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# cGMP-dependent protein kinase I is involved in neurite outgrowth via a Rho effector, rhotekin, in Neuro2A neuroblastoma cells

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ABSTRACT

Although the cGMP/cGMP-dependent protein kinase (cGK) signaling is involved in the regulation of neurite outgrowth, its mechanism remains to be clarified. In this study, we identified a Rho effector, rhotekin, as a cGK-I-interacting protein. Rhotekin was also a substrate for cGK-Iα. In neurite-extended Neuro2A neuroblastoma cells, cGK-Iα and rhotekin were colocalized in the plasma membrane and extended neurites, while treatment with cGMP resulted in translocation of rhotekin to the cytoplasm. In addition, we found that cGK-Iα and rhotekin synergistically suppressed Rho-induced neurite retraction. Our findings suggest that cGK-Iα interacts with and phosphorylates rhotekin, thereby contributing to neurite outgrowth regulation.



## 1. Introduction

Neurite outgrowth is essential for the formation of the nervous system. Several studies have demonstrated that the cGMP signaling pathway regulates neurite outgrowth and neuronal migration [1–4]. cGMP is a second messenger that is synthesized by guanylate cyclases in response to nitric oxide (NO) and natriuretic peptides [5,6]. cGMP-dependent protein kinases (cGKs) are the major intracellular targets for cGMP. Two types of cGKs (cGK-I and cGK-II) encoded by distinct genes have been identified in mammals. Furthermore, cGK-I exists in two isoforms, cGK-Iα and cGK-Iβ, which are produced by alternative splicing and differ only at the amino terminus.

In the nervous system, cGK-Iα is expressed in dorsal root ganglia and cerebellum, while cGK-Iβ is found in hippocampal and olfactory neurons. A study with cGK-I-deficient mice demonstrated that cGK-I was required for the guidance and connectivity of sensory axons [1]. In addition, semaphorin 3A-induced growth cone collapse was overcome by cGMP/cGK-I signaling. A further study found that activation of cGMP signaling by a cell membrane-permeable cGMP analog or by overexpression of cGK-I, promoted branch formation of cultured dorsal root ganglia neurons [2]. Another report showed that treatment of PC12h pheochromocytoma cells with a cell membrane-permeable cGMP analog resulted in increased neurite outgrowth, which was blocked by a cGK inhibitor [3]. Although axon branching resulting from cGMP signaling has been shown to be mediated by the inhibition of glycogen synthase kinase 3 [2], other targets of cGK-I might be involved in the regulation of neurite outgrowth.

Neurite outgrowth is regulated by coordinated changes in the actin cytoskeleton. Rho GTPases are key regulators of the cytoskeleton during neurogenesis [7,8]. While Rac and Cdc42 signaling pathways promote neurite outgrowth, Rho induces neurite retraction. The activities of the Rho GTPases are determined by the ratios of their GTP/GDP-bound forms. This ratio is regulated by guanine nucleotide exchange factors (GEFs), which enhance the exchange of bound GDP for GTP, and the GTPase-activating proteins (GAPs), which increase the intrinsic rate of GTP hydrolysis. Some Rho effectors have been shown to play pivotal roles in Rho-dependent neuronal cellular events. Rhotekin, one of the Rho effectors, that is highly expressed in the brain, has been reported to regulate Rho-dependent gene transcription [9]. The interaction of Rho with rhoGAP is inhibited by the Rho-binding domain of rhotekin [10].

To understand the cGMP signaling in neurite outgrowth, we isolated components of the cGMP/cGK-I signaling pathway including substrates and regulatory proteins for cGK-Is, using a yeast two-hybrid system. In this study, we report evidence that cGK-I isoforms interact with and phosphorylate an effector of Rho, rhotekin, both *in vitro* and *in vivo*. We also found that cGK-I controlled neurite outgrowth via rhotekin in Neuro2A neuroblastoma cells. Our findings highlight that the synergistic action of cGMP/cGK

*Abbreviations:* NO, nitric oxide; cGK, cGMP-dependent protein kinase; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; 8-pCPT-cGMP, 8-(4-chlorophenylthio)-cGMP; RA, *all-trans*-retinoic acid; LPA, 1-oleoyl lysophosphatidic acid; VASP, vasodilator-stimulated phosphoprotein; ROCK, Rho-associated kinase.

