Enzymological Analysis of Mutant Protein Kinase Cγ Causing Spinocerebellar Ataxia Type 14 and Dysfunction in Ca^{2+} Homeostasis

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Spinocerebellar ataxia type 14 (SCA14) is an autosomal dominant neurodegenerative disease caused by mutations in protein kinase Cγ (PKCγ). Interestingly, 18 of 22 mutations are concentrated in the C1 domain, which binds diacylglycerol and is necessary for translocation and regulation of PKCγ kinase activity. To determine the effect of these mutations on PKCγ function and the pathology of SCA14, we investigated the enzymological properties of the mutant PKCγs. We found that wild-type PKCγ, but not C1 domain mutants, inhibits Ca^{2+} influx in response to muscarinic receptor stimulation. The sustained Ca^{2+} influx induced by muscarinic receptor ligation caused prolonged membrane localization of mutant PKCγ. Pharmacological experiments showed that canonical transient receptor potential (TRPC) channels are responsible for the Ca^{2+} influx regulated by PKCγ. Although in vitro kinase assays revealed that most C1 domain mutants are constitutively active, they could not phosphorylate TRPC3 channels in vivo. Single molecule observation by the total internal reflection fluorescence microscopy revealed that the membrane residence time of mutant PKCγs was significantly shorter than that of the wild-type. This fact indicated that, although membrane association of the C1 domain mutants was apparently prolonged, these mutants have a reduced ability to bind diacylglycerol and be retained on the plasma membrane. As a result, they fail to phosphorylate TRPC channels, resulting in sustained Ca^{2+} entry. Such an alteration in Ca^{2+} homeostasis and Ca^{2+}-mediated signaling in Purkinje cells may contribute to the neurodegeneration characteristic of SCA14.

Autosomal dominant SCA14 is a genetically heterogenous group of neurodegenerative disorders characterized by progressive motor incoordination affecting the gait and limbs, cerebellar dysarthria, and nystagmus due to degeneration of cerebellar Purkinje cells. SCA14 is caused by missense or in-frame deletion mutations in the PRKCG gene encoding protein kinase Cγ (PKCγ) (1). PKCγ is a member of the PKC family that plays critical roles in many cellular functions, affecting diverse signal transduction pathways (2). PKCγ is selectively expressed in neurons throughout the brain and is most abundant in cerebellar Purkinje cells (3), which specifically degenerate in SCA14 patients.

One of the characteristic features of PKCγ is its translocation from the cytoplasm to the plasma membrane (4). Translocation is a hallmark of enzyme activation and is triggered by the stimulation of G protein-coupled receptors. It is well known that activation of such receptors causes elevations of DAG and intracellular Ca^{2+} (5). PKCγ contains C1 and C2 domains in its regulatory domain (6). The C1 domain has two zinc-finger motifs, C1A and C1B, that contain highly conserved Cys residues that bind to diacylglycerol (DAG) and tumor promoting phorbol esters. The C2 domain is a Ca^{2+} sensor that binds phosphatidylserine (PS) in the presence of elevated Ca^{2+}. The C1 and C2 domains play crucial roles in PKCγ translocation through binding to DAG and Ca^{2+}, respectively.

The pathogenesis of SCA14 is not well understood. PKCγ knock-out mice exhibit persistent cerebellar multiple climbing fiber innervation and a slight ataxia. However, they do not have cerebellar degeneration (7, 8). Thus, gain-of-function, rather than loss-of-function, of PKCγ may be responsible for the cerebellar neurodegeneration in SCA14. Previously, we have reported that mutant PKCγs aggregate and cause cell death of several cultured cell systems (9, 10). However, the aggregation was not observed in the Purkinje cells of a SCA14 patient who carried the H101Y mutation (1). This suggests that not only the...
aggregated, but also the soluble, fraction of mutant PKCγ may contribute to the pathogenesis of SCA14.

To date, 22 different PKCγ mutations have been found in SCA14 families, 18 of which map to the C1 domain (11–16). This fact strongly suggests that these mutations disturb a fundamental property of PKCγ including membrane translocation and activator-dependent regulation of its kinase activity. In the present study, we tested whether these C1 domain mutations influence membrane translocation and activation of PKCγ. Our results show that C1 domain mutants have a shorter residence time on the plasma membrane. This results in decreased phosphorylation of the TRPC3 channel and alters Ca\(^{2+}\) influx. Our results suggest that alteration in Ca\(^{2+}\) homeostasis induced by mutant PKCγ may contribute to SCA14.

