Dependence of STIM1/Orai1-mediated Calcium Entry on Plasma Membrane Phosphoinositides*

Received for publication, April 23, 2009 Published, JBC Papers in Press, May 29, 2009, DOI 10.1074/jbc.M109.012252

Marek K. Korzeniowski‡, Marko A. Popovic§, Zsofia Szentpetery¶, Peter Varnai§,1, Stanko S. Stojilkovic¶, and Tamas Balla‡2

From the Sections on 4Molecular Signal Transduction and 6Cellular Signaling, Program for Developmental Neuroscience, NICHD, National Institutes of Health, Bethesda, Maryland 20892 and the 5Department of Physiology, Faculty of Medicine, Semmelweis University, H-1094 Budapest, Hungary

Recent studies identified two main components of store-operated calcium entry (SOCE): the endoplasmic reticulum-localized Ca2+ sensor protein, STIM1, and the plasma membrane (PM)-localized Ca2+ channel, Orai1/CRACM1. In the present study, we investigated the phosphoinositide dependence of Orai1 channel activation in the PM and of STIM1 movements from the tubular to PM-adjacent endoplasmic reticulum regions during Ca2+ store depletion. Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) levels were changed either with agonist stimulation or by chemically induced recruitment of a phosphoinositide 5-phosphatase domain to the PM, whereas PtdIns4P levels were decreased by inhibition or down-regulation of phosphatidylinositol 4-kinases (PI4Ks). Agonist-induced phospholipase C activation and PI4K inhibition, but not isolated PtdIns(4,5)P2 depletion, substantially reduced endogenous or STIM1/Orai1-mediated SOCE without preventing STIM1 movements toward the PM upon Ca2+ store depletion. Patch clamp analysis of cells overexpressing STIM1 and Orai1 proteins confirmed that phospholipase C activation or PI4K inhibition greatly reduced ICRAC currents. These results suggest an inositol requirement of Orai1 activation but not STIM1 movements and indicate that PtdIns4P other than PtdIns(4,5)P2 is a likely determinant of Orai1 channel activity.

Store-operated Ca2+ entry (SOCE) is a ubiquitous Ca2+ entry pathway that is regulated by the Ca2+ content of the endoplasmic reticulum (ER) (1). SOCE has been identified as the major route of Ca2+ entry during activation of cells of the immune system such as T cells and mast cells (2, 3), and it is also present and functionally important in other cells such as platelets (4) and developing myotubes (5). The long awaited mechanism of how the ER luminal Ca2+ content is sensed and the information transferred to the plasma membrane (PM) has been clarified recently after identification of the ER Ca2+ sensor proteins STIM1 and -2 (6, 7) and the PM Ca2+ channels Orai1, -2, and -3 (8–10). According to current views, a decrease in the ER Ca2+ concentration is sensed by the luminal EF-hand of the single-transmembrane STIM proteins causing their multimerization. This oligomerization occurs in the tubular ER, where it promotes the interaction of the cytoplasmic C termini of STIM with PM components and association with the PM-localized Orai channels, causing both their clustering and activation in the PM (reviewed recently in Refs. 11–13). Analysis of the interacting domains within the STIM1 and Orai1 proteins suggests that the cytoplasmic domain of STIM1 is necessary and sufficient to activate Orai1 (14), whereas the latter requires its C-terminal membrane-adjacent cytoplasmic tail to be fully activated by the STIM proteins (15, 16). Both STIM1 and -2 contain a polybasic segment in their C termini, and such regions are often responsible for the PM localization of proteins (mostly of the small GTP-binding protein class) via interaction with anionic phospholipids such as phosphatidylserine or PtdIns(4,5)P2 (17). However, the role of this domain in STIM1 function(s) remains controversial. Deletion of the polybasic tail is reported to prevent PM association but not clustering of STIM1 upon ER store depletion (18). In other studies, truncated STIM1 lacking the polybasic domain shows only slightly altered activation (15) or inactivation (19) kinetics without major defects in supporting Orai1-mediated Ca2+ influx. The most recent studies identify the minimal Orai1 activation domain in STIM1 (20, 21) and find that the polybasic domain is not essential for this function but makes electrostatic interaction with classical transient receptor potential channels (22).

PM phosphoinositides have been widely reported as regulators of the activity of several ion channels and transporters (23). However, only a few studies have addressed the inositol requirement of SOCE and none specifically that of the Orai1-mediated Ca2+ entry process. Sensitivity of SOCE to phosphatidylinositol 3-kinases (PI3K) inhibitors has been reported, but this required concentrations that suggested inhibition of targets other than PI3Ks, possibly myosin light chain kinase or the type-III PI4Ks (4, 24–26). Here we have described studies addressing the role of PM phosphoinositides in STIM1 movements as well as in Orai1 channel gating. Our results show that
Phosphoinositides do not have a major role in the prominent reorganization of STIM1 after Ca\(^{2+}\) store depletion but suggest a function of PtdIns4P rather than PtdIns(4,5)P\(_2\) in supporting the Orai1-mediated Ca\(^{2+}\) entry process.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rapamycin and thapsigargin were purchased from Calbiochem. Angiotensin II (human octapeptide) was from Peninsula Laboratories (Bachem, Torrance, CA), and ATP was obtained from Sigma. All other chemicals were of the highest analytical grade.

