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Modulation of intracellular Ca^{2+} via α_{1B}-adrenoreceptor signaling molecules, $G\alpha_h$ (transglutaminase II) and phospholipase C-δ1

Sung Koo Kang,[a] Dae Kyong Kim,[e] Derek S. Damron,[b] Kwang Jin Baek,[a,d,*] and Mie-Jae Im[a,c,1]

[a] *Department of Molecular Cardiology, The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA*

[b] *Department of Anesthesiology, The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA*

[c] *Department of Chemistry, Cleveland State University, Cleveland, OH 44115, USA*

[d] *Department of Biochemistry, College of Medicine, Chung-Ang University, 221 Heuksuk-Dong, Dongjak-Ku, Seoul 156-756, Republic of Korea*

[e] *Department of Environmental & Health Chemistry, College of Pharmacy, Chung-Ang University, 221 Heuksuk-Dong, Dongjak-Ku, Seoul 156-756, Republic of Korea*

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Abstract

We characterized the α_{1B}-adrenoreceptor (α_{1B}-AR)-mediated intracellular Ca^{2+} signaling involving $G\alpha_h$ (transglutaminase II, TGII) and phospholipase C (PLC)-δ1 using DDT1-MF2 cell. Expression of wild-type TGII and a TGII mutant lacking transglutaminase activity resulted in significant increases in a rapid peak and a sustained level of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to activation of the α_{1B}-AR. Expression of a TGII mutant lacking the interaction with the receptor or PLC-δ1 substantially reduced both the peak and sustained levels of $[Ca^{2+}]_i$. Expression of TGII mutants lacking the interaction with PLC-δ1 resulted in a reduced capacitative Ca^{2+} entry. Reduced expression of PLC-δ1 displayed a transient elevation of $[Ca^{2+}]_i$ and a reduction in capacitative Ca^{2+} entry. Expression of the C2-domain of PLC-δ1, which contains the TGII interaction site, resulted in reduction of the α_{1B}-AR-evoked peak increase in $[Ca^{2+}]_i$, while the sustained elevation in $[Ca^{2+}]_i$ and capacitative Ca^{2+} entry remained unchanged. These findings demonstrate that stimulation of PLC-δ1 via coupling of the α_{1B}-AR with TGII evokes both Ca^{2+} release and capacitative Ca^{2+} entry and that capacitative Ca^{2+} entry is mediated by the interaction of TGII with PLC-δ1.

Keywords: Ca^{2+} signaling; α_1-Adrenoreceptor; G-protein; Transglutaminase II; Phospholipase C-δ1

A novel GTPase $G\alpha_h$, known as transglutaminase II (TGII), is a member of the transglutaminase (TGase) family that catalyzes the formation of a ε-(γ-glutamyl) lysine isopeptide bond between peptide-bound glutamyl residues and the lysyl group of polypeptides in a Ca^{2+}-dependent manner [1–3]. On the other hand, the GTPase function of TGII acts as a signal transducer in a receptor-mediated transmembrane signaling pathway that is similar to the function of the α subunits of heterotrimeric G-proteins [2]. TGII-coupled receptors include the α_{1B}-adrenoreceptor (AR) [4–8], α_{1D}-AR [6], α-thromboxane [9], and oxytocin [10] receptors. TGII selectively interacts with these receptors in a subtype-specific manner [6,9]. To date, the known effector for TGII is phospholipase C (PLC)-δ1 [11–14]. TGII also modulates the large conductance Ca^{2+}-activated K^+ channel in the vascular smooth muscle cells [15].

α_1-ARs are classified into three distinct subtypes ($\alpha_{1A=C}$, α_{1B}, and α_{1D}), based on their various ligand-binding properties and physiological responses [16]. These receptors stimulate multiple effectors via multiple G-proteins. G-proteins are G_q family of proteins [17] and pertussis toxin sensitive G-proteins [18], including TGII [2]. The $G\alpha_q$ family proteins stimulate PLC-β, which hydrolyzes phosphatidyinositol 4,5-bisphosphate (PIP_2), producing two second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [19,20].

* Corresponding author. Fax: +822-813-5387.

E-mail addresses: kjbaek@cau.ac.kr (K.J. Baek), imm@ccf.org (M.-J. Im).

[1] Also corresponding author. Fax: +444-216-9263.

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Interaction of IP_3 with its specific receptor releases Ca^{2+} from the intracellular Ca^{2+} stores, resulting in an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [21]. DAG activates protein kinase C, which then phosphorylates a variety of cellular substrates. Stimulation of PLC-δ1 by the coupling of the α_{1B}-AR with TGII also produces these two second messengers [2,19,20]. The α_{1A}-AR, which does not couple with TGII, utilizes $G\alpha_q$ and $G\alpha_{11}$ to regulate intracellular Ca^{2+} signaling through a process of Ca^{2+} release and Ca^{2+} entry [22]. In response to the activation of the receptor, $G\alpha_q$ induces Ca^{2+}-release, whereas $G\alpha_{11}$ is involved in Ca^{2+} entry. The α_{1B}-AR couples with five different G-proteins, four members ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{16}$) of $G\alpha_q$ family [17] and TGII. Studies have demonstrated that activation of the α_{1B}-AR induces Ca^{2+} release and Ca^{2+} entry, causing a sustained rise of $[Ca^{2+}]_i$ [23,24]. However, G-protein involved in α_{1B}-AR-mediated intracellular Ca^{2+} responses has not been identified.

