Critical role of PIP5K1γ87 in InsP₃-mediated Ca²⁺ signaling

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Phosphatidylinositol 4,5-bisphosphate (PIP₂) is the obligatory precursor of inositol 1,4,5-trisphosphate (InsP₃ or IP₃) and is therefore critical to intracellular Ca²⁺ signaling. Using RNA interference (RNAi), we identified the short splice variant of type I phosphatidylinositol 4-phosphate 5-kinase γ (PIP5K1γ87) as the major contributor of the PIP₂ pool that supports G protein–coupled receptor (GPCR)-mediated IP₃ generation. PIP5K1γ87 RNAi decreases the histamine-induced IP₃ response and Ca²⁺ flux by 70%. Strikingly, RNAi of other PIP5K isoforms has minimal effect, even though some of these isoforms account for a larger percent of total PIP₂ mass and have previously been implicated in receptor mediated endocytosis or focal adhesion formation. Therefore, PIP5K1γ87’s PIP₂ pool that supports GPCR-mediated Ca²⁺ signaling is functionally compartmentalized from those generated by the other PIP5KIs.

Introduction

Phosphatidylinositol 4,5-bisphosphate (PIP₂) regulates multiple plasma membrane (PM) functions (Doughman et al., 2003; Yin and Janney, 2003), and it is also a substrate for PLC-mediated inositol 1,4,5-trisphosphate (InsP₃ or IP₃) generation. In spite of PIP₂’s obligatory role as an IP₃ precursor, and the importance of IP₃ mediated Ca²⁺ signaling, surprisingly little is known about the identity of the phosphoinositide kinases involved. Unlike yeast, which has a single type I phosphatidylinositol 4-phosphate 5-kinase (PIP5K1) that synthesizes PIP₂ (Audhya and Emr, 2003), mammals have three major PIP5K isoforms named α, β and γ (Doughman et al., 2003).

PIP5K1γ has two splice variants (PIP5K1γ87 and 90) that are distinguished by a 28–amino acid extension at the COOH terminus of PIP5K1γ90 (Di Paolo et al., 2002; Ling et al., 2002; Fig. 1 A). PIP5K1γ90 is particularly enriched in neurons (Wenk et al., 2001); it is the major PIP₂ synthesizing enzyme at the synapse, where it has been implicated in the regulation of clathrin coat recruitment, actin dynamics (Wenk et al., 2001) and focal adhesion formation (Di Paolo et al., 2002; Ling et al., 2002). In contrast, PIP5K1γ87 is not involved in focal adhesion formation or clathrin-mediated endocytosis (in HeLa cells; Padron et al., 2003).

Here, we examined the role of PIP5K1γ87 in intracellular Ca²⁺ signaling. Previous biochemical studies have shown that cells have agonist-sensitive and -insensitive PIP₂ pools (Koreh and Monaco, 1986). Inhibitor studies suggest that the agonist-sensitive pool can be further classified as constitutive or de novo generated in response to agonists (Nakanishi et al., 1995). Some of these pools are enriched in cholesterol–sphingolipid raft domains (Pike and Casey, 1996) and the stringent spatial and temporal regulation of Ca²⁺ may be specified by assembling key players into supramolecular signaling complexes (Delmas et al., 2004). We now report that PIP5K1γ87 is the major source of the agonist-sensitive PM PIP₂ pool that fuels the initial Ca²⁺ response to external stimuli.

Results and discussion

Knockdown of PIP5K1γ87 by RNA interference

We use small interfering RNA (siRNA) mediated RNA interference (RNAi) to knockdown each PIP5K1 individually (Padron et al., 2003). Anti-PIP5K1γpan antibody recognizes a sharp 87-kD band and a slower migrating diffuse band (Fig. 1 B) which is probably hyperphosphorylated PIP5K1γ87 (Park et al., 2001; Wenk et al., 2001). Anti-PIP5K1γpan stains the PM, the perinuclear region and the nucleus (Fig. 1 D). Low level HA-PIP5K1γ87 overexpression confirms that PIP5K1γ is enriched in the PM and punctate internal structures, but rules out nuclear localization.

Biochemical fractionation shows that 60% of PIP5K1γ87 is sedimented by high speed centrifugation (Fig. 1 E), and
approximately two thirds of this is associated with the PM enriched fraction. PIP5Kια is much more membrane bound, whereas PIP5Kιβ is least membrane associated. Therefore, these PIP5Kιs have different patterns of membrane association. Nevertheless, each can potentially generate PIP₃ at the PM and internal membranes.

