Ca\textsuperscript{2+} influx and protein scaffolding via TRPC3 sustain PKC\(\beta\) and ERK activation in B cells

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Summary

Ca\textsuperscript{2+} signaling mediated by phospholipase C that produces inositol 1,4,5-trisphosphate [Ins(1,4,5)\(P_3\)] and diacylglycerol (DAG) controls lymphocyte activation. In contrast to store-operated Ca\textsuperscript{2+} entry activated by Ins(1,4,5)\(P_3\)-induced Ca\textsuperscript{2+} release from endoplasmic reticulum, the importance of DAG-activated Ca\textsuperscript{2+} entry remains elusive. Here, we describe the physiological role of DAG-activated Ca\textsuperscript{2+} entry channels in B-cell receptor (BCR) signaling. In avian DT40 B cells, deficiency of transient receptor potential TRPC3 at the plasma membrane (PM) impaired DAG-activated cation currents and, upon BCR stimulation, the sustained translocation to the PM of protein kinase C\(\beta\) (PKC\(\beta\)) that activated extracellular signal-regulated kinase (ERK). Notably, TRPC3 showed direct association with PKC\(\beta\) that maintained localization of PKC\(\beta\) at the PM. Thus, TRPC3 functions as both a Ca\textsuperscript{2+}-permeable channel and a protein scaffold at the PM for downstream PKC\(\beta\) activation in B cells.

Key words: B-cell receptor, Ca\textsuperscript{2+} signaling, Diacylglycerol, PKC\(\beta\), TRP channels

Introduction

Calcium signaling evoked by stimulation of plasma membrane (PM) receptors linked to phospholipase C (PLC) plays a central role in lymphocyte activation through production of inositol 1,4,5-trisphosphate [Ins(1,4,5)\(P_3\)] and diacylglycerol (DAG) (Berridge, 1993). In B cells, distinct patterns of Ca\textsuperscript{2+} signaling produced by B-cell receptor (BCR) engagement dictate alternative programs of transcription factor activation and thereby distinct cell fate (Liu et al., 2005). Ins(1,4,5)\(P_3\) receptor mediates Ca\textsuperscript{2+} release from the internal Ca\textsuperscript{2+} stores of endoplasmic reticulum (ER). In addition, Ca\textsuperscript{2+} influx through diverse Ca\textsuperscript{2+}-permeable ion channels is activated by various triggers to control Ca\textsuperscript{2+} signaling (Fasolato et al., 2002). However, it is known that in non-excitable cells, other Ca\textsuperscript{2+}-permeable channels may be activated directly by intracellular messengers such as DAG, Ca\textsuperscript{2+}, Ins(1,4,5)\(P_3\) and arachidonic acid and its metabolites produced downstream of PLC (Fasolato et al., 1994; Bird et al., 2004; Parekh and Putney, 2005). Notably, B cells isolated from the above-mentioned patients with a defect in CRAC activity are capable of mounting normal immune responses (Partiseti et al., 1994; Le Deist et al., 1995; Feske et al., 2001), suggesting that Ca\textsuperscript{2+} influx pathways other than SOCs play essential roles in physiological responses of B cells.

Drosophila transient receptor potential (trp) protein (TRP), which was discovered through genetic studies of a Drosophila visual transduction mutation (Montell and Rubin, 1989), and the vertebrate TRP homologues of the so-called ‘canonical’ subfamily TRPC are channels that may mediate Ca\textsuperscript{2+} influx induced by activation of PLC-coupled receptors (Nishida et al., 2006). TRP homologues were originally hypothesized to encode SOCs, and some supportive evidence for this hypothesis was obtained from cDNA expression and gene knockout experiments for various TRP subtypes (Parekh and Putney, 2005). However, store-independent activation of Ca\textsuperscript{2+} influx and cation currents mediated by TRP channels seem to be the more common function of this channel family, especially the TRPCs (Hofmann et al., 2000; Bird et al., 2004; Parekh and Putney, 2005). Among the seven members of vertebrate TRPCs (TRPC1-7), TRPC2, TRPC3, TRPC6 and TRPC7 have been reported to be activated by DAG (Hofmann et al., 1999; Okada et al., 1999; Lucas et al., 2003). With regard to
Here, we describe an investigation of DACCs and their physiological importance in the context of BCR signaling. In DT40 cells, genetic disruption of translocation of TRPC3 proteins to the PM has revealed that native TRPC3 forms DACCs but not SOCs. Upon BCR activation, the DAG-activated Ca\(^{2+}\) influx via TRPC3 amplifies Ca\(^{2+}\) signals and downstream NFAT activation by controlling PM translocation of PLC\(\gamma\)2 and sustains PKC\(\beta\) translocation and activation. Furthermore, direct physical interaction between TRPC3 and PKC\(\beta\) also regulates the stable retention of PKC\(\beta\) at the PM, leading to the sustained BCR-induced MAP kinase activation. These results suggest that TRPC3 has a dual function in BCR-induced signaling: it is a DACC, which elicits PM translocation of PLC\(\gamma\)2 and PKC\(\beta\), and a scaffolding platform at the PM for PKC\(\beta\).