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and rhotekin provides a novel mechanism for regulating the outgrowth of neurites.

## 2. Materials and methods

### 2.1. Plasmid construction

Constructions of various cGK-Is plasmid were described previously [11]. cDNA encoding mouse full-length rhotekin was obtained by specific primers. PCR product was cloned into TA-cloning vector pGEM-T Easy (Promega), and the fidelity of inserted DNA sequence was confirmed by DNA sequencing. The full-length rhotekin cDNA was subcloned into the mammalian expression vector pcDNA4/His-Max (Invitrogen) which encodes the N-terminal Xpress epitope, and the mammalian GST-expression vector pEBG, to generate pXpress-rhotekin and pEBG-rhotekin, respectively. Site-directed mutagenesis was performed using PrimeSTAR Mutagenesis Basal Kit (Takara) according to the protocol of the manufacturer.

### 2.2. Yeast two-hybrid screening

Yeast two-hybrid screening was performed using MATCHMAKER Two-Hybrid system 3 (Clontech) as previously described [11]. cDNA encoding N-terminal region of cGK-Iα (amino acid residues 1–416) was cloned into the bait vector pGBKT7. pGBKT7-cGK-Iα-N was transformed in yeast strain AH109 (*MATa*), generating GAL4BD-cGK-Iα-N/AH109. Yeast strain Y187 (*MATα*) pretransformed with MATCHMAKER mouse Brain library in pGADT7-Rec GAL4 activation domain vector (Clontech), was mated with GAL4BD-cGK-Iα-N/AH109. Mating yeast mixtures were spread on synthetic dropout plates lacking adenine, histidine, leucine, and tryptophan, and incubated at 30 °C until colonies appeared. The positive colonies were further confirmed with a β-galactosidase filter assay. β-galactosidase positive clones were amplified in synthetic dropout media minus leucine, selecting only for the prey library insert. Finally, the cDNA inserts from positive clones were sequenced.

### 2.3. Cell culture, transfection, and immunofluorescence analysis

Neuro2A cells and COS-7 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% $CO_2$. Transfection was performed by Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Immunofluorescence analysis was performed as previously described [11].

### 2.4. Co-immunoprecipitation and GST pull-down assays

Co-immunoprecipitation assay was described previously [11]. For GST pull-down assay, COS-7 cells were co-transfected with pFLAG-cGK-I and pEBG-rhotekin. After 24 h, cells were scraped in an ice-cold cell lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP40, 1 mM EDTA, 10 μM leupeptin, and 10 μg/ml aprotinin). The cell extracts were centrifuged at 10,000*g* for 10 min at 4 °C, and the supernatants were incubated with glutathione Sepharose (GE Healthcare) over night at 4 °C. The beads were washed five times with wash buffer (lysis buffer with 0.1% NP40 instead of 0.5% NP40). Precipitated proteins were analyzed by immunoblot analysis using mouse anti-FLAG M2 antibody (Sigma).

### 2.5. In vitro and in vivo kinase assays

*In vitro* kinase assay was performed as previously described [12]. For *in vivo* kinase assay, COS-7 cells were co-transfected with pFLAG-cGK-Iα and pFLAG-rhotekin. After 24 h, cells were treated with 100 μM 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP) for 1 h. Cells were scraped in an ice-cold cell lysis buffer supplemented with PhosStop phosphatase inhibitor cocktail (Roche). The cell extracts were subjected to SDS–PAGE, and immunoblotting using anti-phospho RRXS/T (100G7) (Cell Signaling Technology) or anti-FLAG antibodies.

### 2.6. Quantification of neurite outgrowth

For neurite outgrowth, Neuro2A cells were plated at a density of $0.5 \times 10^5$ cells/35-mm dish. The cells were transfected with pFLAG-cGK-Iα and pFLAG-rhotekin together with pEGFP. After 24 h, the medium was changed to differentiation medium comprising DMEM supplemented with 2% FBS and 20 μM of *all-trans*-retinoic acid (RA) for 24 h. After pretreatment with or without 100 μM 8-pCPT-cGMP for 30 min, the cells were treated with 20 μM 1-oleoyl lysophosphatidic acid (LPA) for 20 min and fixed for 20 min in 3.7% formaldehyde. Neuro2A cell with neurite formation is defined as an outgrowth from the cell body with a length longer than the diameter of the cell body. At least 50 cells per treatment were scored.

