Cellular Signalling 23 (2011) 1179–1187

Contents lists available at ScienceDirect

Cellular Signalling

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

Functional regulation of transient receptor potential canonical 7 by cGMP-dependent protein kinase Iα

Keizo Yuasa *[,1], Taito Matsuda [1], Akihiko Tsuji

Department of Biological Science and Technology, The University of Tokushima Graduate School, 2–1 Minamijosanjima, Tokushima 770–8506, Japan

ARTICLE INFO

Article history:
Received 15 January 2011
Accepted 6 March 2011
Available online 11 March 2011

Keywords:
cGMP
cGK
TRPC7
Calcium influx
Interaction

ABSTRACT

The cGMP/cGMP-dependent protein kinase (cGK) signaling pathway is implicated in the functional regulation of intracellular calcium levels. In the present study, we investigated the regulation of transient receptor potential canonical 7 (TRPC7) by the cGMP/cGK-I pathway. TRPC7 contains three putative cGK phosphorylation sites (Arg-Arg/Lys-*Xaa*-Ser/Thr). However, the role of cGK-I in the regulation of TRPC7 activity remains unclear. *In vitro* and *in vivo* kinase assays have revealed that cGK-Iα phosphorylates mouse TRPC7 but not mouse TRPC3. Site-directed mutagenesis analysis revealed that TRPC7 was phosphorylated by cGK-Iα at threonine 15. Phosphorylation of TRPC7 significantly suppressed carbachol-induced calcium influx and CREB phosphorylation. Furthermore, co-immunoprecipitation assay demonstrated that cGK-Iα interacted with the ankyrin repeat domain in the N terminus of TRPC7. cGK-Iβ also bound to TRPC7, while the type II regulatory subunit of cAMP-dependent protein kinase did not bind. These data indicate that cGK-Iα interacts with and phosphorylates TRPC7, contributing to the quick and accurate regulation of calcium influx and CREB phosphorylation.

1. Introduction

cGMP is a second messenger synthesized by guanylate cyclases in response to nitric oxide (NO) and natriuretic peptides [1]. cGMP-dependent protein kinases (cGKs) are the major intracellular targets for cGMP. Two types of cGKs, cytosolic cGK-I and membrane-bound cGK-II, have been identified and are encoded by distinct genes in mammals [2]. 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. cGMP/cGK is known to regulate intracellular calcium levels via several pathways. For example, cGK-Iβ phosphorylates IP_3 receptor-associated cGMP kinase substrate (IRAG) and inhibits IP_3-induced Ca^{2+} release from intracellular stores [3]. Another mechanism that decreases intracellular calcium levels is the phosphorylation of large conductance Ca^{2+}-dependent K^+ (BK_{Ca}) channels by cGK I, which leads to increased opening of this channel [4]. Opening of BK_{Ca} channels hyperpolarizes the membrane and closes some channels, including L-type calcium channels, thereby reducing calcium influx. Furthermore, it has been recently reported that cGK-I inhibits calcium influx via inhibition of the canonical transient receptor potential channel (TRPC) family [5–8]. The TRPC family consists of seven members (designated TRPC1–7). TRPC3, 6, and 7 are activated by diacylglycerol (DAG), generated by G protein-coupled receptors/Gαq/phospholipase C signaling, whereas TRPC1, 4, and 5 are activated by depletion of intracellular calcium stores (store-operated calcium entry). TRPC3 and TRPC6 are phosphorylated by cGK-I, and phosphorylated channels suppress calcium influx [6,7]. On the other hand, cGK attenuates TRPC4-mediated calcium entry via phosphorylation of vasodilator-stimulated phosphoprotein at serine-239 but not direct phosphorylation of TRPC4 [8].

TRPC7 is most closely related to TRPC3 with 81% identity, and demonstrates 75% identity with TRPC6 in mice. The physiological importance of TRPC7 remains elusive. However, it has been suggested that TRPC7 also plays important roles in the calcium signaling pathway because of its unique activation properties such as constitutive activity [9]. Although the regulation of TRPC3 and TRPC6 by cGK-I has been characterized, the regulation of TRPC7 has not.

In this study, we investigated the effect of cGK-Iα on TRPC7-mediated calcium influx. Our results demonstrate that cGK-Iα phosphorylates TRPC7 at threonine-15 and inhibits DAG- and carbachol-induced calcium influx. Furthermore, cGK-Iα interacts with the N terminus of TRPC7 to probably enhance the specificity and efficiency of the phosphorylation. These findings highlight the importance of TRPC7 in regulating calcium signaling, and the inhibitory action of cGMP/cGK on TRPC7 provides a new mechanism to regulate the intracellular calcium concentration.

Abbreviations: cGK, cGMP-dependent protein kinase; TRPC, canonical transient receptor potential channel; 8-CPT-cGMP, 8-(4-chlorophenylthio)-cGMP; DAG, diacylglycerol; OAG, oleyl-acetyl-glycerol; MBP, maltose binding protein; GST, glutathione S-transferase; CREB, cAMP response element-binding protein; PKA, cAMP-dependent protein kinase; RIIα-PKA, type IIα regulatory subunit of PKA; NHERF, Na^+/H^+ exchanger regulatory factor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

* Corresponding author. Tel.: +81 88 656 7527; fax: +81 88 655 3161.
E-mail address: yuasa@bio.tokushima-u.ac.jp (K. Yuasa).

[1] Both authors contributed equally to this work.

0898-6568/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2011.03.005

2. Materials and methods

2.1. Materials

Rabbit monoclonal antibodies against phospho-RRXS/T (100G7), phospho-CREB (Ser-133) (87 G3), CREB (48 H2) were obtained from Cell Signaling Technology. Mouse monoclonal anti-FLAG antibody (M2) was purchased from Sigma. Rabbit anti-HA epitope polyclonal antibody (Y-11) was obtained from Santa Cruz Biotechnology.

2.2. Plasmid construction

cDNAs encoding human cGK-Iα, mouse TRPC3 and TRPC7 were obtained by PCR using the respective specific primers. PCR products were cloned into TA-cloning vector pGEM-T Easy (Promega), and the fidelity of inserted DNA sequences was confirmed by DNA sequencing. Human cGK-Iα cDNA was subcloned into the mammalian expression vector pFLAG-CMV-2 (Sigma) and pHA-CMV-2. cDNAs encoding mouse TRPC3 and TRPC7 were subcloned into the mammalian expression vector pCMV-3Tag-3 (Stratagene) containing a C-terminal 3xFLAG epitope tag, resulting in pCMV-mTRPC3-FLAG and pCMV-mTRPC7-FLAG, respectively. Site-directed mutagenesis was performed using PrimeSTAR Mutagenesis Basal Kit (Takara) according to the protocol of the manufacturer. To introduce desired mutations, the following primers were used (mutation sites are underlined): 5′-CGGCACGCCACCTTGAGGGAGAAGGGG-3′ and 5′-CAAGGTGGCGTGCCGGCGCTGCATGTT-3′ (mTRPC7 T15A); 5′-AAGGACGCCTTCAGTCACTCGCGCTCC-3′ and 5′-ACTGAAGGCGTCCTTGCGCTGCTTCTC-3′ (mTRPC7 S211A); 5′-AAGTTAGCTATGCAATGCAAGGATTTT-3′ and 5′-TTGCATAGCTAACTTCCTGTAATCATT-3′ (mTRPC7 S267A); 5′-CGGCACGACACCTTGAGGGAGAAGGGG-3′ and 5′-CAAGGTGTCGTGCCGGCGCTGCATGTT-3′ (mTRPC7 T15D); 5′-AAGGACGACTTCAGTCACTCGCGC-3′ and 5′-ACTGAAGTCGTCCTTGCGCTGCTT-3′ (mTRPC7 S211D); 5′-AAGTTAGATATGCAATGCAAGGAT-3′ and 5′-TTGCATATCTAACTTCCTGTAATC-3′ (mTRPC7 S267D). In each case, the mutation was confirmed by DNA sequencing analysis.

cDNAs encoding the N-terminal segments of cGK-Iα (amino acids 1–360 and 105–360) were subcloned into the site of the maltose binding protein (MBP) expression vector pMAL (New England Biolabs), generating pMAL-cGK-Iα 1–360 and pMAL-cGK-Iα 105–360, respectively. A cDNA encoding the N-terminal region of TRPC7 (amino acids 35–200) was subcloned into the glutathione S-transferase (GST) expression vector pGEX (GE Healthcare), generating pGEX-TRPC7 35–200.

