Rapidly inducible changes in phosphatidylinositol 4,5-bisphosphate levels influence multiple regulatory functions of the lipid in intact living cells

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Abbreviations used in this paper: [Ca2+]i; cytoplasmic Ca2+; HEK, human embryonic kidney; mRFP, monomeric red fluorescent protein; mTOR, mammalian target of rapamycin; PM, plasma membrane; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; 5-phosphate, phosphatidylinositol 5-phosphate; rapa, rapamycin; Tg, thapsigargin; TRPM8, transient receptor potential melastatin 8.

Rapamycin (rapa)-induced heterodimerization of the FRB domain of the mammalian target of rapa and FKBP12 was used to translocate a phosphoinositide PtdIns(4,5)P2 (5-ptase) enzyme to the plasma membrane (PM) to evoke rapid changes in phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] levels. Rapa-induced PM recruitment of a truncated type IV 5-phosphate containing only the 5-phosphate domain fused to FKBP12 rapidly decreased PM PtdIns(4,5)P2 as monitored by the PLCδ1PH-GFP fusion construct. This decrease was paralleled by rapid termination of the ATP-induced Ca2+ signal and the prompt inactivation of menthol-activated transient receptor potential melastatin 8 (TRPM8) channels. Depletion of PM PtdIns(4,5)P2 was associated with a complete blockade of transferrin uptake and inhibition of epidermal growth factor internalization. None of these changes were observed upon rapa-induced translocation of an mRFP-FKBP12 fusion protein that was used as a control. These data demonstrate that rapid inducible depletion of PM PtdIns(4,5)P2 is a powerful tool to study the multiple regulatory roles of this phospholipid and to study differential sensitivities of various processes to PtdIns(4,5)P2 depletion.

Introduction

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) is the major phosphoinositide species in mammalian cells and has been associated with numerous molecular events critical for cellular signaling. PtdIns(4,5)P2 is hydrolyzed by PLC enzymes to generate diacylglycerol and inositol 1,4,5-triphosphate, two pivotal second messengers (Berridge, 1993), and it is also converted by class I phosphoinositol 3-kinases to PtdIns(3,4,5)P3 (Toker and Cantley, 1997). PtdIns(4,5)P2 directly interacts with several ion channels, transporters (Fuster et al., 2004; Suh and Hille, 2005), and actin binding proteins (Hilpela et al., 2004) and regulates enzymes such as PLC and PLD (Liscovitch et al., 1994; Lomasney et al., 1996). Several molecules within the receptor internalization machinery also contain inositide binding domains, but the exact lipid species that regulates them in the cell has not been firmly established (Itoh et al., 2001). It is a major challenge to understand how a single type of molecule is able to regulate so many processes simultaneously and perhaps independently within the plasma membrane (PM).

Part of the problem in studying the multiple functions of PtdIns(4,5)P2 is that it is difficult to manipulate phosphoinositide levels within the cells. For example, most data on channel regulation rely upon the addition of phospholipids to excised patches and the use of inhibitors such as high concentrations of wortmannin to inhibit PtdIns(4,5)P2 formation (Suh and Hille, 2002; Rohacs et al., 2005). Several attempts have been made to alter the level of PtdIns(4,5)P2 in intact cells by expressing either phosphatidylinositol 4-phosphate 5-kinase or 5-phosphatase (5-phosphate) enzymes (Ono et al., 2004; Chen et al., 2006). However, prolonged changes in PtdIns(4,5)P2 levels initiate several trafficking and signaling events that will alter the disposition of the cells by the time the effects are analyzed (Brown et al., 2001). This makes it difficult to draw firm conclusions regarding direct effect of the lipid on any single process.

To overcome this problem, we developed a strategy to promptly regulate membrane PtdIns(4,5)P2 levels by a drug-inducible membrane targeting of a type IV 5-phosphate enzyme (Kisseleva et al., 2000; Kong et al., 2000) based on the heterodimerization of the FRB (fragment of mammalian target of rapamycin [mTOR] that binds FKBP12) and FKBP12 (FK506
binding protein 12; Muthuswamy et al., 1999). In this approach, the phosphatase is fused to the FKBP12 protein, and upon addition of rapamycin (rapa; or an analogue that does not interact with endogenous mTOR protein) the enzyme rapidly translocates to the membrane where its binding partner, the FRB domain, is targeted. This method has been successfully used to manipulate small GTP binding proteins at the PM (Inoue et al., 2005) and to study the effects of β-arrestin membrane recruitment (Terrillon and Bouvier, 2004). In the present study we show the use of this approach to manipulate PM PtdIns(4,5)P2 levels and demonstrate how these manipulations affect selected processes that are regulated by this phosphoinositide species.