## 3. Results

### 3.1. cGK-I isoforms interact with rhotekin

To determine the molecular mechanism of cGMP/cGK-Iα signaling in neurons, a yeast two-hybrid system was performed. An expression vector that encoded the N-terminal region of cGK-Iα (amino acid residues 1–416) fused to the DNA-binding domain of GAL4 was used as bait in the yeast two-hybrid screening of a mouse brain cDNA library. We obtained some positive clones from the $8.0 \times 10^6$ transformants that were screened. Sequence analysis revealed that two different clones encoded the same protein, rhotekin, one of the Rho effectors [10,13]. The remaining positive clones contained nucleotide sequences for Ras association (RalGDS/AF-6) domain family 1 and sorting nexin 3. Among the positive clones obtained, rhotekin was chosen for further studies.

To investigate the interaction between cGK-Iα and rhotekin in mammalian cells, COS-7 cells were transfected with FLAG-tagged cGK-Iα and Xpress-tagged rhotekin. Cell lysates were immunoprecipitated with either an anti-FLAG or an anti-Xpress antibody followed by anti-Xpress or anti-FLAG immunoblotting, respectively. Immunoblotting analysis revealed that cGK-Iα interacted with rhotekin in COS-7 cells (Fig. 1A). Subsequently, we examined whether the cGK-Iβ isoform interacted with rhotekin, in order to evaluate the interaction between cGK-Iα and rhotekin. Interestingly, the cGK-Iβ isoform interacted more strongly with rhotekin than the cGK-Iα isoform (Fig. 1B). These results demonstrate that cGK-I isoforms specifically interact with rhotekin.

When cGMP binds to the cGMP-binding domains of cGK-I, the kinase undergoes a conformational change that results in activation of its catalytic function [14]. Therefore, we examined whether a conformational change and subsequent activation of cGK-Iα affected the interaction between cGK-Iα and rhotekin. COS-7 cells co-transfected with cGK-Iα and rhotekin were treated with 8-pCPT-cGMP, a cell membrane-permeable cGMP analog. Exposure of the cells to 8-pCPT-cGMP resulted in partial dissociation of the cGK-Iα/rhotekin complex (Fig. 1C). Similar results were obtained with cGK-Iβ (Fig. 1D).

### 3.2. Rhotekin is phosphorylated by cGK-I at serine 93

Amino acid sequence analysis of rhotekin identified a typical phosphorylation site ($RRPS^{93}$) for cGK. To determine whether rhotekin was phosphorylated by cGK-Iα, we coexpressed a FLAG-tagged rhotekin with FLAG-tagged cGK-Iα in COS-7 cells. Cell lysates were immunoprecipitated with an anti-FLAG antibody, and an *in vitro* kinase assay was performed. As shown in Fig. 2A, rhotekin was phos-

