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PI4P And PI(4,5)P_2 Are Essential But Independent Lipid Determinants Of Membrane Identity

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Abstract

The quantitatively minor phospholipid phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P_2] fulfils many cellular functions in the plasma membrane (PM), whereas its major synthetic precursor, phosphatidylinositol 4-phosphate (PI4P), has no assigned PM roles apart from PI(4,5)P_2 synthesis. We used a combination of pharmacological and chemical genetic approaches to probe the function of PM PI4P, which was not required for the synthesis or functions of PI(4,5)P_2. However, depletion of both lipids was required to prevent PM targeting of proteins that interact with acidic lipids, or activation of the transient receptor potential vanilloid 1 cation channel. Therefore, PI4P contributes to the pool of polyanionic lipids that define plasma membrane identity, and to some functions previously attributed specifically to PI(4,5)P_2 that may be fulfilled by a more general polyanionic lipid requirement.

The quantitatively minor phospholipid, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2], is found on the inner surface of the plasma membrane (PM) where it acts as a molecular gate-keeper of both cell signalling and molecular traffic (1-3). Its major route of synthesis is by phosphorylation of phosphatidylinositol (PI) by PI 4-kinases (PI4K or PI4K2), making phosphatidylinositol 4-phosphate (PI4P), which is then phosphorylated at the 5-position by PI4P 5-Kinase (PIP5K). PI4P is generated in many cellular membranes, particularly in the Golgi apparatus, where it is crucial for function (4). Direct evidence for the presence of PI4P in the PM was scarce (5, 6), and the tacit assumption has been that it resides there solely for PI(4,5)P_2 synthesis.

Inhibitors of PI4K activity such as LY294002 and phenylarsine oxide (PAO) cause depletion of cellular PI4P, with only minor effects on the total amount of PI(4,5)P_2 (5, 7, 8). We confirmed this in COS-7 (African green monkey fibroblast) cells using either specific immunocytochemical probes (5), or mass spectrometry (9) (Fig. 1B). As a positive control, activation of PLC with ionomycin (10) caused depletion of both lipids. Although mass spectrometry cannot distinguish regio-isomers, PI4P and PI(4,5)P_2 are the predominant isomers in mammalian cells (11).

To more selectively and acutely manipulate the abundance of PM inositol lipids, we turned to the rapamycin-inducible dimerization of FKBP (FK506 binding protein 12) and FRB

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(fragment of mTOR that binds rapamycin) domains, which can be used to recruit enzymes to the PM (12, 13)(Fig. 1C). We generated an enzymatic chimera of inositol polyphosphate 5-phosphatase E (INPP5E), which converts $PI(4,5)P_2$ to PI4P (12) and the *S. cerevisiae* sac1 phosphatase, which dephosphorylates PI4P (14). We named this fusion protein Pseudojanin (PJ), in reference to its similarity to Synaptojanin (15). PJ recruited to the PM for 2 minutes with rapamycin caused decreased PI4P and $PI(4,5)P_2$ staining (Fig. 1D) and the release of the PI4P and $PI(4,5)P_2$-binding Osh2 tandem pleckstrin homology (PH) domain (PH-Osh2x2) (7, 16) from the PM (Figs 1H and I). Conversely, recruitment of only an INPP5E domain had no effect on PH-Osh2x2 (Fig. 1D), caused small increases in PI4P staining, depleted $PI(4,5)P_2$ staining (Fig.s 1D and S1E) and released PM-bound $PI(4,5)P_2$-biosensors such as the PLCδ1 PH (PH-PLCδ1) or Tubby C-terminal ($Tubby_c$) domains (17) (Figs 1I and S2).

