Quercetin Stimulates NGF-induced Neurite Outgrowth in PC12 Cells via Activation of Na⁺/K⁺/2Cl⁻ Cotransporter

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Key Words

Flavonoid • Solute carrier • Chloride ion • NKCC

incorporation into the intracellular space by activating NKCC1 in PC12 cell.

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Abstract

We have recently reported that Na⁺/K⁺/2Cl⁻ cotransporter isoform 1 (NKCC1) plays an essential role in nerve growth factor (NGF)-induced neurite outgrowth in PC12D cells. On the other hand, it has been reported that dietary flavonoids, such as quercetin, apigenin, and luteolin, stimulate various ion transporters. In the present report, we investigated the effect of quercetin, a flavonoid, on NGF-induced neurite outgrowth in PC12 cells (the parental strain of PC12D cells). Quercetin stimulated the NGF-induced neurite outgrowth in a dose-dependent manner. Knockdown of NKCC1 by RNAi methods abolished the stimulatory effect of flavonoid. Quercetin stimulated NKCC1 activity (measured as bumetanide-sensitive ⁸⁶Rb influx) without any increase in the expression level of NKCC1 protein. The stimulatory effect of quercetin on neurite outgrowth was dependent upon extracellular Cl⁻. These observations indicate that quercetin stimulates the NGF-induced neurite outgrowth via an increase in Cl⁻

Introduction

During differentiation, neuronal cells undergo dramatic shape changes. Typically, neuronal cells extend long membranous processes, named neurites. PC12 cells are derived from rat pheochromocytoma, a tumor arising from chromaffin cells of adrenal medulla [1]. The cell is a useful model for the study on neuronal differentiation, since PC12 cells extend neurites in response to nerve growth factor (NGF), one of neurotrophic factors. We have recently reported that Na⁺/K⁺/2Cl⁻ cotransporter isoform 1 (NKCC1) plays an essential role in NGF-induced neurite outgrowth in PC12D cells, a subclonal cell line of PC12 cell [2, 3].

Chloride ion is one of major anions in our body. Recently, attention has been focused on the physiological importance of intracellular Cl⁻. For example, we have previously reported that reduction of intracellular Cl⁻ concentration ([Cl⁻]_i) is an important signal for Na⁺ reabsorption through an increase in mRNA expression of α-subunit of epithelial Na⁺ channel (α-ENaC) via a

protein tyrosine kinase-dependent pathway in *Xenopus* renal A6 cells [4]. Moreover, Menegazzi et al. have reported that a decrease in $[Cl^-]_i$ due to Cl^- efflux precedes adherence, cell spreading and production of reactive oxygen species in polymorphonuclear leukocyte [5]. These findings indicate that Cl^- is a fundamental signal mediator for regulation of various cellular functions. Generally, homeostasis of $[Cl^-]_i$ is controlled by the concerted action of various Cl^- transporters/channels participating in Cl^- incorporation or excretion. In many cells, including neuronal cells, NKCC participates in the Cl^- incorporation step, and K^+/Cl^- cotransporters (KCCs), Cl^- channels, and/or Cl^- ATPase participate in the Cl^- excretion step [6].

Flavonoids are plant pigments widely distributed in soybeans, vegetables, and fruits. Recent studies have revealed that flavonoids modulate various cellular functions, such as cell cycle progression [7], apoptosis [8], and gene expression [9]. In addition, our previous studies have indicated that some flavonoids stimulate ion transport [10-15]. Specially, flavonoids including quercetin stimulate transcellular Cl^- transport by activating NKCC1 at the basolateral plasma membrane and/or cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel at the apical plasma membrane in renal and airway epithelial cells [13, 14]. These reports suggest that some flavonoids are one of possible activators of NKCC1 and stimulate neurite outgrowth by activating NKCC1, which plays an essential role in NGF-induced neurite outgrowth.

In the present study, we focused our study on clarification of flavonoid action on the NGF-induced neurite outgrowth of PC12 cells, and demonstrate that quercetin stimulates NGF-induced neurite outgrowth via activation of NKCC1.