Because it is not possible to knockdown PIP5Kιγ87 exclusively, we compared the effects of siRNA directed against both PIP5Kι forms (PIP5Kιγ) to that of PIP5Kιγ90 alone. PIP5Kιγ siRNA decreases all PIP5Kι bands in Western blots (Fig. 1 B) and reduces PM and cytoplasmic anti-PIP5Kι immunofluorescence, but not the nonspecific nuclear staining (Fig. 1 D). PIP5Kιγ90 siRNA has little effect on either PIP5Kι bands (Fig. 1 B), even though quantitative real-time PCR established that PIP5Kιγ90 mRNA is decreased by 70% (Fig. 1 C). We conclude that HeLa cells have very little PIP5Kιγ90.

Unexpectedly, PIP5Kιγ siRNA preferentially knocks down PIP5Kιγ87 mRNA relative to PIP5Kιγ90. Because PIP5Kιγ87 is more abundant than PIP5Kιγ90 in HeLa cells and PIP5Kιγ siRNA generates a distinct phenotype (compared with that of PIP5Kιγ90 siRNA), the PIP5Kιγ siRNA effects described here can be attributed primarily to PIP5Kιγ87 knockdown. Importantly, PIP5Kιγ siRNA has almost no effect on PIP5Kια and β protein expression (Fig. 1 B), establishing that the PIP5Kιγ87 knockdown phenotype is not complicated by compensatory changes in the other PIP5Kιs. This was originally a concern, because we have previously found that knockdown of one PIP5Kι induces changes in the mRNA level of some other PIP5Kιs (Padron et al., 2003).

**Figure 1.** PIP5Kιγ RNAi. (A) PIP5Kιγ siRNA design. Pan siRNA is directed against both isoforms. (B) PIP5Kιγ protein knockdown. Effect of PIP5Kιγ RNAi on protein expression of the targeted and nontargeted PIP5Kιs. Western blots were probed with isofom specific antibodies. Additional data are provided in Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200408008/DC1. (C) Quantitative real-time PCR. PCR primers were used to quantitate PIP5Kιγ and PIP5Kιγ90 mRNA and PIP5Kιγ87 mRNA was calculated from the difference. Numbers indicate the amounts of each isoform relative to PIP5Kιγ90 in control cells. Data are the average of duplicate RNAi samples from a single experiment. Similar results were obtained from another experiment. (D) PIP5Kιγ is enriched in the PM. Endogenous PIP5Kιγ was detected with anti-PIP5Kιγ antibody, and overexpressed HA-PIP5Kιγ7 (in cDNA-transfected cells) were stained with anti-HA. Arrows indicate PM. Bars, 50 µm. (E) Differential PIP5Kι membrane association. Fractions obtained after sequential sedimentation were loaded equivalently, except for the cytosol fraction (CYT), which was loaded 10 times less. Western blot band intensity was determined by quantitative densitometry, and expressed as a percent of total recovered, after correcting for differences in fraction of sample loaded.
naling. In control cells, 100 μM histamine induces a rapid and transient rise in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) in 95% of the cells examined (Fig. 3 A). PIP5K1y87 RNAi reduces the percent of responding cells slightly (by 13%), decreases the amplitude of the first Ca$^{2+}$ peak more [by 36%; from 1.14 ± 0.07 (n = 19) to 0.73 ± 0.08 (n = 10) fluorescence ratio unit], and has the most impact on Ca$^{2+}$ flux [76% decrease; from 0.33 ± 0.03 (n = 19) to 0.08 ± 0.01 (n = 10) units/s] (Fig. 3 A). Because Ca$^{2+}$ flux correlates with the open probability of the IP$_3$Rs and hence the rate of IP$_3$ generation (Johenning et al., 2004), our results establish that PIP5K1y87 RNAi depletes the PIP$_2$ pool used for IP$_3$ generation. A similarly large decrease in Ca$^{2+}$ flux was also observed when PIP$_2$ was depleted by overexpressing the PIP$_2$ phosphatase synaptojanin (Johenning et al., 2004).

The weakened Ca$^{2+}$ response by PIP5K1y87 RNAi cells is not due to depletion of Ca$^{2+}$ stores, because thapsigargin releases similar amounts of Ca$^{2+}$ into the cytosol of control and siRNA cells (unpublished data). The [Ca$^{2+}$]$_i$ response to UTP, which binds a different GPCR than histamine (P2Y and H1, respectively), is blunted as well (unpublished data). [Ca$^{2+}$]$_i$ increase in the presence of 1.3 mM of extracellular Ca$^{2+}$ (Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200408008/DC1) is also attenuated, confirming that the lack of PIP$_2$ impacts the entire Ca$^{2+}$ signaling cascade that starts with Ca$^{2+}$ release from internal stores and ends with capacitative Ca$^{2+}$ entry (that usually follows the depletion of intracellular Ca$^{2+}$ stores).