In this study, we elucidated intracellular Ca^{2+} signaling mediated by the coupling of the α_{1B}-AR with TGII. To determine the specific regulation of Ca^{2+} signaling by the coupling, wild-type TGII (wtTG) and various forms of TGII mutants were stably transfected in a smooth muscle cell line, hamster leiomyosarcoma (DDT1-MF2). The cell expresses only the α_{1B}-subtype in α_1-ARs [25] and low levels of TGII and PLC-δ1 [14]. PLC-δ1 is not only a PIP_2 hydrolyzing enzyme but also acts as a guanine nucleotide-exchanging factor (GEF) and GTP hydrolysis-inhibiting factor (GHIF) for TGII [14]. These functions of PLC-δ1 for TGII facilitate TGII-mediated signaling. Therefore, the role of PLC-δ1 in intracellular Ca^{2+} signaling was also investigated. We identified a TGII interaction site in PLC-δ1 located within the C2-domain of the enzyme. Constructs of antisense and the C2-domain of PLC-δ1 and wild-type PLC-δ1 (wtPLCδ1) were stably expressed. The results show that stimulation of PLC-δ1 by the coupling of the α_{1B}-AR with TGII evokes Ca^{2+} release and a sustained elevation of $[Ca^{2+}]_i$. The sustained elevation of $[Ca^{2+}]_i$ is due to capacitative Ca^{2+} entry followed by activation of these three key signaling components. Our data also indicate that TGII is the primary molecule involved in capacitative Ca^{2+} entry and that interaction of TGII with PLC-δ1 via the C2-domain of PLC-δ1 plays a critical role in maintaining the TGII-mediated Ca^{2+} entry.

Materials and methods

Construction of C2-domain and antisense of PLC-δ1. A clone of rat PLC-δ1 cDNA in pIBI20 (provided by Dr. Sue Goo Rhee, Laboratory of Cell Signaling, National Heart, Lung and Blood Institute, NIH) was inserted into pZeoSV2 using Not1 site. A clone in antisense orientation was selected and digested with PvuII to remove most of the coding regions. The remaining coding regions, 5′ and 3′ noncoding regions in pZeoSV2 (Invitrogen) were ligated and used as the antisense constructs of PLC-δ1 (antiPLCδ1). On the basis of the observation that the C2-domain of PLC-δ1 contained a TGII interaction site (Fig. 4), the C2-domain of PLC-δ1 in pZeoSV2 was constructed by removing the coding region of PLC-δ1 DNA in the vector using PvuII. The C2-domain construct contains the 5′ region 184 base pairs of the coding region of the gene.

Transfection and cell culture. DDT1-MF2 cells expressing wtTG and its mutants were utilized for the study [14]. Briefly, DNAs of TGIIs in pcDNA3.1-Neo (Invitrogen) were expressed in DDT1-MF2 cells and grown in the presence of 300 μg/ml G418 in DMEM containing 10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin (growth media). The expression levels of TGIIs were comparable with each other (see [14]). Plasmids of wtPLCδ1, C2-domain, and antiPLCδ1 in pZeoSV2 vector were stably transfected into DDT1-MF2. Cells were selected using 500 μg/ml Zeocin and maintained in the growth media containing 200 μg/ml Zeocin. Expression levels of proteins were determined by immunoblotting using a TGII antibody (CUB7402, NeoMarker) or a PLC-δ1 antibody (Upstate Biotechnology) [7]. Expression and blocking ability of the C2-domain for interaction of TGII with PLC-δ1 were determined by measuring the α_{1B}-AR-mediated $[Ca^{2+}]_i$, as described below.

Measurement of intracellular Ca^{2+} level. $[Ca^{2+}]_i$ was measured using Fura 2-AM (Texas Fluorescence) as previously described [14]. Culture dishes containing fura 2-loaded cells were placed in a temperature-regulated (37 °C) chamber (Bioptechs.) mounted on the stage of an Olympus IX-70 inverted fluorescence microscope (Olympus America). Fluorescence measurements were obtained from either individual cells or from a cluster of neighboring cells in a culture monolayer using a dual-wavelength spectrofluorometer (Deltascan RFK6002, Photon Technology) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The cells were superfused continuously at 1 ml/min with Krebs–Ringer (KR) buffer, which contained (in mM) 125 NaCl, 5 KCI, 1.2 $MgSO_4$, 11 glucose, 2.5 $CaCl_2$, and 25 HEPES, at pH 7.4 adjusted with NaOH. Blockers of α-AR (rauwalscine; 1×10^{-7} M) and β-AR (propranolol; 1×10^{-6} M) were included throughout the study. Just prior to data acquisition, background fluorescence (i.e., fluorescence between cells) was measured and subtracted automatically from the subsequent experimental measurements. Fura 2 fluorescence signals (340, 380, and 340/380 ratio) originating from a cluster of cells ($\leqslant 5$) were continuously monitored to minimize the variability of outcomes due to the different expression levels of proteins in cells at a sampling frequency of 25 Hz. The α_{1B}-AR-mediated $[Ca^{2+}]_i$ was determined by addition of 10^{-5} M (−)epinephrine. All experiments were performed on at least three separate occasions.