2.3. Cell culture, transfection and construction of stable cell lines

COS-7 and HEK293T cells were cultured in Dulbecco's modified Eagle's medium 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. To obtain stably transfected cell lines, the transfectants were selected using medium containing 400 μg/ml G418 for 2 weeks. Non-transfected control cells in 400 μg/ml G418 failed to survive after 2 weeks. From the resulting transfected cell pool, single clonal lines were isolated by limiting dilution.

2.4. In vitro kinase assay

In vitro kinase assay was performed as previously described [10]. COS-7 cells were cotransfected with pFLAG-cGK-Iα and pcDNA-mTRPC3-FLAG or pcDNA-mTRPC7-FLAG. After 24 h, cells were lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP40, 1 mM EDTA, 10 μM leupeptin, and 10 μg/ml aprotinin) and rotated for 30 min at 4 °C. The lysates were centrifuged at 10,000×*g* for 10 min, and supernatants were incubated with anti-FLAG M2 monoclonal antibody and protein G Sepharose 4 (GE Healthcare) for 4 h at 4 °C. The beads were washed five times with wash buffer (lysis buffer with 0.1% NP40 instead of 1% NP40). The *in vitro* kinase reaction was performed in kinase reaction buffer (50 mM Tris–HCl at pH 7.5, 20 mM magnesium acetate, 0.2 mM [γ-^{32}P]ATP, 5 mM glycerophosphoric acid and 1 mM sodium orthovanadate) in the presence or absence of 5 μM cGMP at 30 °C for 30 min. After removing the supernatant, the resins were mixed with an equal volume of 2×SDS loading buffer and heated at 95 °C for 5 min, and the denatured proteins were subjected to SDS-PAGE. The gel was dried and analyzed with a BAS-1500 image analyzer (Fuji-film).

2.5. In vivo kinase assay

COS-7 cells were co-transfected with pFLAG-cGK Iα and pcDNA-mTRPC3-FLAG or pcDNA-mTRPC7-FLAG as described above. After 24 h, cells were treated with 8-(4-chlorophenylthio) cGMP (8-CPT-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 centrifuged at 10,000×*g* for 10 min at 4 °C to remove cellular debris, and the supernatants were immunoprecipitated with anti-FLAG M2 monoclonal antibody with protein G-Sepharose for 4 h at 4 °C by rotation. The beads were washed with cell lysis buffer, and immunecomplexes were eluted by heating at 95 °C in 2×SDS loading buffer, subjected to SDS-PAGE, and immunoblotting using anti-phospho RRXS/T or anti-FLAG antibodies.

2.6. Measurement of intracellular calcium

Intracellular calcium response was measured using Calcium Kit Fluo 4 (Dojindo) according to the manufacture's protocol. Briefly, the HEK293T cells stably expressing TRPC7 were cultured in a 96 well plate for 24 h (2×10^4 cells/well). The culture medium was removed and loading medium containing Fluo 4-AM was loaded. After incubation for 1 h at 37 °C, loading medium was removed and the recording medium was loaded. The change in the fluorescence (490 nm) in response to carbachol (final concentration 100 μM) was monitored using a fluorometric plate reader (Infinite M200, Tecan).

2.7. Immunofluorescence analysis

HEK293T cells grown on poly-L-lysine-coated chamber slides were cotransfected with pEGFP-cGK-Iα and pcDNA-mTRPC7-FLAG. At 24 h post-transfection, cells were washed twice with PBS and fixed for 20 min in 3.7% formaldehyde. Following sequential washes with PBS, cells were permeabilized for 5 min in 0.1% Triton X-100, washed three times with PBS, and then treated with 5% bovine serum albumin for 30 min. Cells were subsequently incubated with mouse anti-FLAG M2 IgG for 2 h. Following three washes with PBS, cells were incubated for 1 h with goat anti-mouse IgG conjugated with Alexa Fluor 555 (Invitrogen). The slides were washed thoroughly with PBS and mounted in fluorescent mounting medium Vectashield (Vector Laboratories). A confocal laser-scanning microscope (Leica TCS SP5, Leica Microsystems) was used to obtain staining profiles.

2.8. Co-immunoprecipitation analysis

COS-7 cells were co-transfected with pHA-cGK-Iα and pcDNA-mTRPC7-FLAG. After 24 h, cells were scraped in an ice-cold cell lysis buffer. The cell extracts were centrifuged at 10,000×*g* for 10 min at 4 °C, and the supernatants were immunoprecipitated with mouse anti-FLAG M2 antibody or rabbit anti-HA polyclonal antibody and protein G Sepharose for 4 h at 4 °C. Immunoprecipitated proteins were analyzed by immunoblot analysis using rabbit anti-HA antibody or mouse anti-FLAG M2 antibody.

2.9. Preparation of recombinant proteins and in vitro binding assay

Each bacterial expression plasmid was introduced into the bacterial strain BL21 Star DE3 (Invitrogene). An overnight culture in LB medium was diluted with fresh LB medium and incubated at 37 °C in a shaking incubator for 2 h. After isopropyl-1-thio-β-D-galactopyranoside was added to the culture to a final concentration of 0.2 mM, the culture was incubated for an additional 2 h. The cells were resuspended in ice-cold soluble buffer (20 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10 mg/ml aprotinin and 10 mM leupeptin). After freezing and thawing, suspended cells were sonicated on ice in short bursts. The lysate was cleared by centrifugation at 10,000 × *g* for 15 min at 4 °C. The supernatant was then incubated with glutathione-Sepharose 4B (GE Healthcare) or amylose resin (New England Biolabs) for 4 h at 4 °C. The beads were washed five times with ice-cold soluble buffer and incubated with 10 mM reduced glutathione or 10 mM maltose at 4 °C to elute fusion protein from the beads. After centrifugation, the supernatant was dialyzed against PBS. The purified proteins were electrophoresed on SDS-PAGE and visualized by Coomassie brilliant blue staining.

MBP, MBP-cGK-Iα 1–360, or MBP-cGK-Iα 105–360 protein bound to amylose resin was incubated with GST-TRPC7 35–200 in ice-cold soluble buffer for 1 h at 4 °C. The beads were washed with ice-cold soluble buffer, and bound proteins were eluted by heating at 95 °C in 2 × SDS loading buffer, separated by SDS-PAGE and immunoblotted with anti-MBP and anti-GST antibodies (Nacalai Tesque).

3. Results

3.1. cGK-Iα phosphorylates threonine-15 of TRPC7

cGMP/cGK signaling is involved in the regulation of intracellular calcium levels. Some potential substrates for cGK-I, which regulate calcium influx, have been identified [3,4,6–8]. Previous reports demonstrated that cGK-Iα phosphorylated TRPC3 and TRPC6 [6,7]. Amino acid sequence analysis of TRPC7, which shares about 70–80% homology with TRPC3 and TRPC6, identified three typical phosphorylation sites (RRHT^{15}, RKDS^{211}, and RKLS^{267}) for cGK in both mice and human (Fig. 1). To determine whether TRPC7 is phosphorylated by cGK-Iα, we coexpressed a C-terminal FLAG-tagged mouse TRPC7 or TRPC3 with FLAG-tagged cGK-Iα in COS-7 cells. The cell lysates were immunoprecipitated with anti-FLAG antibody, and an *in vitro* kinase assay was performed. As shown in Fig. 2A, TRPC7 was phosphorylated by cGMP/cGK-Iα, but TRPC3 was poorly phosphorylated in contrast to previous data [6]. Immunoblot analysis for TRPC7-FLAG showed two bands. TRPC6 is a glycoprotein and detected as two bands by immunoblot analysis [11]. TRPC7 also has a potential glycosylation site (N^{514}VS), and previous immunoblot analysis using anti-TRPC7 antibody showed that a few specific bands were detected in lysates of cells expressing TRPC7 [12].

