 and treatment with NGF reduces the expression level of KCC1 mRNA, and 3) knocking-down of KCC1 with siRNA enhances the NGF-induced neurite outgrowth. Some parts of preliminary data shown in this study were presented at the 88th annual meeting of Physiological Society of Japan [27]. These results strongly suggest that KCC1 negatively regulates the NGF-induced neurite outgrowth in PC12 cells.

Materials and Methods

Materials

D-MEM (high glucose), poly-L-lysine solution, and [(dihydrionindenyl)oxy] alkanoic acid (DIOA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stealth Select RNAiTM siRNA, Lipofectamine 2000 reagent, horse serum (HS), gentamicin solution, Alexa 488-conjugated phalloidin, and *N*-methoxycarbonylmethyl-6-methoxyquinolinium bromide (MQAE) were obtained from Life Technologies (Carlsbad, CA, USA).

Fetal bovine serum (FBS) was purchased from EQUITEC-BIO Inc. (Kerrville, TX, USA). 2.5S mouse nerve growth factor (NGF) was obtained from Alomone Laboratories Inc. (Jerusalem, Israel). RNeasy mini kit was purchased from QIAGEN (Venlo, Netherlands).

Cell culture

PC12 cells, rat pheochromocytoma cells derived from adrenal medulla, were grown in D-MEM supplemented with 10% HS, 5% FBS, and 50 µg/ml gentamicin at 37°C with air containing 5% CO₂. This cell was kindly donated from Dr. M. Sano (Professor Emeritus, Department of Biology, Kyoto Prefectural University of Medicine).

Measurement of neurite outgrowth

PC12 cells were seeded onto glass coverslips pre-coated with poly-L-lysine. DIOA of 0, 10, 20, 50, or 100 µM with NGF (50 ng/ml) was applied, and was continuously present during NGF treatment. The medium containing NGF and DIOA was changed every 2 days. Five days after co-application of DIOA with NGF, the cells were fixed with 3.7% formaldehyde. Then, we permeabilized the cells with 0.1% Triton X-100, and stained them with Alexa488-conjugated phalloidin. After these treatments, we observed neurites with fluorescence microscope BZ-8000 (Keyence, Osaka, Japan), and measured the length of each neurite and the neurite number per cell by using ImageJ software (NIH, Bethesda, MD, USA).

Preparation of RNA and reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was extracted by using RNeasy mini kit according to the manufacturer's protocol [28]. We always performed DNase I treatment to avoid contamination of genomic DNA fragment, and synthesized cDNA by using SuperScript III first strand cDNA synthesis kit (Invitrogen) with TaKaRa *ExTaq* DNA polymerase (TAKARA BIO INC., Otsu, Shiga, Japan). Primer sequences were as follow:

KCC1 sense, 5'-CTGCTGACAGCCATCTCCATG-3';
KCC1 antisense, 5'-GTGGCCACTGTCTCATTGTCC-3';
KCC2 sense, 5'-GCTGGTGGCTCCTACTACATG-3';
KCC2 antisense, 5'-TCATCACAGGTGGCATTGAGG-3';
KCC3 sense, 5'-CTCTTTCTGCGTCTTACCTGG-3';
KCC3 antisense, 5'-CAAGGTGTCTTGATGACAGGG-3';
KCC4 sense, 5'-TTCTGGAGTCCTTCCTCATCG-3';
KCC4 antisense, 5'-AATGCCCTGGATCTCAGTGAC-3'.

These primers were designed to have approximately 500-600 bp PCR amplicon lengths. PCR products were electrophoresed with 1.5% agarose gel. Then, we stained electrophoresed PCR products with ethidium bromide, and visualized them with ChemiDoc XRS Plus molecular imager (BIO-RAD laboratories, Hercules, CA, USA).