As expected, in control cells, 1 μM histamine induces a slower Ca$^{2+}$ flux than 100 μM histamine [0.24 ± 0.01 (n = 63) vs. 0.33 ± 0.03 (n = 19) fluorescence ratio unit/s]. Paradoxically, PIP5K1y87 RNAi cells have the same low Ca$^{2+}$ flux [0.08 ± 0.01 (n = 46) vs. 0.08 ± 0.01 (n = 10) fluorescence ratio unit/s] at both histamine doses. We cannot explain why this is the case. Perhaps because PIP$_2$ is already limiting at sub-maximal stimulation, increasing the intensity of the stimulus does not significantly increase the amount of IP$_3$ generated due to lack of substrate.

Unlike PIP5K1y87 RNAi, PIP5K1y90, α or β RNAi has much less effects (Fig. 3 A), paralleling the trend observed with IP$_3$ production (Fig. 2 B). Thus, the PIP5K1ypan siRNA phenotype can be most simply explained by a decrease in the amount of PLCβ accessible PM PIP$_2$ and that this pool is generated primarily by PIP5K1y87.

If PIP5K1y87 RNAi suppresses Ca$^{2+}$ signaling by depleting PM PIP$_2$, restoring membrane PIP$_2$ should rescue the Ca$^{2+}$ response. We used a membrane permeant polyamine shuttle carrier to deliver exogenous PIP$_2$ into intact cells (Ozaki et al., 2000; Wang et al., 2003). Control HeLa cells respond to sequential histamine challenges identically, and Shuttle PIP$_2$ does not change the Ca$^{2+}$ response significantly (Fig. 3 B). PIP5K1y87 RNAi cells, which are already less responsive to the first stimulus than control cells, have an even more blunted response to the second stimulus in the absence of Shuttle PIP$_2$ (Fig. 3 B, left). This is consistent with depletion of the already small PIP$_2$ pool by the first stimulus, and inadequate refilling before the second stimulus. Significantly, Shuttle PIP$_2$ restores the Ca$^{2+}$ response of PIP5K1y87 RNAi cells to ~74% of that observed in Ctrl RNAi cells (Fig. 3 B). Therefore, the Ca$^{2+}$ signaling defect is due to PIP$_2$ depletion by PIP5K1y RNAi.

Together, our results demonstrate that PIP5K1y87 has a critical role in GPCR-mediated IP$_3$ signaling in HeLa cells. Interestingly, overexpressed mouse PIP5KIβ (equivalent to human PIP5KIα described in this paper) stimulates tyrosine kinase receptor activated-IP$_3$ generation in B lymphocytes (Saito et al., 2003). Together, these results raise the intriguing possibility that the GPCR- and tyrosine kinase receptor-coupled PIP$_2$ pools may be governed by different PIP5KIs. We plan to determine if this is the case in future studies.

**Effects of PIP5K1y87 RNAi on PIP$_2$ content and distribution**

To understand how PIP5K1y87 uniquely contributes to GPCR mediated IP$_3$ signaling, we estimated the size and location of its

![Figure 3](image-url)

**Figure 3.** PIP5K1ypan siRNA attenuates intracellular Ca$^{2+}$ signaling. Cells loaded with fura2-AM in randomly chosen microscopic fields were ratio imaged (F$_{340}$/F$_{380}$) to obtain baseline Ca$^{2+}$ values. Histamine was recorded as a function of time. (A) Ca$^{2+}$ response to 100 μM histamine [0.24 ± 0.01 (n = 63) vs. 0.33 ± 0.03 (n = 19) fluorescence ratio unit/s]. Paradoxically, PIP5K1y87 RNAi cells have the same low Ca$^{2+}$ flux [0.08 ± 0.01 (n = 46) vs. 0.08 ± 0.01 (n = 10) fluorescence ratio unit/s] at both histamine doses. (B) In vivo rescue of intracellular Ca$^{2+}$ signaling in PIP5K1ypan siRNA-treated cells by Shuttle PIP$_2$. Top panels are representative tracings. (Bottom) The Ca$^{2+}$ flux of the transients elicited by the second histamine addition was plotted and 10 cells were analyzed per condition.
Figure 4. Effect of PIP5KI RNAi on PIP_2. (A) PIP_2 mass (HPLC) and ^32P-incorporation (TLC). Means ± SEM of independent experiments are shown. (B) PIP_2 distribution as detected with anti-PIP_2 and overexpressed GFP-PLC_6-PH. Cross-sectional plots of fluorescence intensity are shown next to the image. Bars, 50 μm. (C) Analysis of PIP_2 quantitation. (Left) The average intensities of anti-PIP_2 at the PM and inside the cell are expressed in arbitrary units (mean ± SEM). 10 cells were analyzed per RNAi condition. (Right) PM/cytoplasmic intensity ratios of anti-PIP_2 and GFP-PLC_6-PH were shown. 10 cells were analyzed per label per RNAi condition.

