Amplification of receptor signalling by Ca²⁺ entry-mediated translocation and activation of PLC γ 2 in B lymphocytes

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In non-excitabile cells, receptor-activated Ca²⁺ signalling comprises initial transient responses followed by a Ca²⁺ entry-dependent sustained and/or oscillatory phase. Here, we describe the molecular mechanism underlying the second phase linked to signal amplification. An *in vivo* inositol 1,4,5-trisphosphate (IP₃) sensor revealed that in B lymphocytes, receptor-activated and store-operated Ca²⁺ entry greatly enhanced IP₃ production, which terminated in phospholipase C γ 2 (PLC γ 2)-deficient cells. Association between receptor-activated TRPC3 Ca²⁺ channels and PLC γ 2, which cooperate in potentiating Ca²⁺ responses, was demonstrated by co-immunoprecipitation. PLC γ 2-deficient cells displayed diminished Ca²⁺ entry-induced Ca²⁺ responses. However, this defect was canceled by suppressing IP₃-induced Ca²⁺ release, implying that IP₃ and IP₃ receptors mediate the second Ca²⁺ phase. Furthermore, confocal visualization of PLC γ 2 mutants demonstrated that Ca²⁺ entry evoked a C2 domain-mediated PLC γ 2 translocation towards the plasma membrane in a lipase-independent manner to activate PLC γ 2. Strikingly, Ca²⁺ entry-activated PLC γ 2 maintained Ca²⁺ oscillation and extracellular signal-regulated kinase activation downstream of protein kinase C. We suggest that coupling of Ca²⁺ entry with PLC γ 2 translocation and activation controls the amplification and co-ordination of receptor signalling.

Keywords: Ca²⁺ influx/C2 domain/PLC γ 2/signal amplification/TRPC

Introduction

In non-excitabile cells, stimulation of surface membrane receptors induces Ca²⁺ signals via activation of phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DG) (Berridge, 1993; Clapham,

1995). IP₃ triggers rapid Ca²⁺ release from internal Ca²⁺ stores of the endoplasmic reticulum (ER) by activating IP₃ receptors (IP₃R) and a consequent transient increase in cytosolic Ca²⁺ concentration ([Ca²⁺]_i). The initial phase of receptor-activated Ca²⁺ signalling is followed by sustained and/or oscillatory [Ca²⁺]_i changes. The molecular mechanism underlying the second phase is still elusive (Feske *et al.*, 2001; Mori *et al.*, 2002), despite the vital role played by this phase in various biological responses (Miyazaki *et al.*, 1992; Thorn *et al.*, 1993; Dolmetsch *et al.*, 1998; Li *et al.*, 1998).

The second phase of Ca²⁺ signalling is critically controlled by receptor-activated Ca²⁺ entry processes such as capacitative Ca²⁺ entry (CCE) mediated by store-operated Ca²⁺ channels (SOCs) coupled to IP₃-induced Ca²⁺ release or consequent depletion of Ca²⁺ stores (Putney and Bird, 1993). In electrophysiological measurement, SOCs and other Ca²⁺ channels activated by DG in a membrane-delimited and protein kinase C (PKC)-independent manner have been identified (Hofmann *et al.*, 1999; Okada *et al.*, 1999; Inoue *et al.*, 2001; Gamberucci *et al.*, 2002). In [Ca²⁺]_i measurement, it has been postulated that CCE is distinguished as an 'off' response elicited by readministration of extracellular Ca²⁺ to cells after pretreatment with store-depleting agents such as thapsigargin (TG) in the absence of Ca²⁺ (Parekh *et al.*, 1997). Importantly, there is a large body of evidence that the properties of IP₃R Ca²⁺ release channels affect the temporal patterns of Ca²⁺ signals/oscillations (Tsien and Tsien, 1990; Miyazaki *et al.*, 1992; Miyakawa *et al.*, 1999). Thus, together with direct elevation of [Ca²⁺]_i by the Ca²⁺ entered through SOCs, the 'capacitative' function of CCE, which refills Ca²⁺ stores to maintain Ca²⁺ release, is a major role played by Ca²⁺ entry in eliciting the second phase.

In receptor-induced Ca²⁺ signalling, activation of PLC isoforms is controlled through distinct coupling mechanisms (Rhee, 2001). It has been commonly understood that receptor and non-receptor tyrosine kinases are responsible for activation of PLCs. Recent evidence has added the novel view that PLCs have multifunctional properties, and directly interact with numerous target proteins (Smith *et al.*, 1994; Kim *et al.*, 2000; Rebecchi and Pentylä, 2000; Rhee, 2001; Patterson *et al.*, 2002; Putney, 2002; Runnels *et al.*, 2002; Ye *et al.*, 2002; Trebak *et al.*, 2003). Here, we reveal that PLC γ 2, beyond its role in initiating the Ca²⁺ response upon stimulation of tyrosine kinase-coupled receptors, mediates the second phase of Ca²⁺ signals in B lymphocytes. B cell receptor (BCR)-induced and store-operated Ca²⁺ entry elicited the translocation of PLC γ 2 towards the plasma membrane in a lipase-independent manner, and enhanced PLC γ 2 activation to maintain IP₃ production and IP₃-induced Ca²⁺ release. We suggest that Ca²⁺ entry is coupled with PLC γ 2

translocation and activation to control physiological coordination and amplification of receptor-mediated signals.

Results

Activation of PLC γ 2 by BCR-induced Ca $^{2+}$ entry

In chicken DT40 B lymphocytes, ligation of BCR with anti-IgM antibody (anti-IgM) induced rapid rises in $[Ca^{2+}]_i$ followed by sustained elevation of $[Ca^{2+}]_i$ in the presence of extracellular Ca $^{2+}$ (Figure 1A). Since the sustained phase was eliminated by omitting extracellular Ca $^{2+}$, the sustained phase must derive from Ca $^{2+}$ entry. Real time changes in $[IP_3]_i$ were assessed in individual cells, using a new IP $_3$ -selective sensor R9-PHIP $_3$ -D106 constructed based on the pleckstrin homology (PH) domain of PLC δ 1 (Mori *et al.*, 2002). Stimulation of BCR triggered a rapid decrease in R9-PHIP $_3$ -D106 fluorescence, indicative of an initial $[IP_3]_i$ increase in the wild-type (WT) cells (Figure 1B). The increase in $[IP_3]_i$ was sustained or slowly enhanced for >5 min in the presence of extracellular Ca $^{2+}$, but was transient when Ca $^{2+}$ was removed. Population IP $_3$ measurement confirmed the observation (Supplementary figure 1C, available at *The EMBO Journal* Online). Since PLC γ 2 mediates BCR-induced Ca $^{2+}$ signalling (Takata *et al.*, 1995), the PLC γ 2-deficient (PLC γ 2 $^{-}$) DT40 cell line was employed to examine whether the observed IP $_3$ production is in fact due to PLC γ 2 activation. The BCR-induced $[IP_3]_i$ and $[Ca^{2+}]_i$ rises terminated in the PLC γ 2 $^{-}$ cells (Figure 1C and D). The defects were resolved by heterologous expression of rat PLC γ 2 in the PLC γ 2 $^{-}$ cells, but not by a lipase-dead PLC γ 2 mutant (LD; see also Figure 4A).