Capacitative Ca^{2+} entry was determined using thapsigargin which increases $[Ca^{2+}]_i$ via irreversible inhibition of endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) Ca^{2+}-ATPase [21]. In the absence of extracellular Ca^{2+}, thapsigargin (2 μM) was applied to deplete ER/SR Ca^{2+} stores. With thapsigargin still present capacitative Ca^{2+} entry was then induced by restoring the extracellular Ca^{2+} concentration (2.5 mM).

Measurement of IP_3 production in cells. Production of IP_3 was determined in cells using IP_3 [^{3}H]-radioimmunoassay kit (Du Pont NEN, Boston MA). COS-1 cells transfected with the α_{1B}-AR were permeabilized using streptolysine O (10 μM) for 30 min, washed, and then incubated with peptides derived from the C2-domain of PLC-δ1 at 37 °C for 30 min (Fig. 4A). The α_{1B}-AR-mediated IP_3 production was determined in the presence and absence of 10^{-5} M epinephrine. IP_3 formation in the cells was stopped at 8 min by adding ice-cold 20% TCA after removing the media as recommended by the manufacturer [7]. Changes in the levels of IP_3 by the thapsigargin-induced Ca^{2+} entry were performed under the same conditions used to determine capacitative Ca^{2+} entry. Prolonged treatment of the cells with Ca^{2+}-containing KR buffer did not change the IP_3 level among the cell lines examined.

Preparation of peptide affinity gel. Equimolar concentration of peptides was cross-linked to CNBr-activated Sepharose 4B using a protocol provided by the manufacturer (Pharmacia). Peptides were synthesized at the Molecular Biotechnology Core Facility of the Lerner Research Institute at the Cleveland Clinic Foundation. The purity of the synthesized peptides was determined by HPLC and mass spectroscopy.

Results

α_{1B}-AR-evoked rise of $[Ca^{2+}]_i$ is mediated by stimulation of PLC-δ1 via TGII

Activation of the α_{1B}-AR results in a rapid peak and a sustained elevation of $[Ca^{2+}]_i$ in various cell types [23,24]. To examine the roles of TGII and PLC-δ1 in α_{1B}-AR-mediated Ca^{2+} signaling, DDT1-MF2 cells were stably expressed with wtTG and a TGII mutant (Δδ1TG), which lacks the interaction with PLC-δ1 by deleting the interaction site [7,14]. The effect of coupling between the α_{1B}-AR and TGII on the intracellular Ca^{2+} signals was determined in the presence and absence of extracellular Ca^{2+} (Fig. 1). To examine whether the sustained elevation of $[Ca^{2+}]_i$ is due to Ca^{2+} entry, cells were washed with Ca^{2+}-free buffer when the sustained Ca^{2+} signal had stabilized. Fig. 1A and B demonstrate Ca^{2+} fluorescence levels in the presence of extracellular Ca^{2+}. Activation of the α_{1B}-AR in the control (vector) cells resulted in a rapid peak and sustained elevation in $[Ca^{2+}]_i$. Both the peak and sustained increases in $[Ca^{2+}]_i$ were greatly increased in the cells expressing wtTG. The levels of $[Ca^{2+}]_i$ in the wtTG cells were ~152% (peak) and ~35% (sustained at 290 s) higher than those in the control cells, respectively. The cells expressing Δδ1TG showed the decreased levels of the peak (~43% reduction) and the sustained $[Ca^{2+}]_i$ (~55% reduction) as compared to those in the vector cells. Depletion of extracellular Ca^{2+} by washing the cells with the Ca^{2+}-free KR buffer resulted in an immediate decrease in the sustained Ca^{2+} signal and returned to the baseline in all cell lines. These results show that the sustained rise in $[Ca^{2+}]_i$ is caused by Ca^{2+} entry.

Release of Ca^{2+} from the stores was determined in the absence of the extracellular Ca^{2+}. Activation of the α_{1B}-AR resulted in a transient increase in $[Ca^{2+}]_i$ in all cell lines (Fig. 1C). The transient rise of $[Ca^{2+}]_i$ in the wtTG cells was increased by ~94%, and the Ca^{2+} rise in the Δδ1TG cells was reduced by ~61% as compared to that in the control cells. The residual increase in $[Ca^{2+}]_i$ was consistently observed with the Δδ1TG cells, probably due to an incomplete block of the functional complex formation consisting of the α_{1B}-AR, endogenous TGII, and endogenous PLC-δ1 by Δδ1TG. The increase in $[Ca^{2+}]_i$ was completely blocked by incubation of the cells with a nonspecific PLC inhibitor U73122, showing that the increase in $[Ca^{2+}]_i$ is a result of PLC stimulation