**DNA Constructs**—The YFP- and mRFP-STIM1 plasmids as well as the Orai1 constructs used in this study have been described previously (27). The plasmids designed for the rapamycin-induced PM recruitment of the type IV 5-phosphatase domain as well as those for PLC\(\delta\), PH-GFP and OSH2-2xPH-GFP have also been described elsewhere (28, 29). For siRNA-mediated knockdown of the various PI4K isoforms, the duplexes and treatment protocols have been described previously (29).

**Cytoplasmic Ca\(^{2+}\) [Ca\(^{2+}\)]i and TIRF Measurements in Single Cells**—COS-7 cells were cultured on glass cover slips (3 \times 10^5 cells/35-mm dish) and transfected with the indicated constructs (0.5 \(\mu\)g of DNA/dish) using Lipofectamine 2000 for 24 h as described previously (27). TIRF analysis was performed at room temperature in an Olympus IX81 microscope-based through-the-lens dual-launch TIRF system equipped with a Hamamatsu EM-CCD camera and a PlanApo 60\(\times\)/1.45 objective. Excitation with 488 or 568 nm lasers were used for the YFP or Fluor and mRFP, respectively, and scans were performed at every 10 s. For data acquisition OpenLab Software (Improvision) was used, and the pictures were exported as TIFF files for processing with the MetaMorph software (Molecular Devices). Quantification of the membrane intensities was determined after defining the regions of individual cells and thresholding. Because of the large variations in the intensities of individual cells due to different footprint size and translocation responses, these responses were normalized and their maximal value due to different footprint size and translocation responses. The responses were then averaged and their S.E. calculated and plotted against time.

For calcium experiments cells were loaded with Fura2/AM (3 \(\mu\)M for 45 min, room temperature). Calcium measurements with Fura2 were performed in modified Krebs-Ringer solution (see Ref. 27 for composition) supplemented with 200 \(\mu\)M sulfipyrazone. Calcium studies were also performed in individual cells attached to coverslips at room temperature using an Olympus IX70 inverted microscope equipped with a Lambda-DG4 illuminator and a MicroMAX-1024BFT digital camera and the appropriate filter sets. MetaFluo (Molecular Devices) software was used for data acquisition. When cells were studied in suspension, they were removed from the culture plates with mild trypsinization and loaded with 3–5 \(\mu\)M Fura2/AM at room temperature as described previously (30). Loaded cells were kept in HEPES-buffered M199, Hanks’ salt solution containing 0.1% bovine serum albumin and 200 \(\mu\)M sulfipyrazone, and an aliquot of the cells was centrifuged immediately before [Ca\(^{2+}\)]i measurement in the modified Krebs-Ringer solution containing sulfipyrazone but not bovine serum albumin. [Ca\(^{2+}\)]i measurements in suspension were performed at 34 °C in a PTI DeltaScan fluorescence spectrophotometer (Photon Technol. International).

**Electrophysiological Recordings**—All voltage clamp recordings were performed at room temperature using an Axopatch 200 B patch clamp amplifier (Axon Instruments, Foster City, CA) and were low-pass filtered at 2 kHz. Ramp generation and acquisition data were done with a PC equipped with a Digidata 1322A A/D interface in conjunction with Clampex 10 (Axon Instruments). The standard HEPES-buffered saline solution contained (mM): 140 NaCl, 2.5 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 15 glucose, and 10 HEPES (pH to 7.4 with NaOH). Fire-polished pipettes fabricated from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) with 3–5-megohm resistance were filled with the following (mM): 100 cesium methanesulfonate, 20 BAPTA (dissolved in 0.3 M CsOH), 10 HEPES, 10 NaCl, and 6 MgATP (pH to 7.2 with CsOH). In all experiments, the pipette also contained 25 \(\mu\)M inositol 1,4,5-trisphosphate (InsP\(_3\), hexasodium salt; Sigma). Voltage ramps (–100 to +100 mV) of 250 ms were recorded every 2 s immediately after gaining access to the cell from a holding potential of 0 mV, and the currents were normalized based on cell capacitance. Leak currents were subtracted by taking an initial ramp current before IC\(_{\text{CRAC}}\) developed and subtracting this from all subsequent ramp currents. Access resistance was typically between 5 and 10 megohms. Wortmannin (Wm) and angiotensin II (AngII) were applied in some experiments using a gravity-driven microperfusion system, RSC-200 (Bio-Logic SAS, Claix, France).

