The Class II Phosphatidylinositol 3 kinase C2β Is Required for the Activation of the K⁺ Channel KCa3.1 and CD4 T-Cells

Shekhar Srivastava,† Lie Di,‡ Olga Zhdanova,†† Zhai Li,‡† Santosh Vardhana,‡§ Qi Wan,‡§ Ying Yan,¶ Rajat Varma,¶¶ Jonathan Backer,‖ Heike Wulff,ª Michael L. Dustin,§ and Edward Y. Skolnik‡‡

†Division of Nephrology, *Departments of Pharmacology and §Molecular Pathogenesis, The Helen L. and Martin S. Kimmel Center for Biology and Medicine at the †Skirball Institute for Biomolecular Medicine, New York University Langone Medical Center, New York, NY 10016; ‡T-Cell Biophysics Unit, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892; ¶Department of Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461; ‖Department of Pharmacology, University of California Davis, Davis, CA 95616

Submitted May 13, 2009; Revised June 19, 2009; Accepted June 30, 2009

Monitoring Editor: Carl-Henrik Heldin

The Ca²⁺-activated K⁺ channel KCa3.1 is required for Ca²⁺ influx and the subsequent activation of T-cells. We previously showed that nucleoside diphosphate kinase beta (NDPK-B), a mammalian histidine kinase, directly phosphorylates and activates KCa3.1 and is required for the activation of human CD4 T lymphocytes. We now show that the class II phosphatidylinositol 3 kinase C2β (PI3K-C2β) is activated by the T-cell receptor (TCR) and functions upstream of NDPK-B to activate KCa3.1 channel activity. Decreased expression of PI3K-C2β by siRNA in human CD4 T-cells resulted in inhibition of KCa3.1 channel activity. The inhibition was due to decreased phosphatidylinositol 3-phosphate [PI(3)P] and the histidine kinase nucleoside diphosphate kinase B (NDPK-B, also known as nm23 H2) are also required for KCa3.1 activation (Srivastava et al., 2005, 2006a,b).

INTRODUCTION

The Ca²⁺-activated K⁺ channel KCa3.1 and the voltage-activated K⁺ channel Kv1.3 play a critical role in the activation of a number of immune cells including T- and B-lymphocytes and mast cells. By modulating the efflux of K⁺, these channels function to maintain a negative membrane potential, which is critical for sustained calcium entry into these cells via calcium release-activated Ca²⁺ channels (CRAC) after antigen receptor activation (Cahalan et al., 2001; Wulff et al., 2003a; Srivastava et al., 2006b). Sustained Ca²⁺ entry then leads to the activation of calcineurin, which by dephosphorylating the transcription factor NFAT (nuclear factor of activated T-cells) induces the production of NFAT-dependent cytokines and the subsequent proliferation of these cells (Crabtree and Olson, 2002; Cahalan et al., 2007; Feske, 2007).

Although both channels are expressed in human T- and B-cells, Kv1.3 and KCa3.1 have been reported to play quite different roles in the activation of different T- and B-cell subsets. For example, in resting naive T-cells Kv1.3 is the dominating channel and is required for maximal Ca²⁺ influx into these cells. In contrast, KCa3.1 channels are expressed at low levels in resting naive T-cells and are not required for activation of these cells. However, KCa3.1 channels are rapidly up-regulated after T-cell activation through AP-1–dependent transcription and are required for maximal Ca²⁺ influx and proliferation during the reactivation of naive T-cells (Ghanshani et al., 2000). KCa3.1 channels are also expressed in central memory T-cells, whereas Kv1.3 channels are expressed in effector memory T-cells, where they play pivotal roles in Ca²⁺ influx and the activation of these cells (Wulff et al., 2003b; Beeton et al., 2006).