**EXPERIMENTAL PROCEDURES**

**Materials**—12-O-Tetradecanoylphorbol 13-acetate (TPA), anti-FLAG M2 monoclonal antibody, and anti-FLAG M2 Affinity Gel Freezer-Safe were purchased from Sigma. [γ-\(^{32}\)P]ATP and monosodium \(^{32}\)Pphosphate were products of MP Biomedicals, Inc. (Irvine, CA). Carbocyl (Cch), GF 190203X, SKF-96395, and nifedipine were purchased from Calbiochem. 2-aminoethoxydiphenyl borate was obtained from Alexis Biochemicals (San Diego, CA). SEA0400 was a gift from Dr. Toshio Matsuda, Osaka University. The following antibodies were purchased: anti-cPKCγ (C-19) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-GFP polyclonal antibody (Molecular Probes, Leiden, Netherlands), peroxidase-conjugated AffiniPure goat anti-mouse IgG and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). All the other chemicals used were of analytical grade.

**Plasmid Constructions**—Wild-type and mutant PKCγs were cloned into pcDNA3 with GFP or DsRed monomer (DsRed) as described previously (9). All mutant PKCγ cDNAs were verified by sequencing. For cDNA cloning of human TRPC3, total RNA was reverse-transcribed using random primers and the ThermoscriptTM RT-PCR System (Invitrogen). PCR primers were designed based on a published nucleotide sequence of human TRPC3 (GenBankTM accession number NM_003305). Amplified TRPC3 gene was cloned into p3XFLAG-CMV-10 (Sigma). TRPC3 cDNA was verified by sequencing.

**Cell Culture and Transfection**—COS-7, SH-SY5Y, and HEK-293 cells were purchased from the Riken Cell Bank (Tsukuba, Japan). CHO cells expressing human muscarinic acetylcholine receptor 1 (CHOhm1) were a gift from Dr. Tatsuya Haga. COS-7 and HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (Wako, Osaka, Japan), SH-SY5Y cells were maintained in Dulbecco’s modified Eagle’s medium/ Ham’s F-12 medium (Invitrogen), and CHOhm1 cells were cultured in minimum essential medium α medium (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO\(_2\). All media contained 10% fetal bovine serum, penicillin (500 units/ml), and streptomycin (500 μg/ml). For SH-SY5Y cells, 1% GlutaMAXTM-I (Invitrogen) was added. Transient expression in COS-7 cells was performed by electroporation (4), and SH-SY5Y, HEK-293, and CHOhm1 cells were transfected by lipofection using FuGENETM 6 Transfection Reagent (Roche Applied Science) as described previously (18). SF9 cells were cultured in EX-CELL TM 420 (JRH Biosciences) containing 10% fetal bovine serum and 0.5% antibiotic/antimycotic (Invitrogen) in a temperature-controlled orbital shaker operating at 150 rpm at 27.5 °C.

**Observation of PKCγ Translocation**—The culture medium of CHOhm1 cells expressing wild-type or mutant PKCγ-GFPs (or PKCγ-DsReds) was replaced with Ringer’s solution (135 mM NaCl, 5.4 mM KCl, 1 mM MgCl\(_2\), 5 mM HEPES pH 7.35, 10 mM glucose). CaCl\(_2\) was added to the appropriate Ca\(^{2+}\) concentration depending on the experimental conditions. The fluorescence of the GFP was monitored with a confocal laser scanning fluorescent microscope (LSM510, Carl Zeiss, Jena, Germany) at 488-nm argon excitation with a 515–535-nm band pass barrier filter, DsRed was excited with a 543-nm HeNe laser and detected using a 560-nm long pass filter. All experiments were done at 37 °C. Image analysis was performed using the Zeiss LSM 510 software, and the membrane fluorescence ratio was calculated as described previously (19).