Ca $^{2+}$ entry induced by G protein-coupled receptor stimulation activates PLC γ 2

To characterize the extracellular Ca $^{2+}$ -dependent sustained phase separately from the initial BCR-evoked phase in PLC γ 2 activation, heterologously expressed M1 muscarinic acetylcholine receptor (M1R) (Sugawara *et al.*, 1997) was stimulated with carbachol (CCh). This is because the different isoform PLC β mediates PIP $_2$ hydrolysis to trigger Ca $^{2+}$ release upon M1R and G $_q$ protein activation (Parekh *et al.*, 1997). In the WT and PLC γ 2 $^{-}$ cells, stimulation of M5R triggers Ca $^{2+}$ mobilization via G $_q$ and PLC β (Patterson *et al.*, 2002). Stimulation of M1R induced smaller $[Ca^{2+}]_i$ increases in the PLC γ 2 $^{-}$ cells compared with those in the WT cells (Supplementary figure 2). However, omission of extracellular Ca $^{2+}$ abolished the difference between the WT and PLC γ 2 $^{-}$ cells in M1R-activated Ca $^{2+}$ responses (Figure 1E). The data support the view that PLC γ 2 is responsible for external Ca $^{2+}$ -dependent enhancement of Ca $^{2+}$ responses, and that Ca $^{2+}$ entry induced by PLC β -coupled receptor leads to late sustained PLC γ 2 activation. Furthermore, the defect in Ca $^{2+}$ entry-induced Ca $^{2+}$ response in PLC γ 2 $^{-}$ cells was partially restored by the LD mutant in the PLC γ 2 $^{-}$ cells (Figure 1E), suggesting the lipase-independent (Patterson *et al.*, 2002) and lipase-dependent roles of PLC γ 2 in receptor-activated Ca $^{2+}$ response.

Activation of PLC γ 2 by store-operated Ca $^{2+}$ influx

To analyse further the Ca $^{2+}$ entry-induced mechanism in BCR-induced PLC γ 2 activation, we used SERCA Ca $^{2+}$ -

Fig. 1. Extracellular Ca $^{2+}$ elicits sustained PLC γ 2 activation and receptor-evoked $[Ca^{2+}]_i$ mobilization. (A) Left, representative time courses of Ca $^{2+}$ responses in DT40 cells upon BCR stimulation with anti-IgM (1 μg/ml) in 2 mM Ca $^{2+}$ -containing external (n = 32 cells) or EGTA-containing, Ca $^{2+}$ -free (n = 32) solution. Right, peak BCR-induced $[Ca^{2+}]_i$ rises and $[Ca^{2+}]_i$ increases sustained after 5 min stimulation. (B) Left, representative time courses of BCR-induced changes in fluorescence intensities of IP $_3$ indicator R9-PHIP $_3$ -D106 (n = 12–15). F is the fluorescence intensity and F $_0$ is the initial F. Right, BCR-induced $[Ca^{2+}]_i$ rises (C) and $[IP_3]_i$ rises (D) at peak and sustained after 6 min stimulation in WT cells expressing GFP (GFP/WT), and in PLC γ 2 $^{-}$ cells expressing GFP (GFP/PLC γ 2 $^{-}$), PLC γ 2 (PLC γ 2/PLC γ 2 $^{-}$) or LD mutant (LD/PLC γ 2 $^{-}$) (n = 22–60). Ca $^{2+}$ is present in external solution. (E) PLC γ 2 enhances Ca $^{2+}$ responses induced by Ca $^{2+}$ entry upon M1R stimulation. Ca $^{2+}$ release was first evoked in Ca $^{2+}$ -free solution, and Ca $^{2+}$ entry-induced Ca $^{2+}$ responses were induced by readministration of 2 mM Ca $^{2+}$ in WT cells expressing GFP and in PLC γ 2 $^{-}$ cells expressing GFP, PLC γ 2, LD or Δ SH3 (n = 5–13). Left, average time courses. Right, peak $[Ca^{2+}]_i$ rises in Ca $^{2+}$ -free solution and after Ca $^{2+}$ readministration. Significance difference from control: *P < 0.05.

ATPase inhibitor TG, which activates SOCs via passive depletion of Ca $^{2+}$ stores. In the presence of extracellular Ca $^{2+}$, TG produced dramatic $[Ca^{2+}]_i$ increases in the WT cells, but only moderate $[Ca^{2+}]_i$ increases in the PLC γ 2 $^{-}$ cells (Figure 2A and B). In the absence of Ca $^{2+}$, TG caused indistinguishable transient Ca $^{2+}$ rises in the WT and PLC γ 2 $^{-}$ cells, indicating that TG-sensitive Ca $^{2+}$ stores are not affected by the loss of PLC γ 2. As shown in Figure 2C, treating the WT cells with TG induced marked $[IP_3]_i$ rises

Fig. 2. Store-operated Ca^{2+} entry induced by passive depletion of Ca^{2+} stores activates PLC γ 2. (A) Average time courses of Ca^{2+} -responses induced by TG (2 μM) in WT (left) and PLC γ 2 $^{-}$ (right) cells in Ca^{2+} -free or Ca^{2+} -containing external solution ($n = 18$ –33). (B) Peak $[\text{Ca}^{2+}]_i$ rises induced by TG in WT and PLC γ 2 $^{-}$ cells. (C) Typical time courses of TG-induced changes in F/F_0 of R9-PHIP3-D106 in WT (left) and PLC γ 2 $^{-}$ cells (right) in the presence (top) or absence (bottom) of extracellular Ca^{2+} ($n = 7$ –15). (D) Peak TG-induced $[\text{IP}_3]_i$ rises. (E) Dependence of TG-induced 'off' Ca^{2+} responses on extracellular Ca^{2+} concentrations in WT and PLC γ 2 $^{-}$ cells. The 'off' responses were induced after 12.5 min exposure to TG in Ca^{2+} -free solution ($n = 34$ –53). Right, peak $[\text{Ca}^{2+}]_i$ rises plotted against extracellular Ca^{2+} concentration.

in the presence of extracellular Ca^{2+} , but elicited small $[\text{IP}_3]_i$ rises in the absence of Ca^{2+} . The $[\text{IP}_3]_i$ increase induced by TG ($1.43 \pm 0.1 \mu\text{M}$) was about half that induced by BCR ($2.70 \pm 0.3 \mu\text{M}$) (Figure 2D). However, in the PLC γ 2 $^{-}$ cells, TG produced small $[\text{IP}_3]_i$ increases, regardless of extracellular Ca^{2+} (Figure 2C and D). These results suggest that Ca^{2+} entry activated by TG-induced store depletion leads to IP_3 production by PLC γ 2.