PIP_2 pool relative to those of other PIP5Ki's. PIP5KIγapan siRNA reduces PIP_2 mass, determined by HPLC (Nasuhoglu et al., 2002), by 14% [from 377 ± 90 (n = 3) to 325 ± 95 (n = 3) pmol/mg protein] and ^32P-incorporation into PIP_2, determined by TLC, to a similar extent (Fig. 4 A). PIP5KIγ90 siRNA has no statistically significant effect. PIP5KIγRNAi decreases PIP_2 by 34% (Fig. 4 A), which is consistent with the large decrease in [^32P]PIP_2 reported previously (Padron et al., 2003). Paradoxically, although PIP5KIα RNAi does not decrease [^32P]PIP_2 (Padron et al., 2003), it decreases PIP_2 mass by 33%. The difference between the TLC and HPLC estimates may be because they measure different parameters. ^32P-labeling/TLC detects PIP_2 that turns over during the labeling period, whereas the HPLC method does not involve radiolabeling (Nasuhoglu et al., 2002) and measures PIP_2 mass. It is possible that the 4-h labeling interval we used was not long enough to completely equilibrate a particularly stable PIP_2 pool, and therefore underestimates its size.

PIP5KIγ87 RNAi decreases PM PIP_2 significantly. PIP_2 was detected by single cell fluorescence imaging using overexpressed GFP-PLC_6-PH (Varnai and Balla, 1998; Watt et al., 2002) and anti-PIP_2 (Laux et al., 2000; Matsuda et al., 2001; Fig. 4 B). Although it is generally accepted that the PM is par-
particularly enriched in PIP2 and that GFP-PLC6-PH labels the PM intensely, internal GFP-PLC6-PH labeling has also been reported in at least some types of cells (Matsuda et al., 2001). However, because GFP-PLC6-PH was overexpressed and also binds IP3 (Hirose et al., 1999), it is difficult to determine if the internal GFP-PLC6-PH is bound to PIP2/IP3 or represents unliganded PH. This issue is clarified somewhat by a quantitative immuno-electron microscopic study which shows that the PM accounts for 40% of total GST-PLC6-PH labeling, and internal organelles account for the remainder (Watt et al., 2002).

Our anti-PIP2 staining results clearly shows that PIP2 is present in internal membranes as well as PM in HeLa cells. Using image quantitation (Fig. 4, B and C), we estimate that anti-PIP2 fluorescence in the vicinity of the PM accounts for 12.3 ± 1.2% (n = 10) of total, and its intensity is 1.54 ± 0.13 times (n = 10) higher than in internal sites (PM/cytosol ratio; Fig. 4 C). Although these cross-sectional analyses underestimate the size of the PM pool (compared with morphometric analysis by electron microscopy, as described by Watt et al., 2002), it can be used to compare the effects of different PIP5KI RNAi.

In conclusion, PIP5KIy87 is the major source of the GPCR mobilized PIP2 pool. This specialized PIP2 accounts for a small fraction of total PIP2, a significant fraction of PM PIP2 and most of the histamine induced IP3 response. The exquisitely selective effect of PIP5KIy87 RNAi on Ca2+ signaling suggests that the cell’s PIP2 is functionally compartmentalized in a PIP5KI-dependent manner. This study provides a mechanistic understanding of how PIP2 can regulate multiple PM functions independently. Additional studies will determine if PIP5KIy87 is part of the supramolecular PLCβ signaling scaffold that specifies rapid local Ca2+ generation and propagation (Delmas et al., 2004), and whether the functionally compartmentalized PIP2 is physically segregated in the PM.

Materials and methods

Antibodies

Anti-PIP5Kα was purchased from Santa Cruz Biotechnology, Inc. Anti-PIP5Kβα and PIP5Kβypan were gifts from C. Carpenter (Harvard Medical School, Boston, MA) and P. De Camilli (Yale University, New Haven, CT; Wenk et al., 2001), respectively. Monoclonal anti-PIP2 (Fukami et al., 1988) was a gift from K. Fukami (University of Tokyo, Tokyo, Japan).