Quantitative realtime PCR analysis of mRNA copy number for KCC isoforms

mRNA expression levels of KCCs were examined by a quantitative realtime PCR method with TaqMan gene expression assay (Life Technologies). We used pre-designed, ready-made solutions containing primers and probe. IDs of TaqMan gene expression assays were as follow: rat KCC1, Rn00570248_m1; rat KCC2, Rn00592624_m1; rat KCC3, Rn01514943_m1; rat KCC4, Rn01537520_m1. We first tried to test glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein L29 (RPL29) as reference genes for normalization of mRNA expression levels of KCCs. However, NGF treatment increased mRNA expression levels of these genes by 1.5 - 2-fold (data not shown). Therefore, we generated standard curves by using serial dilutions of specific plasmid, and evaluated mRNA expression levels of KCCs as "copy number / ng RNA". For this purpose, we constructed specific plasmids for quantification reference. TaqMan gene expression assay consists of unlabeled PCR primers (forward and reverse primers) and a TaqMan probe (reporter) with a FAM fluorescent dye on the 5' end and a nonfluorescent quencher dye on the 3' end, and information on only amplicon length and sequence of reporter is available. So we designed that all plasmids included the TaqMan PCR amplicon plus 5' and 3' surrounding sequences. Amplicon length and sequence of reporter of TaqMan probe Rn00570248_m1 is 83 and 5'-GCCGCCAAGGCACCCAGCATGGGCA-3' (corresponding to the nucleotide sequence 334-358 of open reading frame of rat KCC1), Rn00592624_m1 is 79 and 5'-TCGAGATCCTGCTGGCTTACCTCTT-3' (corresponding to the nucleotide sequence 599-623 of open reading frame of rat KCC2), Rn01514943_m1 is 157 and 5'-GAGCACAGCCAGCTGTTAGATGATG-3' (corresponding to the nucleotide sequence 298-322 of open reading frame of rat KCC3), Rn01537520_m1 is 72 and 5'-TGTGCTGCACCTGTACAATGCTGAC-3' (corresponding to the nucleotide sequence 476-500 of open reading frame of rat KCC4). Then, we purified total RNA from PC12 cells by using RNeasy Mini Kit, and generated first strand cDNA by using SuperScript III first strand cDNA synthesis kit. Next, we amplified

cDNA fragments of rat KCC1-4 containing the amplicon of TaqMan Gene Expression assays by polymerase chain reaction by using KOD plus DNA polymerase (TOYOBO).

Primer sequences were as follow:

KCC1 sense, 5'-GGGGTACCATGCCTCACTTCACCGTG-3';

KCC1 antisense, 5'-GGGGTACCGGAGCAATGTAGGTCAGC-3';

KCC2 sense, 5'-GGGGTACCATCCTCTTTCTGCGGCTC-3';

KCC2 antisense, 5'-GGGGTACCACAGGTGGCATTGAGGAG-3';

KCC3 sense, 5'-GGGGTACCTCCTCCAGAAACCACCAC-3';

KCC3 antisense, 5'-GGGGTACCTGAAGGACTCCAGCTGTG-3';

KCC4 sense, 5'-GGGGTACCGAGAGAGCTACTTCGAGG-3';

KCC4 antisense, 5'-GGGGTACCACCAGCATAGATGGCCAG-3'.

Underline indicates *KpnI* site. Each amplified fragment was digested with *KpnI*, and was inserted into the same site of pEGFP-N1 vector. Each plasmid contains the DNA fragment corresponding to the nucleotide sequence 1-689 for KCC1, 343-993 for KCC2, 6-656 for KCC3, and 179-882 for KCC4.

We confirmed the sequence of each one by DNA sequencing. Next, we quantified expression levels of KCCs. Total RNA was extracted by using RNeasy mini kit (with DNase I treatment). Reverse transcription was performed with High capacity cDNA reverse transcription kit (Life Technologies). Fluorescence changes during realtime PCR were measured with ABI 7300 real-time PCR system or StepOne Plus real-time PCR system (Life Technologies).

siRNA-transfection

For knockdown of KCC1, PC12 cells were transfected with siRNA. PC12 cells grown on coverslips pre-coated with poly-L-lysine were transfected with siRNA using Lipofectamine 2000 reagent according to the manufacturer's protocol. We used three independent Stealth Select RNAi™ siRNAs against rat KCC1 (Stealth Select Oligo ID ; RSS334485, RSS334486, and RSS334487). BLOCK-iT red fluorescent oligo (Invitrogen) was used as control oligo. We treated the cells with NGF (50 ng/ml) 24 h after transfection, and further cultured the cells for 5 days with NGF. The length of each neurite and the neurite number per cell were measured by using ImageJ software (NIH) as described above. The expression level of KCC1 mRNA was quantified by realtime PCR as described above.