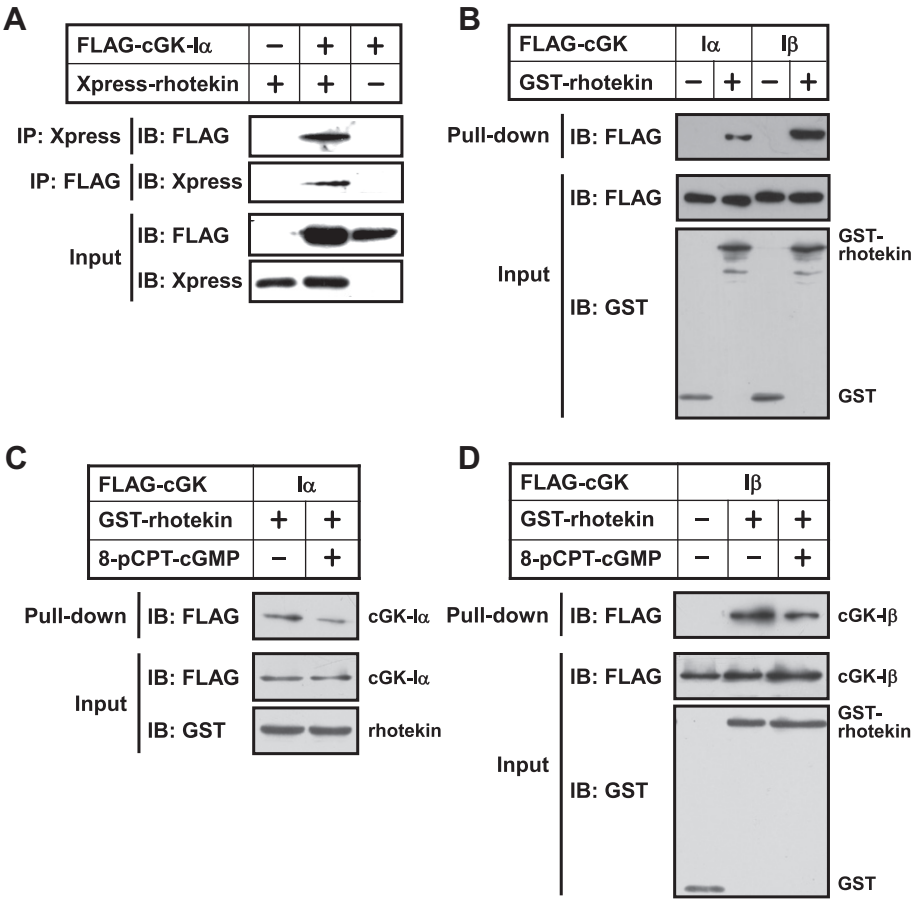


**Fig. 1.** Interaction between cGK-Is and rhotekin. (A) COS-7 cells were co-transfected with pFLAG-cGK-Iα and pXpress-rhotekin. After 24 h, cell lysates were immunoprecipitated with anti-Xpress or anti-FLAG antibody, and immunoprecipitates were detected by immunoblotting with anti-FLAG or anti-Xpress antibody. (B) COS-7 cells were transiently transfected with FLAG-cGK-Iα or FLAG-cGK-Iβ together with GST-rhotekin. Whole cell lysates were pull-downed with glutathione resin, and analyzed by immunoblotting using anti-FLAG antibody. (C and D) COS-7 cells transfected with FLAG-cGK-Iα or FLAG-cGK-Iβ together with GST-rhotekin, were treated with 100 μM 8-pCPT-cGMP for 1 h. The cell lysates were used in a pull-down assay. IP; immunoprecipitation, IB; immunoblot.

phorylated by cGK-Iα in a cGMP-dependent manner. Furthermore, rhotekin phosphorylation by cGK-Iα was confirmed by an *in vivo* kinase assay using an anti-phospho RRXS/T antibody, which detects proteins containing a phosphorylated serine or threonine residue with an arginine at the −3 and −2 positions. COS-7 cells transiently transfected with FLAG-tagged rhotekin and cGK-Iα were treated with 8-pCPT-cGMP. Cell lysates were immunoprecipitated with an anti-FLAG antibody, and then immunoblotted with an anti-phospho RRXS/T antibody. The phosphorylation of vasodilator-stimulated phosphoprotein (VASP), one of the substrates for cGK, by cGMP/cGK-Iα was detected using this phospho-antibody (Fig. 2B). Rhotekin was also efficiently phosphorylated by cGMP/cGK-Iα *in vivo*. These results indicated that rhotekin was phosphorylated by cGK-Iα in a cGMP-dependent manner.

To identify phosphorylation sites in rhotekin, we created a rhotekin mutant, in which serine 93 of the putative cGK phosphorylation site ($RRPS^{93}$) was replaced by alanine (rhotekin S93A), and tested it for phosphorylation by cGK-Iα. As shown in Fig. 2C and D, both *in vitro* and *in vivo* kinase assays revealed that phosphorylation of the S93A mutant was almost completely suppressed compared with that of wild-type rhotekin. This indicated that serine 93 is a potential site of rhotekin for phosphorylation by cGK-Iα.