Materials and Methods

Materials

D-MEM (with high glucose), quercetin, and bumetanide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Low Cl^- medium (Cl^- -replaced medium) was obtained from Cell Science & Technology Institute Inc. (Sendai, Japan). Stealth™ small interfering RNA (siRNA), Lipofectamine 2000, Alexa 488-conjugated phalloidin, gentamicin solution, and horse serum (HS) were obtained from Invitrogen (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). BCA protein assay kit was obtained from Pierce Chemical Co. (Rockford, IL, USA). Immobilon-P polyvinylidene difluoride

(PVDF) membrane was purchased from Millipore Corp. (Billerica, MA, USA). ECL western blotting detection reagent was obtained from GE healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England). Polyclonal anti-GAPDH and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Bio Technology (Santa Cruz City, CA, USA). Anti-TrkA antibody was purchased from Abcam Inc. (Cambridge, UK). Anti-phosphorylated ERK1/2 antibody was obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Monoclonal anti-NKCC1 (T4) antibody developed by C. Lytle and B. Forbush [16] was obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by Department of Biological Science, the University of Iowa (Iowa City, IA, USA). Rubidium-86 (^{86}Rb) radionuclide was purchased from Perkin Elmer (Yokohama, Japan).

Cell culture

PC12 cells, kindly donated from Dr. M. Sano (Professor Emeritus, Department of Biology, Kyoto Prefectural University of Medicine), were grown in D-MEM supplemented with 10% HS, 5% FBS, and 50 μ g/ml gentamicin at 37°C with air containing 5% carbon dioxide.

Cl^- replaced culture medium

To incubate PC12 cells under a low Cl^- condition, we used Cl^- -replaced D-MEM, which was prepared by respectively substituting NaCl, KCl and $CaCl_2$ with $NaNO_3$, KNO_3 , and $Ca(NO_3)_2$. The medium was supplemented with 10% HS, 5% FBS, and 50 μ g/ml gentamicin. Actual Cl^- concentration ($[Cl^-]$) in each medium was measured with a Corning Chloride Analyzer Model 925 (Corning, Corning, NY, USA). The $[Cl^-]$ in normal medium was 124.3 mM. The $[Cl^-]$ in Cl^- -replaced (low Cl^-) medium was 13.0 mM. Furthermore, we mixed the low Cl^- medium with the normal medium at various ratios to set the $[Cl^-]$ in media at various levels, and the actual $[Cl^-]$ in each medium was also measured.

Measurement of neurite outgrowth

PC12 cells were seeded onto glass coverslips, pre-coated with poly-L-lysine. Quercetin was applied 30 min before addition of NGF (50 ng/ml), and was continuously present during NGF treatment. Five days after application of quercetin and NGF, the cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and stained with Alexa488-conjugated phalloidin, and observed with a confocal microscope FV1000 (Olympus, Tokyo, Japan). Processes greater in length than one cell diameter (approximately 10-15 μ m) were defined as neurite, and the length of each neurite was measured using FV1000 software (Olympus) or ImageJ software (NIH, Bethesda, MD, USA).

Western blotting

PC12 cells were lysed with lysis buffer (25 mM Hepes-NaOH, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), pH7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.5 μ g/ml leupeptin, and 2 μ g/ml aprotinin) and

phosphatase inhibitors (2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 5 mM sodium fluoride). Protein concentrations were determined using BCA protein assay kit. Proteins of 10 µg separated by SDS-PAGE (8% gel) were transferred onto PVDF membrane. Protein bands were visualized with ECL Kit. Protein bands corresponding to NKCC1 were quantified by Western blotting using T4 monoclonal antibody with Kodak 1D software (Eastman Kodak, New Haven, CT, USA).

siRNA-transfection and quantification of neurite outgrowth

For knockdown of NKCC1, PC12 cells were transfected with siRNA, siRNA1660 and siRNA1921, respectively corresponding to nt 1660-1684 (TGT ACT TCT GCA GCC TGC AAA TTAA) and nt 1921-1945 (GAA CCT CTT CGT GGT TAC ATC TTA A) of open reading frame of rat NKCC1. 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. BLOCK-iT fluorescent oligo (Invitrogen) was used as transfection control. Twenty-four hours after transfection, the cells were treated with NGF (50 ng/ml), and further cultured. Five days after NGF application the cells were fixed with 3.7% formaldehyde, stained with Alexa 488-conjugated phalloidin. The length of each neurite was measured by a confocal microscopy FV1000 (Olympus, Tokyo, Japan). The expression level of NKCC1 protein was quantified by Western blotting using T4 monoclonal antibody as described above.