Over the past several years, it has become increasingly clear that KCa3.1 activity is regulated by various mechanisms. It has been appreciated for some time that the carboxy-terminus of KCa3.1 is constitutively bound to calmodulin, and channel opening occurs only after binding of Ca²⁺ to calmodulin (Xia et al., 1998; Keen et al., 1999; Fanger et al., 2001; Maylie et al., 2004). This finding made physiological sense as it would provide a mechanism whereby the initial influx in Ca²⁺ could feed forward to stimulate sustained calcium entry by activating KCa3.1. We recently found that, in addition to Ca²⁺, phosphatidylinositol 3-phosphate [PI(3)P] and the histidine kinase nucleoside diphosphate kinase B (NDPK-B, also known as nm23 H2) are also required for KCa3.1 activation (Srivastava et al., 2005, 2006a,b).
These studies demonstrated that NDPK functions downstream of PI(3)P and activates Kcα3.1 by phosphorylating histidine (H) 358 in Kcα3.1’s carboxy-terminal (CT) tail (Srivastava et al., 2006b). In addition, we identified two new negative regulators of Kcα3.1, the PI(3)P phosphatase myotubularin-related protein 6 (MTRM6) and the histidine phosphatase, phosphohistidine phosphatase-1 (PHPT-1), which inhibit Kcα3.1 by dephosphorylating PI(3)P and Kcα3.1, respectively (Srivastava et al., 2006a, 2008). These molecules also play a critical role in the reactivation of human CD4 T-cell; NDPK-B is required for T-cell receptor (TCR)-stimulated Ca2+ flux and proliferation, whereas both MTRM6 and PHPT-1 inhibit these responses.

One of the unanswered questions has been the identification of the phosphatidylinositol 3-kinase (PI3K) responsible for generating the pool of PI(3)P that mediates activation of Kcα3.1 in CD4 T-cells. PI3Ks are composed of a family of lipid kinases that phosphorylate the 3’ position of the inositol head group of d-myo-phosphatidylinositol (Cantley, 2002; Foster et al., 2003). Members of this family have been divided into three classes (I, II, and III) based on sequence homology and substrate specificity. Most of the previous work on PI3Ks in lymphocyte activation have focused on the homology and substrate specificity. Previous studies of knockout mice have demonstrated deficits in T-cell development and survival, indicating that class I PI3Ks have partially redundant functions (Varshavsky et al., 2000; Okkenhaug et al., 2006; Fruman, 2007; Patton et al., 2007). Previous studies of knockout mice have demonstrated diminished TCR signaling and PI3K activation in peripheral T-cells from p110α, p110β, and p110δ single knockout mice (Sasaki et al., 2000; Okkenhaus et al., 2002; Rodriguez-Borlando et al., 2003). In addition, mice lacking both p110α and p110δ have a profound defect in T-cell development and survival, indicating that class I PI3Ks have partially redundant functions (Webb et al., 2005; Swat et al., 2006). Surprisingly, we found that the class II PI3K-C2γ, and not the class I PI3Ks, is required for the activation of Kcα3.1 in T-cells. This is the first demonstration for a role of a class II PI3K in lymphocyte activation.

MATERIALS AND METHODS

Reagents

Cells. Jurkat T-cells were cultured in RPMI + 10% FBS. Jurkat T-cells were purchased from the ATCC (Manassas, VA) and then transfected with a flag-tagged Kcα3.1 and neo-resistant cell lines overexpressing Kcα3.1 (Jurkat-Kcα3.1) were obtained. Green fluorescent protein (GFP)-tagged PI3K-C2α and PI3K-C2β were kindly provided by J. Dumin (Imperial College, London). Jurkat-Kcα3.1 cells overexpressing PI3K-C2α and PI3K-C2β were obtained by transfection using AMAXA reagents (Amaxa Biosystems, Gaithersburg, MD). CD4 T-cells were isolated from peripheral adult blood buffy coats (NY Blood Center) using the CD4 isolation kit from Miltenyi Biotec (Auburn, CA) according to manufacturers protocol. We routinely obtained >95% CD4 T-cells as assessed by FACS using this procedure.

For small interfering RNA (siRNA) transfection, unstimulated human CD4 T-cells or Jurkat T-cells were electroporated using Amaxa reagents (Amaxa Biosystems, Gaithersburg, MD) according to manufacturers protocol. We routinely obtained >95% CD4 T-cells as assessed by FACS using this procedure. To silence PI3K-C2β in Jurkat T-cells was performed as described above. To assess whether TCR stimulation leads to an increase in Kcα3.1 channel activity, Jurkat T-cells were cultured together with Raji B-cells as an antigen presenting cells (at a ration of 5:1) that had been preincubated with (activated) or without (control) the superantigen staphylococcal enterotoxin (SEB) for 30 min at 37°C as previously described (Varma et al., 2005). Activated Kcα3.1 channel activity was assessed on Jurkat T-cells 15 min after forming a stable synapse with SEE-pulsed Raji B-cells. To assess whether overexpression of GFP-PI3K-C2α or PI3K-C2β affected Kcα3.1 channel, whole-cell patch-clamp was performed on GFP-positive Jurkat cells conjugated to Raji B-cells. To verify that silencing of PI3K-C2β led to decreased Kcα3.1 channel activity by inhibiting the production of PI(3)P, we determined whether the addition of PI(3)P (100 nM) into the pipette solution during patch-clamping restored channel activity as previously described (Srivastava et al., 2005). PI(3)P diC₁₆ [C₆H₁₄Na₃O₆P₂] was purchased from Echelon Biosciences (Salt Lake City, UT) and used according to specifications. PI(3)P diC₁₆ was resuspended in water and flash frozen in liquid nitrogen and used at a concentration of 100 nM in the pipette solution.