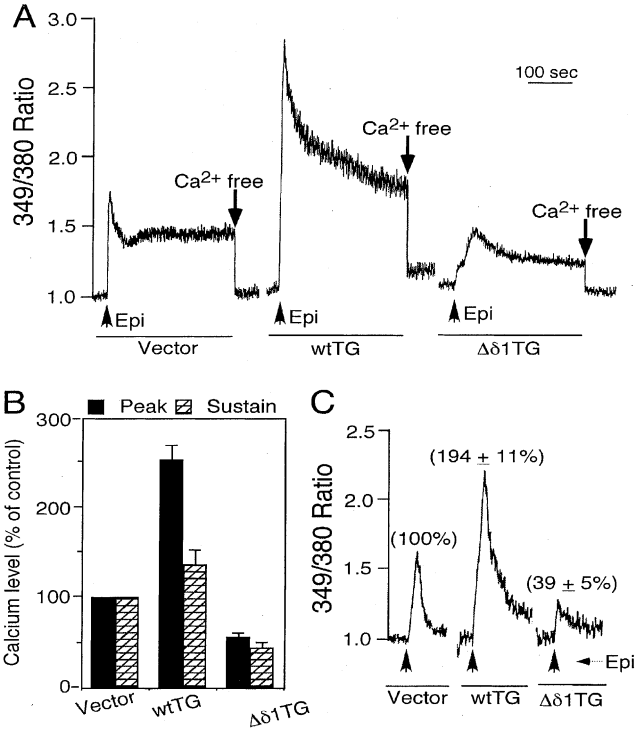

Fig. 1. α_{1B}-AR-evoked intracellular Ca^{2+} signaling in the cells expressing wtTG and Δδ1TG. (A) The α_{1B}-AR-induced peak and sustained rises of $[Ca^{2+}]_i$ are mediated by the stimulation of PLC-δ1 by TGII. Ca^{2+} responses were obtained for successive application of epinephrine (Epi, 1×10^{-5} M) in KR buffer containing 2.5 mM Ca^{2+}. At the indicated time (300 s, the cells were washed with Ca^{2+}-free KR buffer to determine the source of the sustained Ca^{2+} response. (B) Comparison of the peak and the sustained levels of $[Ca^{2+}]_i$ among cell lines expressing vector (control), wtTG, and Δδ1TG. The level of $[Ca^{2+}]_i$ in the vector (control) cells was taken as 100%. The Ca^{2+} level at 290 s was taken as the sustained elevation of $[Ca^{2+}]_i$ using each baseline reached by application of Ca^{2+}-free KR buffer. (C) The α_{1B}-AR-evoked Ca^{2+} signal in the absence of extracellular Ca^{2+}. The changes in $[Ca^{2+}]_i$ are presented on top of the peak Ca^{2+} response. The vector control was taken as 100%, and data shown in (B) and top of peak in (C) are means ± SD from three experiments.

(data not shown). Both the transient and the sustained increases in $[Ca^{2+}]_i$ were also blocked by the α_1-antagonist prazosine (10 nM), demonstrating that the Ca^{2+} signals are mediated by the activation of the α_{1B}-AR. Taken together, stimulation of PLC-δ1 by coupling the α_{1B}-AR with TGII evokes both Ca^{2+} release and Ca^{2+} entry.

The α_{1B}-AR-evoked Ca^{2+} cycling is mediated by G-protein function of TGII, but not by TGase

TGII is a bifunctional enzyme possessing GTPase and TGase activities [5]. The TGase activity is involved in posttranslational modification by cross-linking a variety of proteins in a Ca^{2+}-dependent manner. To delineate GTPase versus TGase activity of TGII in the α_{1B}-AR-evoked Ca^{2+} cycling, aTGII mutant ($^{C-S}$TG)

was stably expressed. This TGII mutant exhibits only G-protein function due to mutation of cysteine277 for serine in the TGase active site [26]. To ensure that the coupling of the α_{1B}-AR with TGII triggers Ca^{2+} cycling, another TGII mutant (m3TG) lacking the interaction with the α_{1B}-AR was also utilized. Expression of $^{C-S}$TG resulted in a rapid peak and a sustained increase in $[Ca^{2+}]_i$ in response to activation of the α_{1B}-AR, which were similar to those observed with the wtTG cells (see Fig. 1A and B). The increases in the peak and sustained levels of $[Ca^{2+}]_i$ in $^{C-S}$TG cells were ~195% and ~65% higher than those in the vector cells, respectively. Depletion of the extracellular Ca^{2+} again resulted in an immediate return to the baseline Ca^{2+} level in all cell lines. These data demonstrate that G-protein function of TGII, but not TGase, is involved in this α_{1B}-AR-mediated Ca^{2+} signaling process. In the cells expressing m3TG, the peak increase in $[Ca^{2+}]_i$ was small (~65% reduction as compared to the control cells), accordingly the sustained $[Ca^{2+}]_i$ was also greatly reduced (~76% reduction at 290 s), again showing that both Ca^{2+} release and Ca^{2+} entry are mediated by the coupling of the α_{1B}-AR with TGII.