**Results**

**Disruption of PM expression of endogenous TRPC3 channels in DT40 B cells**

The expression of DAG-activated TRPC3 and TRPC7 channels was previously demonstrated in DT40 cells (Nishida et al., 2003). Furthermore, recombinant expression studies suggested that TRPC3 channels are in part responsible for Ca\(^{2+}\) entry associated with translocation and sustained activation of PLC\(\gamma\)2 (Nishida et al., 2003). Therefore, to study the possible physiological significance of DAG-activated Ca\(^{2+}\)-permeable TRPC3 channels in PLC\(\gamma\)2-mediated BCR signaling, the TRPC3 gene locus was disrupted by deletion of the exon encoding amino acid residues (a.a.) 681-750, containing the well conserved TRP domain (Okada et al., 1999), through homologous recombination in DT40 B cells (Fig. 1A,B). RT-PCR revealed that TRPC3-mutant (MUT) DT40 cells expressed truncated TRPC3 transcripts in which the targeted exon was deleted (Fig. 1C), in accordance with immunoblotting detecting a slightly smaller band in MUT cells (Fig. 1D). Evaluation of channel function of mouse TRPC3 (mC3) with the corresponding deletion [mC3(A667-736): a.a. 667-736 in mC3 corresponds to a.a. 681-750 in chicken TRPC3] revealed that it lacks Ca\(^{2+}\) influx channel activity upon stimulation by ATP, carbacol (CCh), and the membrane permeable DAG analogue, 1-oleoyl-2-acetyl-sn-glycerol (OAG), when expressed in the HEK293 cell system (supplementary material Fig. S1A). Confocal images revealed an intracellular localization of a mC3(A667-736)-EGFP fusion construct, in contrast to WT mC3-EGFP which was localized in the PM (supplementary material Fig. S1B). These results clearly indicate that deletion of the TRP domain ablates targeting of TRPC3 proteins to the PM. Consistent with these results, immunofluorescence staining using anti-TRPC3 antibody revealed intracellular localization of TRPC3 proteins in MUT cells but PM localization in WT cells, suggesting that the endogenous truncated mutant of TRPC3 in MUT cells has a defect in PM expression (supplementary material Fig. S1C). The level of cell surface expression of BCR examined by staining with FITC-conjugated anti-chicken IgM antibody on MUT clones was indistinguishable from that of parental DT40 cells (supplementary material Fig. S1D).

**TRPC3 constitutes a DACC but not SOC in DT40 B cells**

DAG-induced ionic currents in WT and MUT DT40 cells were analyzed using the whole-cell patch-clamp technique (Fig. 1E-G). In WT cells, bath application of 10 \(\mu\)M OAG induced relatively sustained inward currents (1.74±0.45 pA/pF, \(n=7\)) at a holding potential of -60 mV (Fig. 1G). The current-voltage (\(I-V\)) relationship showed a slight outward rectification similar to the recombinant
Regulation of BCR signaling by TRPC3

TRPC3 current (Lintschinger et al., 2000) with reversal potential of 0 mV (Fig. 1F). The time between OAG challenge and current activation varied among WT cells: the time to maximum current amplitude after OAG application was from 78 to 301 seconds and the average time to maximum was 196±31 seconds. In contrast to WT cells, MUT cells showed significantly reduced OAG-induced currents (0.51±0.27 pA/pF, n=8; Fig. 1E-G). BCR ligation induced rapid release of Ca$^{2+}$ from internal stores and depletion of Ca$^{2+}$ stores subsequently activating store-operated Ca$^{2+}$ entry (SOCE), a major Ca$^{2+}$ entry pathway in B cells (Mori et al., 2002). We compared Ca$^{2+}$ release-activated Ca$^{2+}$ currents ($I_{\text{CRAC}}$) that correspond to SOCE in WT and MUT cells. Intracellular dialysis with 10 μM Ins(1,4,5)P$_3$ via the patch pipette elicited inward currents that showed the salient features of $I_{\text{CRAC}}$: a positive reversal potential and inward rectification over the voltage range from –150 to 50 mV (Fig. 1H). Peak current densities and activation kinetics of $I_{\text{CRAC}}$ recorded in MUT cells were comparable to those in WT cells (Fig. 1I,J). In [Ca$^{2+}$]$_i$ measurements, in the absence of extracellular Ca$^{2+}$, ionomycin, which fully depletes intracellular Ca$^{2+}$ stores, caused a transient Ca$^{2+}$ rise in both WT and MUT cells, indicating that stores are not affected by loss of TRPC3.

Readmission of extracellular Ca$^{2+}$ led to a comparable [Ca$^{2+}$]$_i$ increase in both WT and MUT cells (supplementary material Fig. S2). These results clearly indicate that DACCs are ablated, but SOCs are unaffected by the expression defect of functional TRPC3 channels at the PM in DT40 B cells.

TRPC3 plays a critical role in BCR-induced Ca$^{2+}$ signaling in DT40 B cells

We next analyzed [Ca$^{2+}$]$_i$ mobilization in response to physiological stimuli via BCR in WT and MUT DT40 cells (Fig. 2). In the presence of 2 mM extracellular Ca$^{2+}$, [Ca$^{2+}$]$_i$ increases, induced by BCR ligation, were significantly suppressed in MUT cells (Fig. 2A,C). BCR-induced Ca$^{2+}$ mobilization in MUT cells was indistinguishable from that in WT cells in the absence of extracellular Ca$^{2+}$ (Fig. 2B,C), indicating that the Ca$^{2+}$ response defect in MUT cells is attributable to Ca$^{2+}$ influx defect. In support of this observation, the compromised Ca$^{2+}$ influx in MUT cells was restored in MUT cells stably transfected with mC3 cDNA (Fig. 2A-C). The stable MUT transfectant exhibited immunolocalization of TRPC3 at the PM and intracellular TRPC3, which may represent exogenous mC3 and an endogenous TRPC3 truncation mutant, respectively (supplementary material Fig. S1C;
The level of TRPC3 expression was nearly doubled in the stable MUT transfectant compared with the original MUT cells (supplementary material Fig. S3B). Thus, disruption of functional TRPC3 expression at the PM, which mediates DAG-activated currents, elicits a Ca\textsuperscript{2+} influx defect in DT40 B cells. We have previously shown that BCR stimulation induces the initial Ca\textsuperscript{2+} responses followed by Ca\textsuperscript{2+}-entry-dependent sustained and/or oscillatory responses (Nishida et al., 2003). In MUT cells, the amplitudes of Ca\textsuperscript{2+} oscillations were reduced compared with those in WT cells (Fig. 2D-F) when the same concentration of anti-IgM (1 µg/ml) as Fig. 2A-C was employed for BCR stimulation. By contrast, the frequency of Ca\textsuperscript{2+} oscillation was indistinguishable between WT and MUT cells. This suggests that Ca\textsuperscript{2+} influx via DAG-activated TRPC3 channels is crucial for the modulation of Ca\textsuperscript{2+} oscillations.