In Figure 2E, the Ca^{2+} entry-evoked 'off' Ca^{2+} response was induced separately from the preceding passive Ca^{2+} release/depletion by TG (Parekh *et al.*, 1997). In the physiological range of extracellular Ca^{2+} concentrations (1–10 mM), the Ca^{2+} entry-induced 'off' responses were significantly higher in the WT cells than those in the PLC γ 2 $^{-}$ cells. Replacing Ca^{2+} with Ba^{2+} failed to discriminate the 'off' responses between the WT and the PLC γ 2 $^{-}$ cells (Supplementary figure 3A), suggesting a specific positive role of Ca^{2+} in PLC γ 2 activation or a lack of Ba^{2+} permeability of the Ca^{2+} entry channel. We also tested the possibility that the suppressed 'off' response in the PLC γ 2 $^{-}$ cells derives from a decrease in the electrical driving force

for Ca^{2+} by membrane depolarization through cation channels such as TRPM7, which associates with PLC β via the C2 domain (Runnels *et al.*, 2002). In the solution containing K^{+} ionophore valinomycin, which maintains cell membrane potential at about -75 mV (Mori *et al.*, 2002), and in the membrane-depolarizing high K^{+} (30 mM) solution, the 'off' $[\text{Ca}^{2+}]_i$ increase remained significantly smaller in the PLC γ 2 $^{-}$ cells than in the WT cells (Supplementary figure 3B). Thus, the reduced 'off' response in the PLC γ 2 $^{-}$ cells was not due to membrane depolarization.

IP₃-mediated Ca^{2+} release is involved in TG-induced 'off' Ca^{2+} responses

We next examined whether IP_3 R-mediated Ca^{2+} release is involved in TG-induced 'off' Ca^{2+} responses in DT40 B cells. Importantly, larger 'off' Ca^{2+} responses in the WT cells compared with those in the PLC γ 2 $^{-}$ cells were not observed after pretreatment with the IP_3 R antagonists, Xestospongin C (Xest C) (Figure 3A and B) and 2-aminoethoxydiphenyl borate (2-APB) (Supplementary figure 3C). The involvement of IP_3 -mediated Ca^{2+} release in the 'off' responses was confirmed by using ionomycin (IM), which depletes internal Ca^{2+} stores more indiscriminatingly and efficiently than TG, which selectively targets SERCA-containing stores (Supplementary figure 3D) (Parekh *et al.*, 1997). The WT cells treated with IM showed greater Ca^{2+} release, but a smaller 'off' Ca^{2+} response compared with the WT cells treated with TG (Figure 3C). IM elicited nearly identical 'off' Ca^{2+} responses in the WT and the PLC γ 2 $^{-}$ cells, suggesting that thorough store depletion by IM nullified the contribution of IP_3 -induced Ca^{2+} release to the 'off' responses in the WT cells. Interestingly, IM elicited significant PLC γ 2 translocation (Table I) and $[\text{IP}_3]_i$ increase ($1.55 \pm 0.1 \mu\text{M}$). In the cells deficient in three IP_3 R subtypes (IP_3 R-KO) (Sugawara *et al.*, 1997), TG and IM induced nearly identical 'off' Ca^{2+} responses (Figure 3D). The data support the view that 'off' responses are promoted by IP_3 -induced Ca^{2+} release from Ca^{2+} stores, when Ca^{2+} stores are partially depleted (Supplementary figure 3D).

Contrary to the above notion, the IP_3 R-KO cells showed TG-induced 'off' Ca^{2+} responses indistinguishable from those in the WT cells (Supplementary figure 4A). This discrepancy seems partly due to a compensatory enhancement in DG-sensitive Ca^{2+} channel expression; notable La^{3+} -sensitive 'off' $[\text{Ca}^{2+}]_i$ increases due to the DG analogue 1-oleoyl-2-acetyl-sn-glycerol (OAG, 30 μM) were observed in about half of the IP_3 R-KO cells (18/41) (Supplementary figure 4B), while fewer WT cells (15/50) showed small, transient OAG-induced 'off' responses. As reported previously (Venkatachalam *et al.*, 2001), extracellular application of Ba^{2+} for Ca^{2+} elicited a tiny increase in the fluorescence ratio (data not shown), suggesting the importance of extracellular Ca^{2+} in differentiating WT cells from IP_3 R-KO cells in the OAG-induced 'off' response. Thus, TG-induced 'off' Ca^{2+} responses in IP_3 R-KO cells may result from store-operated Ca^{2+} entry and Ca^{2+} entry activated by DG via PLC γ 2 activation, while the contribution of the latter pathway may be minor in WT cells.

Fig. 3. Critical involvement of IP_3 -induced Ca^{2+} release in 'off' Ca^{2+} responses. (**A** and **B**) The IP_3 R blocker Xest C significantly suppresses TG-induced 'off' Ca^{2+} responses in WT (**A**) but not in $PLC\gamma 2^{-/-}$ (**B**) cells ($n = 23-48$). Xest C (50 μM) was loaded with fura-2/AM using 0.1% Pluronic F-127 (Molecular Probes) for 30 min prior to $[Ca^{2+}]_i$ measurements. During measurements, Xest C (50 μM) was added to perfusion solution for 20 min, and was omitted from Ca^{2+} readministration solution. Left, average time courses. Right, peak $[Ca^{2+}]_i$ rises in Ca^{2+} -free or Ca^{2+} -containing external solution. (**C**) Left, average time courses of Ca^{2+} responses induced by TG and IM (1 μM) in WT and $PLC\gamma 2^{-/-}$ cells ($n = 16-33$). Right, comparison between peak IM- and TG-induced $[Ca^{2+}]_i$ rises in Ca^{2+} -free or Ca^{2+} -containing external solution. (**D**) Left, average time courses of Ca^{2+} responses induced by TG or IM in IP_3R knockout cells. Right, peak IM- and TG-induced $[Ca^{2+}]_i$ rises in Ca^{2+} -free or Ca^{2+} -containing solution.

C2 domain is responsible for Ca^{2+} influx-dependent activation of $PLC\gamma 2$

The domains critical for Ca^{2+} entry-dependent activation were determined in $PLC\gamma 2$. Three potential Ca^{2+} -binding domains (EF-hand, catalytic domain and C2 domain), and the PH and SH3 domains are distinguished in $PLC\gamma 2$ (Bairoch *et al.*, 1990; Essen *et al.*, 1996; Rhee, 2001; Ananthanarayanan *et al.*, 2002). In $PLC\gamma 1$, PH mediates membrane-targeting through phosphatidylinositol 3,4,5-trisphosphate (PIP_3) binding, while SH3 acts as a physiological guanine nucleotide exchange factor (GEF) for the