**RESULTS**

**Rapid Dephosphorylation of PtdIns(4,5)P\(_2\) to PtdIns4P Does Not Inhibit SOCE and Has Only Minor Effects on STIM1 Translocation from the Tubular to PM-adjacent ER**—First we wanted to determine whether isolated changes in PtdIns(4,5)P\(_2\) could alter STIM1 movements or the activation of Orai1-mediated Ca\(^{2+}\) influx in intact cells. To this end, we used the recently described chemically (rapamycin) induced recruitment of the 5-phosphatase domain of the type IV phosphoinositide 5-phosphatase enzyme (5-PTase domain) to rapidly reduce PtdIns(4,5)P\(_2\) levels in the PM (28). This method allows changing the level of PtdIns(4,5)P\(_2\) without setting off the signaling cascade downstream of PLC activation. For these studies we used COS-7 cells, as they show very robust elimination of PtdIns(4,5)P\(_2\) upon rapamycin treatment (28). Cells were transfected with the PM-targeted FRB construct and the mRFP-FKBP12-fused 5-phosphatase domain, which is cytosolic under basal conditions but becomes recruited to the PM after the addition of rapamycin causing rapid depletion of PtdIns(4,5)P\(_2\) from the membrane. This process can be followed in TIRF experiments, where both the recruitment of the phosphatase and the release of the PtdIns(4,5)P\(_2\) reporter PLC\(\delta\), PH-GFP from the membrane can be monitored simultaneously (see Ref. 31).

In previous studies we showed that PtdIns(4,5)P\(_2\) elimination from the PM by this method does not prevent Tg-induced STIM1 translocation to PM-adjacent regions (27). However, analysis of STIM1 translocation from many cells in TIRF experi-
**Phosphoinositides in Store-operated Calcium Entry**

In parallel experiments, the translocation response of the PLCβ1-PH-GFP was also followed using the same sequence of stimulation. This showed that ATP/Tg only slightly reduced PtdIns(4,5)P₂ levels, which showed a large decrease only after rapamycin addition (see on Fig. 3). These experiments indicated that PM PtdIns(4,5)P₂ may contribute to the stabilization of STIM1-PM interaction.

We also determined the effect of PtdIns(4,5)P₂ removal on the cytosolic Ca²⁺ signal after store depletion. For this, [Ca²⁺], was monitored with Fura2 in COS-7 cells expressing the 5-phosphatase recruitment system either alone or with mRFP-FKBP12 and untagged Orai1 to boost SOCE. The endogenous P2Y purinergic receptors of COS-7 cells were stimulated with ATP together with Tg to rapidly release and deplete the ER Ca²⁺ stores and activate SOCE. This was followed by rapamycin addition to recruit the 5-phosphatase and deplete PtdIns(4,5)P₂. As shown in Fig. 2, the addition of rapamycin failed to affect either the endogenous SOCE or the one enhanced by overexpression of STIM1/Orai1. These results suggested that change in the PM PtdIns(4,5)P₂ was not a major factor in the regulation of SOCE in these cells, despite its minor effect on STIM1 translocation.

**PI4K Inhibition Affects SOCE but Not STIM1 Movements**—Previous data had shown that PI3K inhibitors inhibit SOCE at concentrations that could also inhibit PI4Ks (26). To investigate whether PtdIns4P might be a regulatory factor of SOCE activity, the PI 3K inhibitor LY294002 was added to the cells at concentrations that inhibit type III PI4Ks (32). Our experience with the use of Wm in microscopy studies suggests that this inhibitor is not reliable because illumination with 488 nm (or shorter wavelengths) on the microscope stage rapidly inactivates this compound⁴ (also see Ref. 33). For this reason we used LY294002 in these experiments, first studying its effects on the movements of STIM1 in TIRF experiments. LY294002 was added after STIM1 translocation had already been induced by ATP/Tg treatment and PtdIns(4,5)P₂ had been eliminated by recruitment of the 5-phosphatase. As shown in Fig. 1, the addition of LY294002 (30–300 μM) to such pretreated COS-7 cells failed to affect the STIM1 signal in the TIRF plane, suggesting that PtdIns4P is not a factor in keeping STIM1 at the PM. (A delayed increase in the TIRF signal was observed in both channels after LY294002 addition in this set of studies, and we attributed it to changes in the attachment of the cells or a slight change in focus.) In contrast, LY294002 addition rapidly inhibited both endogenous SOCE and that enhanced by Orai1/STIM1 expression in a dose-dependent manner (Fig. 2). Importantly, this effect was also observed without prior elimination of PtdIns(4,5)P₂ when LY294002 was applied to naive, untransfected COS-7 cells (data not shown).