Measurement of NKCC1 activity

The activity of NKCC1 was assessed by measuring bumetanide-sensitive ^{86}Rb uptake as a tracer for K^+ . PC12 cells were seeded onto poly-L-lysine-coated 24-well plate. Quercetin (10 µM) or DMSO (0.1%) was applied 30 min before application of NGF (50 ng/ml), and were continuously present during NGF treatment. One day after application of quercetin and NGF, the medium was replaced with a solution (140 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 2 mM MgCl_2 , 5 mM glucose, and 10 mM HEPES, pH7.4) for 15-30 min. Then, the solution was replaced with experimental solution (140 mM NaCl, 5 mM $^{86}\text{RbCl}$ (1 µCi/well), 1 mM CaCl_2 , 2 mM MgCl_2 , 5 mM glucose, and 10 mM HEPES, pH7.4) containing 1 mM ouabain and with or without 250 µM bumetanide. The cells were incubated at room temperature for indicated time periods. Experiments were terminated by aspirating the solution and washing the cells with the ice-cold isotonic solution twice, and the cells were immediately lysed with lysis buffer (2% SDS, 10 mM HEPES, pH7.4). Radioactivity of ^{86}Rb in lysate was determined by β -scintillation counting (TRI-CARB 2007TR Liquid Scintillation Analyzer, Packard Japan, Osaka, Japan). The protein concentration of lysate was measured with BCA protein assay kit. The activity of NKCC1 was estimated as bumetanide-sensitive ^{86}Rb uptake per mg protein (nmol/mg protein).

Statistics

All data are represented as means $\pm$ SEM. The ANOVA, Tukey's HSD, and Student's *t*-test were used for statistical

Fig. 1. Effect of NGF on NKCC1 expression and activity. (A) PC12 cells were treated with or without 50 ng/ml NGF for indicated days. Total cell lysates were prepared, proteins of 10 µg were separated by 8% SDS-PAGE, transferred onto PVDF membrane, and detected with monoclonal anti-NKCC1 and polyclonal anti-GAPDH antibodies. * indicates a non-specific band. (B) Statistical results of relative NKCC1 protein expression under conditions described above. Values are represented as means $\pm$ SEM. *, $p < 0.05$ vs. NGF (-) at each time point and control (0 day). $n = 4$. (C) PC12 cells were treated with or without NGF for 1 day. NKCC1 activity was estimated by measuring the bumetanide-sensitive ^{86}Rb uptake. Values are represented as means $\pm$ SEM. *, $p < 0.05$ vs. NGF (-). $n = 3$.

Fig. 2. Effect of NKCC1 knockdown on the NGF-induced neurite outgrowth. PC12 cells were transfected with the indicated siRNA. At 24 h after transfection, the cells were treated with 50 ng/ml NGF for further 5 days. NKCC1 was detected with a monoclonal anti-NKCC1 antibody as described in the legend for Fig. 1. (A-a) Representative Western blotting image. * indicates a non-specific band. (A-b) Relative NKCC1 protein expression under conditions described above. Values are represented as means $\pm$ SEM. *, $p < 0.05$ vs. mock. $n = 4$. (B-a) Representative micrographs of mock-transfected and siRNA1921-transfected cells. Cells were fixed with formaldehyde, stained with Alexa488-phalloidin. The micrographs were taken by a confocal microscopy. Scale bar, 10 μ m. (B-b) The length of each neurite in cells with transfected with siRNA was measured, and the mean neurite length was calculated. Values are represented as means $\pm$ SEM. *, $p < 0.01$ vs. mock. $n = 106$ -125.

analysis as appropriate and a p value < 0.05 was considered statistically significant.