Measurement of intracellular Cl⁻ concentration ([Cl⁻]_i)

For measurement of [Cl⁻]_i, we used halide-sensitive fluorescent dye MQAE. Fluorescence of this dye is quenched by halide ions, including Cl⁻. PC12 cells grown on glass-bottom dish were cultured in media containing 50 ng/ml NGF and with or without 20 μM DIOA for 5 days; culture media containing NGF with or without DIOA were changed every two days. Alternatively, the cells were transfected with control oligo (Block-iT red fluorescent oligo) or RSS334487, and treated with 50 ng/ml NGF for 5 days. The cells were incubated in the experimental solution (115 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 5 mM glucose, 10 mM HEPES, and 25 mM NaHCO₃, pH7.4) containing 5 mM MQAE for 60 min at 37°C with air containing 5% CO₂ for uptake of the dye into the cells. Then, the solution was replaced with the experimental solution (without MQAE), and the cells were placed into microscope stage incubator on a ZEISS 510 META confocal microscope equipped with a tunable titan-sapphire laser (Spectra-Physics MaiTai ultrafast laser system, Newport Corp. Irvine, CA, USA). MQAE was excited by 780 nm two-photon laser. We measured the fluorescence intensities of MQAE in the cell body and neurite tip, with 12bit depth (intensity 0-4095).

Statistics

All data are represented as means ± SEM. Student's *t*-test, ANOVA, and Tukey's HSD were used for statistical analysis as appropriate. The difference between groups was evaluated with ANOVA. If ANOVA indicated a significance difference, Tukey's HSD was performed to determine the significance between the means. A *p* value < 0.05 was considered statistically significant.

Results

To examine if KCC1 contributes to NGF-induced neurite outgrowth in PC12 cells, we first studied effects of DIOA, an inhibitor of KCCs, on NGF-induced neurite outgrowth. Representative images are shown in Fig. 1. DIOA itself did not have any effect on neurite outgrowth of PC12 cells without NGF treatment. Treatment with DIOA enhanced the NGF-induced neurite outgrowth in a dose-dependent manner (Fig. 2A). DIOA showed its maximum

Fig. 1. Effects of DIOA on NGF-induced neurite outgrowth: Representative micrographs of cells treated with or without DIOA (50 μ M) for 5 days in NGF (50 ng/ml)-treated or -untreated cells. Cells were fixed with 3.7% formaldehyde and stained with Alexa488 phalloidin. Bar indicates 20 μ m.

DIOA (μ M)	Number of neurite / cell body
0	3.78 ± 0.13
10	4.25 ± 0.15 ^{NS}
20	4.23 ± 0.16 ^{NS}
50	3.85 ± 0.16 ^{NS}
100	4.24 ± 0.13 ^{NS}

Table 1. The number of neurites per cell treated with DIOA. The number of neurite per cell was counted and represents as mean $\pm$ SEM ($n = 81$ -88). Statistical significance was determined by ANOVA followed by Tukey's HSD test. NS indicates that there was no statistically significant difference vs. control (DIOA = 0 μ M).

Fig. 2. Effects of DIOA on NGF-induced neurite outgrowth. PC12 cells were treated with 0 (0.1% DMSO alone), 10, 20, 50, or 100 μ M DIOA in the presence of 50 ng/ml NGF for 5 days. (A) The length of each neurite was measured, and the mean values of neurite lengths were calculated. Values are represented as means $\pm$ SEM. Statistical significance was determined by ANOVA followed by Tukey's HSD test. *, $p < 0.01$ vs. control (0 μ M). #, $p < 0.01$ vs. DIOA of 10, 20 or 100 μ M. $n = 309$ -336. (B) Distribution of the neurite lengths in cells treated with DMSO (closed squares) or 50 μ M DIOA (open circles). The vertical (Y) axis indicates the percentage of neurites longer than the value indicated in the horizontal (X) axis.