To deplete PI4P specifically, we inactivated PJ's INPPE domain by mutation, making a chimera we call PJ-Sac. Recruitment of this enzyme to the PM caused depletion of PM PI4P staining, but had no effect on PM $PI(4,5)P_2$ staining (Fig. 1D) or localisation of PH-Osh2x2, PH-PLCδ1 or $Tubby_c$ (Figs 1H, I and S2). In fact, cells showing the largest degree of PI4P depletion induced by LY294002, PAO or PJ-Sac had scarcely altered $PI(4,5)P_2$ abundance (Figs S1C and S1D). The effects of the chimeras depended on rapamycin-induced membrane recruitment (Fig. S1B), and were not observed with PJ-Dead, a chimera with inactivated sac and INPP5E domains (Fig. S1B). PJ did not affect Golgi PI4P or endosomal PI3P staining (Fig. S3).

These observations demonstrate that most PM PI4P is not required to maintain the steady-state $PI(4,5)P_2$ pool. However, PI4P may still act as a reserve for cellular functions associated with continued consumption, and therefore replenishment, of PM $PI(4,5)P_2$. Such processes include clathrin mediated endocytosis of transferrin (18), continued generation of the lipid second messengers $PI(3,4,5)P_3$ and $PI(3,4)P_2$, and generation of Ca^{2+}-mobilising IP_3. Indeed, PM recruitment of PJ or INPP5E inhibited all of these processes (Figs 2A, B, C and S4). Depletion of PM PI4P with PJ-Sac, on the other hand, had no effect (Figs 2 and S4) and PI4P is thus dispensable for maintaining the functionally relevant $PI(4,5)P_2$ pool.

One possible explanation for this lack of effect is that a small fraction of the total PM $PI(4,5)P_2$ pool is consumed during endocytosis or signalling. In contrast, activation of PLC by muscarinic M1 (8, 19) or angiotensin II receptors (7) leads to consumption of up to 90% of $PI(4,5)P_2$. We therefore used transient over-expression of M1 receptors in COS-7 cells to investigate re-synthesis of $PI(4,5)P_2$ (Fig. 3A). Stimulation of M1-expressing cells led to reduced $PI(4,5)P_2$ and PI4P staining, which returned to pre-stimulation levels after addition of the M1 receptor antagonist atropine (Fig. 3B). PM-recruited PJ-Sac had no effect on this recovery of $PI(4,5)P_2$ staining, despite sustained depletion of PM PI4P (Fig. 3B). Likewise, $PI(4,5)P_2$ biosensors showed translocation from the PM upon PLC activation, but their return to the PM after atropine addition was unaffected by PJ-Sac recruitment (Figs 3C, S6).

These data indicate that PM PI4P seems to be redundant for synthesis of $PI(4,5)P_2$. Intuitively, such a result seems contradictory, given the known requirements for PI4K in this pathway. Indeed, the PI4K inhibitor PAO prevented re-synthesis of $PI(4,5)P_2$ assayed with $PI(4,5)P_2$ staining (Fig. 3B) or the $Tubby_c$ and PH-PLCδ1 reporters (Fig. 3C) (7, 10). These experiments show that despite a requirement for PI4K, $PI(4,5)P_2$ production continues in the absence of PM PI4P, either due to the efficiency of PIP5K in consuming residual PI4P (i.e. the PI4P used for $PI(4,5)P_2$ synthesis is synthesised *ad hoc* by PI4Ks), or else PI4P is supplied from other membranes (20). Either way, we conclude that the majority of PM PI4P is not required for $PI(4,5)P_2$ synthesis.