Intracellular Ca2+ Activity. Cells were loaded at 1 × 10⁶ cells/ml with 5 μM Fura-2 AM ester (Molecular Probes, Eugene, OR) in RPMI medium for 30 min at room temperature, washed, and then resuspended in RPMI. Cells were attached to poly-L-lysine–coated coverslips for 20 min in a KC-20 bath flow chamber (Warner Instrument, Hamden, CT) and fura-2 fluorescence was recorded (Delta Ram; PTL, South Brunswick, NJ) at excitation wavelengths of 340 and 380 nm. Background fluorescence was obtained by treating the cells with 100 mM MgCl₂ at the end of the experiment. Data are represented as the ratio of 340 to 380 after background subtraction. Cells were perfused with the bath solution (composition described before) in the presence or absence of extracellular calcium and stimulated with 5 μg/ml anti-CD3 cross-linked with 5 μg/ml rat anti-mouse IgG.

Quantitative RT-PCR. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and then reverse-transcribed using random hexamer primers. Quantitative PCR was then assessed using SYBR Green 1 by iCycler IQ (Bio-Rad, Richmond, CA) using gene-specific primers purchased from Qiagen (Chatsworth, CA).

Planar Lipid Bilayers. Glass-supported lipid bilayers were generated as described previously (Campi et al., 2005). Briefly, glass-supported dioleoylphosphatidylincholine bilayers incorporating ICAM-1 (intercellular adhesion molecule 1; 300 molecules/μm²) and 0.1% cap-biotin were prepared in a Biopuchs (Butler, PA) flow cell. Unlabeled streptavidin (8 μg/ml) and mono-biotinylated anti-human CD3, OKT3 clone (10 μg/ml) without a fluorophore (to assess localization of Cherry-Zap-70) or conjugated to Cy3, were loaded sequentially in HBS/HSA buffer.

Imaging of TCR, Zap-70, PI3K, and Phosphoprotein Immunofluorescence. To assess whether GFP-PI3K-C2α or PI3K-C2β localize with the TCR or Zap-70 at the immunological synapse (IS), Jurkat cells that were transfected with either GFP-PI3K-C2α or GFP-PI3K-C2β with or without ch-Zap-70 were suspended in HEPES-buffered saline supplemented with 5 mM glucose, 2 mM MgCl₂, 1 mM CaCl₂, and 1% human serum albumin (HBS/HSA) and floated onto the lipid bilayer. Total internal reflection fluorescence microscopy (TIRFM) was used to assess localization of the TCR and GFP-PI3K-C2α or PI3K-C2β as previously described (Varma et al., 2006).

PI3K Assays. Jurkat T-cells, transfected with GFP-PI3K-C2β, were stimulated with anti-CD3 and anti-CD28 antibodies for various times. Cells were lysed, and PI3K assay was performed on anti-GFP immunoprecipitates as previously described (Yan et al., 2009).
RESULTS