To demonstrate the effects of LY294002 treatment on PtdIns4P, two approaches were used. First, [³²P]phosphate-labeled COS-7 cells were treated with increasing doses of LY294002 and the labeled phospholipids analyzed by TLC anal-

⁴ P. Varnai and T. Balla, unpublished observation.
Phosphoinositides in Store-operated Calcium Entry

FIGURE 2. Resistance of SOCE-mediated [Ca\(^{2+}\)] elevations to PtdIns(4,5)P\(_2\) removal and sensitivity to PI4K inhibition. COS-7 cells were transfected with a PM-targeted FRB construct and the mRFP-FKBP12-S-phosphatase domain (28) either alone or in combination with the thymidine kinase promoter-driven YFP-STIM1 and Orai1 constructs (27). After 24 h, cells were loaded with Fura2 for single cell [Ca\(^{2+}\)] measurements. Stimulation with ATP/Tg (50 \(\mu\text{M}/200\text{ nM}\)) activates Ca\(^{2+}\) release followed by Ca\(^{2+}\) influx, which is much larger in cells expressing the STIM1/Orai1 proteins (9), Recruitment of the 5-PTase domain by the addition of rapamycin (Rapa, 100 \(\text{nM}\)) has no effect on [Ca\(^{2+}\)]. In contrast, the addition of increasing concentrations of LY294002 strongly inhibits SOCE both in the case of endogenous (A) and enhanced (B) SOCE. Means ± S.E. of at least 20 cells recorded in 3–7 separate experiments are shown.

As shown in Fig. 3A, a 10-min LY294002 treatment reduced the level of labeled PtdIns4P in a dose-dependent manner without a similar decrease in labeled \(^{32}\text{P}\)PtdIns(4,5)P\(_2\), essentially mimicking the effects of 10 \(\mu\text{M}\) Wm as described previously in bovine adrenal and HEK293 cells (29, 30). A slight increase in PtdIns(4,5)P\(_2\) already at a lower LY294002 concentration was also observed. This was attributed to the inhibition of PI3Ks and hence sparing PtdIns(4,5)P\(_2\) usage via that pathway. The potency of LY294002 to inhibit PtdIns4P synthesis was almost identical to that for inhibition of Ca\(^{2+}\) influx (Fig. 3B). Because \(^{32}\text{P}\) could label PtdIns4P pools other than those found in the PM, we also wanted to show that LY294002 acted on the PM pool of PtdIns4P. For this we used the PtdIns4P reporter OSH2–2xPH-GFP (34) that has proven to be a reasonable probe for following changes in PM PtdIns4P levels (29, 35). In these TIRF experiments, we used the same treatment regime as in previous experiments in order to assess the lipid changes evoked by the ATP/Tg treatment. These studies showed negligible changes in OSH2–2xPH-GFP localization by ATP/Tg, but recruitment of the 5-phosphatase to the PM caused a slight decrease in the membrane-bound fraction of the OSH2–2xPH-GFP (Fig. 3C). This can be attributed to a weak PtdIns(4,5)P\(_2\) binding of this reporter, as it was shown to also bind PtdIns(4,5)P\(_2\) in vitro (36). Note the rapid decrease in the localization of the PLC81PH-GFP probe reporting on PtdIns(4,5)P\(_2\) changes. Curiously, we were unable to detect an increased PtdIns4P after 5-phosphatase recruitment with any of the PtdIns4P binding reporter constructs (FAPP1-PH, OSH1-PH, OSBP-PH, OSH2-PH) for reasons that are yet to be understood. Nevertheless, a very significant fraction of OSH2–2xPH-GFP remained associated with the PM after PtdIns(4,5)P\(_2\) elimination, which was then rapidly released after the addition of 100 \(\mu\text{M}\) LY294002 (Fig. 3C). These experiments showed that LY294002 eliminates most of the PtdIns4P from the PM within 5 min of incubation. Thus, although PtdIns(4,5)P\(_2\) level in the PM had only a small impact on STIM1 oligomerization and PM interaction in store-depleted cells, LY294002 had a major impact on SOCE that correlated with PtdIns4P rather than PtdIns(4,5)P\(_2\) depletion.

PI4K Inhibition and PLC Activation Inhibit \(I_{\text{CRAC}}\) —To study the effects of PtdIns4P manipulations directly on \(I_{\text{CRAC}}\), electrophysiological measurements were performed in HEK293 cells stably expressing the Ca\(^{2+}\)-mobilizing AT1a angiotensin receptors (HEK-AT1). Cells were transiently transfected with YFP-STIM1 alone or in combination with Orai1. In cells expressing both proteins, the current developed fully within 50 to 100 s, with a peak amplitude of 0 mV potential, is shown in Fig. 4B. In cells expressing both proteins, the current developed fully within 50 to 100 s, with a peak amplitude of 30.7 ± 3.1 pA/pF; \(n = 30\). Once the current was developed, in a fraction of the cells it stayed unchanged for at least 300 s (Fig. 4B, black trace), whereas in the majority of the cells a slow and linear decay of current was consistently observed (blue trace). The current-voltage relationship indicated strong inward rectification and reversal potential at about +50 mV, both characteristic of \(I_{\text{CRAC}}\) (Fig. 4C, blue trace). Neither untreated cells (data not shown) nor STIM1 alone-expressing cells (Fig. 4C, gray trace) developed any comparable current under the same experimental conditions (the endogenous \(I_{\text{CRAC}}\) current is almost negligible on this scale). These results are consistent with the literature, showing that under our experimental conditions cells displayed a functional \(I_{\text{CRAC}}\) current when co-transfected with STIM1 and Orai1.