**Intracellular Ca\(^{2+}\) Measurement**—CHOhm1 cells expressing wild-type or mutant PKCγ-DsReds were incubated with 4 μM Fluo-4 (Dojindo, Kumamoto, Japan) and 0.02% pluronic F-127 (Dojindo) in Ringer’s solution containing 2 mM EGTA for 25 min at room temperature. Fluo-4 was loaded into SH-SY5Y and HEK-293 cells in Ringer’s solution containing 2 mM Ca\(^{2+}\) (20 min, 37 °C). The cells were then washed twice with Ca\(^{2+}\)-free Ringer’s solution. The fluorescence of Fluo-4 was monitored by confocal microscopy. The time course of Ca\(^{2+}\) measurement was recorded using the Zeiss LSM 510 software. Fluorescence changes (ΔF/F\(_0\)) within the cytoplasm of the cells were quantified as the fraction of the evoked change in Fluo-4 Ca\(^{2+}\) signal divided by the resting fluorescence level in Ca\(^{2+}\) free buffer recorded for 30 s prior to drug application. All cells within the plane of focus that had a change in Ca\(^{2+}\) signal were analyzed. All experiments were done at room temperature.

**In Vitro PKC Kinase Assay**—COS-7 cells expressing wild-type or mutant PKCγ-GFPs were harvested and only the supernatant fraction of the recombinant PKC was used for the PKC kinase assay. Briefly, the cells were centrifuged, and resus-

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**FIGURE 1.** Schematic illustrations of PKCγ protein and the mutated amino acid found in SCA14 families. Twenty-one point mutations and one deletion of SCA14 are listed in the boxes above and below the diagram. With the exception of V692G, all mutations are located in conserved regions and are concentrated in the C1B domain. The numbers below represent the position of amino acids.
pended in 400 μl of homogenization buffer (250 mM sucrose, 10 mM EGTA, 20 mM Tris-HCl, 200 μg/ml leupeptin, 1 mM phenylmethysulfonyl fluoride, pH 7.4). After sonication, samples were centrifuged (200,000 × g for 15 min at 4 °C), and the supernatant was collected. To equalize the amount of PKCγ-GFP or its mutant proteins, GFP fluorescence intensity of the supernatants were measured by Mithras LB 940 (Berthold Technologies, Tokyo, Japan). For immunoprecipitation of PKCγ-GFP, an equivalent amount of GFP from each sample was rotated with anti-GFP antibody (1 h at 4 °C) and then precipitated with Protein A-Sepharose for an additional 1 h. The beads were collected and washed five times with phosphate-buffered saline (−). Finally, 10 μl of suspended pellet was used for kinase assay as described previously (4). Briefly, the kinase activity was assayed by measuring the incorporation of 32P into myelin basic protein from [γ-32P]ATP in the absence of activators (20 mM Tris-HCl and 0.25 mM EGTA only) or in the presence of 8 μg/ml PS (Sigma), 0.8 μg/ml (±)-1,2-didecanoylglycerol (DiC10, BIOMOL, Plymouth Meeting, PA), and 50 μM Ca2+.

In Vitro and in Vivo Phosphorylation of TRPC3—COS-7 cells expressing FLAG-TRPC3 were lysed in 400 μl of homogenization buffer (20 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 200 μg/ml leupeptin, 1 mM phenylmethysulfonyl fluoride, pH 7.4). Samples were centrifuged (200,000 × g for 15 min at 4 °C) and the supernatants were immunoprecipitated with anti-FLAG M2 Affinity Gel for 2 h at 4 °C and the pellet washed 4 times. Recombinant GST and GST-PKCγ were purified from BL21(DE3) pLys cells and SF9 cells, respectively, using glutathione-Sepharose 4B (Amersham Bioscience). Purified FLAG-TRPC3 on beads was incubated with purified GST (0.2 μg) or GST-PKCγ (0.2 μg) in the presence of 8 μg/ml PS, 0.8 μg/ml DiC10, and 500 μM Ca2+ with or without 1 mM GF 109203X for 15 min at 30 °C.

For in vivo TRPC3 phosphorylation, COS-7 cells co-expressing wild-type or mutant PKCγ-GFP and FLAG-TRPC3 were labeled for 2 h with [γ-32P]phosphoric acid (0.33 mCi/ml) in Ringer’s solution containing 2 mM Ca2+ for 2 h at 4 °C and the pellet washed 4 times. Recombinant GST and GST-PKCγ were purified from BL21(DE3) pLys cells and SF9 cells, respectively, using glutathione-Sepharose 4B (Amersham Bioscience). Purified FLAG-TRPC3 on beads was incubated with purified GST (0.2 μg) or GST-PKCγ (0.2 μg) in the presence of 8 μg/ml PS, 0.8 μg/ml DiC10, and 500 μM Ca2+ with or without 1 mM GF 109203X for 15 min at 30 °C.