A previous study revealed that Ca\textsuperscript{2+}-influx-dependent membrane translocation, secondary activation of PLC\textgamma{}2 and secondary production of Ins(1,4,5)P\textsubscript{3} are required for the generation of Ca\textsuperscript{2+} oscillations.
oscillations (Nishida et al., 2003). TRPC3 has been considered as a candidate for the unidentified molecular entity of that Ca²⁺ entry pathway, based on the observation that PLCγ2 was shown to be functionally and physically coupled to TRPC3 in a HEK293 heterologous expression system (Nishida et al., 2003). Thus, we next examined coupling of native TRPC3 channels with PLCγ2 translocation in DT40 cells by observing EYFP-tagged PLCγ2 (PLCγ2-EYFP) with time-lapse confocal laser microscopy. BCR ligation induced PM translocation of PLCγ2-EYFP in WT cells, whereas this was nearly abolished in MUT cells (Fig. 2G,H). Thus, the suppression of BCR-induced Ca²⁺ oscillations in MUT cells may be attributable to the defect in BCR-induced PLCγ2 translocation.

For PM translocation of PLCγ2, Ca²⁺ influx through TRPC3 is particularly important, because expression of red fluorescent protein (mStrawberry)-tagged mC3 (mC3-mStrawberry), but not that of mStrawberry-tagged mutant mC3 with a defect in the pore-forming region (mC3PD-mStrawberry), restored BCR-induced PLCγ2 translocation in MUT cells (supplementary material Fig. S4A). In addition, [Ca²⁺]i elevation by ionomycin also failed to translocate PLCγ2 to the PM (supplementary material Fig. S4B). Interestingly, overexpression of an mStrawberry-tagged mC3 subfragment containing a.a. 23-73 (mStrawberry-mC3(23-73)), which interacts with PLCγ (van Rossum et al., 2005) and suppressed PLCγ2 translocation in WT cells, failed to recover the translocation defect of PLCγ2 in MUT cells (supplementary material Fig. S4C,D). This excludes the possibility that the PLCγ2 translocation defect in MUT cells endogenously expressing the truncated mutant TRPC3 is solely attributable to its dominant-negative effect anticipated from the co-immunoprecipitation of PLCγ2 with the counterpart mC3(Δ667-736) mutant in HEK293 cells (supplementary material Fig. S4E), and supports the importance of functional TRPC3 localized at the PM.

Downstream of Ca²⁺ oscillations, a Ca²⁺-dependent transcription factor NFAT is activated to play a crucial role in lymphocyte activation (Rao et al., 1997; Peng et al., 2001). In addition, BCR stimulation activates NFAT through the BCR-induced PLC-Ca²⁺ signaling pathway (Sugawara et al., 1997). Interestingly, MUT cells showed an approximately 30% reduction in BCR-mediated NFAT activation compared with WT cells (Fig. 2I).

TRPC3-mediated Ca²⁺ influx is required for PM translocation of PKCβ

The secondary PLCγ2 activation should enhance production of DAG as well as Ins(1,4,5)P3, since equimolar DAG and Ins(1,4,5)P3 are generated through phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] hydrolysis by PLC. Therefore, we next examined the impact of TRPC3 deficiency on BCR-induced DAG production and its downstream PKC activation (Fig. 3). Among nine PKC subtypes, our study focused on PKCβs, particularly PKCβII isozymes, as potential target molecules of Ca²⁺ influx via TRPC3 channels, because of the requirement of Ca²⁺ and DAG for their activation as well as the fact that PKCβ isoforms are the major PKC isozymes expressed in B cells (Mischak et al., 1991). PM translocation of endogenous PKCβ was assessed using the membrane fractionation method, since PM translocation of PKC has been considered as the most critical step in its activation and has been frequently used to assess PKC activation (Liu et al., 1998). BCR stimulation caused sustained membrane translocation of PKCβ for at least 30 minutes in the presence of extracellular Ca²⁺ in WT cells (Fig. 3A). As observed for WT cells in the absence of extracellular Ca²⁺, MUT cells exhibited transient PKCβ PM translocation in the presence of extracellular Ca²⁺. The translocation defect was rescued by the recombinant expression of mC3 (Fig. 3A). Levels of BCR-dependent PKCβ activation were compared between WT and MUT cells by analyzing PM translocation of the EGFP fusion protein for PKCβII (PKCβII-EGFP), the most abundant PKCβ isoform in DT40 cells (T. N., unpublished data), at the single cell level using confocal microscopy. PKCβII-EGFP was diffusively distributed throughout the cytosol in resting cells, and rapidly translocated, within 1 minute, from cytosol to the PM upon BCR stimulation. In WT cells, PKCβII-EGFP translocation persisted in the presence of extracellular Ca²⁺ but became transient in the absence of extracellular Ca²⁺ (Fig. 3B,D). By contrast, in the presence of extracellular Ca²⁺, PKCβII-EGFP translocation was transient in MUT cells. Although BCR-induced sustained translocation of PKCβII-EGFP to the PM was recovered by the expression of mC3-mStrawberry, mC3PD-mStrawberry failed to restore the defect in sustained PKCβII PM translocation in response to BCR stimulation in MUT cells (Fig. 3C,D). These studies suggest that TRPC3-mediated Ca²⁺ entry is required for BCR-induced PKCβII PM translocation.