\(^5\) M. Korzeniowski, Z. Szentpetery, and T. Balla, unpublished observations.
We also examined the effects of PI4K inhibition on the amplitude and patterns of $I_{\text{CRAC}}$. For this, Wm was used (as no light exposure was present during the patch clamp recording) at concentrations (10 $\mu$M) that inhibit both PI3Ks and PI4Ks (30). To avoid the effects of dialysis on cells during the whole cell recording, intact cells were treated with Wm for 15 min prior to establishing the current recording, as described above. Fig. 4D illustrates a typical pattern of current response during a 300-s recording. As in untreated cells, the current developed with similar kinetics, but the amplitude of the current was substantially lower (12.3 ± 2.7 pA/pF; $n = 11$). To limit the impact of expression efficiency in different experiments on the amplitude of current, the mean values of current responses recorded in controls and Wm-treated cells were compared from traces generated in the same experiments (Fig. 4E), and a statistical comparison was done at two time points (at 100 and 200 s of recording; Fig. 4F). These results indicated that the inhibition of type III PI4Ks strongly affected the amplitude of the $I_{\text{CRAC}}$ current.

Next we studied whether PLC activation has any effect on the $I_{\text{CRAC}}$ current. For this, AngII was applied using a gravity-driven micropipetation system after the development of the current, and the time of AngII application was varied between experiments. In the majority of cells ($n = 24$), AngII was applied almost immediately after the current had fully developed; this treatment caused a rapid but incomplete inhibition of the current. A representative trace of the AngII-induced change in the current profile is shown in Fig. 5A. In these cells, the amplitude of peak current (31.4 ± 4.6 Pa/pF, $n = 24$) prior to the addition of AngII was almost identical to that observed in controls, suggesting similar levels of the STIM1/Orai1 protein expression. To compare the profiles of currents in control and AngII-treated cells more directly, the mean values of current were generated from traces recorded under the same experimental conditions, normalized for the peak amplitude of current (Fig. 5B). To analyze these results statistically, the mean ± S.E. values for the change in current amplitude were calculated between time points from 100 to 200 s and from 100 to 300 s (Fig. 5C). There was only a small decrease in current amplitude between these time points without AngII addition (Fig. 5C, blue bars), but the current amplitude decreased by ~40% after AngII stimulation (red bars). Importantly, when AngII was applied at a later time (>200 s) after the development of the current, the inhibitory effect of AngII was reduced, and it was practically abolished when the agonist was added >300 s after establishing the whole cell recording (data not shown). These results indicated that in cells with activated $I_{\text{CRAC}}$, stimulation of Ca$^{2+}$-mobilizing AT1 receptors leads to inhibition of the current. The results also suggested that under
Phosphoinositides in Store-operated Calcium Entry

FIGURE 4. Inhibition of store-depletion-induced whole cell current (\(i_{\text{crac}}\)) in HEK293 cells by PI4K inhibition. HEK293 cells stably transfected with AT1 calcium-mobilizing receptors were transiently transfected with cDNAs for Orai1 and STIM1. A, characterization of \(i_{\text{crac}}\). Typical pattern of 25 \(\mu\)M InsP\(_3\)-induced current responses during the initial voltage ramps (250 \(\mu\)s from -100 mV to +100 mV). B, time course of InsP\(_3\)-induced \(i_{\text{crac}}\) with two representative traces extracted from values detected at \(-80\) mV during repetitive ramp applications. The numbers at the top indicate the mean \(\pm\) S.E. from 31 cells recorded in seven independent experiments. C, representative traces of current-voltage (i/V) relationship of \(i_{\text{crac}}\) in cells co-transfected with STIM1 only (gray trace) or STIM1 plus Orai1 (blue trace). D, cells were treated with either Wm (10 \(\mu\)M) or DMSO for 15 min before breaking in with the patch pipette. The pattern and amplitude of the averaged \(i_{\text{crac}}\) Current extracted at \(-80\) mV is shown. E, mean values of current at \(-80\) mV extracted from experiments done under the same experimental conditions for Wm-treated (red) and control (blue) cells. F, statistical comparison of the amplitude of current response at two different time points. *, indicates significant differences (p < 0.01).

The whole cell recording conditions, which lead to dialysis of cells, the pathways responsible for AngII action are affected and that further experiments are warranted to identify these components.

The effects of AngII on the Tg-induced \([Ca^{2+}]\_i\) rise were also studied in HEK-AT1 cells in single cell \([Ca^{2+}]\_i\) measurements. These studies showed an AngII-induced decrease in \([Ca^{2+}]\_i\), both in naïve cells (Fig. 5D) and in cells overexpressing STIM1/Orai1 after store depletion (Fig. 5E). Because AngII stimulation decreases both PtdIns(4,5)P\(_2\) and PtdIns4P levels, these results were compatible with inhibition of SOCE or \(i_{\text{crac}}\) after robust PLC activation and PtdIns4P reduction, although such decreases in \([Ca^{2+}]\_i\) could be caused by other mechanisms(s).