PI3KC2β Is Required for KCa3.1 Channel Activity in Activated Human CD4 T-Cells

We have previously shown that PI(3)P is required for KCa3.1 channel activity in activated human CD4 T-cells (Srivastava et al., 2006a,b). To identify the specific PI3K isoform that is required to generate the PI(3)P pool in CD4 T-cells that mediates activation of KCa3.1, KCa3.1 channel activity was determined in cells treated with various class I isoform-specific p110 kinase inhibitors kindly provided by K. Shokat (UCSF Medical Center; Knight and Shokat, 2007). We found that none of these inhibited KCa3.1 channel activity (Figure 1A), suggesting that a class II or III PI3K is responsible for KCa3.1 activation in T-cells. Of the three class II PI3Ks that have been identified, PI3K-C2α and PI3K-C2β are expressed in lymphocytes (Cantley, 2002; Foster et al., 2003; Traer et al., 2006). To assess whether either of these two PI3Ks generate the PI(3)P pool that mediates activation of KCa3.1, naïve human CD4 T-cells were transfected with a pool of siRNAs to PI3K-C2α or PI3K-C2β mRNA (Dharmacon) using AMAXA reagents and, after resting overnight, were stimulated with antibodies to CD3 and CD28 for 48 h. Whole cell patch-clamping was performed 48 h after stimulation with or without 1 μM of the class 1 PI3K inhibitors shown (Knight and Shokat, 2007). (B, C) Purified CD4 T lymphocytes were transfected with a pool of siRNAs to PI3K-C2α or PI3K-C2β mRNA (Dharmacon) using AMAXA reagents and, after resting overnight, were stimulated with antibodies to CD3 and CD28 for 48 h. Whole cell patch-clamping was performed 48 h after stimulation. (B) Real time PCR showing >80% silencing of PI3K-C2α and PI3K-C2β mRNA. *p < 0.05 as compared to control cells. (C) Whole cell patch-clamping showing the I–V plot of (i) control cells (ii) siRNA PI3K-C2β cells and (iii) siRNA PI3K-C2β cells with 100 nM PI(3)P in the pipette solution. (iv) Bar graph summary of KCa3.1 conductance (pS) measured at −60 mV (N = 12–15 cells). Also shown is 1) decreased expression of PI3K-C2α by siRNA does not affect KCa3.1 conductance and 2) dialyzing PI3K-C2β siRNA transfected cells with 100 nM PI(3)P rescue KCa3.1 conductance. *p < 0.05 as compared to control KCa3.1 conductance and as indicated in the graph.

TCR Stimulation Activates KCa3.1 Channel Activity via a Calcium-independent Mechanism

KCa3.1 channel activity is known to increase after TCR stimulation. The increase in KCa3.1 channel activity has been proposed to be mediated by the rise in intracellular calcium that occurs after TCR stimulation; binding of calcium to calmodulin, which is bound to the carboxy-terminus of KCa3.1, is critical for KCa3.1 channel activation (Fanger et al., 2001). Our finding that PI3K-C2β is also critical for KCa3.1 channel activity suggested that activation of PI3K-C2β by TCR activation could contribute to KCa3.1 activation via the generation of PI(3)P. In contrast to “normal” T-cells, Jurkat T-cells do not contain KCa3.1 channel activity (Figure 2Ai). Instead, they express apamin-sensitive KCa2.2 channels (Fanger et al., 2001). Therefore, to assess whether TCR stimulation contributes to KCa3.1 channel activity via activation of PI3K-C2β, Jurkat T-cells that overexpress KCa3.1 channels were generated (Jurkat-KCa3.1) and found to contain KCa3.1 channel activity by whole-cell patch-clamp as determined by dependence on Ca2+ and inhibition with TRAM-34, a known inhibitor of KCa3.1 channel (Figure 2Aii). To assess whether TCR stimulation activates KCa3.1 with PI(3)P, but not other phosphorylated phosphoinositide (PIs), rescued KCa3.1 channel activity (Figure 1C, iii and iv, and data not shown).
via a Ca\(^{2+}\)-independent mechanism, Jurkat-KCa3.1 cells were cocultured with Raji B-cells that were pulsed with the superantigen SEE and KCa3.1 channel activity was determined 5–15 min after establishing a productive synapse. These findings demonstrated that KCa3.1 channel activity in Jurkat-KCa3.1 cells was increased about twofold after TCR activation when compared with Jurkat-KCa3.1 cells cocultured with Raji B-cells in the absence of SEE (Figure 2A, compare ii and iii, summary vi). The increase in KCa3.1 channel activity was independent of TCR-stimulated increase in intracellular Ca\(^{2+}\) because whole-cell patch-clamp experiments were performed in the presence of 1 \(\mu\)M free calcium in the pipette solution, and therefore intracellular calcium concentrations are not rate-limiting in these experiments.

**PI3K-C2β Mediates KCa3.1 Channel Activation after TCR Stimulation**

To assess whether the increase in KCa3.1 channel activity was due to the activation of PI3K-C2β, KCa3.1 channel activity was performed on Jurkat-KCa3.1 cells that overexpressed PI3K-C2β. Although overexpression of PI3K-C2β did not affect basal KCa3.1 channel activity (Figure 2A, compare ii and iv, summary vii), stimulation with Raji B-cells pulsed with SEE led to a further 1.5-fold increase in KCa3.1 channel activity when compared with Jurkat-KCa3.1 cells not overexpressing PI3K-C2β (Figure 2A, compare iii and v, with vi). In contrast, KCa3.1 channel activity was not increased in Jurkat-KCa3.1 cells that overexpressed PI3K-C2α (Figure 2Avi) despite similar levels of protein expression (Figure 2B).