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For in vivo TRPC3 phosphorylation, COS-7 cells co-expressing wild-type or mutant PKCγ-GFP and FLAG-TRPC3 were labeled for 2 h with [γ-32P]phosphoric acid (0.33 mCi/ml) in Ringer’s solution containing 2 mM Ca2+. After extensively washing, the cells were incubated in Ringer’s solution containing 2 mM Ca2+ with or without 200 nM TPA for 10 min. Cells were then harvested and lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 2 mM EDTA, 150 mM NaCl, 200 μg/ml leupeptin, 1 mM phenylmethysulfonyl fluoride, 1 mM Na3VO4, 1 μM okadaic acid, 1 mM sodium fluoride, 1 μg/ml pepstatin, and 20 mM β-glycerophosphate) followed by sonication. After centrifugation, the pellets were solubilized in Lysis buffer containing 1% Triton X-100, sonicated, and centrifuged. The supernatant was collected and immunoprecipitated with anti-FLAG M2 Affinity Gel. SDS-PAGE and immunoblot analysis were performed as described (18). The
phosphoproteins were detected by autoradiography using BAS2500 (FUJI Film).

Single-molecule Imaging of PKCγ-GFP in Living CHOhm1 Cells—The apparatus, data acquisition, and data analysis will be described in detail. Briefly, individual PKCγ-GFP molecules in the plasma membrane of CHOhm1 cells were visualized using an objective-type total internal reflection microscope constructed on an inverted fluorescence microscope (IX81, Olympus, Japan) (20, 21). Cells were illuminated with 488 nm light from a blue laser (Sapphire 488; Coherent, Japan) using a ×100 objective (PlanApo, NA = 1.45; Olympus, Japan). Fluorescence images were intensified with image intensifier (C8600; Hamamatsu Photonics, Japan) and obtained at video rate with an electron bombardment CCD camera (C7190-23; Hamamatsu Photonics). The fluorescent spots were tracked and analyzed to determine the residence time of PKCγ molecules on the plasma membrane.

RESULTS

SCA14 Mutant PKCγ Exhibits Prolonged Membrane Localization—As 18 of 22 mutations in SCA14 families are located in the C1 domain of PKCγ (Fig. 1) and the C1 domain is responsible for membrane binding in PKC translocation, we first examined whether these C1 domain mutations affect the membrane targeting of PKCγ. CHOhm1 cells expressing wild-type or mutant PKCγ-DsRed (H101Y, G118D, S119P, Q127R, and G128D) were stimulated with Cch and membrane translocation assessed by confocal microscopy. Both wild-type and mutant PKCγ-DsRed translocated in response to Cch (supplemental Fig. S1). However, whereas wild-type PKCγ rapidly returned (“retranslocated”) to the cytoplasm, retranslocation was delayed in the C1 domain mutants. To assess the temporal differences in re-translocation between wild-type and mutant PKCγ, wild-type PKCγ-DsRed and GFP-conjugated PKCγ carrying the G128D mutation were independently expressed in CHOhm1 cells and simultaneously observed (Fig. 2, A and B). Both wild-type and mutant PKCγ rapidly translocated to the plasma membrane in response to Cch. However, whereas re-translocation of wild-type PKCγ was complete by 80 s, membrane levels of the G128D mutant were still maximal. In fact, this prolonged membrane localization continued for at least 90 s; in some cases, it remained more than 3 min after stimulation (data not shown).

Extracellular Ca2+ Is Responsible for Prolonged Membrane Localization of Mutant PKCγ—We have evidence that C1B peptides carrying SCA14 mutations (H101Y, S119P, Q127R, and V138E) have a decreased affinity for phorbol esters (PDBu). Not surprisingly, the G128D mutation also exhibited lower phorbol 12,13-dibutyrate binding (data not shown). Thus, the affinity of the G128D mutant for endogenous DAG is also likely to be attenuated by mutations in the C1B domain. How then, are the mutants retained at the membrane? We tested the hypothesis that one of the other translocation factors, namely Ca2+, is involved in retaining the C1 domain mutants at the plasma membrane. Indeed, addition of the Ca2+ chelator, EGTA, to the extracellular solution resulted in immediate retranslocation of G128D mutant PKCγ-DsRed (Fig. 2, A and B). In addition, in Ca2+-free Ringer’s solution, although G128D mutant exhibited a slight delay (about 10 s) in re-translocation compared with wild-type, both enzymes returned to the cytoplasm within 60 s (Fig. 2, C and D). These results indicate that extracellular Ca2+ is responsible for the prolonged membrane localization of C1 domain mutant PKCγ.