Results and discussion

Targeting of type IV 5-ptsae alters PM PtdIns(4,5)P2 levels

Fig. 1 shows the concept and the constructs used for rapamycin-induced targeting of the type IV 5-ptsae to the PM. For membrane targeting of the FRB domain of mTOR, the palmitoylation sequence of the human GAP43 protein was used (Tanimura et al., 2004). To follow their localization, the FRB protein was also tagged with either CFP or monomeric red fluorescent protein (mRFP). The 5-ptsae (either full-length or only the 5-ptsae domain) was mutated (C641A) to eliminate its C-terminal lipid modification and membrane targeting and was fused to FKBP12 and also tagged with mRFP (Fig. 1). A mutant form of FRB (T2098L of mTOR) that can be heterodimerized with FKBP with a rapa analogue (AP21967; rapalogue) that does not bind to endogenous mTOR has been recommended. However, because of its easier availability and faster action, we mostly used rapa and the wild-type FRB protein in the present studies. Nevertheless, the mutant FRB and the rapalogue have also been tested and their use is recommended for applications where rapa itself could affect the process being investigated.

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To monitor the effects of the 5-ptsae on PtdIns(4,5)P2 levels in the PM, these constructs were transfected together with the PLCδ1PH-GFP plasmid to monitor PtdIns(4,5)P2 in the PM along with the membrane-targeted FRB-CFP (CFP channel is not shown) and the mRFP–FKBP–5-ptsae domain constructs. Addition of 100 nM rapa induces translocation of the 5-ptsae to the membrane, causing a complete loss of PLCδ1PH-GFP localization. Even partial localization of the enzyme (at 30 s) is sufficient to eliminate PtdIns(4,5)P2. FRET analysis of the PLCδ1PH domain translocation in cell suspensions, COS-7 cells were transfected with the CFP- and YFP-tagged forms of the PLCδ1PH domains together with the membrane-targeted FRB and the FKBP–5-ptsae both tagged with mRFP. Cells were trypsinized and analyzed in suspension in a spectrofluorometer as described in Materials and methods. Addition of rapa at the indicated concentrations induces PtdIns(4,5)P2 depletion resulting in a decreased membrane localization of the PH domain reflected in the decreased FRET signal. 10 μM ionomycin (iono) was used to completely eliminate PtdIns(4,5)P2 (Várnai and Balla, 1998). [C] Similar experiment as in B except that the FKBP construct did not contain the phosphatase (FKBP only; black trace) or contained the full-length 5-ptsae–5-ptsae–FL; red trace). Representative data are shown from two identical observations.
However, a small number of cells expressing high levels of the truncated 5-ptase domain showed no PLCδ1PH-GFP localization, indicating that high concentrations of the truncated enzyme could decrease lipid levels even without membrane targeting. 100 nM rapa caused rapid translocation of the fusion protein containing the 5-ptase domain to the PM causing a prompt and complete loss of PLCδ1PH-GFP localization in most cells (Fig. 2 A). Using the full-length phosphatase, however, caused only incomplete translocation of the PLCδ1PH-GFP reporter to the cytosol in some of the cells, and many cells showed no detectable change in PLCδ1PH-GFP localization in spite of efficient enzyme recruitment to the membrane (unpublished data). This important finding is consistent with the notion that a full-length enzyme contains regulatory regions that keep the enzyme activity under control. No changes were observed in PLCδ1PH-GFP distribution upon rapa-induced translocation of the mRFP-FKBP fusion protein that did not contain the 5-ptase. FRET measurements between the CFP- and YFP-tagged PLCδ1PH domain (van Der Wal et al., 2001) used either in single cells (not shown) or in a population of COS-7 cells (Fig. 2 B) have clearly demonstrated the lipid changes evoked by this approach.

Decreasing PM PtdIns(4,5)P₂ levels rapidly terminates Ca²⁺ influx during ATP stimulation

Next, we examined the effects of PtdIns(4,5)P₂ depletion on Ca²⁺ signaling evoked via the endogenous P₂Y receptors in COS-7 cells. Cells were transfected with the PM-targeted FRB-CFP (or -mRFP) together with either the full-length or truncated 5-ptase mRFP-FKBP fusion construct for 1 d. The expression as well as the movements of the 5-ptase were monitored in the red channel simultaneously with single-cell cytoplasmic Ca²⁺ ([Ca²⁺]i) measurements with fura-2. Addition of 50 μM ATP evoked a Ca²⁺ signal in many cells expressing the 5-ptase in the cytosol, but several cells expressing a high level of the phosphatase showed impaired response to ATP. Fig. 3 shows averaged Ca²⁺ recordings from single cells where the truncated 5-ptase domain was expressed and the cells showed a response to ATP. Here, administration of rapa promptly terminated the plateau phase of [Ca²⁺]i, increase with kinetics similar to those of the PtdIns(4,5)P₂ decrease. Notably, these cells failed to respond to a subsequent stimulation with another Ca²⁺-mobilizing agonist, lysophosphatidic acid.