### 3.3. cGK-I colocalizes with rhotekin in Neuro2A cells

To determine the intracellular localizations of cGK-Iα and rhotekin, we used Neuro2A cells, a mouse neuroblastoma cell line. Neuro2A cells are widely used for studies on neurite outgrowth that is induced by retinoic acid (RA) treatment. Neuro2A cells transfected with pFLAG-cGK-Iα and pXpress-rhotekin were incubated with 10% FBS or 2% FBS containing 20 μM RA for 48 h. In Neuro2A cells incubated with 10% FBS, FLAG-cGK-Iα and Xpress-rhotekin were colocalized in the cytoplasm (Fig. 3). When Neuro2A cells were cultured with 2% FBS containing 20 μM RA, cGK-Iα and rhotekin showed a homogenous distribution in the plasma membrane and extended neurites. Interestingly, treatment with 8-pCPT-cGMP resulted in rhotekin translocation from the plasma membrane to the cytoplasm, while cGK-Iα was not translocated. This suggests that stimulation of cGK-I by cGMP results in the dissociation of cGK-I and rhotekin.

### 3.4. cGK-I suppresses lysophosphatidic acid (LPA)-induced retraction via rhotekin in Neuro2A cells

Finally, we investigated the effect of cGK-I/rhotekin signaling on neurite outgrowth. Rhotekin possesses a Rho-binding domain in its N terminus, and this domain interferes with the interaction of Rho with rhoGAP [10]. In neurons, RhoA is a negative regulator of neurite outgrowth and its effector Rho-associated kinase (ROCK) mediates Rho-driven neurite retraction [15]. Therefore, we examined the effects of cGK-I and rhotekin on RhoA-induced neurite retraction in Neuro2A cells. Because LPA causes neurons to retract their neurites and become rounded up through RhoA activation [16], differentiated Neuro2A cells transfected with cGK-Iα, combined with