The Kinase Activity of PKCγ Is Required for Negative Regulation of Ca2+ Entry—We then investigated the effect of overexpression of PKCγ on Cch-induced Ca2+ entry in CHOhm1 cells. Ca2+ mobilization was assessed in Fluo4-loaded cells expressing wild-type PKCγ-DsRed or unconjugated DsRed as a

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control. As shown in Fig. 3A, Cch induced a rapid elevation of intracellular Ca\(^{2+}\) (\([\text{Ca}^{2+}]_i\)) in both cells. However, the kinetics of the Ca\(^{2+}\) decrease in the cells expressing PKC\(\gamma\)-DsRed was distinct from DsRed. Specifically, the 1/2 [Ca\(^{2+}\)] of PKC\(\gamma\)-DsRed was 28.3 ± 5.4 s compared with 70.3 ± 8.6 s in DsRed-expressing cells. In addition, in Ca\(^{2+}\)-free solution, the kinetics of Ca\(^{2+}\) decrease for PKC\(\gamma\)-DsRed and DsRed were identical (Fig. 3B). This data suggests that PKC\(\gamma\) modulates [Ca\(^{2+}\)]

To investigate whether catalytic activity is required for the negative regulation of Cch-induced Ca\(^{2+}\) entry, we examined the effect of PKC inhibitor, GF 109203X, on cells expressing wild-type PKC\(\gamma\)-DsRed or DsRed. The 1/2 [Ca\(^{2+}\)] of PKC\(\gamma\)-DsRed was prolonged in a dose-dependent manner in the presence of GF109203X (Fig. 3C). Moreover, the 1/2 [Ca\(^{2+}\)] for cells expressing a kinase-negative mutant (K380M) was significantly longer than that of wild-type in Ringer’s solution containing Ca\(^{2+}\) (Fig. 3D). These results suggest that kinase activity of PKC\(\gamma\) is necessary for a rapid Ca\(^{2+}\) decay in response to Cch.

**C1 Domain Mutants Cannot Inhibit Ca\(^{2+}\) Entry**—We then examined the effect of 17 missense mutations and one deletion mutation on the regulation of [Ca\(^{2+}\)]

domain mutants, we also observed translocation (supplemental Fig. S1), regulation of [Ca\(^{2+}\)], (Fig. 4A), and kinase activity (Fig. 4B). The V692G mutant was indistinguishable from wild-type. On the other hand, the inhibitory effect of Ca\(^{2+}\) entry in cells expressing constitutively active S361G and F643L mutants was exaggerated compared with wild-type (supplemental Fig. S2). Interestingly, the kinase negative G360S mutant exhibited prolonged membrane localization and sustained [Ca\(^{2+}\)] similar to the C1 domain mutants. These data suggest that in addition to disruption of the C1 domain, a decrease or increase in its kinase activity ablates Cch-induced Ca\(^{2+}\) regulation in CHOhm1 cells.