effect on the NGF-induced neurite outgrowth at 50 μ M. DIOA of 100 μ M also enhanced the NGF-induced neurite outgrowth, but the effect of 100 μ M DIOA was weaker than that of 50 μ M DIOA. This might be due to toxic effects of DIOA at a super-maximum dose including some effects on other types of channels and/or transporters. In contrast to the effect of DIOA on the length of neurites, the number of neurites per cell was not affected by DIOA-treatment (Table 1). Fig. 2B indicates distribution of neurite lengths of control (DMSO-treated) and 50 μ M DIOA-treated cells. DIOA shifted the distribution rightward, meaning that the percentage of neurites longer than the indicated length in DIOA-treated cells was larger than that in control (DMSO).

Four isoforms of KCCs have been identified [29]. All of KCC isoforms are inhibited by DIOA [30-33]. We next examined which isoforms are expressed in PC12 cells. To clarify

Fig. 3. Expression of KCC isoforms in PC12 cells. (A) Total RNA was extracted from PC12 cells by using RNeasy mini kit. cDNA was synthesized using SuperScript III first strand cDNA synthesis kit combined with ExTaq DNA polymerase. PCR products were electrophoresed with 1.5% agarose gel, stained with ethidium bromide, and visualized with molecular imager. (B-E) Expression levels of KCC isoforms in PC12 cells treated with or without NGF for 1, 3, or 5 days. Expression levels of mRNA were quantified by realtime PCR methods (see Materials and Methods): B) KCC1; C) KCC2; D) KCC3; E) KCC4. The vertical (Y) axis indicates copy number / ng RNA. Statistical significance was determined by Student's *t*-test. *, $p < 0.05$ vs. NGF (-) at each time point. No mark between NGF (-) and NGF (+) indicates that there was no statically significant difference. $n = 6-8$.

Fig. 4. Knockdown of KCC1 by RNAi method. PC12 cells were transfected with three independent siRNA against rat KCC1 (RSS334485, RSS334486, and RSS334487). At 24 h after transfection, the cells were treated with NGF (50 ng/ml) and further cultured for 5 days with NGF (50 ng/ml). Expression levels of KCC1 mRNA were quantified by realtime PCR methods. Statistical significance was determined by ANOVA followed by Tukey's HSD test. *, $p < 0.05$ vs. control oligo. $n = 4$.

this point, we first performed conventional reverse transcription (RT)-polymerase chain reaction (PCR). PC12 cells expressed all isoforms, KCC1-4 (Fig. 3A). To investigate which isoform is most abundantly expressed and is responsive to NGF stimulation in PC12 cells, we next performed quantitative realtime PCR. We assessed glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and ribosomal protein L29 (RPL29) as reference genes to normalize KCC gene expression. However, mRNA expression of these genes was increased 1.5 ~ 2 fold

Fig. 5. Effects of knock down of KCC1 on NGF-induced neurite outgrowth: Representative micrographs of cells transfected with control oligo or siRNA against KCC1 (RSS334487) in NGF (50 ng/ml)-treated or -untreated cells. At 5 days after NGF application, cells were fixed with 3.7% formaldehyde and stained with Alexa488 phalloidin. Bar indicates 20 μ m.

siRNA	Number of neurite / cell body
Control oligo	4.25 $\pm$ 0.18
RSS334485	4.62 $\pm$ 0.20 ^{NS}
RSS334486	4.46 $\pm$ 0.15 ^{NS}
RSS334487	4.67 $\pm$ 0.16 ^{NS}

Table 2. The number of neurite per cell transfected with siRNA against rKCC1. The number of neurite per one cell body was counted and represents as mean $\pm$ SEM (n = 76-79). Statistical significance was determined by ANOVA followed by Tukey's HSD test. NS indicates that there was no statistically significant difference vs. control oligo.