To directly assess whether endogenous PI3K-C2β mediates TCR-stimulated increase in KCa3.1 channel activity, KCa3.1 channel activity was assessed in Jurkat-KCa3.1 cells transfected with a siRNA to PI3K-C2β. In comparison to control Jurkat-KCa3.1 cells, TCR-stimulated increase in KCa3.1 channel activity was markedly impaired in siRNA PI3K-C2β–transfected cells (Figure 3B, compare i and ii, summary v). Moreover, the decrease in KCa3.1 channel activity in siRNA PI3K-C2β–transfected cells was due to de-
creased levels of PI(3)P because adding back PI(3)P into the pipette solution rescued KCa3.1 channel activity to levels comparable to cells transfected with PI3K-C2β siRNA-transfected cells. *p < 0.05 compared with control. (B) Jurkat-KCa3.1 cells were transfected with a pool of siRNAs to PI3K-C2β and stimulated 48 h after transfection with Raji B-cells that were either untreated (i) or treated with SEE (ii). Whole-cell patch clamp was then performed as described in Figure 2. (iii) To determine whether inhibition of KCa3.1 channel activity in siRNA PI3K-C2β-transfected cells was due to decreased levels of PI(3)P, siRNA PI3K-C2β-transfected cells were dialyzed with PI(3)P as described in Figure 1. (iv) In addition, KCa3.1 channel activity was rescued by transfecting a GFP-PI3K-C2β mutant (PI3K-C2β mt) that abrogated interaction with the siRNA. (v) Bar graph summary of KCa3.1 conductance (pS) measured at −60 mV (n = 10 cells). *p < 0.05 compared with siRNA PI3KC2β + SEE-transfected cells.

**Figure 3.** siRNA knockdown of GFP-PI3K-C2β inhibits TCR-stimulated activation of KCa3.1 channel activity. (A) Real-time PCR showing >80% silencing of PI3K-C2β in siRNA PI3K-C2β siRNA-transfected cells. *p < 0.05 compared with control. (B) Jurkat-KCa3.1 cells were transfected with a pool of siRNAs to PI3K-C2β and stimulated 48 h after transfection with Raji B-cells that were either untreated (i) or treated with SEE (ii). Whole-cell patch clamp was then performed as described in Figure 2. (iii) To determine whether inhibition of KCa3.1 channel activity in siRNA PI3K-C2β-transfected cells was due to decreased levels of PI(3)P, siRNA PI3K-C2β-transfected cells were dialyzed with PI(3)P as described in Figure 1. (iv) In addition, KCa3.1 channel activity was rescued by transfecting a GFP-PI3K-C2β mutant (PI3K-C2β mt) that abrogated interaction with the siRNA. (v) Bar graph summary of KCa3.1 conductance (pS) measured at −60 mV (n = 10 cells). *p < 0.05 compared with siRNA PI3KC2β + SEE-transfected cells.

PI3K-C2β Also Plays a Critical Role in Augmented Calcium Influx after TCR Stimulation

By mediating the efflux of K⁺, KCa3.1 functions to maintain a hyperpolarized membrane potential that provides the electrochemical gradient that drives Ca²⁺ entry into a subset of T- and B-lymphocytes (Cahalan et al., 2001; Wulff et al., 2003a). Although control Jurkat T-cells do not contain KCa3.1 channels (Figure 2A), they have other K⁺ channels such as KCa2.2, which are not regulated by PI(3)P and can substitute for KCa3.1 to promote anti-CD3 stimulated Ca²⁺ influx (Figure 4i). Overexpression of KCa3.1 in Jurkat T-cells (Jurkat-KCa3.1) led to marked increase in anti-CD3-stimulated Ca²⁺ influx that, upon treatment with TRAM-34, inhibited Ca²⁺ influx to levels seen in control Jurkat T-cells, confirming that the increase in Ca²⁺ influx was due to KCa3.1 (Figure 4i). To address whether PI3K-C2β plays a critical role in TCR-stimulated Ca²⁺ influx by KCa3.1, Ca²⁺ influx was assessed in TCR-stimulated Jurkat-KCa3.1 cells in which PI3K-C2β was overexpressed or silenced with a PI3K-
C2β siRNA (Figure 4, ii and iii). Consistent with the changes seen in KCa3.1 channel activity shown in Figure 3, TCR-stimulated Ca\(^{2+}\) influx was increased in Jurkat-KCa3.1 cells overexpressing PI3K-C2β, whereas Ca\(^{2+}\) influx was inhibited in siRNA PI3K-C2β–transfected cells (Figure 4, ii and iii). The finding that both siRNA PI3K-C2β and TRAM-34 inhibited Ca\(^{2+}\) influx to a similar degree (Figure 4iii), coupled with the findings that TRAM-34 did not further inhibit Ca\(^{2+}\) influx to siRNA PI3K-C2β–transfected cells (data not shown), indicates that PI3K-C2β specifically regulates KCa3.1-dependent Ca\(^{2+}\) influx in these cells.