**PKC\(\gamma\) Negatively Regulates TRPC Channels in Response to Cch**—Cch induces Ca\(^{2+}\) influx through receptor-mediated Ca\(^{2+}\) channels such as TRPC channels (22). Therefore, to test the hypothesis that PKC\(\gamma\) modulates TRPC channel activity to inhibit the Cch-induced Ca\(^{2+}\) entry, we examined the effect of various Ca\(^{2+}\) channel inhibitors on Ca\(^{2+}\) levels in cells expressing wild-type or G128D mutant PKC\(\gamma\)-DsRed. As shown in Fig. 5A, the 1/2 [Ca\(^{2+}\)] of G128D mutant was significantly longer than that of wild-type. Treatment with the TRPC channel inhibitors 2-aminoethoxydiphenyl borate and SKF-96395 completely abolished the excessive Ca\(^{2+}\) entry in cells expressing the G128D mutant. Conversely, blocking the Na\(^+\)/Ca\(^{2+}\) exchanger or the L-type Ca\(^{2+}\) channel with nifedipine and
flag-TRPC3 and wild-type PKCγ-GFP, 200 nM TPA significantly enhanced the phosphorylation of FLAG-TRPC3, an increase of 1.89 ± 0.14-fold in PKCγ overexpressing cells (Fig. 6, B and C). On the other hand, in COS-7 cells co-expressing FLAG-TRPC3 and constitutively active G128D mutant PKCγ-GFP, FLAG-TRPC3 was significantly less phosphorylated, exhibiting an increase only of 1.33 ± 0.25 in response to TPA, which was not significantly different from the GFP control. Similar results were obtained when another constitutively active C1 domain mutant (H101Y) was used (data not shown). These results suggest that C1 domain mutants cannot phosphorylate endogenous substrates, despite their high catalytic activity.

C1 Domain Mutant Decreases the Residence Time of PKCγ on the Plasma Membrane—Inaccessibility of PKCγ to the substrate may explain why the constitutively active C1 domain mutant PKCγ do not phosphorylate TRPC3 channels in the cells. As C1B domain mutations have a decreased affinity for membrane lipids,4 we used total internal reflection fluorescence (TIRF) microscopy to determine how long wild-type and mutant PKCγ-GFP (H101Y, G128D, and G360S) stay on the plasma membrane.

SEA0400, respectively, had no effect. These results suggest that the Cch-induced change in [Ca2+]i, is mediated by TRPC channels. We next examined the effect of PKCγ on the Cch-induced Ca2+ response in HEK-293 and human neuroblastoma SH-SY5Y cells, that endogenously express muscarinic receptors. Similarly to CHO9m1 cells, wild-type, but not the G128D mutant, negatively regulated Ca2+ influx in both cell types (Fig. 5B). This result suggests that the negative regulation of [Ca2+]i by PKCγ likely occurs in neurons that endogenously express muscarinic receptors. Interestingly, although much of the data on Ca2+ responses in HEK-293 cells were similar to CHO9m1 and SH-SY5Y cells, Ca2+ oscillations were frequently observed in cells expressing wild-type but not the G128D mutant (data not shown). Taken together, these results suggest that Ca2+ influx regulated by PKCγ likely depends on plasma membrane Ca2+ channels, especially TRPC channels. However, the G128D mutant lacks the ability to regulate these channels.

A C1 Domain Mutant Fails to Phosphorylate TRPC3 Channel in Vivo—Among the TRPC subfamily, the TRPC3 channel is expressed predominantly in the brain, especially cerebellar Purkinje cells (23, 24), which is rich in PKCγ. In addition, both TRPC3 channels and PKCγ are activated by DAG (25), suggesting that PKCγ could phosphorylate the TRPC3 channel at the plasma membrane of Purkinje cells. In fact, PKCα has been reported to phosphorylate TRPC3 channels in vitro and in vivo (26). We first investigated whether the TRPC3 channel can be phosphorylated by PKCγ in vitro. As shown in Fig. 6A, significant phosphorylation of FLAG-TRPC3 by GST-PKCγ occurred in the presence of PS, DiC10, and Ca2+ and phosphorylation was abolished by the PKC inhibitor GF 109203X. Thus, PKCγ can directly phosphorylate TRPC3 channels in vivo. We next investigated whether wild-type and the G128D PKCγ could phosphorylate TRPC3 channels in intact cells. For this purpose, we first stimulated the CHO1m1 cells co-expressing PKCγ-GFP and FLAG-TRPC3 with Cch. However, we failed to detect significant phosphorylation of TRPC3 by wild-type PKCγ, possibly due to the transient nature of its association with membranes. Indeed, the translocation of wild-type PKCγ elicited by Cch occurs transiently (Fig. 2A). Therefore, TPA was used as it induces irreversible translocation of wild-type PKCγ to the plasma membrane and maximally stimulates PKCγ (4). In COS-7 cells co-expressing
When we imaged the ventral surface of the CHOhm1 cells expressing PKCγ-GFP using TIRF microscopy and a highly sensitive imaging system at the video rate (20, 27), many fluorescent spots appeared on the plasma membrane (supplementary videos 1–4). We could not observe such spots in the mock-treated control cells. Because the distribution of their fluorescence intensity exhibited a single peak and the peak position of the distribution is slightly lower than that for recombinant GFP attached on the coverslip, or for GFP-tagged Ras sensitively imaged at the single molecule level (27) (supplementary Fig. S4), we concluded that each spot mostly represents a single molecule of PKCγ-GFP.