Fig. 6. Effects of knock down of KCC1 on NGF-induced neurite outgrowth. PC12 cells were transfected with control oligo or siRNA against rat KCC1 in the presence of NGF (50 ng/ml) for 5 days, as described in the legend to Figure 4. (A) Neurite lengths in cells transfected with siRNA. Statistical significance was determined by ANOVA followed by Tukey's HSD test. *, $p < 0.01$ vs. control oligo. $n = 314$ -346. (B) Distribution of neurite lengths in cells transfected with control oligo (closed squares) or oligo RSS334487 (open circles). The vertical (Y) axis indicates the percentage of neurites longer than the value indicated in the horizontal (X) axis.

in response to NGF treatment (data not shown). So, we generated standard curves with serial dilution of specific plasmids, and evaluated mRNA expression levels of KCCs as "copy number per ng RNA" (See Materials and Methods). As shown in Figs. 3B-E, the isoform most abundantly expressed in PC12 cells was KCC1. On the other hand, KCC2, an isoform mainly expressed in central neuron, was barely expressed in PC12 cells. Remarkably, quantitative analysis indicates that NGF treatment decreased KCC1 expression to approximately two-thirds of that expressed in NGF-non-treated PC12 cells 3 and 5 days after application of NGF (Fig. 3B). In contrast to KCC1, mRNA expression of other isoforms, KCC2-4, was not affected by NGF treatment (Figs. 3C-E) except an increase in KCC2 5 days after application of NGF.

Fig. 7. Inhibition of KCC1 elevates $[Cl^-]_i$. (A) PC12 cells treated with NGF (50 ng/ml) and with or without DIOA (20 μ M) for 5 days. The cells were stained with halide-sensitive dye MQAE. Fluorescence intensities of MQAE in cell body and neurite tip were measured by a two-photon confocal microscopy. *, $p < 0.05$ vs. DMSO in the same cellular region. #, $p < 0.05$ vs. Neurite tip in DMSO-treated group. $n = 18$ -23. (B) PC12 cells were transfected with RSS334487 or control oligo with NGF for 5 days. The cells were stained MQAE, and fluorescence intensities of MQAE in cell body and neurite tip were measured by a two-photon confocal microscopy. Statistical significance was determined by ANOVA followed by Tukey's HSD test. *, $p < 0.05$ vs. control oligo in the same cellular region. #, $p < 0.05$ vs. Neurite tip in control oligo-transfected group. $n = 12$ -15.

As mentioned above, DIOA inhibits all isoforms of KCCs [30-33]. In addition, it has been reported that DIOA inhibits some kinds of ion transporters, such as Na^+/K^+ ATPase (Na^+/K^+ pump) [34], and Ca^{2+} -activated K^+ channel [35]. Although the reports [30-33] indicate DIOA inhibits KCCs, Lauf et al. have importantly reported that DIOA blocks K^+ channel (K^+ conductance) rather than KCC in human lens epithelial cells [36]. Therefore, we should carefully apply DIOA to various types of cells as a KCC blocker. To study roles of KCC1 in the NGF-induced neurite outgrowth more clearly and specifically, we next tried specific inhibition of KCC1 by using RNAi methods. Three independent Stealth Select RNAiTM siRNAs against rat KCC1 (Stealth Select Oligo ID; RSS334485, RSS334486, and RSS334487) were used. Block-iT red fluorescent oligo, which has no sequence homology to any known mammalian genes, was used as control. As shown in Fig. 4, mRNA expression of KCC1 was reduced to approximately 50% by application of RSS334485 or RSS334486, and to 25% by application of RSS334487. Representative images of siRNA-transfected cells treated with or without NGF are shown in Fig. 5. Transfection with siRNA itself did not have any effect on neurite outgrowth of PC12 cells without NGF treatment (Fig. 5), like DIOA experiment (Fig. 1). Fig. 6A indicates the neurite outgrowth of siRNA-transfected PC12 cells. Although the cells transfected with any siRNA against KCC1 of three different siRNAs used in the present study possessed longer neurites than control cells (control oligo), it is notable that PC12 cells transfected with oligo RSS334487, which most efficiently reduced KCC1 mRNA expression, was most effective in enhancement of NGF-induced neurite outgrowth. Magnitudes of siRNA-induced enhancement of neurite outgrowth seem to be correlated with magnitudes of suppression of KCC1 mRNA expression. Fig. 6B indicates distribution of neurite lengths of control and RSS334487-transfected cells. Transfection with RSS334487 shifted the distribution rightward, meaning that transfection with RSS334487 increases the percentage of neurites longer than the given length shown in Fig. 6A. In contrast to difference in the length of neurites, the number of neurites per cell was not affected by the transfection (Table 2) like DIOA experiment (Figs. 1 and 2, and Table 1).