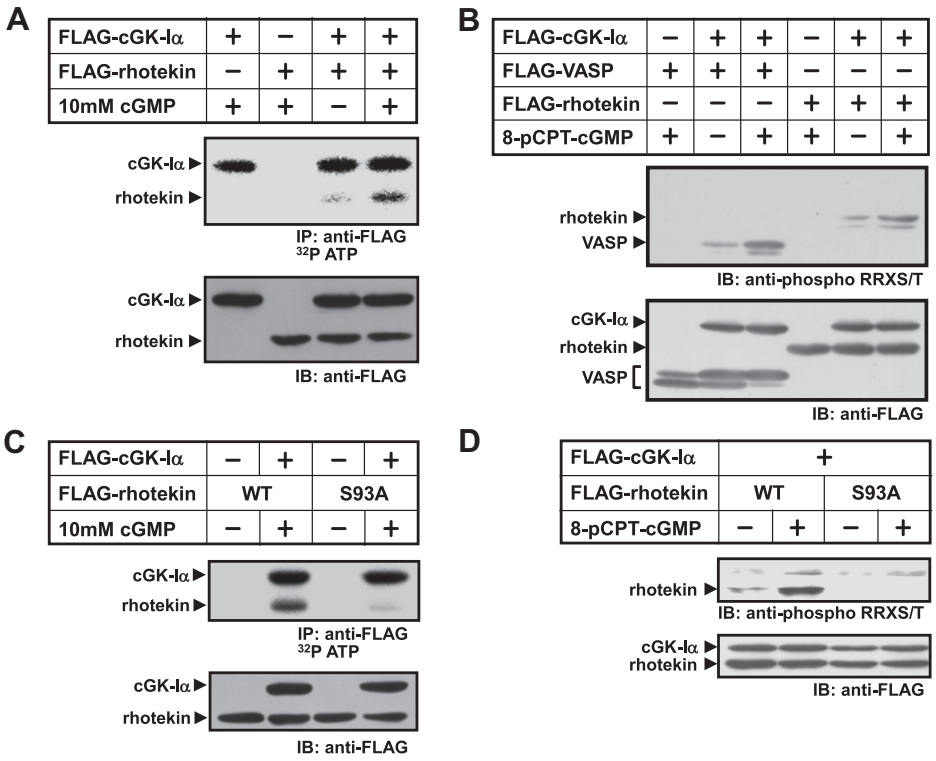


**Fig. 2.** Phosphorylation of rhotekin by cGK-Iα. (A and C) FLAG-rhotekin were expressed in COS-7 cells with FLAG-cGK-Iα. The FLAG-tagged proteins were immunoprecipitated, and then incubated in a kinase buffer containing [$\gamma$-$^{32}$P]ATP with or without cGMP. (B and D) COS-7 cells were transiently transfected with FLAG-rhotekin together with FLAG-cGK-Iα. After 24 h, cells were treated with 100 μM 8-pCPT-cGMP for 1 h. The cell lysates were analyzed by immunoblotting using anti-phospho RRXS/T or anti-FLAG antibodies.

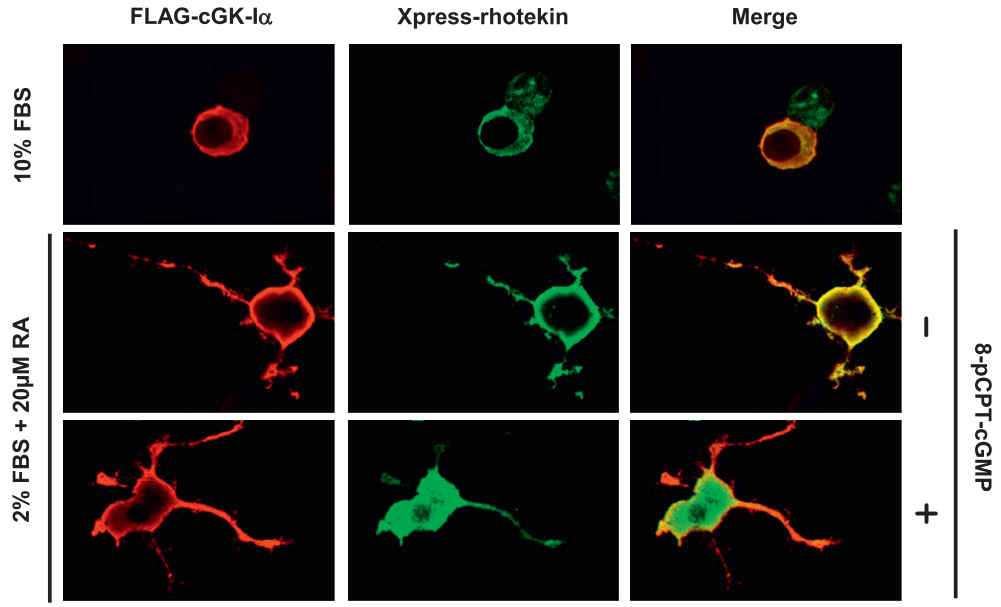


**Fig. 3.** Subcellular localizations of cGK-Iα and rhotekin in Neuro2A cells. pFLAG-cGK-Iα was co-transfected with pXpress-rhotekin into Neuro2A cells. The cells were cultured in 10% FBS or 2% FBS containing 20 μM RA for 48 h. After treatment with 100 μM 8-pCPT-cGMP for 1 h, cells were fixed and incubated with mouse anti-FLAG and rabbit anti-Xpress antibodies. The primary antibody was visualized with FITC-conjugated anti-mouse IgG or Texas Red-conjugated anti-rabbit IgG, followed by fluorescence microscopy. FLAG-cGK-Iα (red) and Xpress-rhotekin (green) is shown with the merged images (merge is in yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rhotekin, were treated with LPA. Stimulation by LPA led to a significant reduction in the average neurite length (Fig. 4A) and in the number of neurite-bearing neurons per culture (Fig. 4B). When cGK-Iα-expressing Neuro2A cells were pretreated with 8-pCPT-cGMP, LPA-induced neurite retraction was significantly suppressed. Cotransfection with rhotekin synergistically inhibited LPA-induced neurite retraction and returned to the control levels in the neurite length and the number of neurite-bearing neurons. We also examined the effect of rhotekin phosphorylation by cGK-Iα on RhoA-driven neurite retraction in Neuro2A cells. A phos-