Cells were stimulated with TPA to analyze membrane targeting through the C1 domain. In response to TPA, a single molecule of wild-type appeared at the plasma membrane and moved around in the plane of the membrane for more than 20 s. The residence time of wild-type on the membrane averaged 22.1 s (Fig. 7, left column, supplementary Videos 1–4). In contrast, the residence time of the H101Y and G128D C1 domain mutants was dramatically short (3.0 and 1.3 s, respectively). On the other hand, the G360S mutant, which has an intact C1 domain, was indistinguishable from wild type. We also measured the residence time of the C1 domain mutant in response to Cch. Compared with wild-type PKCγ, the H101Y and G128D mutants had significantly shorter residence times (~60% of wild type) (Fig. 7, right column), suggesting that C1 domain mutants do not stably associate with their physiological binding partner DAG. The result of this shortened membrane retention time is decreased phosphorylation of PKCγ substrates, such as TRPC3 channels, on the plasma membrane.

DISCUSSION

SCA14 is inherited as autosomal dominant and PKCγ-deficient mice do not exhibit atrophy of the cerebellum or loss of Purkinje cells (8). Therefore, we considered that gain-of-function, rather than loss-of-function, of mutant PKCγ causes neurodegeneration in SCA14. Previously, we demonstrated that, similar to other neurodegenerative disorders, the mutant PKCγ found in SCA14 tends to aggregate and cause cell death (9). However, although immunoreactivity of PKCγ was reduced, the aggregation was not observed in Purkinje cells of an autopsy of a SCA14 patient who carried the H101Y mutation (1). Therefore, we suspected the gain-of-toxic function of the soluble protein in addition to the aggregation that mutant PKCγ may contribute to neurodegeneration in SCA14.

In the present study, we have shown that expression of C1 domain mutants of PKCγ results in sustained Ca2+ influx into the cells due to a decrease in membrane residence time. In addition, cells expressing the G360S mutant PKCγ also caused sustained Ca2+ influx resulting from its kinase inactivity. Wild-type PKCγ negatively regulated Cch-induced Ca2+ entry via phosphorylation of TRPC3 channels. For TRPC3 phosphorylation, both C1 domain-mediated membrane binding and kinase activity of PKCγ are indispensable.

On the other hand, cells expressing S361G and F643L PKCγ with an intact C1 domain and a high kinase activity, were able to suppress Ca2+ entry after physiological stimulation. Interestingly, this inhibitory effect was exaggerated compared with wild-type and Ca2+ influx was completely blocked. This finding suggests that a constitutively active PKCγ with a functional C1 domain may cause hyperphosphorylation of substrates on the plasma membrane. Thus, not only the sustained Ca2+ influx but also inhibition of Ca2+ entry might alter intracellular Ca2+ homeostasis in SCA14 patients (Fig. 8).

By confocal microscopy, TPA and the Ca2+ ionophore induced similar PKCγ localization at the plasma membrane (4, 28). However, electron microscopy revealed that PKCγ has similar, but significantly different, localization depending on stimulation. Whereas DAG or TPA tightly anchored this
enzyme on the plasma membrane, Ca$^{2+}$ ionophore resulted in the accumulation of PKC$\gamma$ to a submembrane region <500 nm from the plasma membrane (28). Thus, we, in these studies, employed TIRF microscopy, which is generally able to visualize the PKC$\gamma$ within 100–150 nm of the plasma membrane. TIRF provides a readout of events occurring essentially at the membrane. These studies revealed that the C1B mutants are at the plasma membrane for significantly less time that wild-type. This results in decreased signaling as measured by the lower phosphorylation of the TRPC3 channels.

PKC$\gamma$ has two DAG binding regions, C1A and C1B, and it is reported that both bind equally to DAG or phorbol ester (29). Although SCA14 mutations affect only one region of the C1 domain, the membrane residence time of mutant PKC$\gamma$s induced by Cch or TPA were significantly shorter than that of wild-type. These results suggest that both C1A and C1B contribute to the stable association of PKC$\gamma$ with membranes; mutations in either decrease membrane residence time.