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If PM $PI(4,5)P_2$ and its functions are independent of PM PI4P, why do cells maintain substantial quantities of PI4P there? Many proteins selectively target the PM though basic amino acid stretches that interact with anionic lipid headgroups (3, 21); monovalent lipids such as PI and phosphatidylserine (PS) are present at high concentrations in several membranes (22), whereas an abundance of polyanionic inositol lipids is unique to the PM (13, 22). These polyanionic lipids concentrate around stretches of polybasic residues through non-specific electrostatic interactions, increasing binding affinity (3). We therefore reasoned that PI4P might contribute to this electrostatic interaction. We screened the localization of short peptide sequences from PM proteins before and after depletion of PI4P and/or $PI(4,5)P_2$ (Figs 4A and S7). These included amphipathic peptides such as the myristoylated alanine-rich C-kinase substrate effector domain (MARCKS-ED) and Rit1 GTPase C-terminus (Rit1-tail), lipid-anchored polybasic sequences such as the C-terminus of K-Ras (K-Ras tail) and the N-terminus of cortical cytoskeleton-associated protein of ~23 kDa ($CAP23_{20}$). We also assayed the kinase-associated 1 (KA1) domain from MAP/microtubule affinity-regulating kinase 1 (MARK1), which interacts non-specifically with acidic lipids (23). In all cases, combined removal of PI4P and $PI(4,5)P_2$ caused depletion of the proteins from the PM (Figs 4A and S7), with little effect when either lipid was depleted alone (13). Proteins that retained a secondary membrane targeting motif, such as prenylated K-Ras tail, were still found in the PM but were no longer enriched there compared to the amounts in other (presumably negatively charged (22)) membranes (Figs 4A and S8A). These effects were due to non-specific electrostatic interactions, because no effect was seen on the PS-specific lactadherin C2 domain (22), or the C-terminus of H-Ras, which interacts with the membrane solely through its hydrophobic lipid moieties (Figs 4A and S7). Measuring K-Ras tail's PM dissociation rate by fluorescence recovery after photobleaching (24) following PI4P and/or $PI(4,5)P_2$ depletion revealed that the two lipids made similar contributions to the protein's electrostatic interactions with the PM *in vivo* (Fig. S8).

$PI(4,5)P_2$ has been proposed to be a molecular switch that restricts activity of several ion channels to the PM (25), a phenomenon that can be highly specific for $PI(4,5)P_2$ (1, 26, 27, 27, 28). We wondered whether this is typical for all channels, or whether some have a more general polyanionic lipid requirement, which can also be fulfilled by PI4P. For example, the heat and capsaicin-activated transient receptor potential vanilloid 1 (TRPV1) cation channel can be both inhibited and activated by $PI(4,5)P_2$ and possibly PI4P (29). Translocation of PJ-Sac or INPP5E had no effect on prolonged (Figs 4B through D) or repetitive (Fig. S9) capsaicin activation of TRPV1, but it was inhibited when both PI4P and $PI(4,5)P_2$ were depleted by PJ (Figs 4B through D, and S9). Therefore, it appears that either lipid is sufficient for TRPV1 channel activity. However, this does not apply to all lipid-activated cation channels. For example, the menthol-activated transient receptor potential melatastatin 8 (TRPM8) channel is specifically dependent on $PI(4,5)P_2$ (12), and was inhibited by $PI(4,5)P_2$ depletion, but not by removing PI4P with PJ-Sac (Figs 4E through G).

Our results reveal an unanticipated role for PI4P at the PM of cells: it is not required to support synthesis of $PI(4,5)P_2$. Rather, PI4P makes an autonomous contribution to the polyanionic lipid pool that defines the inner leaflet of the PM, a function it shares with $PI(4,5)P_2$. We suggest that PI4P fulfils the need of any PM functions that simply require polyvalent anionic lipids. This leaves $PI(4,5)P_2$ free to undergo rapid turnover and regulate its large repertoire of specific effector proteins, which may decrease its effective free concentration, without deleteriously perturbing the unique and defining electrostatic properties of the PM.