Results

NKCC1 is essential for the neurite outgrowth in PC12 cells

We have no information on NGF action on expression and activity of NKCC1 and the role of NKCC1 in the NGF-induced neurite outgrowth in parental PC12 cells, although we reported that in a subclonal cell line of rat pheochromocytoma PC12 cell, PC12D cell, NGF induced expression of NKCC1 without any information regarding NGF action on activity of NKCC1 [2, 3]. In the present study, in parental PC12 cells, we first examined if NGF up-regulates NKCC1 expression and activity. We also studied if NKCC1 is essential for the neurite outgrowth. Figs. 1A and B show the protein expression level of NKCC1 in PC12 cells treated with or without 50 ng/ml NGF for 1-5 days. Expression of NKCC1 protein was increased by NGF treatment (Fig. 1A). Quantitative analysis indicates that NGF treatment increased NKCC1 protein expression approximately 1.5-fold compared with NGF-non-treated cells at each time point (Fig. 1B). Fig. 1C shows the activity of NKCC1 estimated by bumetanide-sensitive 86 Rb uptake in NGF-treated and

NGF-non-treated cells. Bumetanide-sensitive 86 Rb uptake in NGF-treated cells was approximately 1.6-fold higher than that without NGF treatment (8.40 ± 1.97 nmol/mg protein in NGF (-), and 13.65 ± 2.84 nmol/mg protein in NGF (+)).

Next, we examined the effect of knockdown of NKCC1 on the NGF-induced neurite outgrowth in PC12 cells. Two independent siRNA1660 and siRNA1921 [2] were used for the experiment. As shown in Figs. 2A-a and -b, transfection of these siRNAs reduced NKCC1 protein expression level to 5-10% of mock-transfected cells. BLOCK-iT fluorescent oligo (control oligo), which has no sequence homology to any known mammalian genes, was used as transfection control. As shown in Figs. 2B-a and -b, transfection of siRNA1660 or siRNA1921 strongly inhibited the neurite outgrowth. Mock-transfected cells and control oligo-transfected cells extended approximately 40 μ m neurites. In contrast, siRNAs-transfected cells extended short (less than approximately 20 μ m) neurites.

Quercetin, dietary flavonoid, stimulates the NGF-induced neurite outgrowth in PC12 cells via activation of NKCC

We next studied the effect of quercetin on the NGF-induced neurite outgrowth. Quercetin alone did not

Fig. 3. Stimulatory action of quercetin on the NGF-induced neurite outgrowth in PC12 cell. (A) PC12 cells treated with or without 10 μ M quercetin for 30 min. At 30 min after quercetin application, NGF (50 ng/ml) was added. The cells were further cultured for 5 days in the presence or absence of quercetin and/or NGF. The cells with at least one process greater in length than one cell diameter were counted as “neurite forming cells”. Values are represented as means $\pm$ SEM. (B) PC12 cells treated with 0, 2.5, or 10 μ M quercetin for 30 min. At 30 min after quercetin application, NGF (50 ng/ml) was added. The cells were further cultured for 5 days in the presence of quercetin and NGF. The length of each neurite was measured, and the mean neurite length was calculated. Values are represented as means $\pm$ SEM. *, $p < 0.01$ vs. control (quercetin = 0 μ M). #, $p < 0.05$ vs. quercetin = 2.5 μ M. $n = 116$ -132. (C) Distribution of the neurite lengths in cells treated with DMSO (closed squares) or 10 μ M quercetin (open circles). The vertical (Y) axis indicates the percentage of neurites longer than the indicated value in the horizontal (X) axis.

increase the percentage of neurite forming cells (Fig. 3A). Percentage of neurite forming cells without NGF treatment was 0% irrespective of the presence of 10 μ M quercetin (Fig. 3A). NGF (50 ng/ml) treatment induced neurite outgrowth in more than 80% of cells (Fig. 3A), and quercetin did not affect this proportion (84.2% in NGF(+)/Quercetin(-), and 82.4% in NGF(+)/Quercetin(+)) (Fig. 3A). On the other hand, application of quercetin (2.5 μ M and 10 μ M) elongated the neurite length of NGF-treated cells (Fig. 3B) but not NGF-non-treated cells (data not shown). Fig. 3C indicates distribution of neurite lengths in NGF-treated cells with 10 μ M quercetin or without quercetin (DMSO alone). Quercetin shifted the distribution rightward, meaning that quercetin increases the percentage of neurites longer than the given length shown in Fig. 3C.