Since translocation of PKCβ is affected by DAG via the C1 domain (Oancea and Meyer, 1998), we next analyzed the OAG-induced PM translocation of PKCβII-EGFP. Application of OAG to WT cells caused a clear translocation of PKCβII-EGFP to the PM in the presence of extracellular Ca²⁺, whereas in the absence of extracellular Ca²⁺, the OAG-induced translocation was nearly abolished (Fig. 3E,G). Interestingly, in the presence of extracellular Ca²⁺, OAG failed to translocate PKCβII-EGFP to the PM in MUT cells. Although OAG-induced sustained translocation of PKCβII-EGFP to the PM was recovered by the expression of mC3-mStrawberry, mC3PD-mStrawberry again failed to restore the defect in sustained PKCβII PM translocation in response to OAG stimulation in MUT cells (Fig. 3F,G). These results suggest that OAG-activated Ca²⁺ influx mediated by TRPC3 is required for OAG-induced PM translocation of PKCβII.

PKCβ carries the C2 domain that interacts with Ca²⁺ and anionic phospholipids, and cPKC is known to translocate to the PM in response to a rapid, generalized increase in [Ca²⁺], induced by ionomycin (Maasch et al., 2000) through passive leakage from Ca²⁺ stores and Ca²⁺ influx (supplementary material Fig. S2A). In fact, ionomycin evoked the accumulation of PKCβII-EGFP at the PM, which persisted for 10 minutes in WT cells. By contrast, PKCβII-EGFP failed to show significant accumulation at the PM in MUT cells (see data in the presence of U73343, an inactive analogue for the PLC inhibitor U73122 in supplementary material Fig. S5A). These results suggest that TRPC3 mediates localization of PKCβ at the PM induced by a generalized [Ca²⁺], increase. Interestingly, after PKCβII-EGFP transiently accumulated at the PM, it showed cytosolic localization at 10 minutes in WT cells pretreated with the PLC inhibitor U73122 (supplementary material Fig. S5A). Thus, activation of PLCγ2, production of DAG, and Ca²⁺ influx via DAG-activated TRPC3 channels may be involved in PM translocation of PKCβII-EGFP in response to a generalized [Ca²⁺], increase.

TRPC3 functions as a scaffold for BCR-induced sustained translocation of PKCβ to the PM in DT40 B cells

We next tested the possibility that PKCβ might colocalize with TRPC3 at the PM, given PM translocation of PKCβ upon BCR stimulation. PKCβ was co-immunoprecipitated with TRPC3 before BCR stimulation in the presence or absence of extracellular Ca²⁺ in WT and MUT DT40 cells (Fig. 4A). The association between
TRPC3 and PKCβ increased after 15 minutes BCR stimulation in an extracellular-Ca\(^{2+}\)-dependent manner in WT cells. By contrast, no such increase of interaction was observed after 15 minutes BCR stimulation in MUT cells. Interestingly, the increased phase of the interaction between TRPC3 and PKCβ coincided with the sustained phase of PKCβ PM translocation in which TRPC3 plays a critical role (Fig. 3A,B,D). We next assessed in vitro binding of the mC3 constructs with PKCβ in DT40 cell extracts or with the PKCβ

Fig. 3. TRPC3 maintains BCR-induced translocation of PKCβ to the PM in B cells. (A) BCR-induced membrane localization of endogenous PKCβ in WT and MUT cells. The graph depicts time courses of PKCβ membrane translocation relative to that in unstimulated cells. The membrane-bound PKCβ was analyzed by membrane fractionation and subsequent immunoblotting using PKCβ-specific antibody. (B) Confocal images of BCR-induced membrane translocation of PKCβII-EGFP in WT and MUT cells. Scale bars: 2 μm. Enlargements of the boxed regions are also shown in right panels. (C) Confocal images of BCR-induced membrane translocation of PKCβII-EGFP in MUT cells, expressing mC3-mStrawberry or mC3PD-mStrawberry. Scale bars: 2 μm. (D) The time courses of average BCR-induced changes in PKCβII-EGFP fluorescence distribution in PM regions in WT cells stimulated in the presence (n=11) or the absence (n=7) of extracellular Ca\(^{2+}\), in MUT cells stimulated in the presence of extracellular Ca\(^{2+}\) (n=8), and in MUT cells expressing mC3-mStrawberry (n=7) or mC3PD-mStrawberry (n=7) stimulated in the presence of extracellular Ca\(^{2+}\). (E-G) Confocal analyses of OAG-induced changes of subcellular localization of PKCβII-EGFP in WT and MUT cells. The sets of experiments performed in E, F and G are the same as those in B, C and D, respectively, except for the use of OAG for stimulation. In G, nine or five WT cells were analyzed in the presence or the absence of extracellular Ca\(^{2+}\), respectively, while five MUT cells and six MUT cells expressing mC3-mStrawberry or mC3PD-mStrawberry were analyzed. ROI are defined in Fig. 2G. *P<0.05; **P<0.01, versus WT cells in the presence of extracellular Ca\(^{2+}\).
Regulation of BCR signaling by TRPC3