Identity of the molecule that is responsible for Ca^{2+} entry

The above data clearly indicate that sustained rise of $[Ca^{2+}]_i$ is due to Ca^{2+} entry that is evoked by the coupling of the α_{1B}-AR with TGII and subsequent stimulation of PLC-δ1. To determine the molecular entity involved in this Ca^{2+} entry process, we examined capacitative Ca^{2+} entry independent of receptor activation. Capacitative Ca^{2+} entry is triggered by the depletion of intracellular Ca^{2+} stores [21]. A Ca^{2+}-ATPase inhibitor, thapsigargin has been widely used to evaluate capacitative Ca^{2+} entry in a variety of cell types [27–29]. An increase in the Ca^{2+} signal in response to thapsigargin was also assessed in the absence of the extracellular Ca^{2+} to ensure that an equal amount of Ca^{2+} is released. Thapsigargin-induced Ca^{2+} release showed a similar level among the cell lines expressing vector, wtTG, and wtPLCδ1 (Fig. 3A and B). Once the baseline Ca^{2+} signal was stabilized, the extracellular Ca^{2+} was restored in the continued presence of thapsigargin. Restoration of extracellular Ca^{2+} (2.5 mM) resulted in the higher level of $[Ca^{2+}]_i$ in the wtTG cells by ~40% than in the vector or wtPLCδ1 cells. The increase in capacitative Ca^{2+} entry in wtPLCδ1 cells was similar to that in the control cells. When capacitative Ca^{2+} entry was measured in the Δδ1TG cells, the level of Ca^{2+} entry was lower by ~32% than that in the vector cells (Fig. 3C and D). Since activation of PLC-δ1 by capacitative Ca^{2+} entry was

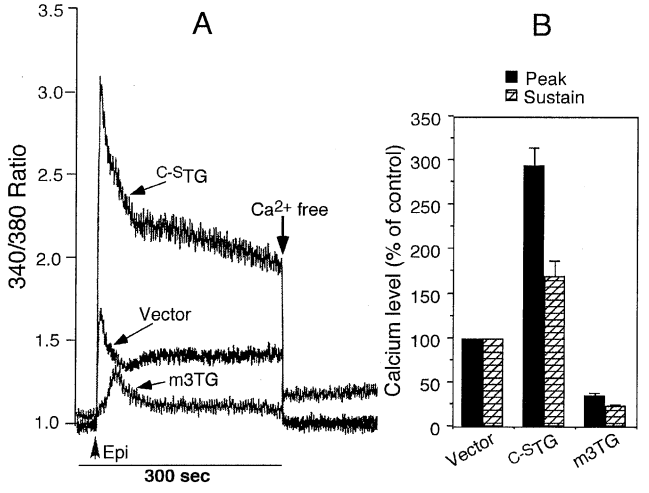

Fig. 2. α_{1B}-AR-evoked intracellular Ca^{2+} signaling in the cells expressing $^{C-S}$TG and m3TG. (A) The α_{1B}-AR-evoked peak and sustained increases in $[Ca^{2+}]_i$ are mediated by the coupling of the receptor with TGII via its G-protein function. The Ca^{2+} responses were obtained under the same conditions as described in (A). Epi, epinephrine. (B) Comparison of the peak and the sustained levels of $[Ca^{2+}]_i$ among cell lines expressing vector (control), $^{C-S}$TG, and m3TG. The vector control was taken as 100%, and the results are means ± SD from three experiments.

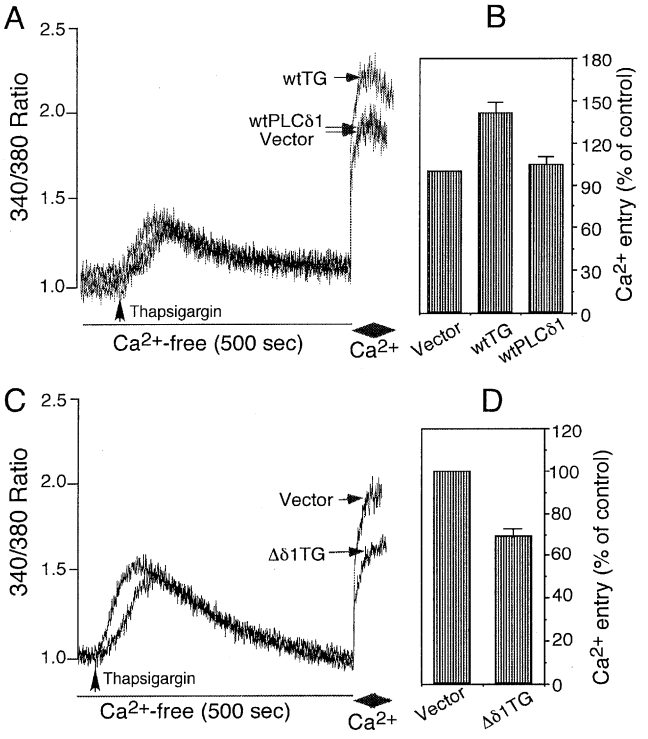

Fig. 3. Comparison of capacitative Ca^{2+} entry in cells expressing vector, wtTG, Δδ1TG, and wtPLCδ1. Calcium in the intracellular stores was depleted by treating the cells with 2 μM thapsigargin in the Ca^{2+}-free KR buffer. When $[Ca^{2+}]_i$ level retrieved to baseline, Ca^{2+} entry was initiated by addition of KR buffer containing 2.5 mM Ca^{2+} and thapsigargin (2 μM). (A and B) Thapsigargin-induced capacitative Ca^{2+} entry in the cells expressing vector, wtTG and wtPLCδ1. (C and D) Thapsigargin-induced capacitative Ca^{2+} entry in the cells expressing vector and Δδ1TG. The results shown in (B) and (D) are means ± SD from three experiments. The vector cell was control (100%).

demonstrated [30], a possibility that the interaction of TGII with PLC-δ1 may stimulate PLC-δ1 in the wtTG cells was also assessed. Capacitative Ca^{2+} entry-induced IP_3 production was measured in these cells under the same conditions used to determine capacitative Ca^{2+} entry. There were no significant differences in IP_3 level among these cell lines, indicating that the increase or decrease in Ca^{2+} entry observed with the wtTG or Δδ1TG cells is not due to the activation of PLC-δ1.