We have reported that quercetin stimulates transepithelial Cl⁻ transport by activating NKCC1 localized at the basolateral plasma membrane in renal and airway epithelial cells [12-14]. Figs. 1 and 2 show that NKCC1 up-regulated by NGF plays an important role in the neurite outgrowth in parental PC12 cells. These observations suggest that quercetin stimulates the NGF-induced neurite outgrowth by activating NKCC1. Therefore, to study if the stimulatory effect of quercetin on the NGF-induced neurite outgrowth of PC12 cells is mediated through activation of NKCC1, we next examined if NKCC1 knockdown affects the stimulatory action of quercetin on neurite outgrowth. As shown in Fig. 4, transfection of siRNA1660 or siRNA1921 abolished the stimulatory action of quercetin on neurite outgrowth. The neurite grew up to around 20 μ m (the onset of neurite outgrowth) even

Fig. 4. Effect of NKCC1 knockdown in the NGF-induced neurite outgrowth of the cells with or without quercetin treatment. PC12 cells were transfected with indicated siRNA. At 24 h after transfection, the cells were treated with 10 μ M quercetin (or DMSO alone as a solvent control) together with NGF (50 ng/ml) for 5 days. The length of each neurite was measured, and the mean value of neurite length was calculated. Values are represented as means $\pm$ SEM. *, $p < 0.01$ vs. DMSO. NS, no significant difference vs. DMSO. $n = 81$ -98.

Fig. 5. Effect of NKCC1 knockdown on the expression of TrkA and phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2). PC12 cells were transfected with indicated siRNA. At 5 days after transfection, the cells were treated with or without NGF (50 ng/ml) for 5 minutes. Total cell lysates were prepared, proteins of 10 μ g were separated by 8% SDS-PAGE, transferred onto PVDF membrane, and detected with monoclonal anti-NKCC1 (T4), polyclonal anti-TrkA and monoclonal anti-phospho-ERK1/2 antibodies. * indicates a non-specific band.

in the NGF-treated cells transfected with NKCC1 siRNA, indicating that the NGF-induced neurite outgrowth up to 20 μ m (the onset of neurite outgrowth) is not mediated via an NKCC1-dependent process. This means that NKCC1 does not participate in the onset of neurite outgrowth up to 20 μ m. Further, quercetin did not stimulate the neurite outgrowth (the length of neurite $\approx$ 20 μ m) in the cells transfected with NKCC1 siRNA (Fig. 4). This means that quercetin has no effect on onset of neurite outgrowth.

To exclude the possibility that transfection of siRNA abolishes the NGF receptor TrkA expression and/or activation of downstream signaling cascade, which are essential for neurite outgrowth, we examined the total expression level of TrkA and phosphorylation level of extracellular signal-regulated kinase (ERK) in siRNA-transfected cells. In PC12 cells, it has been reported that activation of ERK is crucial for the NGF-induced neurite outgrowth [17]. As shown in Fig. 5, transfection of siRNA against NKCC1 had no effect on the expression of TrkA protein. Furthermore, in control cells, treatment of NGF (50 ng/ml) for 5 min induced strong activation of ERK1/2 (see lanes 1-3 in Fig. 5). In siRNA1921-transfected cells, NGF treatment also induced strong activation of ERK1/2 (see lanes 4-6 in Fig. 5), similar to mock-transfected cells. These observations indicate that transfection of siRNA against NKCC1 did not affect expression of NGF receptor TrkA or NGF-induced