KCCs extrude K^+ and Cl^- from the cells. Thus, if KCC1 is expressed and functional, inhibition of KCC1 by DIOA or siRNA may cause elevation of intracellular Cl^- concentration

Fig. 8. mRNA expression levels of all KCC isoforms in KCC1-knocking down cells. PC12 cells were transfected with siRNA against rat KCC1. At 24 h after transfection, the cells were treated with NGF and further cultured for 5 days. mRNA expression levels of all KCC isoforms were determined by realtime PCR method. (A) KCC1, (B) KCC2, (C) KCC3, and (D) KCC4. The vertical (Y) axis indicates copy number / ng RNA. Statistical significance was determined by ANOVA followed by Tukey's HSD test. *, $p < 0.05$ vs. control oligo. $n = 4$.

($[Cl^-]_i$). Therefore, we next measured $[Cl^-]_i$ in the cells treated with DIOA and transfected with siRNA against KCC1 by using halide-sensitive fluorescent dye MQAE. Fluorescence of MQAE is quenched by halide ions, so a decrease in the fluorescence intensity indicates an increase in the chloride ion concentration. As shown in Fig. 7A, treatment with DIOA decreased the fluorescence intensity of MQAE. Moreover, transfection with RSS334487 also decreased the intensity both in cell body and neurite tip areas (Fig. 7B) in the same magnitude as those in DIOA experiment (Fig. 7A).

PC12 cells express KCC2-4 in addition to KCC1 (Fig. 3). Remarkably, the amounts of KCC3 and KCC4 mRNA were relatively higher than the amount of KCC2 mRNA, and the sum of KCC3 mRNA and KCC4 mRNA was comparable to KCC1 mRNA level. To exclude a possibility that KCC2-4 may compensate for KCC1 in siRNA-transfected cells, we assessed the mRNA expression level of KCC2-4 in KCC1 knockdown cells by realtime PCR. As shown in Fig. 8, transfection of siRNA against KCC1 had no effect on the expression level of KCC2-4.

Discussion

In the present study, we demonstrated that: in PC12 cells, 1) inhibition of KCCs by DIOA (an inhibitor of KCCs) enhanced the NGF-induced neurite outgrowth, 2) the most abundantly expressed isoform is KCC1, 3) the expression of KCC1 mRNA was decreased by NGF treatment, whereas expression of other isoforms, KCC2-4, was not changed, 4) knock-down of KCC1 by RNAi elevated $[Cl^-]_i$ in PC12 cells, and 5) knock-down of KCC1 by RNAi enhanced the NGF-induced neurite outgrowth.

Figures 1 and 2 show that treatment with DIOA, an inhibitor of KCCs, stimulated the NGF-induced neurite outgrowth in a dose-dependent manner. The maximum effect of DIOA

was observed at 50 μ M. On the other hand, the number of neurites per cell was not affected with DIOA treatment (Table 1). In addition, DIOA itself did not have any effect on neurite outgrowth without NGF treatment (Fig. 1). In PC12 cells, it has been suggested that neurite outgrowth consists of the neurite formation step (the first step, which determines the number of neurites per cell) and the subsequent elongation step (the second step, which regulates the length of neurites), and that the first and second steps are thought to be controlled by different mechanisms [37, 38]. In the present study, treatment with DIOA elongated the length of neurites, but did not affect the number of neurites per cell, suggesting that KCCs participate in down regulation of the second step of neurite outgrowth (elongation).