Fig. 4. Direct association of PKCβ with TRPC3. (A) Temporal changes of the complex formed between PKCβ and TRPC3. Within each pair of blots, upper and lower panel show co-immunoprecipitated TRPC3 and immunoprecipitated PKCβ, respectively. (B) A schematic representation of GST fusion mC3 subfragments. (C) Pull-down assay of PKCβ using GST fusion mC3 subfragments. DT40 cell extracts (left panels) or purified PKCβ preparations (right panels) were incubated with GST fusion mC3 subfragments immobilized on glutathione-Sepharose beads. Bound proteins were analyzed by immunoblotting using anti-PKCβ antibody. Inputs of each sample are shown in the lower panels. (D) Confocal images indicating BCR-induced membrane translocation of PKCβII-EGFP in WT DT40 cells expressing mCherry or mCherry-mC3(726-753). Subcellular distribution of mCherry and mCherry-mC3(726-753) are also shown. Scale bars: 2 μm. The graph represents the time courses of average changes in fluorescence of PKCβII-EGFP distributed in the PM regions in WT cells expressing mCherry (n=5) or mCherry-mC3(726-753) (n=5). ROI are defined in Fig. 2G. *P<0.05; **P<0.01, versus WT cells expressing mCherry. (E-G) The role of TRPC3 in translocation of PKCβ to the PM in HeLa cells. (E) Confocal images indicating histamine-induced membrane translocation of PKCβII-EGFP in HeLa cells transfected with TRPC3 siRNAs. Scale bars: 10 μm. The insets show enlarged views of the boxed regions. The traces on right show representative time courses of translocation of PKCβII-EGFP to the PM, given as the ratio (relative to time 0) of fluorescence intensity at the PM (FPM) to that in the cytosol (FCyt). ROI on the PM and in the cytosol (cyt) are indicated in the 0 minute panels. The data are representative of five independent experiments. (F) TRPC3 siRNAs disrupt sustained association of PKCβII-EGFP with the PM 15 minutes after histamine stimulation. The changes of PM fluorescence intensity are expressed as ΔF (see Materials and Methods). Images obtained from the experiment performed in E were subjected to analysis. *P<0.05; **P<0.01, versus randomized siRNA-transfected cells stimulated in the presence of extracellular Ca2+. (G) Histamine-induced membrane accumulation of PKCβII-EGFP in HeLa cells co-transfected with TRPC3 siRNAs. Cells were treated for 15 minutes with 100 μM histamine in the presence or absence of extracellular Ca2+. The experiments were performed as in Fig. 3A.
purified preparations in a glutathione S-transferase (GST) pull-down assay (Fig. 4B,C). The TRPC3 C-terminal residues Asn659-Glu836 and Asn659-Phe753 bound to PKCβ, whereas residues of Asn754-Glu836 only showed faint interaction. Furthermore, deletion of Asp667-Arg736 at the C-terminus, which corresponds to deletion of the TRPC3 mutant expressed in MUT cells, failed to affect the interaction of TRPC3 with PKCβ in vitro (Fig. 4B,C). These results suggest that Asn659-Glu666 and/or Leu737-Phe753 (supplementary material Fig. S5B) are essential for the interaction of TRPC3 with PKCβ.

To investigate the functional relevance of the direct interaction between TRPC3 and PKCβ in PKCβ translocation, the effects of the transiently expressed PKCβ-interacting mC3 fragment (Leu726-Phe753) fused to red fluorescent protein mCherry [mCherry-mC3(726-753)] were tested on BCR-induced translocation of PKCβII-EGFP towards the PM in WT DT40 cells. mCherry alone and mCherry-mC3(726-753) were indistinguishable in their subcellular localization in WT cells (Fig. 4D). However, expression of mCherry-mC3(726-753) significantly suppressed the sustained phase of the BCR-induced PKCβ translocation compared with mCherry alone (Fig. 4D). Thus, TRPC3 residues Leu726-Phe753 are sufficient for disruption of PKCβ translocation to the PM, suggesting that TRPC3 is important in anchoring PKCβ to the PM through physical interaction as well as through eliciting Ca²⁺ influx.

### TRPC3 is required for sustained localization of PKCβ at the PM in HeLa cells

PM translocation and activation of PKCβII have been previously analyzed using real-time imaging in HeLa cells (Violin et al., 2003). To examine the precise spatiotemporal patterns of the functional coupling between TRPC3 and PKCβ, we analyzed histamine-induced PM translocation of PKCβII-EGFP in HeLa cells using confocal microscopy. HeLa cells were transfected with small interfering RNAs (siRNAs) specific for human TRPC3 to knockdown endogenous TRPC3 levels (supplementary material Fig. S6). Histamine at a concentration of 100 μM evoked gradual concentration of the PKCβII-EGFP fluorescence at the PM after rapid accumulation in the initial phase, and the PM concentration of PKCβII-EGFP showed oscillations in control HeLa cells (Fig. 4E,F). The removal of extracellular Ca²⁺ reduced both oscillatory translocation and persistent PM localization of PKCβII-EGFP. In TRPC3-deficient cells, although oscillatory movement of PKCβII-EGFP was still observed, the localization of PKCβII-EGFP at the PM was gradually suppressed. The membrane fractionation experiment also showed that both the removal of extracellular Ca²⁺ and the siRNAs against TRPC3 abolished PM accumulation of PKCβII-EGFP after 15 minutes of histamine stimulation (Fig. 4G). These results suggest that TRPC3 channels stabilize PKCβ at the PM also in HeLa cells, raising the possibility that this mechanism is shared by different types of cells.

#### Sustained PM translocation of PKCβ is important for BCR-induced sustained activation of ERK in DT40 B cells

Previous studies have suggested that PKC is required for the BCR-induced activation of ERK, a MAP kinase (Sakata et al., 1999; Cao et al., 2001; Teixeira et al., 2003; Nishida et al., 2003; Aiba et al., 2004). We examined the roles played by TRPC3 channels in BCR-induced ERK activation via PKC in DT40 B cells (Fig. 5). In WT cells, BCR stimulation maintained the increase in ERK phosphorylation over 45 minutes in the presence of extracellular Ca²⁺. Strikingly, removal of extracellular Ca²⁺ resulted in transient ERK activation in WT cells. MUT cells exhibited suppression of ERK activation prominently at the sustained phase and significantly but moderately at the initial phase in the presence of extracellular Ca²⁺. The sustained ERK activation was restored by the heterologous expression of mC3. Hence, in DT40 B cells, Ca²⁺ influx via TRPC3 channels is required for full ERK activation, in which TRPC3 protein also plays an additional role as a platform for signal transduction at the PM.