Interaction of TGII with PLC-δ1, but not stimulation of PLC-δ1, is required for the sustained Ca^{2+} entry evoked by the activation of the α_{1B}-AR

The above data suggest two mechanisms: Δδ1TG may have lost a region which is responsible for capacitative Ca^{2+} entry, because 30 amino acids beginning from the C-terminus of TGII are absent in this mutant [7]; and the other is that the interaction of TGII with PLC-δ1 may stabilize GTP-bound form of TGII, since G-protein function of TGII is involved in both the peak and the sustained rises in $[Ca^{2+}]_i$ (see Fig. 2). PLC-δ1 not only induces GTP binding to TGII via its GEF function but also stabilizes the GTP–TGII via its GHIF action [14]. To evaluate these mechanisms, two approaches were undertaken: identification and expression of the TGII interacting domain in PLC-δ1 and reduced expression of PLC-δ1using an antisense approach.

Based on the structural analyses in the previous reports [31–33], the most probable TGII interaction site is located within the C2-domain of PLC-δ1. It is known that deletion of the C2-domain results in loss of the enzyme activity [33]. Consistent with these observations, the deletion mutants of the C2-domain failed to show any enzyme activity (data not shown). Therefore, a TGII interaction site in PLC-δ1 was identified using synthetic peptides derived from the C2-domain of PLC-δ1 (Fig. 4A). Among five peptides, peptide 4 (P4) was able to bind TGII more (~5-fold) than other peptides (Fig. 4B). The P4-bound TGII was displaced by P4 and intact PLC-δ1 in a concentration-dependent manner (Fig. 4C). To assess the ability of P4 to block the interaction of TGII with PLC-δ1, the α_{1B}-AR was expressed in COS-1 cells, which express TGII and PLC-δ1 [7]. The results revealed that IP_3 formation was inhibited ~50% by P4 (100 μM) but not by other peptides (Fig. 4D). The inhibition was also concentration-dependent on P4 (Fig. 4E).

The C2-domain and antisense constructs of PLC-δ1 were stably expressed (see Materials and methods). Transfection of the antisense construct of PLC-δ1 (antiPLCδ1) reduced the expression level of the protein by ~80% (inset in Fig. 5A). Measurements of the α_{1B}-AR-induced Ca^{2+} signaling showed that the control cells displayed a rapid peak and a sustained rise of $[Ca^{2+}]_i$. In contrast, reduced expression of PLC-δ1 by transfection

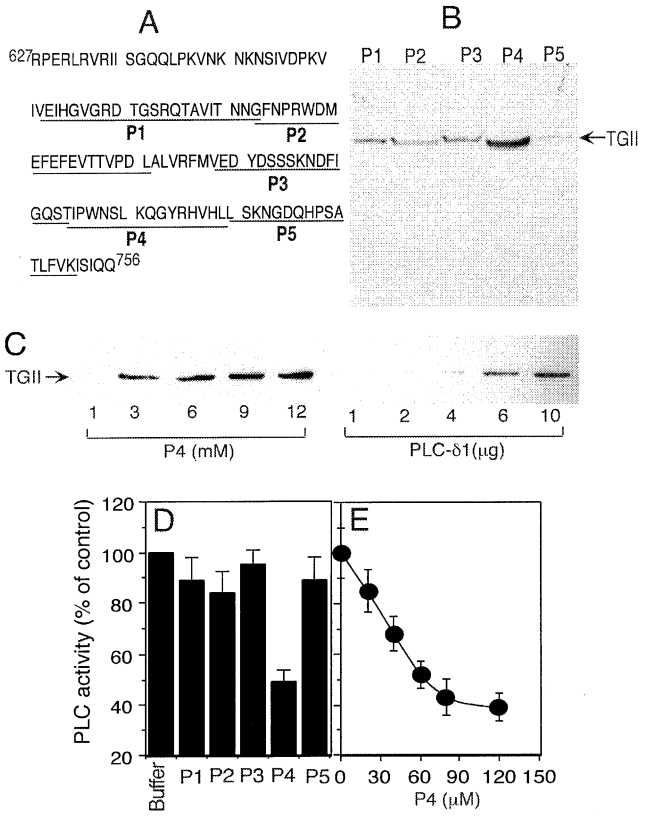

Fig. 4. Interaction site of TGII on PLC-δ1 is located within C2-domain of PLC-δ1. (A) A map of peptide (P) derived from the C2-domain of PLC-δ1. (B) Binding of TGII to the peptide-affinity gels. Cytosol fraction (500 μg) prepared from rat liver was incubated with the peptide-gel (150 μl of 1:1 suspension) overnight at 4 °C. The gels were washed three times (1 ml/wash) with 25 mM HEPES (pH, 7.4) containing 200 mM NaCl and 0.1% sucrose monolaurate. TGII bound to the peptide-gels was visualized by immunoblotting using a TGII antibody. (C) Elution of TGII bound to P4-affinity gel by P4 or intact PLC-δ1. Eluted TGII was visualized by immunoblotting using a TGII antibody. (D) Inhibition of IP_3 formation by P4. The concentration of peptides was 100 μM. Samples added buffer alone (control) was taken as 100%. (E) Inhibition of IP_3 formation as a function of P4 concentration. The results presented are means ± SD from two experiments.