Supplementary Material

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Acknowledgments

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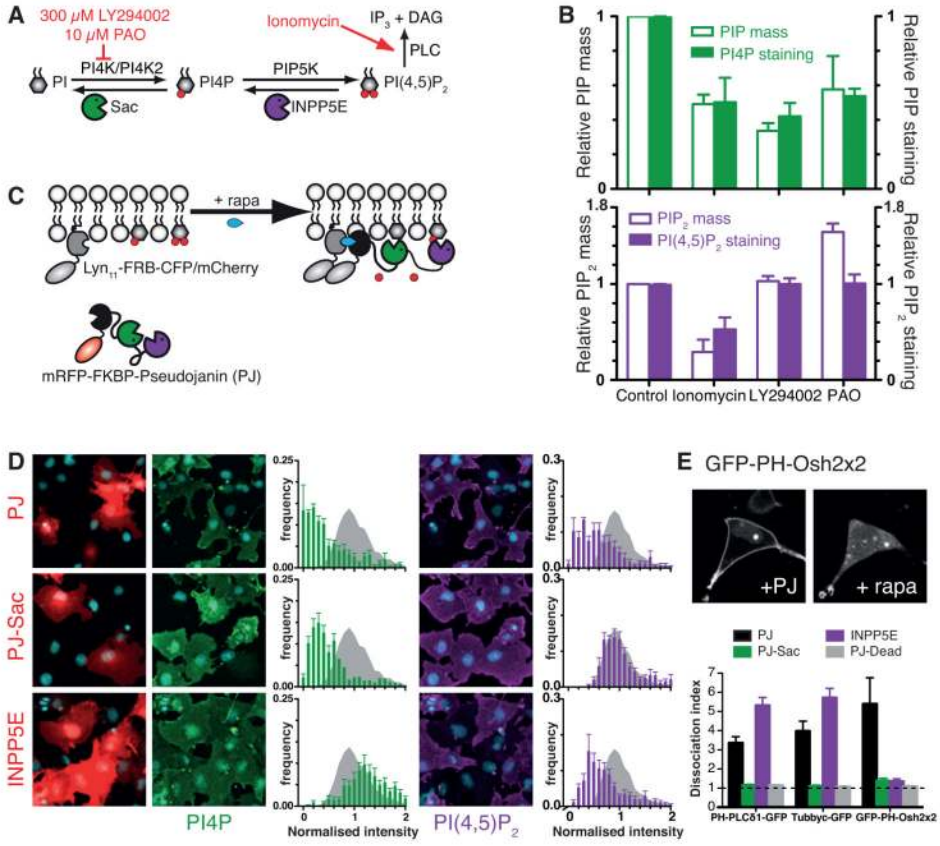

Fig. 1.

Independent depletion of PM PI4P and PI(4,5)P_2. (**A**) Synthesis of PI(4,5)P_2, and effects of inhibitors/activators. (**B**) Effect of LY294002, PAO and ionomycin on PI4P and PI(4,5)P_2 measured by mass spectrometry (open bars) or staining (filled bars; means ± SEM, n = 3-4). (**C**) Generation of Pseudojanin (PJ), a fusion of sac and INPP5E phosphatase domains with FKBP, and its rapamycin-induced recruitment to a PM targeted FRB domain (Lyn_{11}-FRB). (**D**) Effect of PJ, PJ-Sac (with inactivated INPP5E domain) or INPP5E (lacking the sac domain) on PI4P and PI(4,5)P_2 staining intensity after PM recruitment for 2 min with 1 μM rapamycin. Histograms are means ± SEM (n = 4-5); gray peaks are the frequency of occurrence of cells with the indicated staining intensity for mock-transfected cells. (**E**) Effect of PJ constructs on PM recruitment of PI4P/PI(4,5)P_2-binding GFP-PH-Osh2x2 (example images) and the PI(4,5)P_2-selective PH-PLCδ1 and $Tubby_c$ domains (means ± SEM of 10-18 cells).