Fig. 4. The C2 domain mediates Ca^{2+} entry-induced activation of $PLC\gamma 2$. (**A**) Schematic diagram for various mutant constructs of $PLC\gamma 2$. (**B**) Peak BCR-induced $[Ca^{2+}]_i$ rises during 5 min incubation in Ca^{2+} -free solution and after subsequent readministration of Ca^{2+} in $PLC\gamma 2^{-/-}$ cells expressing respective $PLC\gamma 2$ mutants ($n = 11-36$). (**C**) Peak TG-induced $[Ca^{2+}]_i$ rises in Ca^{2+} -free solution and after readministration of Ca^{2+} in $PLC\gamma 2^{-/-}$ cells expressing respective $PLC\gamma 2$ mutants ($n = 12-37$). Protocol is the same as in Figure 2E. (**D**) Left, average time courses of BCR-induced Ca^{2+} responses in $PLC\gamma 2^{-/-}$ cells expressing $PLC\gamma 2$, membrane-bound $PLC\gamma 2$ chimera (m $PLC\gamma 2$) or GFP ($n = 8-14$). m $PLC\gamma 2$ is composed of the human CD16 extracellular domain, the human TCR ζ-chain transmembrane domain and the complete rat $PLC\gamma 2$ as a cytoplasmic domain (Ishiai *et al.*, 1999). Right, BCR-induced $[Ca^{2+}]_i$ rises at peak and sustained after 5 min. (**E**) Left, average time courses of BCR-induced F/F_0 changes of R9-PHIP3-D106. Right, maximal $[IP_3]_i$ elevation and sustained $[IP_3]_i$ rises after 5 min upon BCR stimulation.

nuclear GTPase PIKE, an activator of nuclear phosphatidylinositol 3-kinase (PI3K) (Ye *et al.*, 2002) that produces the PIP_3 required for BCR-induced $[Ca^{2+}]_i$ elevation (Falasca *et al.*, 1998). Mutations were introduced in the $PLC\gamma 2$ domains tagged with yellow fluorescent protein (YFP) (Figure 4A). As expected, expression of the LD

Table I. Changes of PLC γ 2 localization by BCR stimulation (% of total fluorescence)

	PLC γ 2 mutant	Before BCR stimulation (membrane area)	5 min after BCR stimulation (membrane area)
WT	WT (2 mM Ca ²⁺)	37.0 $\pm$ 3.9 (<i>n</i> = 5)	57.9 $\pm$ 2.3*
	LD	32.7 $\pm$ 2.7 (<i>n</i> = 3)	53.4 $\pm$ 2.8*
	LD + U73122	30.1 $\pm$ 2.9 (<i>n</i> = 3)	31.0 $\pm$ 5.0
	LD (EGTA)	35.0 $\pm$ 3.3 (<i>n</i> = 3)	37.6 $\pm$ 4.6
	Δ C2	26.9 $\pm$ 8.4 (<i>n</i> = 3)	22.4 $\pm$ 5.1
	min Δ C2	33.3 $\pm$ 6.4 (<i>n</i> = 3)	42.1 $\pm$ 5.9
	Δ PH	25.5 $\pm$ 4.2 (<i>n</i> = 3)	24.5 $\pm$ 3.5
	E165A	31.8 $\pm$ 4.7 (<i>n</i> = 3)	56.5 $\pm$ 2.9*
	P820L	28.8 $\pm$ 3.1 (<i>n</i> = 3)	46.5 $\pm$ 2.2*
	WT (2 mM Ca ²⁺)	33.8 $\pm$ 4.2 (<i>n</i> = 4)	60.2 $\pm$ 9.3*
PLC γ 2 ⁻	WT (EGTA)	39.4 $\pm$ 5.7 (<i>n</i> = 5)	37.3 $\pm$ 7.5
	WT (2 mM Ba ²⁺)	35.7 $\pm$ 6.1 (<i>n</i> = 3)	39.1 $\pm$ 2.3
	WT + U73122	29.3 $\pm$ 7.9 (<i>n</i> = 3)	35.0 $\pm$ 8.1
	WT + U73343	31.2 $\pm$ 4.0 (<i>n</i> = 5)	56.8 $\pm$ 3.5*
	LD	31.7 $\pm$ 2.1 (<i>n</i> = 4)	34.2 $\pm$ 1.9
	Δ C2	34.7 $\pm$ 8.3 (<i>n</i> = 3)	44.2 $\pm$ 3.8
	min Δ C2	36.7 $\pm$ 5.3 (<i>n</i> = 3)	47.6 $\pm$ 7.7
	Δ PH	27.5 $\pm$ 4.8 (<i>n</i> = 3)	30.2 $\pm$ 8.8
	E165A	29.4 $\pm$ 3.6 (<i>n</i> = 3)	50.0 $\pm$ 5.3*
	P820L	31.3 $\pm$ 5.4 (<i>n</i> = 3)	60.4 $\pm$ 3.7*
	WT (ionomycin)	38.4 $\pm$ 2.0 (<i>n</i> = 4)	44.9 $\pm$ 2.1*

Membrane regions of fluorescent areas in DT40 cells were as described in Figure 5A.

n, number of experiment. **P* < 0.05, significantly different from cells before BCR stimulation.

mutant in PLC γ 2⁻ cells failed to restore either the BCR-induced [Ca²⁺]_i increase or to potentiate a TG-induced 'off' Ca²⁺ response (Figure 4B and C). Strikingly, deletion of C2 domain (Δ C2), which is involved in the formation of the Ca²⁺-phospholipid ternary complex in PLC δ s (Ananthanarayanan *et al.*, 2002), ablated the PLC γ 2 function to restore the BCR- and TG-induced Ca²⁺ responses. Deleting one of the expected Ca²⁺ binding regions (Δ 1138E-1140D) (Essen *et al.*, 1996) in C2 (min Δ C2) similarly blocked the restoration of the 'off' responses but not that of the BCR-induced Ca²⁺ release, strikingly differentiating Ca²⁺ entry-mediated responses from Ca²⁺ release. The PH deletion mutant (Δ PH) also failed to restore the PLC γ 2⁻ deficiency, as reported in PLC γ 1 activation (Falasca *et al.*, 1998). In contrast, the SH3 deletion (Δ SH3) mutant elicited partially impaired the Ca²⁺ responses, and the point SH3 mutant with leucine at proline 820 (P820L) that corresponds to P842 essential for GEF activity in PLC γ 1 (Ye *et al.*, 2002), or the EF-hand mutant (E165A), behaved like WT. Thus, the C2 domain is critical for Ca²⁺ entry-dependent activation of PLC γ 2.

C2 domain mediates Ca²⁺ influx-dependent translocation of PLC γ 2

The above data and the requirement for C2 when various proteins target the plasma membrane (Sutton *et al.*, 1995; Ananthanarayanan *et al.*, 2002; Delmas *et al.*, 2002) motivated us to visualize the localization of YFP-tagged PLC γ 2 (Figure 5). Transfected PLC γ 2 was diffusively distributed in the cytosol from the perinuclear area to the plasma membrane of the WT and the PLC γ 2⁻ cells. Within 4 min, but not within 2 min, stimulation of BCR efficiently concentrated PLC γ 2 near the plasma membrane in the presence of extracellular Ca²⁺ (Figure 5A). This PLC γ 2 translocation was abolished by removing Ca²⁺, using Ba²⁺

for Ca²⁺, using Δ C2 and min Δ C2 in the WT and the PLC γ 2⁻ cells (Figure 5A and B; Table I). Δ PH also suppressed translocation. The EF and SH3 mutants showed significant translocation (Table I). Importantly, even in the absence of extracellular Ca²⁺, expression of membrane-bound PLC γ 2 (mPLC γ 2) (Ishiai *et al.*, 1999) in the PLC γ 2⁻ cells promoted sustained [IP₃]_i and [Ca²⁺]_i increases (Figure 4D and E). Thus, C2 mediates the Ca²⁺ entry-induced transportation of PLC γ 2 for subsequent activation. Furthermore, in the WT cells, but not in the PLC γ 2⁻ cells, the LD mutant of PLC γ 2 showed significant BCR-induced plasma membrane localization inhibited by the PLC blocker, U73122 (Figure 5C). This suggests that transfected PLC γ 2 does not require its own lipase activity for Ca²⁺ entry-induced translocation to the plasma membrane when endogenous lipase-active PLC γ 2 is present.