Translocation of the full-length 5-ptase with 100 nM rapa also caused a rapid inhibition of the ATP-induced Ca²⁺ signal. However, these cells still showed a transient [Ca²⁺]i response to lysophosphatidic acid, indicating that maintenance of the Ca²⁺ signal is more sensitive to small depletion of the PtdIns(4,5)P₂ levels than the initial response of Ca²⁺ mobilization (Fig. 3 C). Because activation of P₂Y receptors leads to InsP₃ production and Ca²⁺ release form ER stores, hence activating capacitative Ca²⁺ influx, we wanted to determine whether capacitative Ca²⁺ influx itself requires PtdIns(4,5)P₂ in the membrane (Broad et al., 2001). To do this, the effect of lipid depletion on thapsigargin (Tg)-induced Ca²⁺ response was examined. Tg depletes Ca²⁺ stores by inhibition of the sarcoplasmic and ER Ca²⁺ ATPase that keeps Ca²⁺ stores filled and therefore activates Ca²⁺ influx without the need for InsP₃. Fig. 3 D shows that the sustained [Ca²⁺]i increase after Tg treatment was not affected by the same manipulations of PtdIns(4,5)P₂ levels that eliminated the ATP-induced sustained Ca²⁺ elevations. This finding suggests that PtdIns(4,5)P₂ depletion interferes with the sustained generation of InsP₃ rather than with the capacitative Ca²⁺ influx mechanism itself. A more detailed analysis of the relationship between these mechanisms is currently under way.

Several controls were used to rule out that the observed effects are caused by rapa itself or by the transfected constructs and/or their translocation to the membrane. First, the response of cells in the same field of view not expressing the phosphatase were monitored and found to show no change in response to rapa. Second, the Ca²⁺ response of cells expressing both the targeting construct and mRFP-FKBP12 without the 5-ptase also showed no change in response to rapa addition (Fig. 3 B).

Decreasing PM PtdIns(4,5)P₂ levels affects the activity of transient receptor potential melastatin 8 (TRPM8) channels

TRPM8 is one of the Ca²⁺ conductive channels that has been shown to require PtdIns(4,5)P₂ for its activity (Liu and Qin, 2005; Rohacs et al., 2005). Therefore, we chose these channels to study their PtdIns(4,5)P₂ dependence by monitoring either
at the right (means SEM from 79 and 76 cells, respectively). Elimination of PtdIns(4,5)P₂ described in Materials and methods. The currents at recording measured in the whole-cell configuration, using the ramp protocol described in Materials and methods. The currents at +100 mV (bottom curves) are shown. The averaged responses to rapa from 14 and 4 recordings for 5-ptase and FKBP-only expressing cells, and were not observed in rapa-treated cells that expressed the same construct without the 5-ptase domain. A more quantitative assessment of this process was obtained by FACS analysis in the case of Tf. Here, the mean green fluorescent intensity of the cells (a measure of internalized Tf) in the population of cells expressing the red (5-ptase) construct showed the changes observed in the confocal pictures (Fig. 5 B). These data suggested that Tf receptors will not internalize when PtdIns(4,5)P₂ is not available in the PM.

Collectively, these data clearly demonstrate that changes in membrane PtdIns(4,5)P₂ levels by themselves without the generation of second messengers can have multiple consequences on a wide range of cellular processes. In a similar manner, modulation of phosphoinositides in defined membrane compartments can be achieved by recruiting other enzymes (phosphatases and kinases) to the PM or to other cellular membrane compartments to analyze the role of PtdIns(4,5)P₂ or other inositol lipids in specific cellular processes. Although this approach has considerable potential, caution and the use of appropriate controls are essential to avoid possible artifacts. Numerous cellular processes are based on FKBP12 interactions, and overexpression of an FKBP12 construct could alter their properties. Similarly, rapa is an inhibitor of mTOR that can exert several effects on its own. This problem is alleviated with the use of the rapalogue that does not bind to the endogenous protein. Lastly, the targeting of the FRB by itself can have its own effects on selected cellular functions. However, if these possibilities are kept in mind
this technique can permit further exploration of the complex regulatory features of phosphoinositides.

Materials and methods

Materials
AP21967 was obtained from Ariad Pharmaceuticals. Rapamycin and Tg were purchased from Calbiochem. Alexa 488–Tf and Alexa 488–EGF were obtained from Invitrogen. All other chemicals were purchased from Sigma-Aldrich and were of highest analytical grade.