Phosphorylation of TRPC7 by cGK-Iα was confirmed by an *in vivo* kinase assay using an anti-phospho RRXS/T antibody. This antibody detects proteins containing a phosphorylated serine or threonine

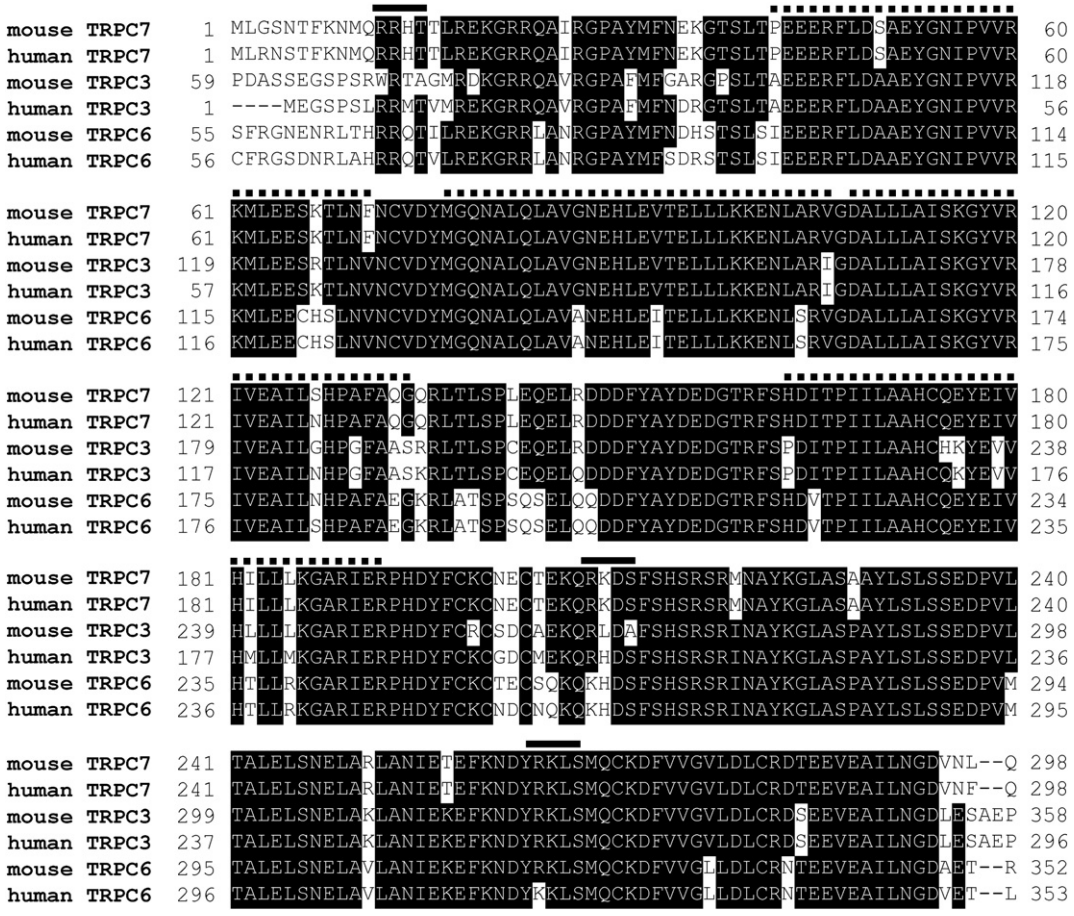

Fig. 1. Sequence alignment of the N-terminal domain of human and mouse TRPC3, 6, and 7. Multiple alignment was performed using GENETYX software. The positions of amino acid residue are shown at each side of the sequence. Identical amino acid residues that are conserved over 50% among the sequences are shown in an outline typeface with black background. Putative cGK phosphorylation sites are shown by continuous lines above the sequences. The four ankyrin domains are shown by broken lines. Accession numbers of the human and mouse TRPC sequences are as follows: mouse TRPC7, NP_036165; human TRPC7, NP_065122; mouse TRPC3, NP_62383; human TRPC3, NP_003296; mouse TRPC6, NP_038866; human TRPC6, NP_004612.

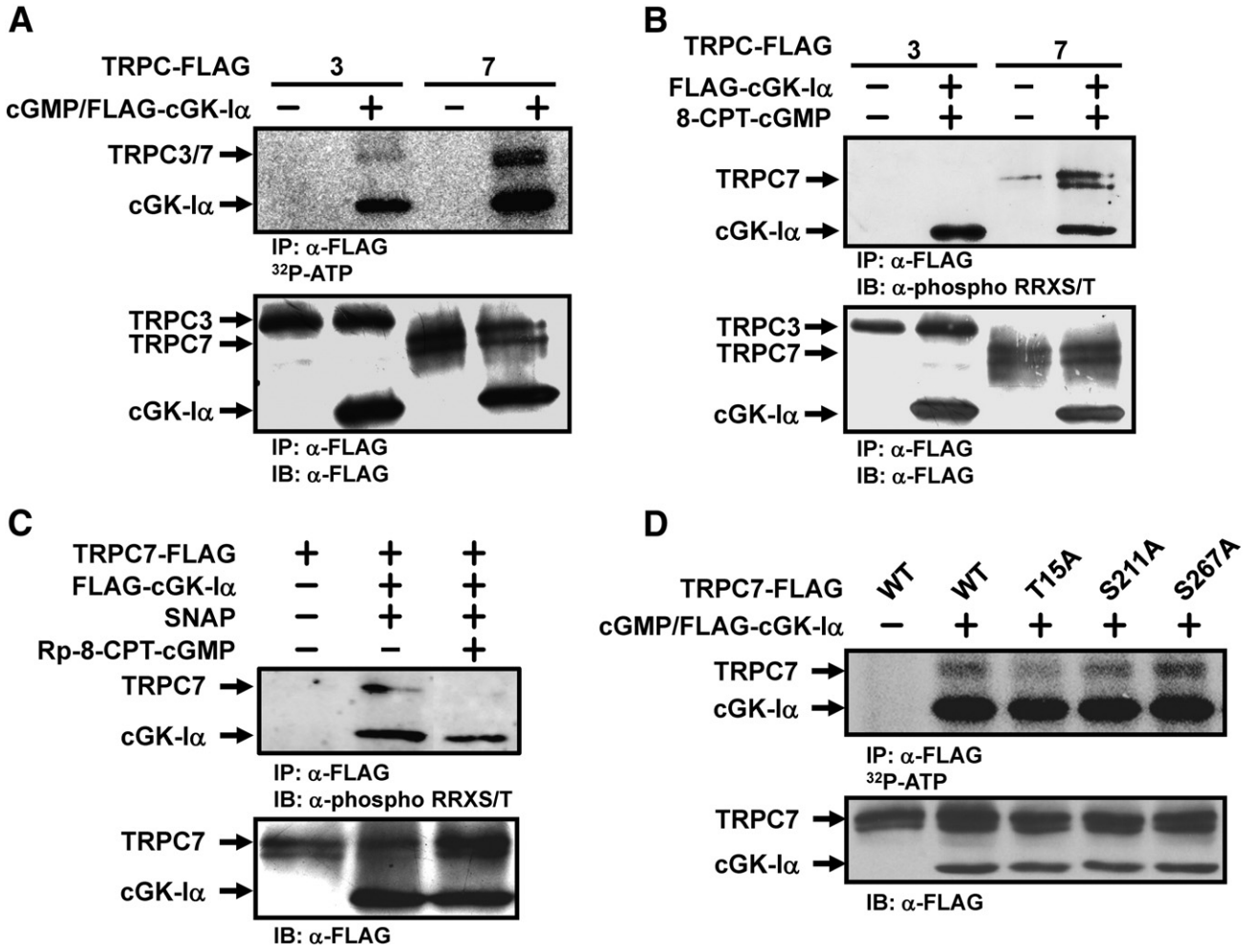

Fig. 2. Phosphorylation of TRPC7 by cGK-Iα. (A) The FLAG-tagged mouse TRPC3 or TRPC7 were expressed in COS-7 cells with FLAG-tagged cGK-Iα. The FLAG-tagged proteins were immunoprecipitated, and then incubated in a kinase buffer containing [γ-^{32}P]ATP with or without cGMP. To monitor the expression level of FLAG-tagged proteins, the immunoprecipitates were blotted with anti-FLAG antibody (IP: α-FLAG, IB: α-FLAG). (B) COS-7 cells were transiently transfected with either TRPC3 or TRPC7 together cGK-Iα. After 24 h, cells were treated with 100 μM 8-CPT-cGMP for 1 h. The cell lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were analyzed by immunoblotting using either anti-phospho RRXS/T (IP: α-FLAG, IB: α-phospho RRXS/T) or anti-FLAG antibodies (IP: α-FLAG, IB: α-FLAG). (C) COS-7 cells expressing TRPC7 and cGK-Iα were exposed to the NO donor SNAP (100 μM) with or without Rp-8-CPT-cGMP (100 μM) for 30 min. The cell extracts were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were blotted with anti-phospho RRXS/T. (D) Three mutants of TRPC7 (T15A, S211A, and S267A) were created and tested in an *in vitro* kinase assay using [γ-^{32}P]ATP.