of antiPLCδ1 resulted in a broad transient rise of $[Ca^{2+}]_i$ that gradually decreased toward the baseline. When the thapsigargin-induced Ca^{2+} entry in the antiPLCδ1 cells was examined, the Ca^{2+} entry was ~42% lower than that in the control cells (Fig. 5A), indicating that PLC-δ1 is involved in this Ca^{2+} entry process. Expression of the C2-domain resulted in a reduced peak elevation in $[Ca^{2+}]_i$, by ~40% as compared to that in the vector cells. Although at the early stage, the sustained rise of $[Ca^{2+}]_i$ was blocked by ~28%, the sustained Ca^{2+} signal gradually reached the level of the vector. Thapsigargin-induced Ca^{2+} entry was similar to the vector (Fig. 5D). The results indicate that the interaction of the C2-domain of PLC-δ1 with TGII is required for TGII to maintain Ca^{2+} entry and that reduced capacitative Ca^{2+} entry in Δδ1TG cells is due to the inability of this TGII mutant to interact with PLC-δ1.

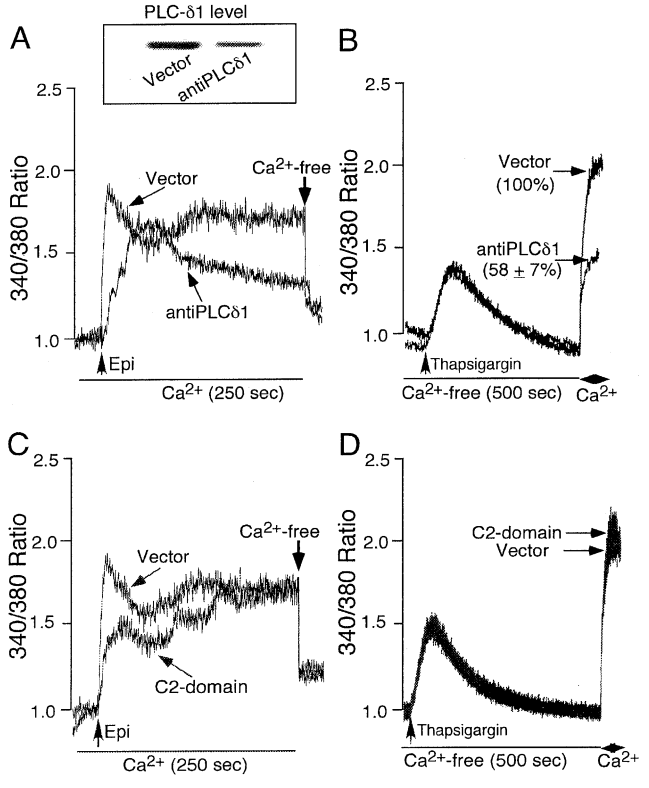

Fig. 5. The role of PLC-δ1 in the α_{1B}-AR-mediated $[Ca^{2+}]_i$ signaling. Inset in (A) shows reduction of PLC-δ1 expression by transfection of antiPLCδ1. Cells lysates (200 μg) prepared from the cells expressing vector or antiPLCδ1 were subjected to immunoblotting using a PLC-δ1 antibody, followed by SDS–PAGE (10% gel). (A) The α_{1B}-AR-evoked Ca^{2+} responses in the cells expressing antiPLCδ1. Epinephrine (Epi, 1×10^{-5} M) was added where indicated. The remaining levels of the sustained $[Ca^{2+}]_i$ were determined by washing the cells with Ca^{2+}-free KR buffer at 250 s. (B) Thapsigargin-induced capacitative Ca^{2+} entry in the cells expressing vector and antiPLCδ1. (C) The α_{1B}-AR-evoked Ca^{2+} responses in the cells expressing C2-domain of PLC-δ1. (D) Thapsigargin-induced capacitative Ca^{2+} entry in the cells expressing C2-domain. Thapsigargin concentration was 2 μM.

Discussion

In this study, we have investigated characteristics of the α_{1B}-AR-mediated intracellular Ca^{2+} signaling involving TGII and PLC-δ1. To determine the specificity and the molecular entity in the Ca^{2+} signaling, DDT1-MF2 cells were stably transfected with wtTG, various forms of TGII mutant, wtPLCδ1, antisense, and C2-domain constructs of PLC-δ1. The studies have revealed that coupling of three key signaling components evokes Ca^{2+} release and Ca^{2+} entry and that TGII is the molecule responsible for the Ca^{2+} entry. The results also indicate that an interaction of TGII with PLC-δ1 is required for TGII-mediated Ca^{2+} entry thereby maintaining a sustained elevation in $[Ca^{2+}]_i$. In addition, we have for the first time revealed an interaction site of TGII in PLC-δ1 that is located at the ^{721}T–^{736}L region of C2-domain.