Fig. 6. Effect of quercetin on NKCC1 expression and activity. (A) and (B) PC12 cells were treated with 10 μ M quercetin or DMSO alone. At 30 min after quercetin application, the cells were treated with NGF (50 ng/ml) for 5 days. Total cell lysates were prepared, proteins of 10 μ g were separated by SDS-PAGE, transferred onto PVDF membrane, and detected with monoclonal anti-NKCC1 antibody. (A) Representative results. (B) Statistical results of relative NKCC1 protein expression level under the conditions described above. Values are represented as means $\pm$ SEM. $n = 4$. (C) PC12 cells were treated with 10 μ M quercetin or DMSO alone. At 30 min after application of quercetin or DMSO alone, the cells were treated with NGF for 1 day. NKCC1 activity was estimated by measuring the bumetanide-sensitive ⁸⁶Rb uptake. Values are represented as means $\pm$ SEM. *, $p < 0.01$ vs. DMSO. $n = 3$ for time of 0 min, $n = 5$ for time of 15 min, $n = 7$ for time of 30 min.

Fig. 7. Stimulatory effect of quercetin on the NGF-induced neurite outgrowth dependent on extracellular Cl^- . PC12 cells were cultured in media containing various Cl^- concentrations (13.0 mM, 27.7 mM, 46.0 mM, 61.6 mM, 88.3 mM, or 124.3 mM). At 30 min after changing medium, quercetin (10 μM) or DMSO (0.1%) with NGF (50 ng/ml) was added. The cells were further cultured for 5 days. The length of each neurite was measured, and the mean value of neurite length was calculated. Values are represented as means $\pm$ SEM. *, $p < 0.05$ vs. DMSO. **, $p < 0.01$ vs. DMSO $n = 144$ –172.

signaling cascade, suggesting that the inhibitory action of siRNA transfection against NKCC1 is not mediated via suppression of NGF receptor TrkA-mediated ERK1/2 activation essentially required for neurite outgrowth.

Next, we examined if quercetin increases the expression level of NKCC1 and/or enhances the transport activity of NKCC1. Figs. 6A and B show the expression levels of NKCC1 protein in control (DMSO alone) and quercetin-treated cells. Quercetin had no effect on the expression level of NKCC1 protein (Figs. 6A and B). Fig. 6C shows the NKCC1 activity (bumetanide-sensitive ^{86}Rb uptake) in control (DMSO alone) and quercetin-treated cells 1 day after NGF application. Quercetin increased the bumetanide-sensitive ^{86}Rb uptake (Fig. 6C). These observations indicate that quercetin activates NKCC1 without any increase in the expression of NKCC1 protein. We have no information if quercetin continuously activates NKCC1 activity during 5 days period, but we speculate that quercetin increases total amount of Cl^- uptake into the cells during 5 days period.

To study the relation between Cl^- incorporation via NKCC1 and stimulatory action of quercetin on the NGF-induced neurite outgrowth, we treated PC12 cells with quercetin in Cl^- -replaced medium (Cl^- was replaced with NO_3^-). As shown in Fig. 7, stimulatory action of quercetin on the NGF-induced neurite outgrowth was abolished

when the Cl^- concentration in culture medium ($[\text{Cl}^-]_0$) was 46.0 mM or less. On the other hand, when $[\text{Cl}^-]_0$ was 61.6 mM or more than 61.6 mM, quercetin stimulated the NGF-induced neurite outgrowth.

Discussion

In the present study, we demonstrated that a dietary flavonoid, quercetin, stimulates the NGF-induced neurite outgrowth via activation of NKCC1 in rat pheochromocytoma PC12 cells. Protein expression and activity of NKCC1 in parental PC12 cells was up-regulated by the NGF treatment (Fig. 1), and knockdown of NKCC1 by RNAi method diminished the NGF-induced neurite outgrowth (Fig. 2).

Application of a dietary flavonoid, quercetin, stimulates the NGF-induced neurite outgrowth in PC12 cells (Fig. 3). In our previous report, in PC12D cells, a subclonal cell line of PC12, NKCC1 is also essential for the NGF-induced neurite outgrowth [2, 3]. PC12D cells extend neurites faster than parental PC12 cells. We also tested the effect of quercetin on the NGF-induced neurite outgrowth in PC12D cells, but stimulatory effect of quercetin was not observed in PC12D cells. In our previous study, PC12D cells express NKCC1 protein approximately two to three-fold higher than parental PC12 cell [18]. The reason why quercetin had no effect on neurite outgrowth in PC12D cells may be high expression (and probably high activity) of NKCC1 at the basal level in PC12D cells.