residue with an arginine at the −3 and −2 positions, and also recognizes −3 arginine-bearing phospho-serine/threonine peptides. COS-7 cells transiently transfected with FLAG-tagged TRPC7 and cGK Iα were treated with a cell-permeable cGMP analog, 8-(4-chlorophenylthio)-cGMP (8-CPT-cGMP). Cell lysates were immunoprecipitated with an anti-FLAG antibody, and then immunoblotted with an anti-phospho RRXS/T antibody. As expected, TRPC7, but not TRPC3, was efficiently phosphorylated by cGMP/cGK-Iα *in vivo* (Fig. 2B). Furthermore, the NO donor S-nitroso-N-acetylpenicillamine (SNAP) also induced TRPC7 phosphorylation, and its phosphorylation was inhibited by a selective and membrane-permeable inhibitor of cGK, R isomer of 8-CPT-cGMP (Rp-8-CPT-cGMP) (Fig. 2C). These results indicated that TRPC7 was phosphorylated by cGMP/cGK-I.

To identify phosphorylation sites of TRPC7 by cGK-Iα, three mutants of TRPC7 in which each of the putative cGK phosphorylation sites was replaced by alanine (T15A, S211A, and S267A) were created and tested by the *in vitro* kinase assay. As shown in Fig. 2D, phosphorylation of the T15A mutant was significantly reduced compared with that of wild-type TRPC7, whereas the S211A and S267A mutants showed phosphorylation intensities equal to that of wild-type TRPC7. These results identify threonine 15 as a potential phosphorylation site of TRPC7 by cGK-Iα.

3.2. cGK-Iα suppresses CREB phosphorylation via phosphorylation of TRPC7

A previous report indicated that TRPC6 increased phosphorylation of Ca^{2+}/calmodulin-dependent kinase and cAMP response element-binding protein (CREB) via calcium influx [13]. Therefore, we examined whether TRPC7 also regulates CREB phosphorylation. COS-7 cells were transiently transfected with a TRPC7 expression plasmid and were treated with a structural analog of DAG, oleyl–acetyl–glycerol (OAG), which is an activator of TRPC7 [14]. When mock-transfected cells were stimulated with OAG, CREB phosphorylation was weakly but significantly enhanced (Fig. 3A, lane 2) because of activation of TRPC family proteins endogenously expressed in COS-7 cells [15]. On the other hand, overexpression of TRPC7 moderately stimulated CREB phosphorylation, even with no OAG treatment (Fig. 3A, lane 3), consistent with evidence that TRPC7 displays constitutive activity [9]. Importantly, OAG treatment of TRPC7-expressing cells strongly promoted phosphorylation of CREB (Fig. 3A, lane 4), indicating that TRPC7 also activated CREB activity. Next, the effect of phosphorylation of TRPC7 by cGK-Iα on CREB activation was investigated. COS-7 cells transiently cotransfected with TRPC7 and cGK-Iα were pretreated with 8-CPT-cGMP, and then stimulated with OAG. cGMP/cGK-Iα markedly inhibited OAG-induced

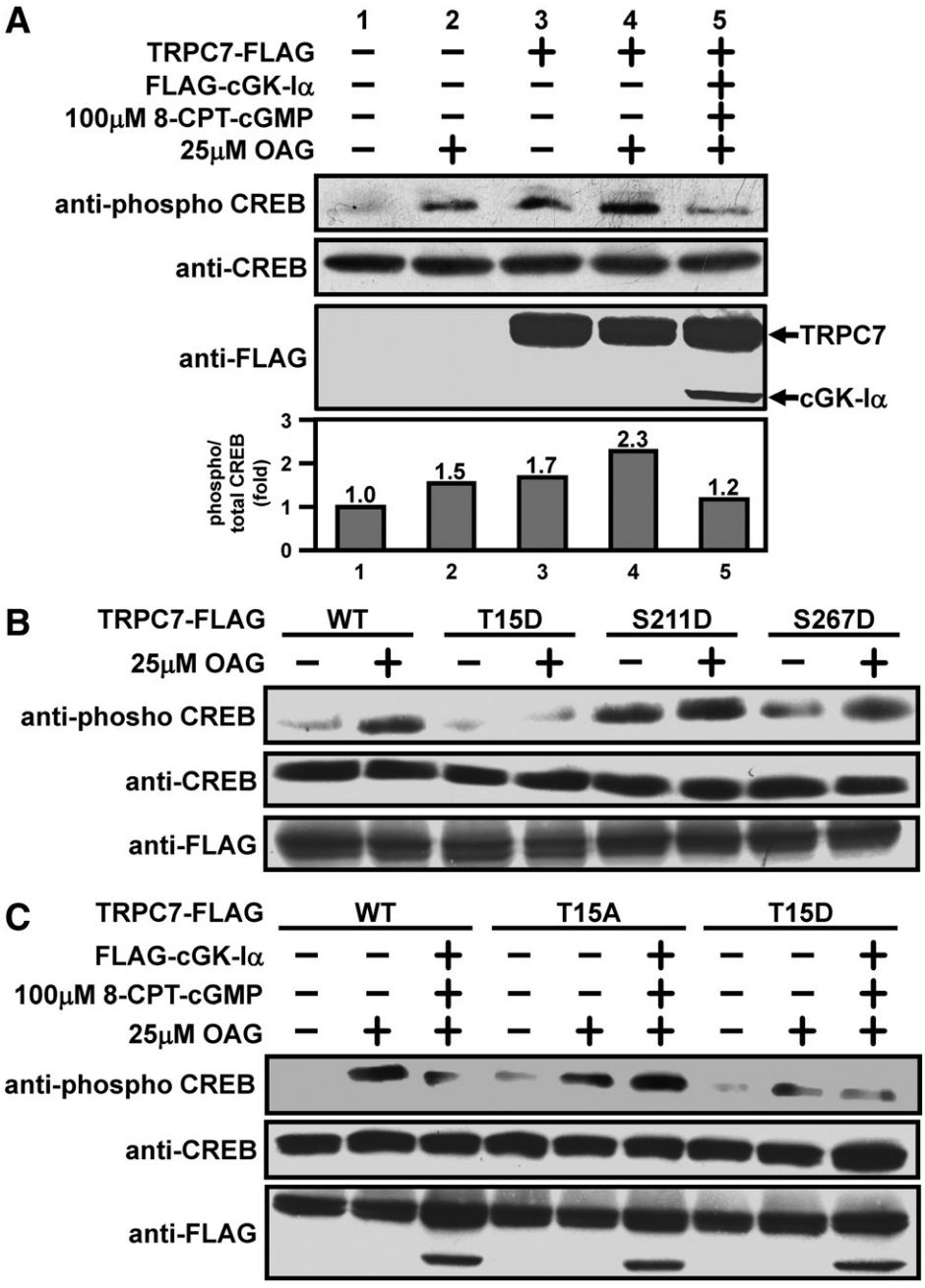

Fig. 3. Regulation of CREB phosphorylation by cGK-Iα. (A) COS-7 cells were transiently cotransfected with the expression plasmids of TRPC7 and cGK-Iα, pretreated with 100 μM 8-CPT-cGMP for 1 h, and then stimulated by 25 μM OAG for 10 min. The total lysates were immunoblotted with antibodies against phosphorylated and total CREB. Expression of FLAG-tagged proteins was verified by immunoblotting with the anti-FLAG antibody. (B) COS-7 cells were transiently transfected with FLAG-tagged wild-type TRPC7 (TRPC7 WT-FLAG) or phospho-mimicking mutants (TRPC7 T15D-FLAG, TRPC7 S211D-FLAG, or TRPC7 S267D-FLAG). Transfected cells were exposed with 25 μM OAG for 10 min. The total lysates were immunoblotted with antibodies against phosphorylated and total CREB. Expression of FLAG-tagged TRPC7 proteins was verified by immunoblotting with the anti-FLAG antibody. (C) COS-7 cells were transiently transfected with FLAG-tagged wild-type TRPC7 (TRPC7 WT-FLAG), phospho-deficient mutant (TRPC7 T15A-FLAG), or phospho-mimicking mutant (TRPC7 T15D-FLAG) in the presence or absence of FLAG-cGK-Iα. Transfected cells were pretreated with 100 μM 8-CPT-cGMP for 1 h and exposed with 25 μM OAG for 10 min. The total lysates were immunoblotted with antibodies against phosphorylated and total CREB. Expression of FLAG-tagged proteins was verified by immunoblotting with the anti-FLAG antibody.