PI4KIII\(\alpha\) Down-regulation Inhibits SOCE—The sensitivity of SOCE to PI4K inhibition or PLC-mediated PtdIns4P depletion raised the question of whether any of the four mammalian PI4Ks was responsible for the production of PtdIns4P in support of Orai1 channel activity. To address this question, we performed \([Ca^{2+}]\_i\) measurements with cells in which each of the four PI4Ks had been individually knocked down by RNA interference-mediated gene silencing. Although in such studies done previously we found that even significantly reduced levels of the PI4Ks had only modest effects on most signaling functions of the cells and complete knockdown could not be accomplished because of cell death (29), we hoped that at least a partial effect of PI4K knockdown could be detected. First, we examined the effects of knockdown of PI4KII\(\alpha\) and -III\(\alpha\) in COS-7 cells and PI4KIII\(\alpha\) in HEK-AT1 cells on the Tg-induced \([Ca^{2+}]\_i\) rise. As shown in Fig. 6B, knockdown of PI4KII\(\alpha\), but not PI4KIII\(\alpha\), partially inhibited the plateau phase of the Tg-induced \([Ca^{2+}]\_i\) increase in COS-7 cells. PI4KIII\(\alpha\) or -II\(\alpha\) knockdown did not inhibit \([Ca^{2+}]\_i\) release by Tg as shown in cells analyzed in \([Ca^{2+}]\_i\)-free medium, but PI4KIII\(\alpha\) knockdown inhibited the \([Ca^{2+}]\_i\) rise upon \([Ca^{2+}]\_i\) readdition (Fig. 6B). In separate experiments the effects of PI4KIII\(\beta\) knockdown were also analyzed in COS-7 cells. However, these effects were more complex and appeared to be dominated by a prolonged disruption of Golgi function and, hence, will require further investigation. Importantly, acute inhibition by PI4KIII\(\beta\) with the more selective inhibitor, PIK93, was without effect on \([Ca^{2+}]\_i\) entry (data not shown).

The effect of PI4KIII\(\alpha\) down-regulation was also examined in single HEK-AT1 cells attached to coverslips. These experiments again showed a modest but significant decrease in the Tg-induced \([Ca^{2+}]\_i\) signal (Fig. 6C). Taken together these studies indicated that PI4KIII\(\alpha\) but not PI4KII\(\alpha\) enzyme is important in the support of Orai function in the PM.

DISCUSSION

The present studies were designed to address the question of whether PM phosphoinositides contribute to the regulation of the STIM1/Orai1-mediated \([Ca^{2+}]\_i\) entry process. The importance of this question is 2-fold. First, only a few ion channels and transporters are known that do not require phosphoinositides for their proper function in the PM (23). Second, the ER-localized STIM1 protein makes a contact with the PM upon ER \([Ca^{2+}]\_i\) store depletion, and it contains a polybasic domain at its C terminus that is perfectly suited to serve as a phosphoinositide-binding module, especially in the multimerized form in which STIM1 exists in the ER \([Ca^{2+}]\_i\)-depleted state (18).

To address this question, we employed several methods to alter PM phosphoinositides. We used PLC activation via stimulation of expressed AT1 receptors by AngII, applied inhibitors or knockdown of PI4Ks, and acutely decreased PM PtdIns(4,5)P\(_2\) levels with the recruitable 5-phosphatase system described recently (28, 37). The effects of these manipulations were studied on STIM1 movements from the tubular to the PM-adjacent ER compartment on \([Ca^{2+}]\_i\) changes after ER \([Ca^{2+}]\_i\) store depletion and on \(i_{\text{crac}}\) recordings. The results of these experiments indicate that STIM1 movements do not depend on PM phosphoinositides, as neither PtdIns(4,5)P\(_2\) depletion nor PI4K inhibition had a major impact on the ability of STIM1 to move and associate with the PM. The slight effect of the removal of PtdIns(4,5)P\(_2\) on STIM1-PM association suggests some contribution of the lipid in the stabilization of these interactions. These data are consistent with findings that STIM1,
lacking the polybasic domain, is fully functional in activating Orai1-mediated SOCE, although its activation and inactivation properties might be impaired (15, 19, 22).

However, in contrast to STIM1 movements, Orai1-mediated store-operated Ca\(^{2+}\) influx was sensitive to PI4K inhibition (but not to PtdIns(4,5)P\(_2\) depletion) and was also inhibited by robust PLC activation, which evokes a simultaneous decrease in PtdIns(4,5)P\(_2\) and PtdIns4P levels in these cells (29). SOCE was also inhibited by PI4K inhibitors under conditions in which PtdIns(4,5)P\(_2\) levels showed no reductions, and similar conclusions could be drawn from the electrophysiological experiments detecting I\(_{\text{CRAC}}\). These data strongly suggest that PtdIns4P rather than PtdIns(4,5)P\(_2\) is required for the optimal function of the Orai1-mediated Ca\(^{2+}\) entry process.