DNA constructs
The PLCδ1 PH-GFP construct and its color variants have been previously described (Varnai and Ballo, 1998; van Der Wal et al., 2001). For PM tethering, the N-terminal localization sequence (MLCMMRTKQVEKNDDBQK) of the human GAP43 (residues 1–20) was fused to the N terminus of the FRB domain of human mTOR1 (residues 2019–2114 amplified from a human EST available from GenBank/EMBL/DDBJ under accession no. 5495577) through a short linker. To visualize the fusion protein, the construct was tagged with CFP or mRFP (mRFP provided by R.Y. Tsien, University of California, San Diego, San Diego, CA). The T2098L mutant version of FRB was generated by exchanging the FRB portion from the plasmid obtained from the Argent heterodimerization kit (Ariad Pharmaceuticals).

The human type IV 5-phosphate enzyme (available from GenBank/EMBL/DDBJ under accession nos. 3504715). All of them contained mRFP fused to the N terminus of FKBP12. The human type IV 5-phosphate enzyme (available from GenBank/EMBL/DDBJ under accession no. NM_019892; provided by P.W. Majerus, Washington University, St. Louis, MO) was then fused to the C terminal of the FKBP12 either as the full-length protein or only its 5-phosphate domain (residues 214–644). In both cases, the C641A mutation was introduced to destroy the lack of internalization of Tf in cells expressing the 5-phosphate domain.

Confocal analysis of single cells and [Ca2+]i measurements
COS7 cells were cultured on glass coverslips (3 × 105 cells/35-mm dish) and transfected with the various constructs (2 μg of total DNA/dish) using Lipofectamine 2000 for 24 h as described elsewhere (Varnai et al., 2005). For [Ca2+]i measurements, cells were loaded with 3 μM fura-2/AM (45 min, room temperature). Ca2+ measurements were performed at room temperature in a modified Krebs-Ringer buffer containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl2, 0.7 mM MgSO4, 10 mM glucose, and 10 mM Na-Hepes, pH 7.4. An inverted microscope (IX70; Olympus) equipped with an illuminator (Lambda-DG4; Sutter Instrument Co.) and a digital camera (MicroMax-1024BFT; Roper Scientific) and the appropriate filter sets were used for Ca2+ analysis. Data acquisition and processing was performed by the MetaFluor software (Molecular Devices). Confocal analysis was performed in the same solution at 35°C using a confocal microscope (LSM 510 META; Carl Zeiss MicroImaging, Inc.).

Patch-clamp recordings
Patch-clamp experiments were performed on HEK293 cells after 2 d of transfection with the respective DNA constructs. Recordings were made using an amplifier (Axopatch 200B; Axon Instruments, Inc.) in an extracellular solution containing 137 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM Hepes, and 10 mM glucose, pH 7.4. The pipette solution contained 135 mM K gluconate, 5 mM KCl, 1 mM MgCl2, 5 mM EGTA, 10 mM Hepes, and 2 mM ATP (Na), pH 7.4. To assess TRPM8 channel activity, voltage ramps were applied from −100 to +100 mV every second. The current values measured at the +100 and −100 mV potential are shown in the recordings. Menthol was used at a concentration of 500 μM and rapamycin at 100 nM.

Cell suspension FRET measurements
COS7 cells were cultured in 10-cm dishes (3 × 105 cells) and transfected with equal amounts of PLCδ1 PH-CFP and -YFP, as well as the mRFP version of the appropriate FRB and FKBP constructs (10 μg of total DNA/dish) using Lipofectamine 2000 for 24 h. Cells were then trypsinized, centrifuged, and resuspended in the same modified Krebs-Ringer solution used in the Ca2+ experiments. Measurements were performed at 35°C using a Deltasonic fluorometer (PTI Technologies, Inc.) with excitation of 425 nm. To monitor the FRET signal, the ratio of the 525- and 475-nm emission was calculated.
FACS measurements

COS-7 cells were cultured in 10-cm dishes (3 × 10⁶ cells) and transfected with equal amounts of the appropriate FRB and FKBP constructs (10 μg of total DNA/dish) using Lipofectamine 2000 for 24 h. Cells were then trypsinized, centrifuged, and resuspended in the same solution that was used in the Ca²⁺ experiments [10³ cells/ml]. After treating the cells with rapsa (3 min) and then with fluorescent transferrin (5 min) they were fixed with 2% PFA. FACS measurements were performed using a FACSScan instrument (Becton Dickinson). To monitor the internalization in the transfected cell populations, the red channel was set to analyze the transfected cells and the mean green fluorescence of these cells was calculated.

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Note added in proof.
While this paper was under review, Suh et al. (2006) described a similar approach to chemically manipulate IP(4,5)P² levels and KCNQ potassium channels.

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