### Discussion

The results of the present study establish TRPC3 as a DACC that plays an important role in B-cell signaling. TRPC3 channels are responsible for Ca²⁺ influx that induces PM translocation of PLCγ2, and amplification of Ca²⁺ signaling and NFAT activation downstream. Importantly, TRPC3 channels also elicit sustained PM translocation and activation of PKCβ by mediating Ca²⁺ influx and acting as platforms at the PM for PKCβ. Sustained PM translocation of PKCβ has significant impact on downstream ERK activation (Fig. 6).

In MUT DT40 cells expressing an endogenous PM-expression-deficient TRPC3 mutant, suppression of BCR-induced Ca²⁺ influx was attributable to the deficit in DAG-activated Ca²⁺ influx (Fig. 1). MUT cells also exhibited impairments in BCR-induced Ca²⁺ oscillations and PLCγ2 accumulation at the PM (Fig. 2), supporting the key role played by DACC TRPC3 in translocation and subsequent secondary activation of PLCγ2 that regulates Ca²⁺ oscillations in the sustained phase of Ca²⁺ signaling in B cells (Nishida et al., 2003). Our previous report demonstrated that the accumulation of PLCγ2 at the PM is completely abolished by the removal of extracellular Ca²⁺ or by treatment with the TRPC3-selective inhibitor Pyr3 (Nishida et al., 2003; Kiyonaka et al., 2009).
Furthermore, overexpression of the PLCγ-interacting mC3(23–73) subfragment significantly suppressed PLCγ2 PM translocation in WT cells, but it failed to restore the defect of PLCγ2 PM translocation in MUT cells (supplementary material Fig. S4C,D). These observations suggest that the accumulation of PLCγ2 at the PM specifically requires both TRPC3-mediated Ca2+ influx and the direct interaction between PLCγ2 and PM-localized TRPC3. The specific requirement of TRPC3-mediated Ca2+ influx for PM translocation of PLCγ2 is also suggested by the additional finding that a generalized [Ca2+]i increase by ionomycin and expression of the mC3PD mutant failed to restore BCR-induced PM translocation of PLCγ2 in MUT cells (supplementary material Fig. S4A,B,D).

It has been known that BLNK, also known as SLP65, is the critical scaffolding protein for PLCγ2 in BCR signaling (Fu et al., 1998; Kurosaki et al., 2000). We have not yet studied the exact molecular composition of the signal complex containing TRPC3 and PLCγ2 in DT40 cells to determine whether BLNK is a constituent of this complex. However, considering the fact that both TRPC3 and BLNK positively regulate PLCγ2 activation, it is more likely that TRPC3 and BLNK cooperate in the same complex to amplify BCR signaling than that they compete and antagonize each other. However, an alternative possibility cannot be excluded that BLNK and TRPC3 share the same binding site on PLCγ2 and regulate BCR signaling with different time dependencies.

In different types of cells, sustained Ca2+ increase and/or oscillations are required for the activation of NFAT (Gwack et al., 2007). It has been suggested that SOCs form the only major Ca2+ influx pathway responsible for NFAT activation in lymphocytes (Feske, 2007). Our previous work in fact demonstrated that disruption of store-operated TRPC1 channels suppresses the frequency of BCR-induced Ca2+ oscillations and NFAT activation in DT40 cells (Mori et al., 2002). However, NFAT activity is also reduced in MUT cells (Fig. 2I). Therefore, our study provides evidence for the first time, that Ca2+ influx via DAG-activated TRPC3 plays a role in Ca2+ oscillations and subsequent NFAT activation in lymphocytes.

With regard to the activation mechanism for cPKC, the coordination of Ca2+ and DAG signals is known to determine the kinetics of translocation and activation (Oancea and Meyer, 1998). Recently, Singh et al. suggested that a DAG-activated TRPC6 channel signals the membrane translocation and activation of PKCβ, and thereby induces RhoA activation and endothelial contraction (Singh et al., 2007). However, the mechanism of PKCβ-mediated Ca2+ entry was not clarified. Our study describes a precise process of cPKC recruitment to the membrane. TRPC3 is capable of providing the sustained Ca2+ influx required for sustained PM localization of PKCβ upon BCR stimulation, because DAG is continuously produced by PLCγ2, and TRPC3 activity is in turn sustained. Importantly, to recruit PKCβ to the PM, OAG per se failed but required Ca2+ influx via OAG-activated TRPC3 channels (Fig. 3E-G). Furthermore, sustained PM translocation of PKCβ evoked by ionomycin became transient following inhibition of DAG production, and was abolished by disruption of TRPC3 expression at the PM (supplementary material Fig. S5A), suggesting that the sustained PM translocation of PKCβ requires TRPC3-mediated Ca2+ influx and persistent production of DAG. Since PKC-mediated phosphorylation has been reported to negatively regulate TRPC3 (Trebak et al., 2003; Venkatachalam et al., 2003; Trebak et al., 2005), the sustained translocation of PKCβ towards PM is the consequence of the TRPC3-mediated Ca2+ influx but is unlikely a requisite of TRPC3 activation. Interestingly, in HeLa cells, histamine stimulation evoked oscillatory translocation on top of gradual accumulation of PKCβII-EGFP at the PM, while knockdown of TRPC3 only suppressed the latter (Fig. 4E,F). This suggests that DAG-dependent persistent localization of PKCβII requires TRPC3-mediated Ca2+ influx, whereas the oscillatory translocation of PKCβII is induced by repetitive Ca2+ spikes in the cytosol. Thus, the persistent localization of cPKCs mediated by DAG-activated Ca2+ influx could be a common mechanism shared by many types of cells.