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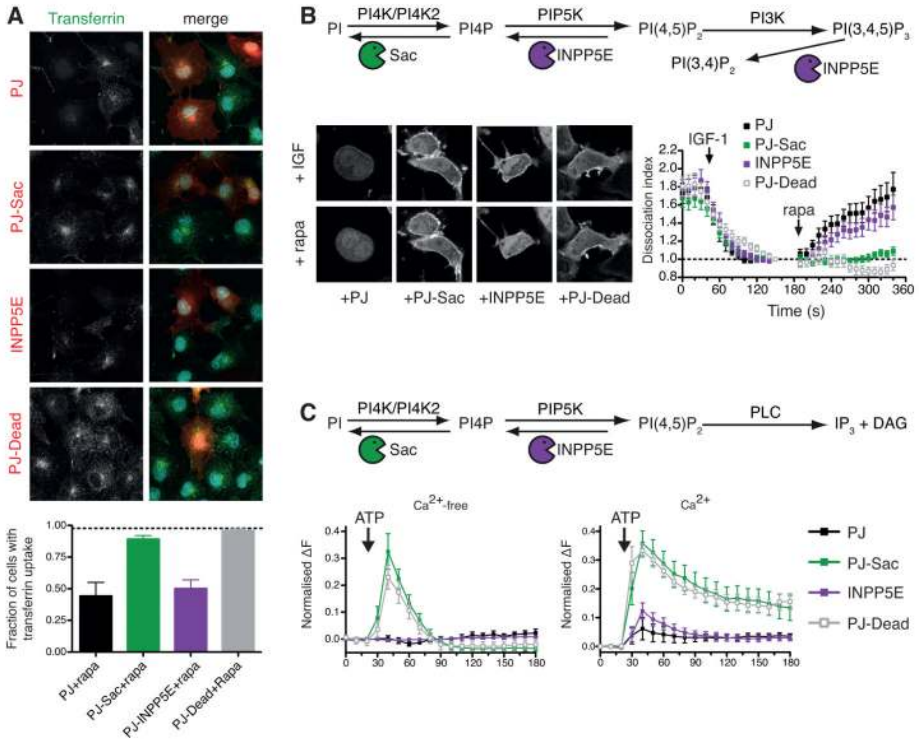

Fig. 2.

Dependence of clathrin-mediated endocytosis, PI3K and PLC signaling on PM PI(4,5)P_2, but not PI4P. (**A**) Effect of PM-recruited PJ constructs (red) on the uptake of transferrin (AlexaFluor®488, 20 μg/ml, green) over 15 min prior to removing surface bound transferrin with a pH 2.5 wash. The bar chart shows the proportion of cells showing uptake of transferrin-associated fluorescence. Data are means ± SEM ($n = 4$). (**B**) Effect of PM recruitment of PJ constructs on the PI(3,4)P_2/PI(3,4,5)P_3 reporter GFP-PH-Akt after stimulation of serum starved cells for 2 min with IGF-1. Data are means ± SEM from 15-26 cells. (**C**) Effect of PM-recruited PJ constructs on PLC-mediated Ca^{2+} signals (monitored with Ca^{2+} indicator Fluo4-AM) after stimulation of endogenous P2Y-receptors with 100 μM ATP in either calcium-free (100 μM EGTA) or Ca^{2+}-containing (1.8 mM) medium. Data are means ± SEM from 11-26 cells.