CREB phosphorylation (Fig. 3A, lane 5). This result suggests that cGMP/cGK-Iα negatively regulates TRPC7.

Furthermore, three phospho-mimic TRPC7 mutants (TRPC7 T15D, S211D, and S267D) in which the putative cGK phosphorylation site was replaced by aspartic acid were generated, and the effect of phospho-mimic mutants on OAG-stimulated CREB phosphorylation was examined. As shown in Fig. 3B, the OAG-induced CREB activation in TRPC7 T15D-expressing cells was markedly decreased compared with that in wild-type TRPC7-expressing cells. On the other hand, TRPC7 S211D and TRPC7 S267D had no effect on the OAG-induced CREB activation, or rather TRPC7 S211D induced CREB phosphorylation even without OAG treatment. The effect of a phospho-null mutant TRPC7 T15A on OAG-stimulated CREB phosphorylation was also investigated. In TRPC7 WT-expressing cells, OAG-induced CREB phosphorylation was suppressed by cGMP/cGK-Iα, while OAG-induced CREB phosphorylation in TRPC7 T15A-expressing cells was unchanged (Fig. 3C). These observations strongly indicated that phosphorylation at threonine 15 is involved in negative regulation by cGK-Iα.

3.3. Effects of wild-type and phospho-mimic TRPC7 on carbachol-induced calcium influx

To further confirm the importance of phosphorylation at threonine 15 for negative regulation by cGK-Iα, HEK293T cells stably expressing either FLAG-tagged wild-type TRPC7 or the phospho-mimic TRPC7 mutant (TRPC7 T15D) were generated. A similar level of FLAG-tagged TRPC7 expression between the two cell lines (TRPC7 WT-FLAG/HEK293T and TRPC7 T15D-FLAG/HEK293T) was confirmed by immunoblot analysis using an anti-FLAG antibody (Fig. 4A). Calcium transients in response to the muscarinic agonist carbachol in these cells were measured using the fluorescent calcium indicator Fluo-4. Stimulation of muscarinic receptors by carbachol increased calcium entry in HEK293T cells (Fig. 4B), because HEK293 cells have some endogenous TRPC proteins including TRPC7 [16]. Overexpression of TRPC7 caused a significant increase in carbachol-evoked calcium influx compared with the parent cells. The calcium influx was almost completely inhibited by the TRPC channel inhibitor SKF96365 (data not shown). Interestingly, carbachol-induced calcium influx in TRPC7 T15D-FLAG/HEK293T was significantly decreased compared with that in TRPC7 WT-FLAG/HEK293T (Fig. 4C). Furthermore, CREB phosphorylation on these cells treated with carbachol was examined. As shown in Fig. 4D, carbachol-induced CREB phosphorylation was suppressed in TRPC7 T15D-FLAG stably expressing HEK293T cells. These results indicate that carbachol-induced calcium influx was negatively regulated by the mutation that mimics phosphorylation at position 15.

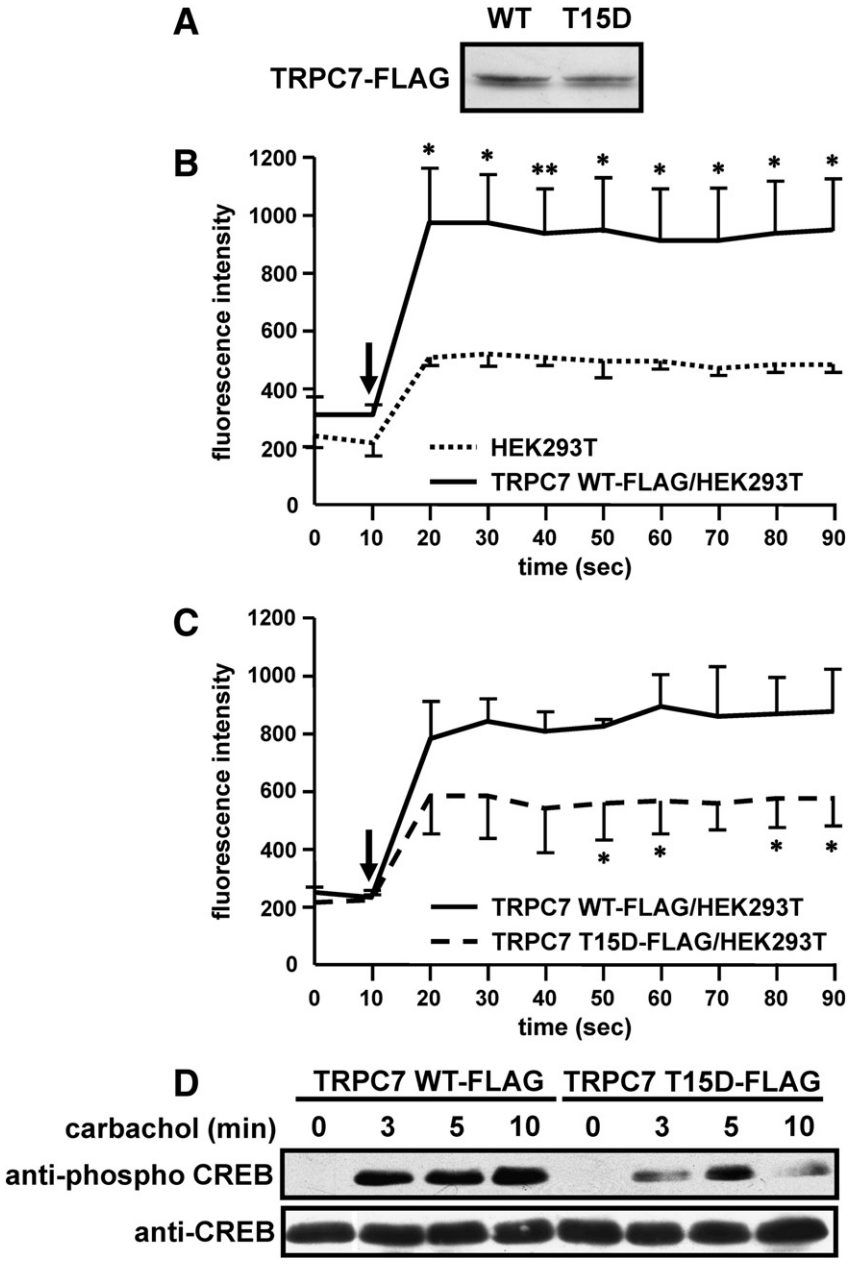

Fig. 4. Regulation of calcium influx via phosphorylation of TRPC7. (A) HEK293T cells stably expressing FLAG-tagged wild-type TRPC7 (TRPC7 WT-FLAG/HEK293T) or phospho-mimic TRPC7 mutant (TRPC7 T15D) (TRPC7 T15D-FLAG/HEK293T) were generated. The expression of FLAG-TRPC7s was confirmed by immunoblot analysis with the anti-FLAG antibody. (B and C) Calcium transients in response to carbachol in TRPC7 WT-FLAG/HEK293T and TRPC7 T15D-FLAG/HEK293T were measured using the florescent calcium indicator, Fluo-4. Carbachol (100 μM final concentration) was added at the time indicated by arrow. Data are the means ± S.D. of three independent experiments. Statistical significances were determined by Student's *t*-test. Single asterisks, $p<0.05$; double asterisk, $p<0.01$. (D) The TRPC7 WT-FLAG or TRPC7 T15D-FLAG stably expressed HEK293T cells were stimulated with 100 μM carbachol for indicated times. Cell lysates were immunoblotted with antibodies against phosphorylated and total CREB.