Previous in vitro studies predicted that binding of two Ca2+ ions to the C2 domain of PKCβ causes slow and low-affinity membrane interaction and an additional third Ca2+ ion subsequently binds to the C2 domain and stabilizes the C2 domain-membrane complex, which allows PKCβ to search for the C1 domain ligand DAG on the membrane (Nalefski and Newton, 2001; Kohout et al., 2002). Notably, it has been proposed that the binding affinity of the third Ca2+ ion is too low to promote occupancy except when Ca2+ levels reach the millimolar range, or additional groups for Ca2+ coordination are provided, as in the presence of phospholipids (Nalefski and Newton, 2001). Since the local Ca2+ concentration ([Ca2+]i) within nanometers of the mouths of Ca2+ channels is orders of magnitude larger than bulk cytosolic [Ca2+]i (Marsault et al., 1997), TRPC3 may sufficiently augment [Ca2+]i to provide the third Ca2+ ion to the C2 domain, once PKCβ directly interacts with TRPC3 (Fig. 3A-D). In addition, since TRPC3 also directly interacts with PLCγ2 and regulates PM translocation and activation of PLCγ2, DAG also should be concentrated near TRPC3 and PKCβ. Thus, TRPC3 may increase local concentrations of Ca2+ and DAG in organizing the nanodomain that supports sustained PM localization of PKCβ and stabilization of a ternary complex of PKCβ, Ca2+ and lipid. Interestingly, it has recently been demonstrated that TRPC3 interacts with a receptor for activated C-kinase-1, which is known to be a scaffolding protein for PKCβ via its N-terminus (Bandyopadhay et al., 2008). In the Drosophila phototransduction system, TRP functions both as a Ca2+-permeable channel and as a...
molecular anchor for signalplexes (Li and Montell, 2000). Notably, Leu737-Phe753, the binding region of TRPC3 to PKCβ, is not highly conserved in TRPC6 and TRPC7 (supplementary material Fig. S5B), suggesting a specific function of TRPC3 in anchoring PKCβ at the PM. Thus, TRPC3 stabilizes PKCβ PM localization directly, as a platform, and also indirectly as an amplifier of Ca2+ and DAG signals for organizing specific signal complex to achieve specificity and efficiency in BCR-induced signaling.

We can propose a mechanism underlying enhancement of TRPC3 interaction with PKCβ after 15 minutes of BCR stimulation (Fig. 4A). A recent study suggested a control of surface expression of mature B cells but an increase of intracellular Ca2+ levels is induced in the relative absence of PtdIns(4,5)2 hydrolysis in immature B cells (King and Monroe, 2000). These effects can be addressed using TRPC3 knockout mice, in which B-cell function has not been demonstrated yet (Hartmann et al., 2008; Kim et al., 2009). Hence, a combination of genetic and pharmacological approaches can reveal the importance of the in vivo DAG-TRPC3 channel and its associated mechanism in the context of the developmental maturation of B cells.

**Materials and Methods**

**Cell cultures and cdNA expression**

EGFP-fused chicken PKCβ1 cdNA (Aiba et al., 2004) was first established in the pEGFP-N1 vector (Clontech), and then transfected into pa-puro expression vector (Takata et al., 1994). mC3 cdNA was subcloned into pA-puro vector. DT40 cells were transfected with these constructs by electroporation (550 V, 25 μF) and selected in the presence of 0.5 μg/ml puromycin WT and PD mutant (in which Leu609, Phen610, Trp611 were changed to alanines) of mC3 (mouse TRPC3) were fused at the C-terminal with mStrawberry and then transferred into pMXΔA (Mori et al., 2002). PKCβ-interacting (Leu726-Phe753) or PLCγ2-interacting (Ser23-Glu73) regions of mC3 were amplified using PCR and fused at the N-terminus with mCherry or mStrawberry, respectively, and cloned into pMXA. Cell cultures and cdNA expression in DT40 cells using the vesicular stomatitis virus glycoprotein pseudotyped retroviruses were performed as described previously (Mori et al., 2002). An mC3 mutant with deletion of amino acids 667-736 (mC3[667-736]) was constructed using PCR and cloned into pcI-neo (Promega) or pEGFP-N1 to be tagged with EGFP-C-terminally. HeLa and HEK293 cells, grown in DMEM supplemented with 10% FBS, were transfected using Superfect (Qiagen) according to the manufacturer’s instructions.

**Generation of TRPC3-deficient DT40 cells**

The chicken genomic TRPC3/CDNA was obtained by PCR using pairs of primers chTRPC3-P1 and chTRPC3-P14, chTRPC3-P3 and chTRPC3-P10, respectively (supplementary material Table S1). The targeting vector was constructed by replacing the genomic sequence, contains the exon corresponding to the sequence distal to the H8 transmembrane region containing TRP domain (EWKFAR) of chicken TRPC3, with a histidinol (HisD) or neomycin (neo) resistance cassette as shown in Fig. 1A (Takata et al., 1994). The upstream 2.1-kb and downstream 4-kb genomic sequences of TRPC3 were used as a targeting vector. The targeting vector transfection and isolation of several clones were performed as described previously (Mori et al., 2002). Clones were further screened by Southern blot analysis of XbaI-digested genomic DNA hybridized with a cDNA probe using Gene Images random prime labeling and detection system (GE Healthcare) according to the manufacturer’s instructions.

**Measurement of changes in [Ca2+]i**

DT40 cells were fixed with 4% paraformaldehyde for 5 minutes, immobilized on slides using cytospin centrifugation, and permeabilized with 0.2% Triton X-100 for 5 minutes. After blocking with 5% BSA, cells were incubated with anti-TRPC3 antibody for 2 hours (Nishida et al., 2003). The primary antibodies were detected using anti-rabbit secondary antibodies labeled with Alexa Fluor 488 (Invitrogen).