**PI3K-C2β Is Recruited to the IS Where it Colocalizes with the TCR and ZAP-70**

To explore the mechanism whereby PI3K-C2β is activated by TCR stimulation, we initially determined whether PI3K-C2β’s enzymatic activity was increased after TCR stimulation. Jurkat-KCa3.1 cells were transfected with GFP-PI3K-C2β, and PI3K-C2β enzymatic activity was determined on anti-CD3 immunoprecipitates at various times after treatment with anti-CD3 antibodies. These studies demonstrated that TCR cross-linking did not increase PI3K enzyme activity (Figure 5i). PI3K activity was not detected in untransfected cells, indicating that the PI3K activity detected was specific. In addition, anti-phosphotyrosine Western blots demonstrated an increase in tyrosine-phosphorylated proteins, indicating that the cells were stimulated (data not shown).

We next determined whether TCR stimulation affected the subcellular localization of PI3K-C2β; recruitment of GFP-PI3K-C2β to the plasma membrane (PM) could be an alternative mechanism whereby TCR stimulation could lead to an increase in PM PI(3)P. It is now known that organization of signaling molecules in T-cells at the IS is central to their regulation (Dustin, 2006; Varma et al., 2005). The formation of a stable IS with a ring of adhesion molecules is a common molecular pattern associated with T-cell activation and effector functions. A third structure in the IS distal to the pSMAC has been referred to as the distal SMAC (dSMAC), which is rich in CD45 (Freiberg et al., 2002b). In addition, TCR microclusters, which contain active signaling molecules such as Lck and Zap-70, form early after TCR stimulation and continually form during sustained signals (Krummel et al., 2000; Campi et al., 2005; Seminario and Bunnell, 2008). TCR microclusters eventually converge on the cSMAC where elements involved in sustained signaling and signal termination are segregated (Varma et al., 2006; Cemerski et al., 2008). The balance between T-cell receptor signaling and degradation at the center of the IS is determined by antigen quality and spatiotemporal regulation of T-cell costimulation by TCR-CD28 microclusters and protein kinase C translocation (Yokosuka et al., 2008).

To determine whether GFP-PI3K-C2β is recruited to the IS, Jurkat T-cells transfected with GFP-PI3K-C2β were floated over planar lipid bilayers that were preloaded with ICAM-1 and Cy-3–labeled anti-CD3 antibodies. Cells were then visualized at various time points using TIR-FIM as previously described (Varma et al., 2006). We found that within 5 min after being exposed to the planar lipid bilayer PI3K-C2β colocalizes with CD3 in the microclusters, which over time converge in cSMAC (Figure 5ii, A). The recruitment was specific because under the same conditions, GFP-PI3K-C2α, which does not play a role in activation of KCa3.1, was not recruited (Figure 5ii, C). The tyrosine kinase Zap-70 is rapidly recruited to the microclusters where it also colocalizes with the TCR. To assess whether GFP-PI3K-C2β also colocalizes with proximal signaling molecules downstream of TCR, experiments were carried out in the Jurkat T-cells transfected with GFP-PI3K-C2β cherry-Zap-70. In similar experiments, we found that GFP-PI3K-C2β also colocalized with Zap-70 (Figure 5ii, B). Thus, these findings suggests...
that recruitment of PI3K-C2β, to the IS may play a critical role in its regulation.