3.4. cGK-Iα specifically interacts with TRPC7

To determine the subcellular localization of cGK-Iα and TRPC7, the plasmids encoding GFP fused to cGK-Iα, FLAG-tagged TRPC7, or both were transfected into HEK293T cells and analyzed by immunofluorescence microscopy. TRPC7-FLAG was present at the plasma membrane and nuclear envelope (Fig. 5B). On the other hand, cGK-Iα was distributed diffusely in the cytoplasm in cells transfected with GFP-cGK-Iα alone (Fig. 5A). Interestingly, GFP-cGK-Iα was colocalized in the plasma membrane when coexpressed with TRPC7-FLAG (Fig. 5C–E). These observations stimulated us to examine whether cGK-Iα interacts with TRPC7.

The localization of the kinase near its substrates may ensure the rapid phosphorylation of specific substrates in response to cellular stimulation. Therefore, we carried out co-immunoprecipitation experiments to test whether cGK-Iα is associated with TRPC7. The HA-tagged cGK-Iα was coexpressed with the FLAG-tagged TRPC7 in COS-7 cells, and the cell extracts were subjected to co-immunoprecipitation analysis. The ability of an antibody against either the FLAG or HA-epitope to precipitate a complex of cGK-Iα and TRPC7 indicates that these two proteins interact with each other *in vivo* (Fig. 6A). To evaluate the interaction between cGK-Iα and TRPC7, we examined whether the cGK-Iβ isoform or type IIα regulatory subunit (RIIα) of cAMP-dependent protein kinase (PKA) interacts with TRPC7. As shown in Fig. 6B, cGK-Iβ isoform was associated with TRPC7 as well as cGK-Iα, whereas PKA-RIIα did not bind. These results demonstrated that cGK I isoforms are specifically interacted with TRPC7.

Furthermore, we constructed deletion mutants of TRPC7 to determine the domains of TRPC7 responsible for binding to cGK-I. Immunoprecipitation of the FLAG-tagged TRPC7 full length (TRPC7 1–862) and N-terminal segment (TRPC7 1–351) coprecipitated HA-tagged cGK-Iα (Fig. 6C, left panel). In contrast, immunoprecipitation of the C-terminal segment (TRPC7 673–862) did not result in the coprecipitation with cGK-Iα. Further detailed deletion analysis revealed that TRPC7 interacted with cGK-Iα via its amino acids 35–200 whose region contains four ankyrin repeat domains (Fig. 6C, right panel). On the other hand, cGK-Iα was associated with TRPC7 via two cGMP-binding domains (amino acids 105–360) (Fig. 6D).

Finally, we performed *in vitro* binding assay to test whether cGK-Iα directly binds to TRPC7. Two N-terminal segments of cGK-Iα (cGK-Iα 1–360 and cGK-Iα 105–360) were expressed as a fusion protein with maltose binding protein (MBP) in *E. coli*. The N-terminal region of TRPC7 (TRPC7 35–200) was expressed as a fusion protein with glutathione S-transferase (GST). Each fusion protein was expressed in *E. coli* and purified by affinity chromatography. The purified protein showed a nearly single band on SDS-PAGE stained with Coomassie brilliant blue (Fig. 7A). Using these purified proteins, *in vitro* binding assay was carried out. GST-TRPC7 35–200 specifically bound to MBP-cGK-Iα 1–360 and cGK-Iα 105–360 immobilized on amylose resin but not to MBP alone (Fig. 7B), demonstrating that cGK-Iα directly binds to TRPC7.

4. Discussion

The present study clearly demonstrates that cGK-Iα phosphorylates TRPC7 at threonine-15 (RRHT[15]) and attenuated TRPC7-mediated calcium transients and CREB phosphorylation. On the other hand, although a previous report revealed cGK-dependent TRPC3 phosphorylation [6], the

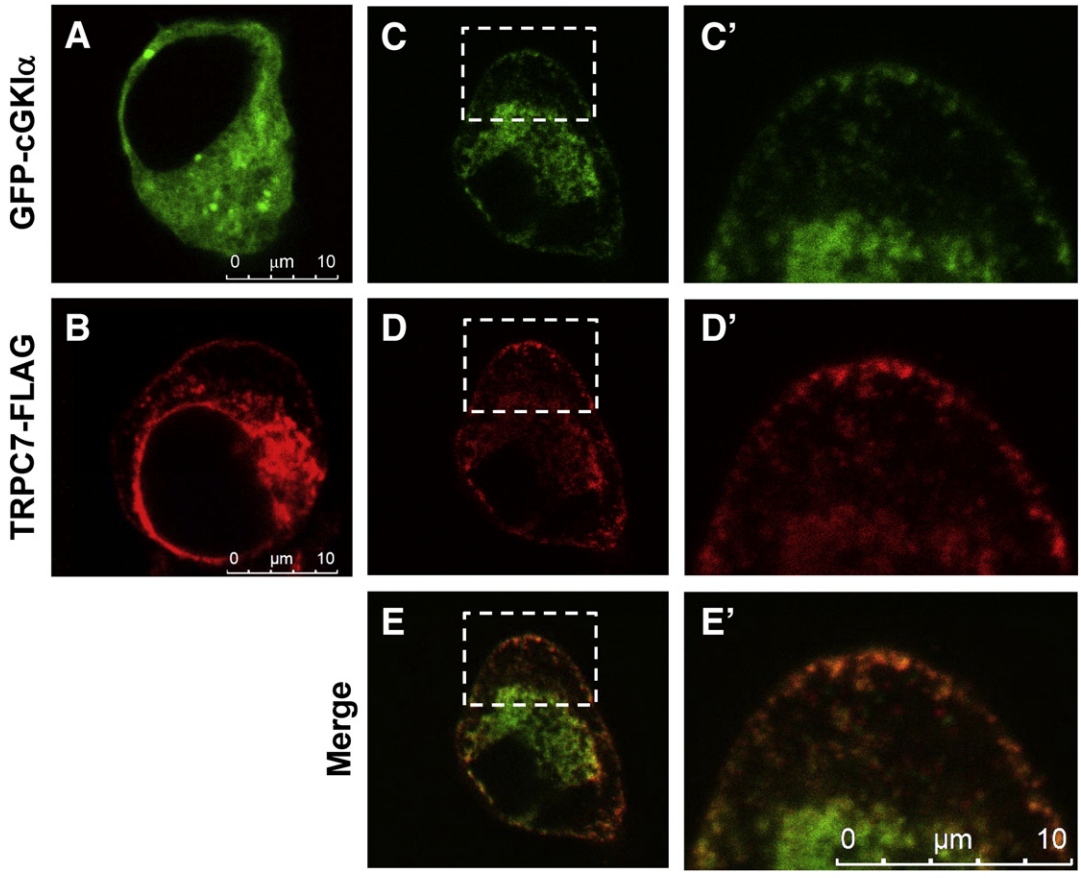

Fig. 5. Subcellular localization of cGK-Iα and TRPC7 in HEK293T cells. HEK293T cells were transiently transfected with pEGFP-cGK-Iα (A), pcDNA-TRPC7-FLAG (B), or a combination of both (C–E). Cells were fixed and incubated with mouse anti-FLAG antibody. The primary antibody was visualized with Alexa Fluor 555-conjugated anti-mouse IgG, followed by fluorescence microscopy. Fluorescence for GFP-cGK-Iα (green) (A, C) and TRPC7-FLAG (red) (B, D) is shown with the merged images (merge is in yellow) (E). C'–E' are the high-magnified images of C–E.