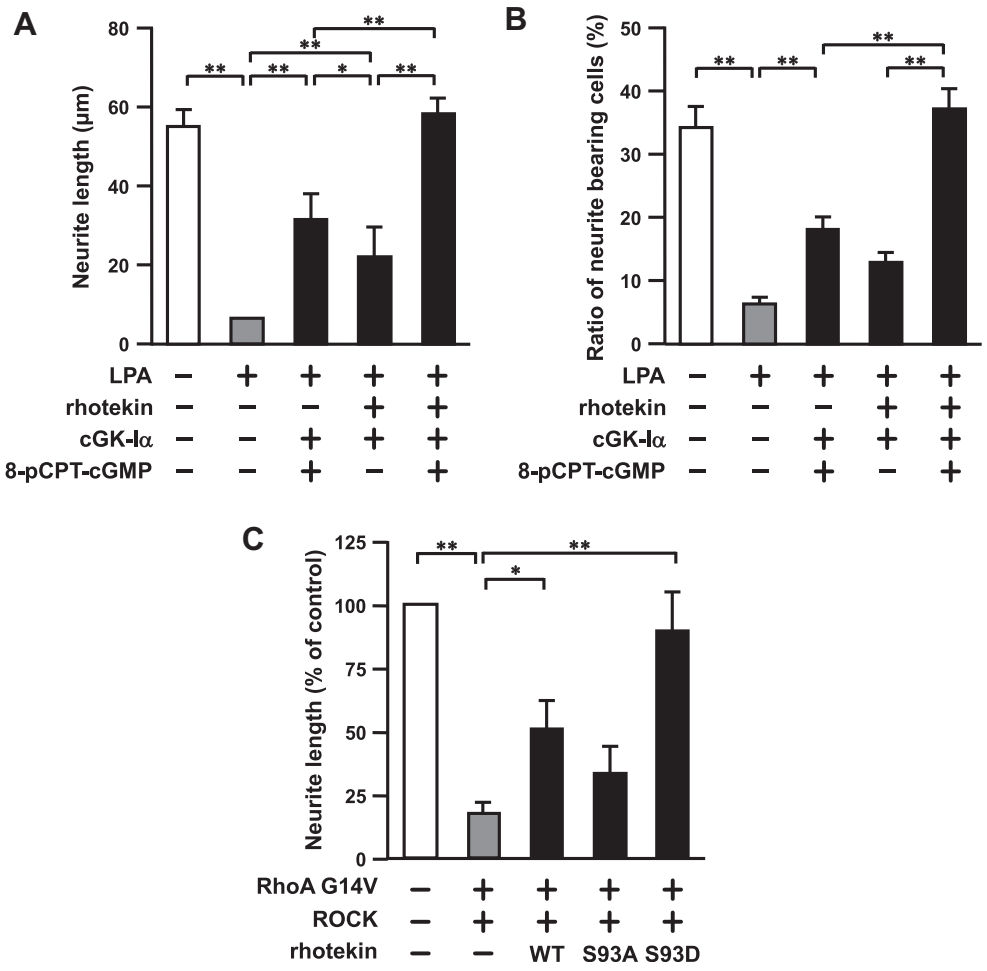


**Fig. 4.** cGK-I suppresses RhoA-induced retraction via rhotekin in Neuro2A cells. (A and B) Neuro2A cells were transfected with pFLAG-cGK-Iα and pFLAG-rhotekin together with pEGFP. After incubation in a medium containing 2% FBS and 20 μM RA for 24 h, cells were exposed to 20 μM LPA for 20 min with or without pretreatment of 100 μM 8-pCPT-cGMP. The morphology of cells was observed under a microscope. Neurite lengths were measured in randomly chosen fields of each dish (A). The cells bearing neurites longer than 1 diameter of cell soma were counted as differentiated cells (B). (C) Neuro2A cells were transfected by rhotekin S93D in combination with the constitutively active RhoA (RhoA G14 V) and ROCK together with pEGFP. After treatment with RA, neurite lengths were measured in randomly chosen fields. Values are expressed as means ± S.E. Statistical significance was determined by Student's t-test. $^{*}P < 0.05$, $^{**}P < 0.01$.