**Electrophysiology**

Measurements of OAG-activated currents and I_{KCa} were carried out as described previously (Inoue et al., 2001; Mori et al., 2002). For measurement of OAG-activated current, DT40 cells were allowed to settle in the perfusion chamber for 5 minutes in the external solution (in mM): 140 NaCl, 5 KCl, 1.5 MgCl2, 1 CaCl2, 10 Hepes, 10 glucose, 20 OAG (adjusted with Tris base). For experiments shown in Fig 4B, a pipette solution containing (in mM): 120 CsOH, 20 aspartate, 20 CsCl, 5 creatine, 2 MgSO4, 5 EGTA, 2 ATP, 5 Hepes (pH 7.2 adjusted with Tris base). For measurements of I_{KCa}, DT40 cells were suspended in standard external, modified Ringer’s solution (in mM): 135 NaCl, 2.8 KCl, 10 CsCl, 2 MgCl2, 10 CaCl2, 10 glucose, 5 Hepes (pH 7.4 adjusted with NaOH).

**Confocal microscopy and image analysis**

Fluorescent protein-expressing DT40 or HeLa cells were plated onto poly-L-lysine-coated glass coverslips. Fluorescence images were acquired with an inverted microscope (FV500; Olympus) using the 488-nm line of an Ar laser for excitation and a 560-nm long-pass filter for diaminonaphthalene detection. Confocal microscopy images were recorded for 15 minutes at 10-second intervals. The standard pipette solution contained (in mM): 132 CsOH, 132 glutamate, 6 NaCl, 1 MgCl2, 10 EGTA, 2 MgATP, 0.2 GTP, 0.01 Ins(1,4,5)P_3, 5 Hepes (pH 7.2 adjusted with CsOH).

**Immunofluorescence staining**

DT40 cells were fixed with 4% paraformaldehyde for 5 minutes, immobilized on slides using cytospin centrifugation, and permeabilized with 0.2% Triton X-100 for 5 minutes. After blocking with 5% BSA, cells were incubated with anti-TRPC3 antibody for 2 hours (Nishida et al., 2003). The primary antibodies were detected using anti-rabbit secondary antibodies labeled with Alexa Fluor 488 (Invitrogen).
increased in the PM by 15 minutes of histamine stimulation in HeLa cells (shown in Fig. 4E), the fluorescence intensities of the PM were averaged and normalized according to the following equation: \( R_\text{PM} / R_\text{cell} \), where \( R_\text{PM} \) is the ratio of fluorescence intensity of the PM to that of the whole cell at 15 minutes after histamine stimulation, and \( R_\text{cell} \) is the ratio at time 0.

NFAT reporter assay

NFAT activity was quantified with 1420 ARVOx (Wallac) using NFAT luciferase (Stratagene) and the Dual-Luciferase system (Promega) as described previously (Sugawara et al., 1997).

Separation of membrane and cytosolic fractions

DT40 or HeLa cells were stimulated with 10 μg/ml anti- IgM or 100 μM histamine in serum-free PSS, respectively. Membrane fractionation was performed as described previously (Krotova et al., 2003). Samples were resolved by SDS-PAGE and subjected to immunoblotting with anti-PKCβ monoclonal antibody (BD Transduction Laboratories). The bands were scanned and the density of each band was determined using ImageJ software.

Immunoprecipitation

DT40 cells were stimulated with 10 μg/ml anti-IgM, and lysed in NP-40 lysis buffer (137 mM NaCl, 20 mM Tris-HCl, pH 8.0, 10% glycerol, 1% NP-40, 2 mM EDTA, 1 mM PMSF, 20 μg/ml leupeptin, 0.1 μg/ml aprotinin and 5 mM sodium orthovanadate). HEK293 cells expressing PLCβ2-EGFP with mC3 or mC3A667-736) were lysed as described previously (Kiyonaka et al., 2009). The cell lysate was further subjected to immunoprecipitation as described previously (Nishida et al., 2003) using anti-PKCα antibody (BD Transduction Laboratories) which cross-reacts with PKCβ (Fig. 4A) or anti-TRPC3 (supplementary material Fig. S4A). The immunocomplexes were characterized by immunoblotting with anti-TRPC3 antibody (Fig. 4A) or with anti-GFP antibody (supplementary material Fig. S4A).

GST pull-down assay

cDNAs for mC3 fragments and the GST were cloned into the pET23 vector (Novagen). Purification of GST fusion proteins and pull-down assays were performed as described previously (Kiyonaka et al., 2007). DT40 cells were lysed in NP-40 lysis buffer. Purified human PKCβ1 was obtained from Sigma. The samples were subjected to immunoblotting using anti-PKCβ antibody.

RNA interference

HeLa cells were transfected 72 hours prior to confocal analysis with siRNA duplex using Oligofectamine (Invitrogen) according to manufacturer’s instructions. TRPC3 was subjected to immunoblotting using anti-PKCα antibody or anti-GFP antibody (supplementary material Fig. S4A).

Expression analysis

The PCR protocol used for the expression analysis was described previously (Inoue et al., 2001). The PCR primers used are listed in supplementary material Table S1.

Analysis of ERK activity

DT40 cells were stimulated with 10 μg/ml anti-IgM in serum-free PSS and then lysed as described previously (Nishida et al., 2003).

Flow cytometric analysis

Cell surface expression of BCR on WT and MUT cells was analyzed with Epics Altra (Beckman Coulter) using a FITC-conjugated anti-chicken IgM antibody.

Analysis of ERK activity

DT40 cells were stimulated with 10 μM histamine and analyzed with FACS Calibur (Becton Dickinson) using a FITC-conjugated anti-chicken IgM antibody.

Statistical analysis

All data are expressed as means ± s.e.m. The data represent at least three independent experiments for each condition. Statistical significance was evaluated using the Student’s t-test for comparisons between two mean values. Multiple comparisons between more than three groups were carried out using ANOVA followed by Tukey-Kramer test.

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