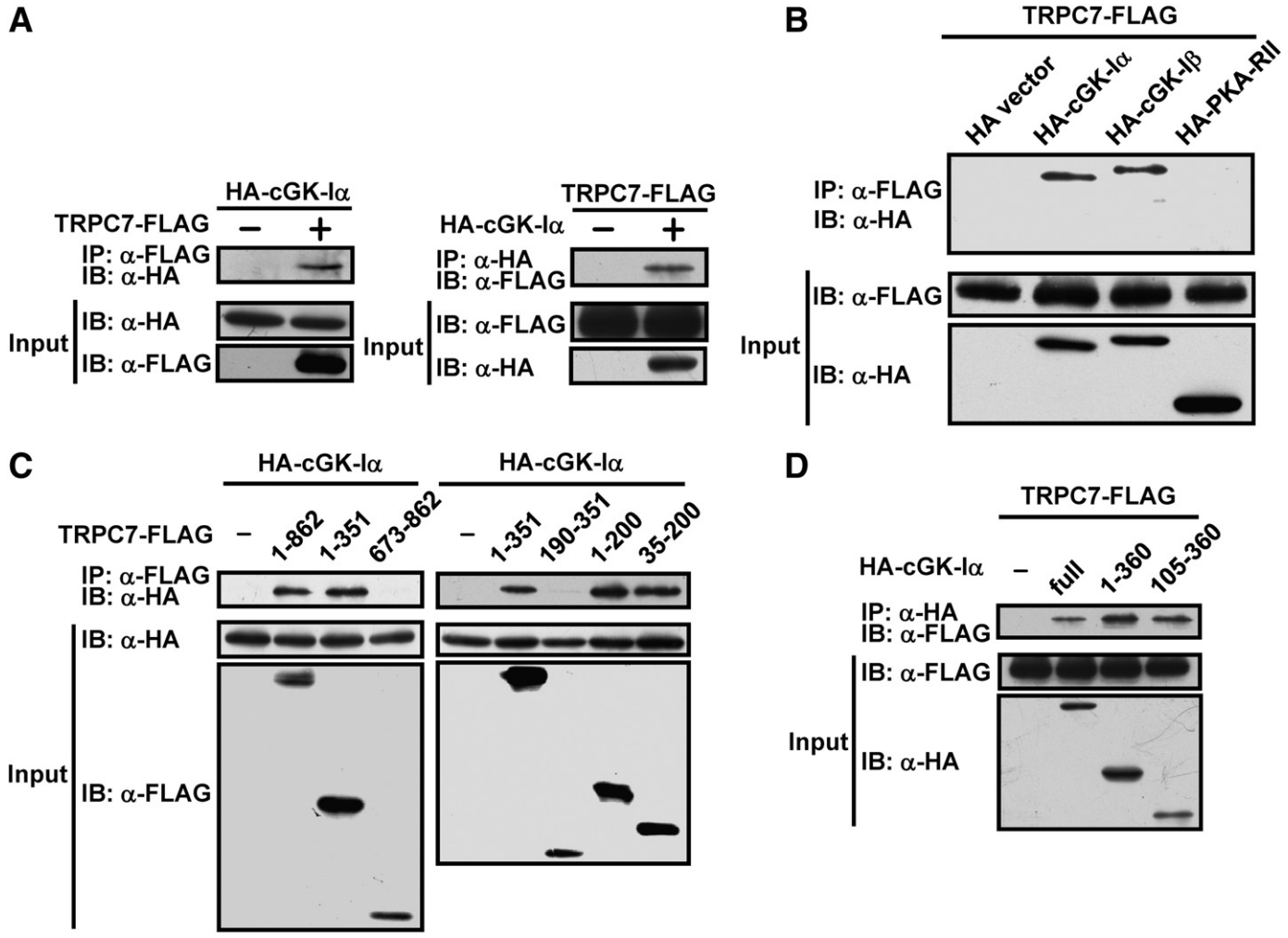

Fig. 6. Interaction between cGK-Iα and TRPC7. COS-7 cells were cotransfected with pHA-cGK-Iα and pcDNA-TRPC7-FLAG. After 24 h, cell lysates were immunoprecipitated with anti-FLAG antibody, and HA-tagged protein in immunoprecipitates were detected by immunoblotting with anti-HA antibody (IP: α-FLAG, IB: α-HA). The same lysates were separated by SDS-PAGE and then immunoblotted with anti-FLAG or anti-HA antibody.

present study failed to confirm this observation. This discrepancy may be due to species differences. Human TRPC3 is phosphorylated by cGK at threonine-11 (RRMT^{11}) and serine-263 (RKLS^{263}), but mouse TRPC3 has only a consensus phosphorylation site (RKLS^{325}) corresponding to amino acids 260–263 of human TRPC3 (Fig. 1). The amino acid sequence corresponding to amino acids 8–11 (RRMT^{11}) of human TRPC3 is RWRT^{72}

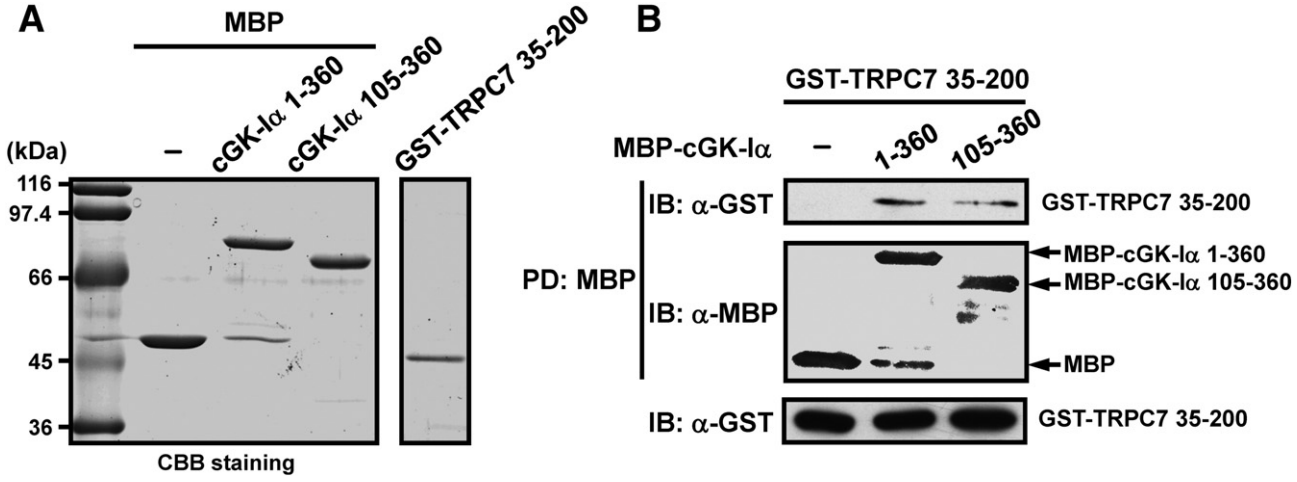

Fig. 7. In vitro binding of cGK Iα and TRPC7. (A) MBP, MBP-cGK-Iα 1–360, MBP-cGK-Iα 105–360, and GST-TRPC7 35–200 were expressed in *E. coli* and purified by affinity chromatography. Purified proteins were electrophoresed on SDS-PAGE and visualized by Coomassie brilliant blue (CBB) staining. (B) MBP, MBP-cGK-Iα 1–360, or MBP-cGK-Iα 105–360 protein bound to amylose resin was incubated with GST-TRPC7 35–200. The bound proteins were eluted by heating at 95 °C in 2 x SDS loading buffer, separated by SDS-PAGE and immunoblotted with anti-GST (PD: MBP, IB: α-GST) and anti-MBP (PD: MBP, IB: α-MBP) antibodies. PD; pull down, IB; immunoblot.

in mice. Interestingly, cGK-I phosphorylates TRPC6 at threonine-69 ($RRQT^{69}$, corresponding to threonine-11 of human TRPC3) but not at serine-321 [7]. Together with our data showing that cGK-Iα phosphorylated TRPC7 at threonine-15 but not at serine-267, cGK-I may preferentially phosphorylate at threonine-15 rather than serine-263 of human TRPC3, resulting that cGK-I fails to phosphorylate mouse TRPC3.