Recent studies have demonstrated that flavonoids modify various cellular functions, such as proliferation [7], apoptosis [8], and transcription and/or translation [9], in manners dependent on or independent of its antioxidant properties. In addition, it has been reported that some flavonoids and flavonoid derivatives affect ion transport [10–14, 19, 20]. Especially, we have reported that quercetin stimulates Cl^- secretion by activating NKCC1 localized at the basolateral plasma membrane in renal and airway epithelia [12, 14]. Knockdown of NKCC1 by RNAi abolished the stimulatory effect of quercetin on the NGF-induced neurite outgrowth (Fig. 4). In PC12 cells and several other subclonal cell lines, it has been suggested that neurite formation step (the first step) and subsequent elongation step (the second step) are controlled by different mechanisms [21, 22]. In the present study, we observed short neurite (approximately 15–20 μm ; onset of neurite outgrowth) even in NKCC1 knockdown cells (Fig. 2B-b), and that quercetin had no effects on the length

of neurite in NKCC1 knockdown cells (Figs. 2 B-b and 4). These observations strongly suggest that NKCC1 participates in the second step, extension from short neurite to long one, but not in the first formation step (the onset of neurite outgrowth), and quercetin stimulates the second elongation step via activation of NKCC1.

In cells with NKCC1 knockdown, expression of TrkA protein and NGF-induced phosphorylation of ERK1/2 are comparable to those in mock-transfected cells (Fig. 5). NGF bound to its specific receptor TrkA transduces growth factor signalings by activating downstream MAPK signaling cascade. Neurite outgrowth from neurons and neuronal cell lines induced by neurotrophic factors, such as NGF, absolutely requires the activation of ERK [17]. These results suggest that up-regulation of NKCC1 expression and/or activity triggered by NGF treatment is essential for the second step of NGF-induced neurite outgrowth (extension from short neurite (about 20 μm) to long one) but not for the first step of neurite outgrowth (extension up to short neurite (about 20 μm); the onset of neurite outgrowth), and treatment with quercetin enhances the second step of NGF-induced neurite outgrowth (extension from short neurite (about 20 μm) to long one) via up-regulation of NKCC1 activity.

Quercetin increased NKCC1 activity (estimated by bumetanide-sensitive ^{86}Rb uptake) without elevation of the total expression of NKCC1 protein (Fig. 6). We have previously reported that quercetin elevates NKCC1 activity in epithelial cells [12, 14]. There are at least two mechanisms activating NKCC1; 1) increment in expression of NKCC1 protein at the plasma membrane via modulation of insertion and/or endocytosis (including degradation) [18], and 2) increment in activity of individual NKCC1 located in the plasma membrane. Carmosino et al. [23] have reported that the carboxy-terminal dileucine motif of NKCC1 is a basolateral sorting signal and the amino-terminal cytosolic region of NKCC2 contains an apical sorting signal. Moreover, Del Castillo et al. [24] have reported that NKCC1 is internalized via a protein kinase C- ϵ (PKC- ϵ)-dependent pathway. However, there is little information on molecular mechanisms participating in intracellular trafficking of NKCC protein itself or NKCC regulatory proteins. Further studies are necessary to clarify if quercetin promotes intracellular trafficking of NKCC1 regulatory proteins in PC12, since at least in human airway epithelial cells quercetin activates intracellular trafficking of NKCC1 regulatory proteins without any changes in NKCC1 expression at the basolateral membrane [14]. On the other hand, the activity of NKCC1 (and also NKCC2) is regulated by various

kinases and phosphatases [25-28]. It is well known that Ste20-related Proline/Alanine-rich kinase (SPAK) and oxidative stress responsive 1 (OSR1) activate NKCC1 by phosphorylating three threonine residues of NKCC1 [26]. On the other hand, certain protein phosphatases such as PP1 and PP2 dephosphorylate NKCC1, attenuating the activity [27, 28]. Recent studies have demonstrated that flavonoids modulate various cellular kinases and phosphatases [29, 30]. In the present study, quercetin may modulate directly or indirectly activity of these kinases/phosphatases leading to stimulation of NKCC1 activity. Further studies are necessary to clarify if quercetin influences the phosphorylation status of NKCC1 via activation or inhibition of these enzymes.