As shown in Figure 3, PC12 cells expressed mRNA of all four KCC isoforms. The isoform most abundantly expressed in PC12 cells was KCC1. Only a small amount of KCC2, mainly expressed in central neurons, was expressed in PC12 cells. KCC2 is known as a neuronal marker and it has been reported that expression of KCC2 increases with maturation of central nerve cells [39]. Recently, KCC2a, novel splice variant of KCC2, has been reported. Just N-terminal of KCC2a is different from the previously known KCC2, KCC2b. Uvarov et al. have reported that Slc12a5 gene generates two isoforms by using alternative promoter and exon 1, and that KCC2a is co-expressed with traditional KCC2b in central nerve cells [40]. On the other hand, several studies have revealed that KCC2a is expressed in non-neuronal cells. Lauf et al. have reported that only KCC2a but not KCC2b is expressed in human lens epithelial cell line [41]. Moreover, Payne et al. have reported that KCC2a is expressed in cardiomyocyte of chicken [42]. In the present study, we observed that small amounts of KCC2 mRNA were expressed in PC12 cells, and that NGF treatment increased the expression of KCC2 5 days after NGF application (Fig. 3C). Both the primers for conventional RT-PCR and the TaqMan gene expression assays used in this study can detect not only KCC2b but also KCC2a. The primers for RT-PCR amplify the region corresponding to exon 6 to exon 8, and TaqMan assays amplify the region corresponding to exon 6 to exon 7. Previously, Payne et al. have reported that PC12 cells would not express KCC2 mRNA [43]. However, their methods can detect only KCC2b, since the primers used in their study [43] amplify the region containing exon 1 of KCC2b. Thus, we speculate that the weak expression of KCC2 observed in the present study would be KCC2a, although we could not neglect a possibility of the ectopic transcription / illegitimate transcription [44] of KCC2b.

In the present study, treatment with NGF reduced mRNA expression of KCC1 (Fig. 3B). Rivera et al. [45] have reported that in hippocampal slice, brain derived neurotrophic factor (BDNF) down-regulates KCC2 protein expression via activation of TrkB (BDNF receptor). The NGF-induced down-regulation of KCC1 mRNA observed in the present study would be mediated via mechanisms similar to the TrkB-dependent mechanism by which BDNF down-regulates KCC2 in central nerve as mentioned above [45]. Reduction in mRNA expression is, in general, caused by at least one of the following two mechanisms (or combination of both mechanisms): 1) reduction of mRNA stability (reduction of mRNA half-life time), or 2) reduction of transcription. In the present study, we observed NGF-induced reduction of KCC1 mRNA expression. In PC12 cells and several subclonal cells, it has been reported that NGF treatment increases transcription of various genes, such as cytosolic phospholipase A₂ α [46] and tyrosine hydroxylase [47]. On the other hand, Naranjo-Suarez et al. [48] have reported that NGF treatment reduces mRNA and protein expression of hypoxia inducible factor-2 via reduction of mRNA stability. Although the present study indicates that NGF decreases mRNA expression of KCC1, further studies are necessary to clarify if NGF treatment regulates which process, reduction of mRNA stability or transcription, or both processes.

Knockdown of KCC1 with siRNA enhanced neurite outgrowth (elongation), but did not affect the number of neurites per cell (Figs. 4, 5 and 6, and Table 2) like DIOA experiment (Figs. 1 and 2, and Table 1). DIOA inhibits all isoforms of KCCs [30-33]. Furthermore, it has been reported that DIOA inhibits other Cl⁻ transporters (e.g., anion exchanger [29] and volume

sensitive Cl^- channels [49]), ion-transporting ATPase (e.g., Na^+/K^+ ATPase, H^+/K^+ ATPase and Ca^{2+} ATPase [34]), and other ion channels (e.g., Ca^{2+} -activated K^+ channel [35]). So, we tried specific inhibition of KCC1, which was most abundantly expressed, and responsive to NGF treatment. As shown in Fig. 6, knockdown of KCC1 increased neurite lengths in an extent similar to application of DIOA. These observations indicate that in PC12 cells, effects of KCC1 knock-down on neurite lengths and the number of neurites per cell are same as those of DIOA, which inhibits all isoforms of KCCs. Namely, these observations mean that inhibition of only KCC1 by using siRNA influences neurite lengths without any effects on the number of neurites per cell in PC12 cells similar to inhibition of all isoforms of KCCs. When KCC1 is knocked down, other isoforms of KCCs might compensate for lack of Cl^- efflux via KCC1. If so, DIOA inhibiting all isoforms of KCCs should have larger effects on elongation of neurites compared with knockdown of KCC1 only affecting KCC1 but not other isoforms of KCCs (KCC2-4). However, effects of DIOA on elongation of neurites were identical to those of knockdown of KCC1. In addition, knocked-down of KCC1 decreased the MQAE fluorescence to the same level in DIOA-treated cells (Fig. 7) without any significant change in the mRNA expression levels of other KCC isoforms (Fig. 8). Therefore, we conclude that PC12 cells mainly express KCC1 in function, but may not functionally express other isoforms up to significant levels compared with KCC1.