Functional and physical association of PLC γ 2 with TRPC3

Among the TRPC channels responsible for receptor-activated Ca²⁺ entry (Montell *et al.*, 2002), expression of TRPC1, C3, C4, C5 and C7 was identified in DT40 cells (Figure 6A). The respective TRPC channels were tested for functional coupling with PLC γ 2 by co-expressing PLC γ 2 in HEK cells. Upon stimulation of the endogenous muscarinic acetylcholine receptors (MRs) with CCh, TRPC3, C5, C6 and C7 alone promoted increased [Ca²⁺]_i (Figure 6C), while co-expression of PLC γ 2 exerted further potentiation only in the TRPC3-expressing cells (Figure 6B and C). LD elicited the intact potentiation of peak Ca²⁺ responses but failed to sustain Ca²⁺ response. In the patch-clamp recording, PLC γ 2 significantly sustained CCh-activated TRPC3 currents (Figure 6D). Upon MR stimulation, PLC γ 2-YFP fluorescence near the plasma membrane showed increases that indicate translocation in

TRPC3-expressing cells (47.8 ± 0.7 to $52.3 \pm 0.3\%$, $P < 0.05$). Furthermore, immunoprecipitation analysis revealed a physical association between PLC γ 2 and TRPC3 (Figure 6E), which was impaired by Δ SH3, but not by LD or min Δ C2 (Figure 6F), in the HEK cells. In the DT40 cells, co-immunoprecipitation of TRPC3 with PLC γ 2 enhanced by BCR stimulation was observed (Figure 6G). These results suggest that receptor-activated TRPC3 channels functionally and physically interact with PLC γ 2 via SH3 to enhance Ca^{2+} response and to maintain their own channel activity.

Critical involvement of Ca^{2+} entry-dependent PLC γ 2 activation in receptor-evoked Ca^{2+} oscillation

Periodic increases in $[\text{Ca}^{2+}]_i$ (Ca^{2+} oscillation) are important for the efficiency and specificity of gene expression in lymphocytes (Dolmetsch *et al.*, 1998) and other biological responses (Miyazaki *et al.*, 1992; Thorn *et al.*, 1993). Figure 7A depicts a typical Ca^{2+} oscillation induced by stimulation of BCR in DT40 cells. LD and Δ C2 expression almost eliminated the late oscillatory phase of the BCR-induced Ca^{2+} response in the WT cells, while they only

Fig. 5. Ca^{2+} influx induces translocation of PLC γ 2 via the C2 domain. (A) Confocal fluorescence images indicating BCR-induced changes of subcellular localization of YFP-tagged PLC γ 2 in PLC γ 2⁻ cells in Ca^{2+} -containing or Ca^{2+} -free solution ($n = 5$). Right, representative fluorescent changes of PLC γ 2-EYFP distributed in $1 \mu\text{m}$ widths peripheral regions (Mem.) and in the rest the cytosolic areas (Cyt.). (B) Confocal images indicating changes of localization of PLC γ 2 constructs (WT, LD, Δ C2 or Δ PH) in WT and PLC γ 2⁻ cells by 5 min BCR stimulation ($n = 5$). (C) PLC inhibitor U73122 blocks BCR-induced localization changes of LD in WT cells. Treatment with U73122 (3 μM) or its inactive analogue U73343 was started 20 min prior to BCR stimulation. Lower left, average time courses of BCR-induced F/F_0 changes of R9-PHIP₃-D106 in WT cells expressing GFP or LD, or pretreated with U73122 or U73343. Lower right, maximal $[\text{IP}_3]_i$ elevation upon BCR stimulation.

elicited partial suppression of the initial transient Ca^{2+} response (Figure 7B and C). Considering the translocation of LD to the plasma membrane in the WT cells (Figure 5B), this evidence supports the view that lipase activity is essential for the translocated PLC γ 2 in maintaining downstream Ca^{2+} oscillation. The expression of PLC γ 2 restored Ca^{2+} oscillation in the PLC γ 2⁻ cells (Figure 7F). However, ΔSH3 incompletely restored Ca^{2+} oscillation, and ΔC2 failed to elicit a detectable Ca^{2+} response in the PLC γ 2⁻ cells (Figure 7G and H). The results demonstrate the physiological significance of Ca^{2+} influx-dependent PLC γ 2 activation in BCR-induced Ca^{2+} oscillation.

Amplification of DG signalling promotes ERK activation

The PLC γ 2-mediated signal amplification should have a significant impact on the responses downstream of DG, since DG production is also enhanced by Ca^{2+} entry-induced PLC γ 2 activation and PIP₂ hydrolysis. Activation of PKC by DG promotes activation of extracellular signal-regulated kinase (ERK) through phosphorylation in DT40 cells (Kurosaki, 2002). Figure 8A depicts sustained BCR-induced ERK activation in an extracellular Ca^{2+} -dependent manner. The ERK activation was inhibited by a PKC inhibitor, bisindolylmaleimide (BIM, 10 μM), and more prominently by PLC γ 2 deficiency, which was partially reversed by a PKC activator phorbol-12-myristate-13-acetate (PMA) (Figure 8B). Hence, signal cascades other than PKC activation may be evoked by PLC γ 2 to activate ERK. Furthermore, TG gradually increased the phosphorylated levels of ERK in an extracellular Ca^{2+} - and PLC γ 2-dependent manner (Figure 8C and D). Thus, Ca^{2+} entry-dependent PLC γ 2 activation is important in DG-induced activation via PKC of ERK in mitogen-activated protein kinase (MAPK) pathways.