The data in Fig. 2A suggested that C1 domain mutants stay longer on the plasma membrane than wild-type. Thus, we predicted increased substrate phosphorylation. However, this was not the case. Mutations in the C1 domain decrease PKC$\gamma$-DAG binding, membrane retention time, and substrate phosphorylation. Indeed, the G128D mutant could not phosphorylate TRPC3 on the plasma membrane. These results emphasize the importance of the C1 domain for membrane binding and substrate phosphorylation.

It is reported that of the 50 amino acids in the C1B domain in PKC$\gamma$, residues 101–143 mediate phorbol ester binding (30). Although C150F mutation is outside this core region, the C150F mutant could not control intracellular Ca$^{2+}$ concentrations and kinase activity. This indicates that C150F does not have a functional C1B domain and cannot associate with DAG. The substitution of phenylalanine for cysteine is thought to induce a conformational change in the C1B domain that inhibits DAG/phorbol ester binding. Increased kinase activity of the C150F mutant was also reported by Verbeek et al. (31). Moreover, they demonstrated that, in COS-7 cells, Ca$^{2+}$ ionophore induces more rapid translocation of the C150F mutant than wild-type PKC$\gamma$. We also observed that Ca$^{2+}$ ionophore induces more rapid and enhanced translocation of PKC$\gamma$s carrying the C1 domain mutation in the CHOhm1 cell (data not shown). PKC is known to enhance Ca$^{2+}$ efflux through phosphorylation of the plasma membrane Ca$^{2+}$-ATPases (32). As is the case with the lower phosphorylation of the TRPC3 channel by the G128D mutant, these C1 domain mutants may also fail to phosphorylate the plasma membrane Ca$^{2+}$-ATPases. Therefore, enhancement of the Ca$^{2+}$ efflux by PKC$\gamma$ may not occur in cells expressing C1 domain mutant, thus, an increase of intracellular Ca$^{2+}$ would induce strong translocation of mutant PKC$\gamma$.

In Purkinje cells, TRPC3 is the most abundantly expressed member of the TRPC family (24). However, its specific roles in the neurons are largely unknown. It has been reported that the mGluR-dependent slow excitatory postsynaptic currents require the activation of TRPC channels in Purkinje cells (33). PKC$\gamma$ also plays a key role in mGluR signaling. Therefore, the loss of inhibition of TRPC activity due to its decreased phosphorylation by mutant PKC$\gamma$ may indirectly activate slow excitatory postsynaptic currents. Interestingly, an ataxic phenotype is observed in various knock-out mice that are deficient in the mGluR cascade (e.g. mGlur1, G$q$, PLC$\beta$, inositol 1,4,5-trisphosphate receptor, and PKC$\gamma$) (34–37). Moreover, TRPC3 knock-out mice also exhibit abnormal motor coordination. Therefore, abnormal regulation of TRPC channels, as seen in cells expressing C1B mutants of PKC$\gamma$, may contribute to cerebellar dysfunction.

Although intracellular Ca$^{2+}$ signals are critical for the regulation of synaptic functions in the central nervous system, sustained high levels of intracellular Ca$^{2+}$ can be toxic for neurons. For example, dysregulation of intracellular Ca$^{2+}$ has been reported in aging brain and been implicated in vulnerability to

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A. Konnerth, personal communication.
Mutant PKCy and Ca\(^{2+}\) in SCA14

In summary (Fig. 8), we have studied the effects of SCA14 PKCy mutations on Cch-induced Ca\(^{2+}\) influx and TRPC channel phosphorylation. We have shown that the phosphorylation of TRPC channels is reduced in cells expressing the C1 domain mutant PKCy, resulting in sustained high levels of intracellular Ca\(^{2+}\) upon cell stimulation. Surprisingly, the mutant PKCy have higher kinase activity \textit{in vitro} but cannot stably associate with the plasma membrane. These results suggest that mutant PKCy fail to phosphorylate TRPC3 channels leading to sustained high levels of intracellular Ca\(^{2+}\) and aberrant intracellular signaling. Such an alteration in Ca\(^{2+}\) homeostasis and Ca\(^{2+}\)-mediated signaling in Purkinje cells may be responsible for the neurodegeneration characteristic of SCA14.

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