DISCUSSION

In contrast to the class I and III PI3K, little is still known about either the biological functions regulated by the class II PI3Ks or the mechanism whereby they are regulated. We now find that the class II PI3K-C2β is activated after TCR stimulation and plays a central role in generating the PI(3)P pool that mediates activation of the Kα channel KCa3.1. Moreover, although TCR stimulation does not increase PI3K-C2β catalytic activity, it does stimulate the recruitment of PI3K-C2β to the IS where it colocalizes with both the TCR and Zap-70. These findings suggest that calcium influx in some T-cell subsets requires the simultaneous activation of two parallel pathways after TCR stimulation (Figure 6). On the one hand, TCR stimulation leads to the activation of PLCγ leading to the generation of IP3 and diacylglycerol. Binding of IP3 to its receptor in the ER leads to release of Ca2+, which in turn results in opening of CRAC channels and the influx of Ca2+ (Cahalan et al., 2007; Feske, 2007). Ca2+ influx, via binding to calmodulin bound to the CT of KCa3.1, also activates KCa3.1. TCR stimulation also activates PI3K-C2β, which generates the pool of PI(3)P required for KCa3.1 activation. The combined effect of increased intracellular calcium, together with PI(3)P-stimulated activation of NDPK-B, ensures that KCa3.1 channels are fully active to facilitate sufficient calcium entry via CRAC channels to activate NFAT-dependent signaling pathways.

Previous work on PI3 kinase in T-cells has focused on the class I PI3Ks for which the biological function and regulation in multiple biological processes are well described. Previous studies using knockout mice have demonstrated important roles for both p110δ and p110γ in T-cell activation (Webb et al., 2005; Swat et al., 2006; Fruman, 2007; Patton et al., 2007). These studies demonstrated that TCR-stimulated activation of these class I PI3Ks generate predominantly PI(3,4)P2 and PI(3,4,5)P3, which function to bind and activate PH-domain-containing proteins such as AKT. Our demonstration that PI3K-C2β is activated after TCR stimulation and generates the PI(3)P pool in response to TCR stimulation which mediates KCa3.1 activation, adds to the increasing role for class II PI3Ks in regulating agonist-stimulated intracellular functions. This is consistent with other studies demonstrating that PI and to a lesser extent PI(4)P are the preferred sub-

Figure 5. PI3K-C2β colocalizes with the TCR and Zap70 in the immunological synapse (IS) after stimulation with anti-CD3 antibodies. (i) PI3K enzyme activity is not increased after TCR stimulation. Jurkat T-cells overexpression PI3K-C2β were stimulated with anti-CD3 and anti-CD28 antibodies. Cells were then lysed at various time points, and PI3K activity was determined on anti-GFP immunoprecipitates. Shown are phosphorimager units incorporated into PI. The data shown are ±SEM done in triplicate. (ii) Jurkat T-cells that were transfected with GFP-PI3K-C2β (A) or PI3K-C2α (C) were floated onto planar lipid bilayers incorporating GPI-linked ICAM-1 and Cy3-conjugated anti-human CD3 (10 μg/ml). Total internal reflection fluorescence microscopy (TIRFM) was used to assess localization of CD3 and PI3K-C2β at various times after adhering to the bilayer. (B) Jurkat T-cells transfected with GFP-PI3K-C2β together with Cherry-Zap70, were floated onto planar lipid bilayers containing unlabeled CD3, and cellular localization of PI3K-C2β and Zap70 was determined as described in A.

Figure 6. Schematic for TCR-stimulated activation of KCa3.1. Activation of two signaling pathways is required for TCR-stimulated activation of KCa3.1. Signal 1, TCR stimulation leads to the activation of PLCγ leading to the generation of IP3, stimulating release of Ca2+ from the ER, resulting in opening of CRAC channels and the influx of Ca2+; signal 2, TCR stimulation also leads to the activation of PI3K-C2β leading to the generation of PI(3)P at the plasma membrane, which is required for NDPK-B to phosphorylate histidine 358 in the carboxy-terminus of KCa3.1. Both binding of Ca2+ to the calmodulin bound to the CT of KCa3.1 and phosphorylation of H358 in CT of KCa3.1 by NDPK-B is required for KCa3.1 activation.
strate for class II PI3Ks, and that one function of class II PI3Ks is to increase the level of PI(3)P at the PM (Maffucci et al., 2005; Falasca et al., 2007). These studies demonstrated that generation of PM PI(3)P by PI3K-C2β is critical for lysophosphatidic acid (LPA)-stimulated cell migration (Maffucci et al., 2005) and that the generation of PM PI(3)P by PI3K-C2α is critical for insulin-stimulated GLUT4 translocation (Falasca et al., 2007). PM PI(3)P is also critical for activation of KCa3.1 (Srivastava et al., 2005). Moreover, our finding that KCa3.1 channel activity in T-cells transfected with an siRNA to PI3K-C2β is restored by dialyzing these cells with PI(3)P, and not other phosphorylated PIs, confirms that PI(3)P is the in vivo product generated by PI3K-C2β in T-cells. The ability of agonist activation of the class II PI3K to increase PM PI(3)P levels is therefore distinct from the role for the class III PI3K Vps34, which generates the majority of the constitutive PI(3)P in the cell that is localized primarily to the endosomal compartment (Gilfooly et al., 2001; Yan and Backer, 2007).