Discussion

Ca^{2+} entry amplifies BCR-induced Ca^{2+} signalling via PLC γ 2 translocation and activation

This study reveals a novel molecular mechanism that amplifies cellular signals in the second phase of the Ca^{2+} response in B lymphocytes. The amplification mechanism is a positive feedback cycle, in which a key event is the Ca^{2+} entry-evoked translocation towards the plasma membrane and the subsequent PLC γ 2 activation/IP₃ production then leads to Ca^{2+} release and entry (Figure 9). The C2 domain mediates the translocation of PLC γ 2 (Figure 5B). Importantly, C2 is also found in PLC δ (Ananthanarayanan *et al.*, 2002), phospholipase A₂ (PLA₂) (Clark *et al.*, 1991), synaptotagmin I (Sutton *et al.*, 1995) and PKC isoforms (Schaefer *et al.*, 2001; Delmas *et al.*, 2002), among which PLA₂ and PKCs are translocated by Ca^{2+} entry (Clark *et al.*, 1991; Schaefer

Fig. 6. Functional and physical association of PLC γ 2 with the TRPC3 Ca^{2+} channel promotes receptor-evoked TRPC3 currents and Ca^{2+} responses. (A) RT-PCR analysis of RNA expression of TRPC homologues in DT40 cells. (B) Average time courses of Ca^{2+} responses upon stimulation of MRs in HEK cells expressing TRPC3 and/or PLC γ 2 (WT or LD) ($n = 32$ –38). (C) Peak MR-activated $[\text{Ca}^{2+}]_i$ rises in HEK cells expressing respective TRPC1–C7 channels alone or along with PLC γ 2. (D) Left, representative traces of ionic currents recorded from cells expressing TRPC3 alone or plus PLC γ 2 at a holding potential of -60 mV. Right, peak and sustained (30 s after reaching peak) amplitudes of the MR-activated TRPC3 currents ($n = 9$ –10). (E) Co-immunoprecipitation of TRPC3 and PLC γ 2 via SH3. Immunoprecipitates (IP) with anti-GFP polyclonal antibody from TRPC3/GFP- and TRPC3/PLC γ 2-YFP-expressing HEK cells (upper) or GFP/PLC γ 2- and TRPC3-GFP/PLC γ 2-expressing HEK cells (lower) were separated on 8% SDS-PAGE, and blotted with anti-PLC γ 2 or anti-TRPC3 antibody, respectively, in western blot analysis (WB). (F) IP with the anti-GFP antibody from LD/TRPC3-, min ΔC2 /TRPC3- and ΔSH3 /TRPC3-expressing HEK cells. Anti-TRPC3 antibody was used to assess co-immunoprecipitation in WB. All the PLC γ 2 constructs were tagged with YFP. (G) IP with the anti-GFP antibody from TRPC3-GFP-expressing WT and TRPC3-GFP-expressing PLC γ 2⁻ cells with (+) or without (–) 5 min BCR stimulation (anti-IgM). Co-immunoprecipitation was assessed using anti-PLC γ 2 antibody in WB.

Fig. 7. PLC γ 2 requires its lipase activity and C2 domain to elicit BCR-evoked Ca^{2+} oscillation. (A–D) Representative time courses of BCR-induced Ca^{2+} responses in WT and PLC γ 2 $^{-}$ cells in Ca^{2+} -containing (A–C and E–H) ($n = 16$ –72) or Ca^{2+} -free (D) ($n = 50$) solution (long time course, 60 min). PLC γ 2 constructs were the same as those in Figure 4.

et al., 2001). For targeting membrane microdomains and activating PLC γ 2, its PH domain is essential (Figure 5B). PIP_3 by PI3K is required for receptor-stimulated Ca^{2+} mobilization (Bae *et al.*, 1998), and PIP_3 binds to the PH with the highest affinity among the phosphoinositides to activate PLC γ 1 through membrane targeting (Falasca *et al.*, 1998). In fact, our data demonstrate that the ΔPH mutant failed to restore the Ca^{2+} responses (Figure 4B and C), and that wortmannin (1 μM), which suppresses PIP_2 and PIP_3 synthesis by inhibiting PI3K and PI4K, significantly suppressed BCR-stimulated Ca^{2+} oscillation, and attenuated the TG-induced $[\text{Ca}^{2+}]_i$ increase in the WT cells, but not in the PLC γ 2 $^{-}$ cells (Supplementary figure 5).

The second key process in the amplification mechanism is successive Ca^{2+} release. We observed a striking difference in the ‘off’ response between the WT and the PLC γ 2 $^{-}$ cells (Figure 3C) when TG was used to selectively deplete SERCA-containing stores, but not when IM was employed for more indiscriminate and thorough store depletion. This suggests that IP_3 -induced Ca^{2+} release from partially depleted stores significantly contributes to the TG-induced ‘off’ response, being consistent with the previous report in which TG frequently failed to induce Ca^{2+} release-activated Ca^{2+} currents in patch-clamp recording despite its efficiency in inducing ‘off’ responses in $[\text{Ca}^{2+}]_i$ measurement (Parekh *et al.*, 1997). Efficient activation of Ca^{2+} entry channels via IP_3 -induced store depletion (Zhu *et al.*, 1996) following PLC γ 2 activation is the third key process. This process may be elicited from the physical association of TRPC3 with PLC γ 2 and IP_3Rs (Boulay *et al.*, 1999; Kiselyov *et al.*, 1999) (Figure 6E). Activation of type 3 IP_3R in the plasma membrane (Vazquez *et al.*, 2002) and direct DG action on Ca^{2+} channels (Hofmann *et al.*, 1999; Trebak *et al.*, 2003) are also important processes to be considered. Ultimately, determining the composition of the signalplex that

contains PLC γ 2, TRPC3 and other proteins such as BLNK (Ishiai *et al.*, 1999) is essential for fundamental understanding of the amplification mechanism.

The transfected LD PLC γ 2 mutant was translocated to the plasma membrane area in the WT cells, but not in the PLC γ 2 $^{-}$ cells (Figure 5B). This result suggests that Ca^{2+} influx is mediated by endogenous PLC γ 2 to elicit subsequent translocation of LD in WT cells, and that PLC γ 2 does not require its own lipase activity in the translocation process. Independent of the hydrolysis of PIP_2 , the translocated PLC γ 2 may exert positive regulatory action on TRPC3 Ca^{2+} channel activity at the plasma membrane through physical interaction via SH3 (Figure 6) (Patterson *et al.*, 2002). It is also possible that PLC γ 2 translocation is accompanied by associated TRPC3 targeting the plasma membrane (Figure 9). In fact, on M1R stimulation, we observed partial but significant restoration of Ca^{2+} entry-induced $[\text{Ca}^{2+}]_i$ elevation by the ΔSH3 mutant in the PLC γ 2 $^{-}$ cells (Figure 1E). Thus, the enzyme activity-independent translocation and consequent interaction of PLC γ 2 with TRPC3 at the plasma membrane may underlie a non-enzymatic role of PLC γ 2 (Patterson *et al.*, 2002; Putney, 2002), cooperating with the second lipase activation in enhancing Ca^{2+} entry to fully restore the Ca^{2+} deficiency in mutant cells. This view is consistent with a recent report by Patterson *et al.* (2002) in which LD restored Ca^{2+} entry-induced $[\text{Ca}^{2+}]_i$ increases in M5R-stimulated PLC γ 2 $^{-}$ cells, although the restoration was comparable to that by WT. The difference in peak responses between our data using M1R and the data of Patterson *et al.* (2002) using M5R is attributable to different activation and/or desensitization speeds of receptor-activated Ca^{2+} entry and subsequent lipase activity-dependent PLC γ 2 signalling, considering smaller Ca^{2+} release but larger Ca^{2+} entry-induced Ca^{2+} response upon M1R stimulation (Figure 1E) compared with that upon

Fig. 8. Requirement of Ca²⁺ influx-dependent PLC γ 2 activation for sustained BCR-induced activation of ERK in DT40. (A) WT or (B) PLC γ 2⁻ cells were stimulated with anti-IgM (1 μg/ml) for indicated time in the presence or absence of extracellular Ca²⁺. Treatment with BIM (10 μM) was started 10 min before BCR stimulation. PMA (50 nM) was applied to anti-IgM in PLC γ 2⁻ cells (B). (C and D) Average time courses of ERK activation evoked by TG (2 μM) in WT (C) and PLC γ 2⁻ (D) cells. The ERK activities were assessed by the quantification of its phosphorylation using phospho-specific antibody.