pho-mimic rhotekin mutant (rhotekin S93D) in which serine 93 was replaced by aspartic acid, was transfected along with constitutively active RhoA (RhoA G14 V) and ROCK into Neuro2A cells, and the neurite outgrowth was induced by RA treatment. As shown in Fig. 4C, overexpression of constitutively active RhoA and ROCK resulted in significant retraction of the neurites that formed after RA treatment. Cotransfection with wild-type rhotekin significantly, but modestly, suppressed RhoA-induced neurite retraction. The phospho-mimic rhotekin mutant S93D inhibited RhoA-induced neurite retraction, which returned to the control level. These results suggest that cGMP/cGK-Iα regulates neurite outgrowth by phosphorylating rhotekin.

## 4. Discussion

Several studies have demonstrated that cGK-I regulates neurite formation [1–4], although the detailed mechanism remains unclear. In this study, rhotekin, a Rho effector, was identified as a cGK-Iα-interacting protein by yeast two-hybrid screening. Rhotekin is classified together with rhophilin and protein kinase N, which have class I Rho-binding motifs. Rhotekin has been suggested to capture the active form of Rho and inhibit Rho GTPase hydrolysis [13]. A number of cGK-interacting proteins have been identified, and most of these proteins are phosphorylated by cGK [17,18]. Rhotekin is also phosphorylated by cGK-I isoforms at serine 93. Interestingly, a pull-down assay revealed that cGMP induced the release of cGK-I isoforms from rhotekin. Furthermore, although cGK-Iα and rhotekin were colocalized in the plasma membrane and extended neurites in neurite-extended Neuro2A cells, treatment with cGMP resulted in translocation of rhotekin to the cytoplasm. Because cGMP binding to cGK-I has been shown to produce distinct conformational changes in the enzyme [14], the stability of the interaction between cGK-Is and rhotekin might be dependent on rhotekin phosphorylation and/or cGK-I conformational changes. In addition, cGK-Iβ binds more tightly to rhotekin than cGK-Iα. Previous studies indicated that cGK-Iα is slightly autophosphorylated and activated in the absence of cGMP, compared with cGK-Iβ [19,20]. Thus, the interaction between cGK-Is and rhotekin appears to be affected by the activation state of cGK-I.

RhoA belongs to the Rho GTPase protein family, whose members are key regulators of the actin cytoskeleton and play essential roles in orchestrating the development and remodeling of synapses [7,8]. Although RhoA is primarily regulated by GEFs and GAPs, RhoA phosphorylation also regulates the activity of RhoA. RhoA has been shown to be phosphorylated and inactivated by cGK-I

[21]. However, most cellular RhoA is found in the cytosol and complexed with a GDP dissociation inhibitor (GDI). Because its phosphorylation site is masked by GDI, membrane-bound RhoA, which is presumably in a GTP state and free from GDI, can be phosphorylated [22]. A previous study demonstrated that Rho interaction with rhoGAP was inhibited by the Rho-binding domain of rhotekin [10]. A more recent study demonstrated that rhotekin phosphorylation at serine 435 by protein kinase D modulated the anchoring of RhoA in the plasma membrane, which resulted in increased RhoA activity [23]. In contrast, sodium nitroprusside, an NO donor, caused the translocation of activated RhoA from the membrane to the cytosol [24]. We also found that cGMP treatment resulted in the translocation of rhotekin from the membrane to the cytoplasm. Thus, cGK-I might regulate RhoA activity directly via phosphorylation and indirectly via the rhotekin pathway.

In conclusion, the results of this study demonstrated that cGK-I isoforms interacted with rhotekin. Site-directed mutagenesis analysis revealed that cGK-I phosphorylated rhotekin at serine 93. cGK-I was colocalized with rhotekin in the plasma membrane and extended neurites, and suppressed LPA-stimulated neurite retraction by phosphorylating rhotekin. These observations suggest that rhotekin is one of the targets of cGMP/cGK signaling during neurite outgrowth. Further investigations will attempt to determine the molecular mechanisms underlying cGMP/cGK-I-mediated neurite formation.

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