RNAi

We used the human PIP5KI isoforms designation, which is different from the mouse designation. siRNA oligonucleotides were performed as described previously (Padron et al., 2003). HeLa cells were transfected with the siRNA and used 48–72 h later.

Quantitative real-time PCR

RNA extracted from Hela cells transfected with siRNA were reverse transcribed and used for PCR in a sequence detection system (Prism 7000; Applied Biosystems). Primers directed at nucleotides 414–478 (pan) and 1993–2048 (unique to PIP5Kiy90) were used (Padron et al., 2003).

PIP2 measurements

PIP2 mass was determined by a nonradioactive HPLC detection system (Nasuhoglu et al., 2002). Incorporation into PIP2 was determined by labeling cells for 4 h with [32P]PO4 (NEN Life Science Products), resolving lipids by TCC, and quantitation by phosphorimager analysis (Wang et al., 2003). PM PIP2 was determined by image analysis of cells overexpressing low amounts of GFP-PLCβ-PH (Varnali and Balla, 1998), or labeling with anti-PIP2 (Laux et al., 2000; Matsuda et al., 2001). Fluorescence images were captured by confocal microscopy (model LSM5; Carl Zeiss Microlmaging, Inc.) and intensity plots were analyzed by MetaMorph Offline software (Varnali and Balla, 1998). The average fluorescence intensity of the two cell edges and between the cell edges were defined as PM and cytoplasmic PIP2, respectively, and are expressed in arbitrary units.

Immunofluorescence microscopy

For most purposes, 0.4% formaldehyde fixed cells were permeabilized with Triton X-100 and processed for confocal microscopy as described previously (Wang et al., 2003). Anti-PIP2 staining was detected by permeabilizing fixed cells with 10 μg/ml digitonin, which preserves the lipid signal better than Triton X-100.

Multistep membrane fractionation

Cells were homogenized by 25 strokes in a prechilled steel homogenizer and homogenates were centrifuged sequentially to obtain the crude organelle/membrane fractions as described previously (Wei et al., 2002). LSP is enriched for Golgi membranes and early endosomes, and HSP is enriched for lysosomes and late endosomes. The PM fraction was obtained by placing the 19,000 g pellet on top of a sucrose cushion, and collecting the membranes at the top after centrifugation at 100,000 g.

IP3 measurement

Cell monolayers incubated in Ca2+-free Hank’s buffer supplemented with 0.1% BSA were stimulated with 100 μM histamine (Sigma-Aldrich) for 0–25 s at RT and the reaction was stopped with PCA. IP3 content was assayed by competition with exogenous [3H]IP3 to bind calf cerebellar microsomes (Sun et al., 1995).

Single cell Ca2+ imaging

Cells plated on glass-bottom culture dishes (Mat Tek) were loaded with fura2/AM, washed and incubated for 30 min at RT to allow de-esterification of the loaded dye. The dish was mounted on the stage of an inverted fluorescence microscope (Axiovert 200; Carl Zeiss MicroImaging, Inc.) with a 40× objective. Cells were excited at 340 and 380 nm and the change in fluorescence ratio values (F340/F380) as a function of time in individual cells within a field was recorded simultaneously. The percent of responding cells was obtained by dividing those with a Ca2+ signal to total cells recorded. The ratio of maximal F340/F380 induced by histamine to basal F340/F380 is defined as the Ca2+ peak. Ca2+ flux is defined as the slope of a line between the initiation of a persistent increase in F340/F380 and the maximal F340/F380.

Intracellular delivery of PIP2 by Shuttle PIP2

The intracellular delivery of PIP2 by Shuttle PIP2 sRNA-treated cells were stimulated with 1 μM histamine in the absence of extracellular Ca2+ and ratio imaged. Histamine was washed out and cells were loaded with a mixture of 1 μM diC16-PIP2 and 1 μM of carrier 2 (Shuttle PIP2; Echelon Biosciences, Inc.) diluted in the Hank’s buffer (PIP2) or buffer only (Mock; Wang et al., 2003) for 10–15 min on the microscope stage. Cells were then reexposed to 1 μM histamine and imaged again.

Online supplemental material

Table S1 illustrates the effect of PIP5KI RNAi on PIP5KI protein expression. HeLa cells transfected with siRNA for each PIP5KI was lysed and subjected to Western blotting with specific antibodies. Table S2 illustrates the effect of PIP5KI RNAi on histamine induced Ca2+ transients. HeLa cells trans-
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