M5R stimulation. The slower signal transduction through PLC γ 2 may result in the Ca²⁺ entry and lipase activity-dependent response that persisted throughout the protocol (Figure 1E), in contrast to the almost complete suppression of Ca²⁺ entry and absence of lipase activity-dependent response in the M5R-expressing PLC γ 2-deficient cells (Figure 6 of Patterson *et al.*, 2002). Since deleting SH3 also terminated the enhancing effect of PLC γ 1 on the Ca²⁺ entry-induced Ca²⁺ responses in the PC12 cells and the TRPC3-expressing HEK cells (Patterson *et al.*, 2002), PLC γ 1 and PLC γ 2 may share an enzyme-independent function in receptor-induced Ca²⁺ responses.

Physiological impact of Ca²⁺ entry-induced PLC γ 2 activation in BCR signalling

Our finding is contrary to the prevailing view that receptor-activated Ca²⁺ entry channels amplify Ca²⁺ responses predominantly through the direct effect of Ca²⁺ permeation and replenishing Ca²⁺ stores. The

PLC γ 2-mediated amplification of the Ca²⁺ signal would therefore dramatically change the view of the physiological roles played by receptor-activated Ca²⁺ entry. The PLC γ 2-mediated signal amplification should have a significant eventual impact on downstream Ca²⁺-dependent responses, including regulation of gene expression via transcription factors such as NF-AT (Dolmetsch *et al.*, 1998), cell proliferation and apoptosis (Verkhatsky and Petersen, 2002; Irvine, 2002). Importantly, PIP₂ hydrolysis and DG production are also enhanced in the amplification mechanism. Activation of PKC by DG promotes ERK activity in the MAPK pathway to induce phosphorylation of the transcription factor CREB, which interacts with an enhancer cAMP-response element (Kurosaki, 2002). Our results provide direct evidence that ERK activation is promoted by Ca²⁺ entry via PLC γ 2 and PKC. DG is also known to activate the TRPC3, C6 and C7 channels in a PKC-independent manner (Hofmann *et al.*, 1999; Okada *et al.*, 1999; Trebak *et al.*, 2003), while PIP₂ is linked to the

Fig. 9. Model for amplification of Ca^{2+} signalling by $\text{PLC}\gamma 2$ in B lymphocytes. BCR stimulation induces activation of $\text{PLC}\gamma 2$ via phosphorylation by Syk tyrosine kinase, and the activated $\text{PLC}\gamma 2$ hydrolyses PIP_2 to produce IP_3 . IP_3 leads to Ca^{2+} release from ER and Ca^{2+} influx via TRPC3 -containing receptor-activated Ca^{2+} channels (RACCs), which may correspond to SOCs activated through interaction with IP_3R or to DG-activated Ca^{2+} channels. Ca^{2+} influx via RACC promotes lipase-independent $\text{PLC}\gamma 2$ translocation from the cytosol to the plasma membrane (PM) by acting on the C2 domain. The targeted $\text{PLC}\gamma 2$ is activated to hydrolyse PIP_2 and produce IP_3 and DG, which in turn induces Ca^{2+} release from ER and Ca^{2+} influx via Ca^{2+} entry channels such as TRPC3 . $\text{PLC}\gamma 2$ may also positively regulate TRPC3 channels via direct interaction.

pathway composed of PI3K, Bruton's tyrosine kinase and the serine threonine kinase Akt/PKB (Kurosaki, 2002). Hence, the model predicts extensive regulatory roles of Ca^{2+} entry-induced $\text{PLC}\gamma 2$ activation in the amplification and/or co-ordination of multiple BCR-induced signalling pathways.

$\text{PLC}\gamma 2$ is also expressed in the anterior pituitary and cerebellar Purkinje and granule cells (Rebecchi and Pentyla, 2000). Previously, regarding Ca^{2+} oscillation in REF52 fibroblasts, Harootunian *et al.* (1991) proposed that a dominant feedback mechanism is the stimulation of PLC by the $[\text{Ca}^{2+}]_i$ increase through population IP_3 measurement, while pathways that evoke the $[\text{Ca}^{2+}]_i$ increase remained unidentified. Recent research has also demonstrated production of IP_3 induced by voltage-dependent Ca^{2+} influx (Okubo *et al.*, 2001) and regulation of metabotropic glutamate receptor-mediated neuronal signalling by extracellular Ca^{2+} in Purkinje neurons (Tabata *et al.*, 2002). When eggs are fertilized by sperm, the Ca^{2+} oscillations essential for embryo development are mediated by $\text{PLC}\gamma$ (Sato *et al.*, 2000) or homologous $\text{PLC}\zeta$ (Saunders *et al.*, 2002). Furthermore, the activation of recombinantly overexpressed $\text{PLC}\delta 1$ by Ca^{2+} entry that follows the $\text{PLC}\beta$ activation upon bradykinin stimulation has been reported in PC12 cells (Kim *et al.*, 1999). Since the ubiquitous $\text{PLC}\gamma 1$ and $\text{PLC}\delta$ also contain C2 domains (Rebecchi and Pentyla, 2000), the signal amplification mediated by Ca^{2+} entry-induced PLC translocation and activation may be universal among various cell types.

Materials and methods

Cell cultures and cDNA expression

The $\text{PLC}\gamma 2$ mutants were constructed using PCR techniques. The WT $\text{PLC}\gamma 2$ (Takata *et al.*, 1995), LD (H327F) (Patterson *et al.*, 2002) and other mutated constructs were fused at the C-terminus with YFP using an EYFP-N1 vector (Clontech). In the ΔC2 , ΔPH and ΔSH3 mutants, residues 1043M-1237V, 20R-131Q and 810D-829A were deleted,

respectively. Cell cultures and cDNA expression in the DT40 cells using the vesicular stomatitis virus and in the HEK293 cells were performed as described previously (Hara *et al.*, 2002; Mori *et al.*, 2002).

Measurement of changes in $[\text{Ca}^{2+}]_i$

The cells on coverslips were loaded with $1\text{ }\mu\text{M}$ fura-2/AM in a physiological salt solution (PSS) containing (in mM): 150 NaCl, 8 KCl, 2 CaCl_2 , 1 MgCl_2 , 5 HEPES, and 5.6 glucose, adjusted to pH 7.4 with NaOH. Fluorescence images of the cells were recorded and analysed with a video image analysis system (ARGUS CA-20; Hamamatsu Photonics) as described previously (Mori *et al.*, 2002). All ratio data were calculated to $[\text{Ca}^{2+}]_i$ using an *in vivo* calibration method.