Inhibition of agonist-induced Ca\(^{2+}\) influx by PI4K inhibitors has been described in several studies (25, 38, 39), but this effect could be explained by the gradual depletion of the agonist-sensitive phosphoinositide pools and hence the calcium-mobilizing messenger, InsP\(_3\) (30, 40). Fewer studies have noted an effect of PI4K inhibition on SOCE activated by Ca\(^{2+}\) store depletion (4, 26). Broad et al. (26) has reported the most thorough analysis of the effects of a high concentration of Wm and LY294002 on SOCE and endogenous I\(_{\text{CRAC}}\) in rat basophilic leukemia cells. These authors showed that Wm treatment strongly inhibits SOCE and I\(_{\text{CRAC}}\), noting that this is not caused by the lack of InsP\(_3\) or diacylglycerol and that it correlates better with changes in the level of PtdIns4P than of PtdIns(4,5)P\(_2\) (26). The present results have confirmed and extended these observations, showing that the PtdIns4P requirement is not at the level of STIM1 movements but at the activity of the Orai1 channels. Rosado et al. (4) also analyzed the effects of LY294002 on SOCE in platelets and concluded that the inhibitory effect is not due to depolarization. This is an important question that should always be asked with [Ca\(^{2+}\)]\(_i\) measurements, as SOCE is very sensitive to changes in the driving force of Ca\(^{2+}\) entry to the cells and hence to a drop in the membrane potential (41). Because a number of potassium channels require phosphoinositides for their proper function, depletion of phosphoinositides could, in theory, inhibit some of these channels leading to depolarization. Although this mechanism may contribute to the effects of phosphoinositide depletion on SOCE in intact cells, the current patch clamp studies and those reported by Broad et al. (26) clearly showed that I\(_{\text{CRAC}}\) is dependent on phosphoinositide levels regardless of the membrane potential.

Our studies also suggest that the PI4K relevant for generating PtdIns4P is the type III\(\alpha\) enzyme. Although the knockdown of PI4KIII\(\alpha\) did not show strong inhibition, this was the only PI4K that had a consistent effect on SOCE, and knockdown of the PI4KIII\(\alpha\) enzyme or pharmacological blockade of PI4KIII\(\beta\) was without effect on Tg-induced [Ca\(^{2+}\)]\(_i\) elevations. These results are in agreement with our previous studies in which the PI4KIII\(\alpha\) enzyme was found to generate the PM phosphoinositide-signaling pool (29). This finding has been somewhat puz-
is not at the level of STIM1 protein movement but rather at the level of Orai1 activation. Surprisingly, the regulatory lipid is not the most abundant PtdIns(4,5)P₂ but rather its precursor, PtdIns4P, most likely synthesized by the PI4KIIIα enzyme. Further studies are needed to determine the molecular means by which PtdIns4P regulates the activity of the Orai1 channel or the mechanism by which the ER-localized PI4KIIIα enzyme supplies the PM with its lipid product, PtdIns4P.

Acknowledgments—We are grateful to Dr. Roger Y. Tsien for the monomeric red fluorescent protein and to Dr. Philip W. Majerus for the human type IV 5-PTase clone. TIRF imaging was performed at the Microscopy and Imaging Core of the National Institutes of Health, NICHD, with the kind assistance of Drs. Vincent Schram and James T. Russell.