There are three mammalian class II PI3Ks: PI3K-C2α, PI3K-C2β, and PI3K-C2γ, of which PI3K-C2α and PI3K-C2β are widely expressed (Cantley, 2002; Foster et al., 2003; Traer et al., 2006). In contrast to class I PI3K, all the class II PI3Ks contain an extended C-terminus composed of tandem PX and C2 domains and lack regulatory domains. A number of studies have demonstrated activated PI3K-C2α or PI3K-C2β by a number of agonists including epidermal growth factor (EGF), integrins, insulin, LPA, stem cell factor (SCF), and chemokines, as well as via the interaction with clathrin (Arcaro et al., 2000, 2002; Gaidarov et al., 2001; Maffucci et al., 2005; Traer et al., 2006; Falasca et al., 2007), and although insulin has been shown to activate both isoforms (Brown and Shepherd, 2001), for the most part either PI3K-C2α or PI3K-C2β is activated by these stimuli. Our finding that PI3K-C2β and not PI3K-C2α generates the pool of PI(3)P that is required for KCa3.1 channel activity and T-cell activation reinforces the idea that each class II PI3K isoform mediates distinct biological functions. This was further supported by the finding that only PI3K-C2β, and not PI3K-C2α, is recruited to the peripheral microclusters in the IS after TCR (activation). Although potential mechanisms whereby only one class II PI3K isoform couples to a specific upstream signal are still poorly defined, both the N- and C-terminal extension have been proposed to play critical role in their regulation (Traer et al., 2006). Recruitment of PI3K-C2β to peripheral microclusters, by localizing PI3K-C2β to the PM, is likely to play an important role in generating PM PI(3)P after TCR stimulation. In addition, the finding that PI3K-C2β colocalizes with the TCR and Zap70 in peripheral microclusters containing active tyrosine kinases, such as Zap-70 and Lck, suggests that TCR signaling may also directly activate PI3K-C2β. However, so far we have been unable to demonstrate that these recruitment functions to either stimulate the tyrosine phosphorylation of PI3K-C2β as has been described in insulin, EGF, and SCF-stimulated cells, or to increase PI3K-C2β’s enzymatic activity as has been described for insulin (Brown and Shepherd, 2001).

Another hypothesis we are now testing is that spatial arrangement in the IS of the various molecules that have been shown to regulate KCa3.1 is critical to KCa3.1 regulation. For example, our previous work has demonstrated that generation of PI(3)P is required to enable NDPK-B to phosphorylate the CT of KCa3.1, leading to KCa3.1 activation (Srivastava et al., 2006b). Thus, one plausible hypothesis is that recruitment of NDPK-B, PI3K-C2β, and KCa3.1 to peripheral microclusters in the IS is critical for the activation of KCa3.1. This is at least partly supported by a previous report showing that KCa3.1 is recruited to the IS, although the exact location of in the IS was not identified (Nicolaou et al., 2007). On the flip side, segregation of the negative regulators MTMR6 and PHPT1 away from peripheral microclusters would be one way of ensuring continuous signaling in the context of sustained TCR activation. As discussed above, it is also now recognized that signaling molecules in peripheral microcluster stream into the cSMAC, where TCR signaling is terminated by dephosphorylation and incorporation into multivesicular bodies (Campi et al., 2005; Varma et al., 2006), while an active signaling compartment is maintained (Yokosuka et al., 2008). Localization of MTMR6 and PHPT1 to the cSMAC could provide one possible mechanism that would enable both these molecules to inhibit KCa3.1 activity once TCR stimulation abates, without interfering with channel activation in the context of ongoing TCR stimulation. In fact, such a model has been proposed for the tyrosine phosphatase CD45, which is both excluded from peripheral microclusters and concentrated in the cSMAC where it is positioned to contribute to the dephosphorylation of active signaling molecules associated with the TCR (Varma et al., 2006).

ACKNOWLEDGMENTS

We thank J. Domin (Imperial College, London) for the GFP-PI3K-C2α and PI3K-C2β constructs and K. Shokat (UCSF Medical Center) for the class I PI3K inhibitors. E.Y.S. is supported by National Institutes of Health Grants ROI GM084195 and ROI AI052459.

REFERENCES