Involvement of TGII and PLC-δ1 in the α_{1B}-AR-evoked Ca^{2+} release and sustained elevation of $[Ca^{2+}]_i$ is substantiated by the observations that expression of wtTG or $^{C-S}$TG results in significant increases in both Ca^{2+} signals and that these Ca^{2+} signals are greatly reduced by expression of m3TG, which lacks the interaction with the α_{1B}-AR and of Δδ1TG, which lacks the interaction with PLC-δ1. In this α_{1B}-AR-mediated Ca^{2+} cycling process, stimulation of PLC-δ1 by the coupling of the receptor with TGII induces Ca^{2+} release via the IP_3 receptor, since expression of Δδ1TG reduces Ca^{2+} release. In an intracellular Ca^{2+} replenishment process, it appears that TGII induces capacitative Ca^{2+} entry. Thus, thapsigargin-induced Ca^{2+} entry was higher in the cells expressing wtTG than in cells expressing vector control or wtPLCδ1. In this TGII-mediated Ca^{2+} entry, PLC-δ1 is likely to play a critical role. Three lines of observations support this conclusion: (a) expression of Δδ1TG reduced the sustained rise of $[Ca^{2+}]_i$ and capacitative Ca^{2+} entry; (b) reduced expression of PLC-δ1 resulted in a transient increase in $[Ca^{2+}]_i$ and decrease in capacitative Ca^{2+} entry; and (c) expression of C2-domain of PLC-δ1 resulted in a small reduction of the sustained elevation of $[Ca^{2+}]_i$ that gradually returned to control level. The rapid peak rise of $[Ca^{2+}]_i$ was significantly attenuated in this cell line. Furthermore, in the cells expressing the domain, thapsigargin-induced capacitative Ca^{2+} entry was similar to that observed in the control cells. However, it should not be overlooked that the gradual increase in the sustained $[Ca^{2+}]_i$ in this cell line may be due to slow displacement of the interaction of TGII with the C2-domain by endogenous PLC-δ1. The GEF activity of PLC-δ1 for TGII plays an important role in the activation of TGII by facilitating the α_{1B}-AR role (the receptor is prime GEF) at initiation step of the receptor signaling [14]. Since G-protein function of TGII evokes the sustained elevation of $[Ca^{2+}]_i$, it is likely that the GHIF activity of PLC-δ1 is involved in TGII-mediated Ca^{2+} entry by stabilizing the GTP-bound form of TGII.

It appears that in the Ca^{2+} entry process, interaction of TGII with PLC-δ1, but not stimulation of the enzyme, is required for TGII to maintain Ca^{2+} entry. This finding suggests that when intracellular Ca^{2+} level reaches a certain level, the primary function of PLC-δ1 that is PIP_2 hydrolysis is latent. Although there is no direct evidence to support the mechanism, some spurs have pointed out that this possibility may exist. An increase in IP_3 by the capacitative Ca^{2+} entry is not observed among the cell lines expressing vector, wtTG and wtPLCδ1. We have previously reported that stimulation of PLC-δ1 (69 kDa fragment) by GTPγS-TGII exhibits a biphasic modulation of the enzyme activity: at low concentrations of Ca^{2+}, the enzyme was stimulated, whereas at high concentrations of Ca^{2+}, the enzyme was inactivated [34]. Inhibition of PLC stimulation via TGII

has been observed when the level of TGII expression is increased [35]. Consistent with these observations, high expression of TGII in vivo has resulted in no stimulation of PLC [36]. A recent study has indicated that a small portion of PLC-δ1 expressed in PC-12 cells is activated by capacitative Ca^{2+} entry [30]. All of these observations suggest that Ca^{2+} and TGII may interact with each other to define PLC-δ1 functions as a PIP_2 hydrolyzing enzyme, GEF and GHIF for TGII, and others which are yet to be identified. In addition, results from our study indicate that the association of GTP–TGII with PLC-δ1 stimulates PLC-δ1. To date, two mechanisms of PLC-δ1 stimulation via TGII are proposed: one is the release of GTP–TGII from PLC-δ1 upon binding of GTP to TGII thereby PLC-δ1 released from TGII is activated by Ca^{2+} when the intracellular Ca^{2+} level is increased [13]. The other mechanism is an association of GTP–TGII with PLC-δ1 to activate the enzyme [7,11,14, and present study]. In the case of the former mechanism, the α_{1B}-AR-evoked increase in Ca^{2+} release and Ca^{2+} entry in the cells overexpressing wtTG or $^{C-S}$TG would not be observed, since it is likely that the level of PLC-δ1 associated with TGII is increased. Moreover, reduction of Ca^{2+} release and Ca^{2+} entry by the expression of Δδ1TG would not be observed, because PLC-δ1 is present as a free form.

In summary, our studies have indicated that stimulation of PLC-δ1 by the coupling of the α_{1B}-AR with TGII regulates intracellular Ca^{2+} signaling through mechanisms involving receptor-mediated Ca^{2+} release and capacitative Ca^{2+} entry. TGII and PLC-δ1 seem to play a dual role in these Ca^{2+} release and refilling processes. Stimulation of PLC-δ1 by TGII releases Ca^{2+} from the intracellular stores, and association of TGII with PLC-δ1 maintain Ca^{2+} entry. The mechanism by which TGII stimulates Ca^{2+} entry remains unknown. Studies have indicated that tyrosine kinases are involved in the α_{1B}-AR-mediated capacitative Ca^{2+} entry [23,24]. In this regard, it is of interest to determine whether TGII activates one of these kinases.

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