REFERENCES

1. Parekh, A. B., and Putney, J. W., Jr. (2005) Phys. Rev. 85, 757–810
2. Hoth, M., and Penner, R. (1992) Nature 355, 353–356
3. Prakriya, M., and Lewis, R. S. (2002) J. Gen. Physiol. 119, 487–507
4. Rosado, J. A., and Sage, S. O. (2000) J. Biol. Chem. 275, 9110–9113
5. Stiber, J., Hawkins, A., Zhang, Z. S., Wang, S., Burch, J., Graham, V. W., Ward, C. C., Seth, M., Finch, E., Malouf, N., Williams, R. S., Eu, J. P., and Rosenberg, P. (2008) Nat. Cell Biol. 10, 688–697
6. Liou, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrell, J. E., Jr., and Meyer, T. (2005) Curr. Biol. 15, 1235–1241
7. Roos, J., DiGregorio, P. J., Yeromin, A. V., Ohlsen, K., Lioudyny, M., Zhang, S., Safrina, O., Kozak, J. A., Wagner, S. L., Cahalan, M. D., Velieceli, G., and Staudermayer, K. A. (2005) J. Cell Biol. 169, 435–445
8. Feske, S., Gwack, Y., Prakriya, M., Srikanta, S., Suh, H., Tanasa, B., Hogan, P. G., Lewis, R. S., Daly, M., and Rao, A. (2006) Nature 441, 179–185
9. Vig, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Koblman-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R., and Kinet, J. P. (2006) Science 312, 1220–1223
10. Zhang, S. L., Yeromin, A. V., Zhang, X. H., Yu, Y., Safrina, O., Penna, A., Roos, J., Staudermayer, K. A., and Cahalan, M. D. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 9357–9362
11. Hewavitharana, T., Deng, X., Soboloff, J., and Gill, D. L. (2007) Cell Calcium 42, 173–182
12. Putney, J. W., Jr. (2007) Cell Calcium 44, 103–110
13. Lewis, R. S. (2007) Nature 446, 284–287
14. Huang, G. N., Zeng, W., Kim, J. Y., Yuan, J. P., Han, L., Mulleman, S., and Worley, P. F. (2006) Nat. Cell Biol. 8, 1003–1010
15. Li, Z., Lu, J., Xu, P., Xie, X., Chen, L., and Xu, T. (2007) J. Biol. Chem. 282, 29448–29456
16. Muik, M., Frischauf, I., Derler, I., Fahrner, M., Besgmann, I., Eder, P., Schindl, R., Resch, C., Polzinger, B., Fritsch, R., Kahr, H., Madl, J., Gruber, H., Grosschner, K., and Romanin, C. (2008) J. Biol. Chem. 283, 8014–8022
17. Heo, W. D., Inoue, T., Park, W. S., Kim, M. L., Park, B. O., Wandless, T. I., and Meyer, T. (2006) Science 314, 1458–1461
18. Liu, J., Fivaz, M., Inoue, T., and Meyer, T. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 9301–9306
19. Spassova, M. A., Soboloff, J., He, L. P., Xu, W., Dziadek, M. A., and Gill, D. L. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 4040–4045
20. Yuan, J. P., Zeng, W., Dorwart, M. R., Choi, Y. J., Worley, P. F., and Muallem, S. (2009) Nat. Cell Biol. 11, 337–343
21. Park, C. Y., Hooper, P. J., Mullins, F. M., Bachhawat, P., Covington, E. D., Raunser, S., Walz, T., Garcia, K. C., Dolmetsch, R. E., and Lewis, R. S. (2009) Cell 136, 876–890
22. Zeng, W., Yuan, J. P., Kim, M. S., Choi, Y. J., Huang, G. N., Worley, P. F., and Muallem, S. (2008) Mol. Cell 32, 439–448
23. Gamper, N., and Shapiro, M. S. (2007) Nat. Rev. Neurosci. 8, 921–934
24. Takahashi, R., Watanabe, H., Zhang, X. X., Kakizawa, H., Hayashi, H., and
25. Hashimoto, Y., Ogihara, A., Nakanishi, S., Matsuda, Y., Kurokawa, K., and Nonomura, Y. (1992) J. Biol. Chem. 267, 17078–17081
26. Broad, L. M., Braun, F. J., Lievremont, J. P., Bird, G. S., Kuroski, T., and Putney, J. W., Jr. (2001) J. Biol. Chem. 276, 15945–15952
27. Várnai, P., Tóth, B., Tóth, D. J., Hunyady, L., and Balla, T. (2007) J. Biol. Chem. 282, 29678–29690
28. Varnai, P., Thyagarajan, B., Rohacs, T., and Balla, T. (2006) J. Cell Biol. 175, 377–382
29. Balla, A., Kim, Y. J., Varnai, P., Szentpetery, Z., Knight, Z., Shokat, K. M., and Balla, T. (2008) Mol. Biol. Cell 19, 711–721
30. Nakanishi, S., Catt, K. J., and Balla, T. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 5317–5321
31. Balla, T. (2007) J. Physiol. 582, 927–937
32. Downing, G. J., Kim, S., Nakanishi, S., Catt, K. J., and Balla, T. (1996) Biochemistry 35, 3587–3594
33. Warashina, A. (1999) Arch. Biochem. Biophys. 367, 303–310
34. Roy, A., and Levine, T. P. (2004) J. Biol. Chem. 279, 44683–44689
35. Yeung, T., Terebiznik, M., Yu, L., Silvius, J., Abidi, W. M., Philips, M., Levine, T., Kapus, A., and Grinstein, S. (2006) Science 313, 347–351
36. Yu, J. W., Mendrola, J. M., Audhya, A., Singh, S., Keleti, D., DeWald, D. B., Murray, D., Emr, S. D., and Lemmon, M. A. (2004) Mol. Cell 13, 677–688
37. Suh, B. C., Inoue, T., Meyer, T., and Hille, B. (2006) Science 314, 1454–1457
38. Nakanishi, S., Catt, K. J., and Balla, T. (1994) J. Biol. Chem. 269, 6528–6535
39. Watanabe, H., Takahashi, R., Zhang, X. X., Kakizawa, H., Hayashi, H., and Ohno, R. (1996) Biochem. Biophys. Res. Commun. 225, 777–784
40. Willars, G. B., Nahorski, S. R., and Challiss, R. A. (1998) J. Biol. Chem. 273, 5037–5046
41. Sarkadi, B., Tordai, A., and Gárdos, G. (1990) Biochim. Biophys. Acta 1027, 130–140
42. Balla, A., and Balla, T. (2006) Trends Cell Biol. 16, 351–361