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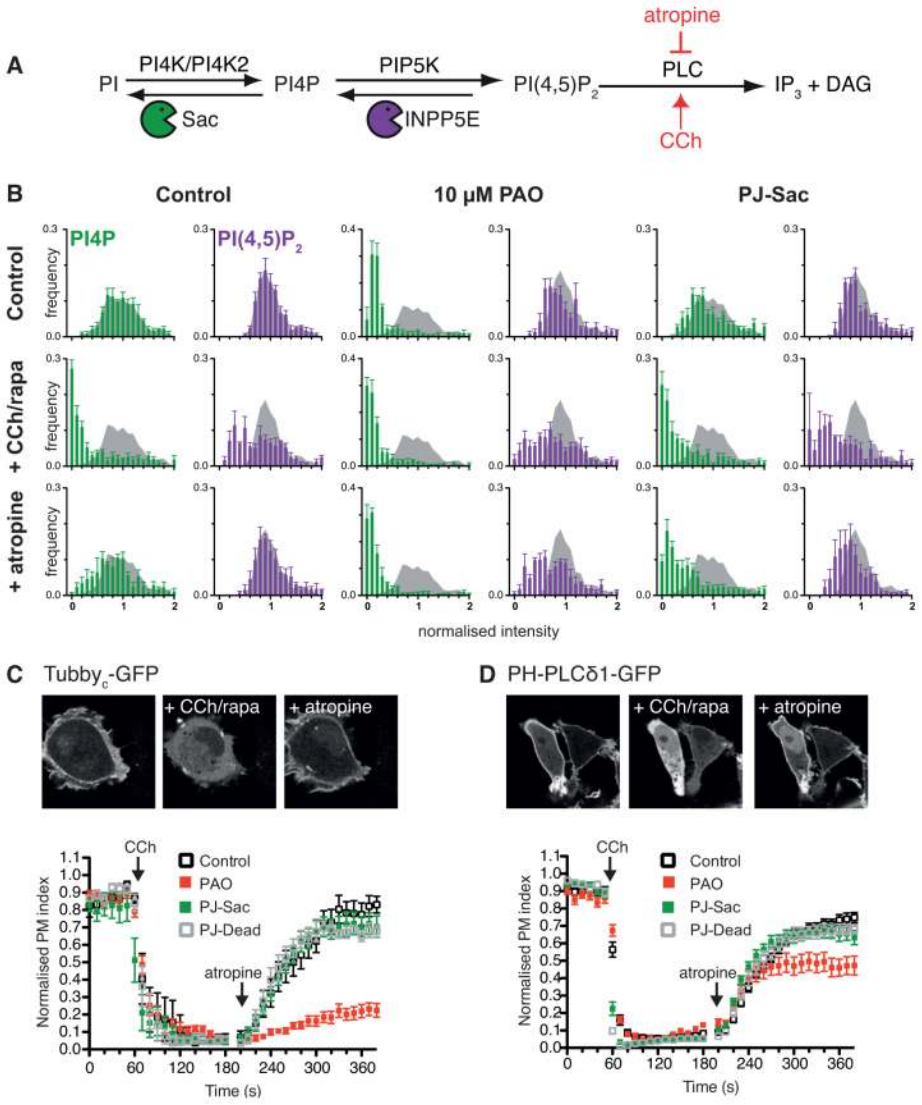

Fig. 3.

Requirements of PI4K activity but not PM PI4P for re-synthesis of $PI(4,5)P_2$ after robust PLC activation. (**A**) In muscarinic M1 receptor expressing cells, PLC activity is stimulated with carbachol (CCh) and inactivated by atropine. (**B**) Effect of PAO or PJ-Sac (recruited to the PM with rapamycin) on PI4P and $PI(4,5)P_2$ staining before, after 2 min 1mM CCh stimulation or after a further 3 min of 10 µM atropine treatment. Histograms are the relative frequency of occurrence of PI4P and $PI(4,5)P_2$ staining intensities expressed as means ± SEM ($n = 4$). Gray peaks are data from control, un-stimulated cells. (**C and D**) Effect of PAO or PJ-Sac recruited to the PM on $PI(4,5)P_2$ re-synthesis assayed with the $PI(4,5)P_2$ biosensors Tubby$_c$-GFP (**C**) or PH-PLCδ1-GFP (**D**) during stimulation with CCh and subsequent inhibition with atropine. Data are means ± SEM of 8-26 cells. Images show cells co-expressing the reporters with Lyn$_{11}$-FRB-CFP and PJ-Sac.