Fluorometric measurement of changes in $[\text{IP}_3]_i$

The basic strategy in designing the optical direct IP_3 sensors is described in Morii *et al.* (2002). Conjugation with fluorophore 6-bromoacetyl-2-dimethyl-aminonaphthalene (DAN) at Cys106 introduced near the mouth of the IP_3 -binding site prevents semi-specific ligand binding (Morii *et al.*, 2002), conferring an IP_3 -selective sensitivity to the PH protein. The dissociation constant was only slightly affected by Ca^{2+} and ranged from 2.2 to 3.3 μM in 0–1 mM Ca^{2+} . The protein was further fused to arginine (Arg) nonapeptide for improved cellular incorporation. The modified sensor, R9-PHIP3-D106 [Arg (R)₉-PH IP_3 sensor conjugated with DAN at residue 106], showed an efficient *in vivo* IP_3 -sensing ability. In *in vivo* measurements, cells were plated onto poly-L-lysine-coated glass coverslips. After a 2 h incubation for cell adhesion, the cells were transferred to Hank's balanced salt (HBS) solution containing 1 μM of IP_3 sensor and 0.1% F-127 (Molecular Probes) for 10 min at 37°C. After the cells were washed, changes in the fluorescence images of the cells were observed using a video image analysis system at an emission wavelength of 510 nm (bandwidth 20 nm) at room temperature by excitation at 380 nm (bandwidth 11 nm) in PSS. $[\text{IP}_3]_i$ increases were detected as fluorescence quenching of R9-PHIP3-D106 due to IP_3 binding. All traces were displayed after subtracting the averaged time courses of fluorescence reduction due to photobleaching or photodegradation.

For the *in vivo* calibration of $[\text{IP}_3]_i$, synthesized IP_3 (Dojindo) was applied to R9-PHIP3-D106-preloaded DT40 cells after treatment with β -escin (1 μM ; Sigma) (Supplementary figure 1). The initial decrease in fluorescence was fitted to a binding isotherm:

$$\Delta F = \Delta F_{\max} \{ [\text{IP}_3] / (K_d + [\text{IP}_3]) \} \quad (1)$$

where ΔF is the change in fluorescence intensity. $\Delta F_{\max}$ is ΔF at saturation, K_d is the dissociation constant, and $[\text{IP}_3]$ is the concentration of IP_3 . $[\text{IP}_3]_i$ was calculated based on Equation 1, where the K_d value is 1.24 μM .

Table II. Sequences of antisense and sense oligonucleotides used

Gene	Orientation	Pair of primers for first PCR (5' to 3')	Pair of primers for nested PCR (5' to 3')
TRPC1	Sense	TGGATTATTGGGATGATTGGTCAGAC	
	Antisense	TCGGGCGAATTTCCATTCTTATCTTC	
TRPC3	Sense	GTCTGAAGTAACCTCTGTGTCCTG	GAGAACATGGATATGTTCTCTATGGC
	Antisense	CACATCTCAACATATTGGGATTGAG	ATGGCTAACTCTTCTGTGCGCTGGGAC
TRPC4	Sense	GAGCTGGGACATGTGGCATCCAACGC	GAGGCACTGTTTGCAATTGCAAAACATC
	Antisense	GCACAAGGTGTCTCCATTATCCACC	GCTTGGTATGACATTGAAAGAGTGGGC
TRPC5	Sense	TTGAGGTCTTCTGAAGGACCACGCAG	CTGCAAGTGGATTGCAATCAGCTGTGC
	Antisense	GGCTTCTCTTCGCCCATGTCAATCCAG	GTTTAAATTAGCGCAGACACATTGAAGTC
TRPC7	Sense	ACGCCTGATTAAGCGGTATGTGCTGAAG	CTCAGGTGGACCGAGAAAATGATGAA
	Antisense	GCCAAAAGAGAGGCTCGGTGTGTG	GCAAAGAGGAAGTTTTGGCTTGAATC

Electrophysiology

The whole-cell patch-clamp technique was performed as described previously (Mori *et al.*, 2002). The cells on coverslips were perfused in an external solution containing in (mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with NaOH (300 mOsm). The pipette solution contained in (mM): 75 CsOH, 75 aspartate, 40 CsCl, 4 MgCl₂, 10 BAPTA, 2 ATP Na₂, 5 HEPES, and 8 phosphocreatine, adjusted to pH 7.4 with CsOH. mTRPC3-pCIneo (Okada *et al.*, 1999) was co-transfected with pIRES2-EGFP (Clontech) or pIRES2-EGFP that contained PLC γ 2 cDNA in the HEK cells, and GFP-positive cells were selected for recording.

Confocal visualization of PLC γ 2-YFP fusion constructs

PLC γ 2-YFP-expressing cells were plated onto poly-L-lysine coated glass coverslips after 72 h of retroviral infection. Fluorescence images were acquired with a confocal laser-scanning microscope (FV500; Olympus) using the 488 nm line of an argon laser for excitation and a 505 nm long-pass filter for emission. The specimens were viewed at high magnification using plan oil objectives (60 $\times$, 1, 40 NA; Olympus).

Expression analysis

The PCR protocol used for the expression analysis is as described previously (Inoue *et al.*, 2001). The PCR primers used are listed in Table II.

Immunoprecipitation and western blot analysis

TRPC3-GFP was first established in the EGFP-N1 vector (Clontech), and transferred into pMXA. HEK or DT40 Cells ($\sim 3 \times 10^6$) were lysed in 1 ml of RIPA buffer (pH 8.0) containing 150 mM NaCl, 0.5% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 2 mM EGTA, 1 mM PMSF and 10 μ g/ml leupeptin. GFP-tagged mTRPC3 or YFP-tagged PLC γ 2 protein was immunoprecipitated in an RIPA buffer with 3 μ l of anti-GFP polyclonal antibody (Clontech) in the presence of a 25 μ l bed volume of Sepharose G-agarose beads (Amersham Pharmacia) rocking overnight at 4°C (Patterson *et al.*, 2002). The immune complexes were washed three times with the RIPA buffer for 5 min at room temperature, and resuspended in 50 μ l of the SDS sample buffer. The protein samples (5 μ l) were fractionated by 8% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (Inoue *et al.*, 2001). The blots were incubated with an anti-PLC γ 2 (Ishiai *et al.*, 1999) or an anti-mouse TRPC3 antiserum, and stained using the ECL system (Amersham Pharmacia). The anti-mouse TRPC3 rabbit antiserum was directed against the C-terminus (EELAILHKLSEKLNPSVLRCE).

Analysis of ERK activity

Activation of ERK was determined by western blot with anti-phosphospecific ERK antibody (Cell Signaling). A total of $\sim 5 \times 10^5$ cells were stimulated in serum-free PSS, and then lysed in 50 μ l of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 100 μ M Na₃VO₄, 2 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin and 5 μ g/ml aprotinin) for 10 min on ice. After centrifugation at 22 000 g for 10 min, 10 μ l of supernatants were subjected to 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The total amount of ERK was detected by using anti-ERK polyclonal antibody (Santa Cruz Biotech). The bands were scanned and the density of each band was determined by NIH image software.

Statistical analysis

All data are expressed as means $\pm$ SEM. The data were accumulated under each condition from at least three independent experiments. The *P* values are the results of Student's *t*-tests.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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