Interestingly, the NGF-induced neurite outgrowth was significantly inhibited when the NGF-treated cells were cultured in a low $[\text{Cl}^-]$ medium, and the stimulatory effect of quercetin on the NGF-induced neurite outgrowth was dependent on the extracellular Cl^- concentration (Fig. 7). Quercetin of 10 μM stimulated the NGF-induced neurite outgrowth in culture medium containing 61.6, 88.3, or 124.3 mM Cl^- ($[\text{Cl}^-]$ of normal culture medium is 124.3 mM). By contrast, when cells were cultured in a medium with low $[\text{Cl}^-]$ (13.0, 27.7, or 46.0 mM Cl^-), the stimulatory effect of quercetin was abolished. It is predicted that culture in a medium with low $[\text{Cl}^-]$ would lower $[\text{Cl}^-]_i$. Cl^- -dependent molecular mechanisms regulating the NGF-induced neurite outgrowth are still unclear. However, as mentioned above, we and other investigators have indicated that intracellular Cl^- affects various cellular functions, such as cell cycle progression and proliferation via up- or down-regulation of cell-cycle-related enzymes [31-40], gene expression of epithelial sodium channel via a PTK-dependent pathway [4], and cell migration via remodeling of cytoskeleton [41-43]. In addition, Menegazzi and colleagues [5, 44] have reported that physiological functions of neutrophil (e.g. respiratory burst and phagocytosis) are associated with a change in $[\text{Cl}^-]_i$. On the other hand, it is suggested that differentiation of PC12 cells and several other subclonal cell lines is regulated by various events, such as regulation of gene expression [45], activation of many signaling cascades [46], remodeling of cytoskeleton [47], and membrane trafficking [48]. Changes in $[\text{Cl}^-]_i$ may directly or indirectly affect the steps mentioned above. For example, in our preliminary experiment [49], Cl^- influences *in vitro* microtubule polymerization through modulating intrinsic GTPase activity of tubulin. In growing neurites, it is suggested that the formation of long microtubule polymer is based on the polymerization of α/β -tubulin heterodimers, and

the polymerization of microtubule promotes outgrowth of neurite. Both α -tubulin and β -tubulin are GTP-binding proteins that possess GTPase activity. In particular, the GTP/GDP cycle of β -tubulin is crucial for its polymerization forming to microtubule. On the other hand, Nakajima et al. [50] have reported that Cl^- inhibits GTPase activity of heterotrimeric G-protein G_κ . Furthermore, α -subunit of $\text{G}_{q/11}$ protein is highly associated with cytoskeletons including microtubules. These observations suggest that Cl^- directly or indirectly affects GTPase activity of tubulin, controlling dynamic behavior of neuritic microtubule.

Our observations in the present study suggest that incorporation of Cl^- via NKCC1 is essential for the NGF-induced neurite outgrowth, and that quercetin stimulates neurite outgrowth by increasing Cl^- uptake via NKCC1, although further studies are necessary to clarify how intracellular Cl^- regulates the NGF-induced neurite outgrowth in detail.

Flavonoids are widely distributed in plants such as vegetables, fruits, and soybean, and have their variety

and relatively low toxicity compared with other plant compounds (e.g. alkaloids). Many animals including humans ingest significant quantities of flavonoids in their diet. Our present study suggests that: 1) quercetin, a flavonoid, is one of possible activators of NKCC1, 2) the activation of NKCC1 stimulates the neurite outgrowth by increasing $[\text{Cl}^-]_i$, and 3) quercetin enhances the neurite outgrowth via an increase in $[\text{Cl}^-]_i$ by activating NKCC1.

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