To date, a number of cGK-interacting proteins have been identified [3,10,17]. Most of the interacting proteins are phosphorylated by cGK. Colocalization of the kinase with substrates is important for its specificity and efficiency. cGK-Iα was colocalized with TRPC7 in the plasma membrane, although cGK-Iα was localized to the cytoplasma in cells transfected with cGK-Iα alone. Importantly, cGK-I isoforms are specifically associated with TRPC7, but PKA-RIIα is not. Although threonine-15 ($RRHT^{15}$) of TRPC7 could be a target for phosphorylation by PKA, colocalization with cGK-I strongly suggests that TRPC7 is a substrate for cGK-I. A previous report demonstrated that TRPC4 bound to a PDZ domain-containing protein, NHERF (Na^+/H^+ exchanger regulatory factor), via its last three C-terminal amino acids [18]. Furthermore, TRPV5 also interacted with NHERF2 through the TRPV5 carboxyl tail [19]. On the other hand, three cGK isoforms interacted with NHERF2, and cGK-II efficiently inhibited Na^+/H^+ exchange activity of NHE3 (Na^+/H^+ exchange 3) via its interaction [20]. These observations suggest that cGK-Iα also interacts with the C terminus of TRPC7 through interaction with anchoring proteins such as NHERF. However, contrary to our expectations, cGK-Iα directly bound to the N terminus of TRPC7. The N-terminal segment of TRPC7 contains four ankyrin repeat domains, and TRPC7 seem to associate with cGK-Iα via the ankyrin repeat domains. Ankyrin repeat is a motif that mediates specific protein–protein interactions [21]. In fact, it has been shown that the ankyrin repeat domains of TRPC7 are involved in the interaction with MxA, a member of the dynamin superfamily [22]. Moreover, it is interesting that other TRPC family members including TRPC3 and TRPC6 also encompass 3–4 ankyrin repeats. Further experiments on the interaction between the cGK-I and TRPC family led to a better understanding of the regulation of TRPC family by cGMP/cGK signaling.

Although TRPC7 is expressed in the heart, lung and eye in mice [9], the physiological role of TRPC7 is still unknown. A recent study demonstrated that TRPC7 mediated angiotensin II-induced myocardial apoptosis [14]. However, another report revealed that TRPC3 and TRPC6, but not TRPC7, were essential for angiotensin II-induced cardiac hypertrophy [23]. TRPC6 positively regulates calcineurin-NFAT (nuclear factor of activated T cells) signaling, resulting in cardiac hypertrophy [24]. Importantly, TRPC7 displays some functions via association with other proteins, such as TRPC6 [12,25]. TRPC7 may form heteromeric channels with TRPC6, and be involved in cardiac failure. On the other hand, the TRPC7 transcript level is highest in cerebellar Purkinje cells [9]. cGK-Iα is also highly expressed in Purkinje cells. To date, only G-substrate has been identified as a substrate for cGK-I in Purkinje cells [26]. Phosphorylated G-substrate inhibited protein phosphatase 1/2A, leading to enhanced phosphorylation and internalization of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor, contributing to long-term depression. TRPC calcium signaling has also been implicated in AMPA receptor trafficking [27]. cGK-I may regulate AMPA receptor trafficking not only via G-substrate phosphorylation, but also via TRPC7 phosphorylation.

In conclusion, the present study demonstrates that TRPC7 activity is suppressed via phosphorylation by cGMP/cGK-I. Site-directed mutagenesis analysis revealed that this suppression was due to the direct phosphorylation of TRPC7 at threonine-15 by cGK-I. Furthermore, cGK-Iα was interacted with the N-terminal segment of TRPC7, and was localized in the plasma membrane. These observations suggest that TRPC7 is one of the targets of the cGMP/cGK signaling, which regulates intracellular calcium levels.

Acknowledgments

The authors thank Miss Yurika Kitashimizu and Mr Masaki Muroshita for their technical assistance. This work was supported in part by Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

[1] T. Münzel, R. Feil, A. Mülsch, S.M. Lohmann, F. Hofmann, U. Walter, Circulation 108 (2003) 2172–2183.
[2] S.M. Lohmann, A.B. Vaandrager, A. Smolenski, U. Walter, H.R. De Jonge, Trends Biochem. Sci 22 (1997) 307–312.
[3] J. Schlossmann, A. Ammendola, K. Ashman, X. Zong, A. Huber, G. Neubauer, G.X. Wang, H.D. Allescher, M. Korth, M. Wilm, F. Hofmann, P. Ruth, Nature 404 (2000) 197–201.
[4] M. Fukao, H.S. Mason, F.C. Britton, J.L. Kenyon, B. Horowitz, K.D. Keef, J. Biol. Chem. 274 (1999) 10927–10935.
[5] X. Yao, H.Y. Kwan, Y. Huang, Neurosignals 14 (2005) 273–280.
[6] H.Y. Kwan, Y. Huang, X. Yao, Proc. Nat. Acad. Sci. U.S.A. 101 (2004) 2625–2630.
[7] S. Takahashi, H. Lin, N. Geshi, Y. Mori, Y. Kawarabayashi, N. Takami, M.X. Mori, A. Honda, R. Inoue, J. Physiol. 586 (2008) 4209–4223.
[8] X. Wang, J.L. Pluznick, D.C. Settles, S.C. Sansom, Am. J. Physiol. 293 (2007) F1768–F1776.
[9] T. Okada, R. Inoue, K. Yamazaki, A. Maeda, T. Kurosaki, T. Yamakuni, I. Tanaka, S. Shimizu, K. Ikenaka, K. Imoto, Y. Mori, J. Biol. Chem. 274 (1999) 27359–27370.
[10] K. Yuasa, H. Michibata, K. Omori, N. Yanaka, J. Biol. Chem. 274 (1999) 37429–37434.
[11] A. Dietrich, M. Mederos y Schnitzler, J. Emmel, H. Kalwa, T. Hofmann, T. Gudermann, J. Biol. Chem. 278 (2003) 47842–47852.
[12] Y. Maruyama, Y. Nakanishi, E.J. Walsh, D.P. Wilson, D.G. Welsh, W.C. Cole, Circ. Res. 98 (2006) 1520–1527.
[13] Y. Tai, S. Feng, R. Ge, W. Du, X. Zhang, Z. He, Y. Wang, J. Cell Sci. 121 (2008) 2301–2307.
[14] S. Satoh, H. Tanaka, Y. Ueda, J. Oyama, M. Sugano, H. Sumimoto, Y. Mori, N. Makino, Mol. Cell. Biochem. 294 (2007) 205–215.
[15] A. Riccio, A.D. Medhurst, C. Mattei, R.E. Kelsell, A.R. Calver, A.D. Randall, C.D. Benham, M.N. Pangalos, Mol. Brain Res. 109 (2002) 95–104.
[16] T.K. Zagranichnaya, X. Wu, M.L. Villereal, J. Biol. Chem. 280 (2005) 29559–29569.
[17] K. Yuasa, K. Omori, N. Yanaka, J. Biol. Chem. 275 (2000) 4897–4905.
[18] L. Mery, B. Strauss, J.F. Dufour, K.H. Krause, M. Hoth, J. Cell Sci. 115 (2002) 3497–3508.
[19] M. Palmada, S. Poppendieck, H.M. Embark, S.F. van de Graaf, C. Boehmer, R.J. Bindels, F. Lang, Cell. Physiol. Biochem. 15 (2005) 175–182.
[20] B. Cha, J.H. Kim, H. Hut, B.M. Hogema, J. Nadarja, M. Zizak, M. Cavet, W. Lee-Kwon, S.M. Lohmann, A. Smolenski, C.M. Tse, C. Yun, H.R. de Jonge, M. Donowitz, J. Biol. Chem. 280 (2005) 16642–16650.
[21] J. Li, A. Mahajan, M.D. Tsai, Biochemistry 45 (2006) 15168–15178.
[22] M.P. Lussier, S. Cayouette, P.K. Lepage, C.L. Bernier, N. Francoeur, M. St-Hilaire, M. Pinard, G. Boulay, J. Biol. Chem. 280 (2005) 19393–19400.
[23] N. Onohara, M. Nishida, R. Inoue, H. Kobayashi, H. Sumimoto, Y. Sato, Y. Mori, T. Nagao, H. Kurose, EMBO J. 25 (2006) 5305–5316.
[24] M. Nishida, K. Watanabe, Y. Sato, M. Nakaya, N. Kitajima, T. Ide, R. Inoue, H. Kurose, J. Biol. Chem. 285 (2010) 13244–13253.
[25] M. Ju, J. Shi, S.N. Saleh, A.P. Albert, W.A. Large, J. Physiol. 588 (2010) 1419–1433.
[26] S. Endo, F. Shutoh, T.L. Dinh, T. Okamoto, T. Ikeda, M. Suzuki, S. Kawahara, D. Yanagihara, Y. Sato, K. Yamada, T. Sakamoto, Y. Kirino, N.A. Hartell, K. Yamaguchi, S. Itohara, A.C. Nairn, P. Greengard, S. Nagao, M. Ito, Proc. Nat. Acad. Sci. U.S.A. 106 (2009) 3525–3530.
[27] H. Nakata, S. Nakamura, FEBS Lett. 581 (2007) 2047–2054.