Recent studies have revealed that KCCs play important roles in tumor cell growth. Shen et al. have reported that in cervical and ovarian cancer cells, insulin-like growth factor stimulates cancer cell growth via expression and activation of KCC, and inhibition of KCC diminished the cell growth [7, 8]. On the other hand, in the present study, expression of KCC1 was decreased by NGF treatment and inhibition of KCC1 stimulated NGF-induced neurite outgrowth. Namely, KCC has a positive effect on cell growth in cancer; whereas KCC has a negative effect on neurite outgrowth in PC12 cells. In PC12 and other subclonal cell lines, it has been suggested that the cells stop their cell cycle and growth, and differentiate into neuron-like cell in response to NGF stimulation. We speculate that KCCs would act as a positive regulator for cell growth in a growing state, but KCCs including KCC1 would play no significant role in cell cycle and growth in a differentiating state of PC12 cells stimulated by NGF. Alternatively, down-regulation of KCC1 expression in response to NGF stimulation may correlate with the arrest of cell growth.

Changes in $[\text{Cl}^-]_i$ modify various cellular functions, such as cell proliferation of gastric cancer cells [11-13, 50], prostatic cancer cells [51] and osteoblastic cells [52], and gene expression of epithelial Na^+ channel α -subunit (α -ENaC) in renal epithelial cells [16]. In addition, we have recently revealed that Cl^- is essentially required for the NGF-induced neurite outgrowth of PC12 cells and its subclonal PC12D cells [21-23]. In PC12 and PC12D cells, incorporation of Cl^- via NKCC1 plays important roles in the neurite outgrowth. KCCs extrude K^+ and Cl^- from the intracellular space to the extracellular space causing lowered $[\text{Cl}^-]_i$, while inhibition of KCC would elevate $[\text{Cl}^-]_i$. On the other hand, NKCC incorporates Na^+ , K^+ , and Cl^- into the intracellular space from the extracellular space, elevating $[\text{Cl}^-]_i$. In the present study, we indicate that inhibition of KCC1 (with DIOA or siRNA) enhanced the NGF-induced neurite outgrowth. In contrast, our previous studies indicate that inhibition of NKCC diminishes the neurite outgrowth [21-23]. These observations suggest that $[\text{Cl}^-]_i$ is one of the important factors regulating the NGF-induced neurite outgrowth, and that higher $[\text{Cl}^-]_i$ may be an favorite ionic circumstance for efficient neurite outgrowth. Recent studies have revealed that ion transporters regulate various cellular functions, dependent on or independent of their ion-transporting activities. For example, it has been reported that KCCs play important roles in proliferation, migration, invasion, and epithelial to mesenchymal transition (EMT) in cervical and ovarian carcinoma cells by regulating local cell volume, dynamics of cytoskeleton and activity of matrix metalloproteinase [6-9]. As mentioned above, recent studies have revealed that changes in $[\text{Cl}^-]_i$ modulate various cellular functions, such as gene expression

and cell proliferation [11-14, 16, 27, 50-58]. In addition, intracellular Cl^- has been reported to act as a second messenger to regulate β_2 integrin activation in leukocytic cells in response to stimulation by tumor necrosis factor [17, 18]. Therefore, KCCs would play essential roles in various cell functions by modulating $[\text{Cl}^-]_i$, although further studies are necessary to clarify how KCC1 is involved in mechanisms regulating processes of neurite outgrowth.

In conclusion, we demonstrate here that KCC1 negatively regulates the NGF-induced neurite outgrowth of PC12 cells. It may be possible that inhibition of KCC1 promotes regeneration of injured peripheral neuron in various pathological situations.

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