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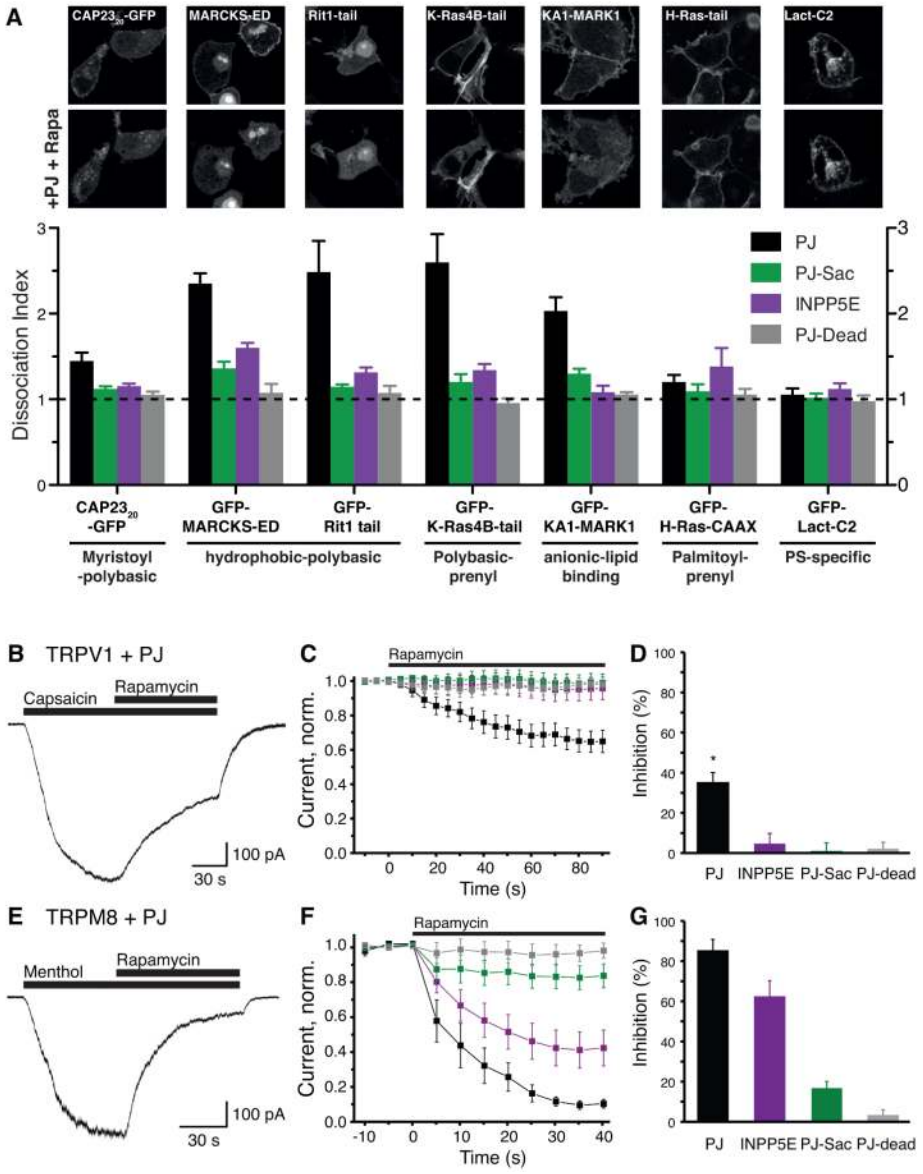

Fig. 4.

(**A**) Targeting of proteins with polybasic motifs to the PM by PI4P and PI(4,5)P_2. Representative images before and after rapamycin treatment, and dissociation index of cells transfected with the indicated GFP-tagged motifs, Lyn_{11}-FRB-CFP and the indicated PJ construct. Data are means ± SEM for 8-19 cells. (**B-D**) Capsaicin-induced currents in HEK293 cells expressing TRPV1 are enabled by the presence of either PI4P or PI(4,5)P_2. (**B**) Specimen showing rapamycin induced inactivation in the presence of Pseudojanin. (**C**) Time course of the capsaicin-induced current in the presence of Pseudojanin, INPP5E, PJ-Sac or PJ-Dead. (**D**) Percentage of inhibition at the end of the 90 s co-application of rapamycin 1 μM ($n = 8$). (**E-G**) Menthol-induced currents in HEK293 expressing TRPM8 depend primarily on the presence of PI(4,5)P_2. (**E**) Representative specimen showing the effect of Pseudojanin. (**F**) Time course of the menthol-induced current in the presence of Pseudojanin, INPP5E, PJ-Sac or PJ-Dead. (**G**) Percentage of inhibition at the end of the 90 s co-application of rapamycin 1 μM ($n = 7$).