Phosphatidylinositol 4,5-Biphosphate (PIP₂) Modulates Interaction of Syntaxin-1A with Sulfonylurea Receptor 1 to Regulate Pancreatic β-Cell ATP-sensitive Potassium Channels*

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**Background:** PIP₂ actions on activating K<sub>ATP</sub> channels are not only on Kir6.2 but may be also on syntaxin-1A, to modulate syntaxin-1A actions on SUR1.

**Results:** PIP₂ disrupts Syn-1A:SUR1 interactions by reducing syntaxin-1A availability to inhibit of K<sub>ATP</sub> channels.

**Conclusion:** PIP₂ modulates syntaxin-1A:SUR1 interactions.

**Significance:** Membrane phospholipid composition in health and diabetes profoundly affect β-cell K<sub>ATP</sub> channels by several mechanisms to influence insulin secretion.

In β-cells, syntaxin (Syn)-1A interacts with SUR1 to inhibit ATP-sensitive potassium channels (K<sub>ATP</sub> channels). PIP₂ binds the Kir6.2 subunit to open K<sub>ATP</sub> channels. PIP₂ also modifies Syn-1A clustering in plasma membrane (PM) that may alter Syn-1A actions on PM proteins like SUR1. Here, we assessed whether the actions of PIP₂ on activating K<sub>ATP</sub> channels is contributed by sequestering Syn-1A from binding SUR1. In vitro binding showed that PIP₂ dose-dependently disrupted Syn-1A:SUR1 complexes, corroborated by an in vivo Forster resonance energy transfer assay showing disruption of SUR1(-EGFP)/Syn-1A(-mCherry) interaction along with increased Syn-1A cluster formation. Electrophysiological studies of rat β-cells, INS-1, and SUR1/Kir6.2-expressing HEK293 cells showed that PIP₂ dose-dependent activation of K<sub>ATP</sub> currents was uniformly reduced by Syn-1A. To unequivocally distinguish between PIP₂ actions on Syn-1A and Kir6.2, we employed several strategies. First, we showed that PIP₂-insensitive Syn-1A-5RK/A mutant complex with SUR1 could not be disrupted by PIP₂, consequently reducing PIP₂ activation of K<sub>ATP</sub> channels. Next, Syn-1A-SUR1 complex modulation of K<sub>ATP</sub> channels could be observed at a physiologically low PIP₂ concentration that did not disrupt the Syn-1A:SUR1 complex, compared with higher PIP₂ concentrations acting directly on Kir6.2. These effects were specific to PIP₂ and not observed with physiologic concentrations of other phospholipids. Finally, depleting endogenous PIP₂ with polyphosphoinositide phosphatase synaptotagmin-1, known to disperse Syn-1A clusters, freed Syn-1A from Syn-1A clusters to bind SUR1, causing inhibition of K<sub>ATP</sub> channels that could no longer be further inhibited by exogenous Syn-1A. These results taken together indicate that PIP₂ affects islet β-cell K<sub>ATP</sub> channels not only by its actions on Kir6.2 but also by sequestering Syn-1A to modulate Syn-1A availability and its interactions with SUR1 on PM.

Pancreatic β-cell regulates glucose-stimulated insulin secretion through association with ATP-sensitive potassium channels (K<sub>ATP</sub> channels). The K<sub>ATP</sub> channel is a hetero-octamer of four Kir6.2 (inward rectifier K⁺ 6.2) subunits forming a conduction channel surrounded by four regulatory SUR1 subunits (1). β-Cell plasma membrane (PM) excitability and insulin secretion are set by concentration of nucleotides, ATP, and ADP (2, 3). The physiologic β-cell secretagogue is glucose, which, upon entry and metabolism in β-cells, increases ATP production, causing K<sub>ATP</sub> channel closure leading to cellular depolarization (4), which activates L-type voltage-dependent Ca<sup>2⁺</sup> channels, with resulting Ca<sup>2⁺</sup> influx triggering exocytotic fusion of insulin granules with PM (4, 5). Conversely, when plasma glucose levels fall, increase in ADP and decrease in ATP concentrations lead to K<sub>ATP</sub> channel activation, with ensuing PM hyperpolarization, which reduces insulin release.

In addition to adenine nucleotides, K<sub>ATP</sub> channels are regulated by other endogenous factors in β-cells, particularly phosphatidylinositol 4,5-bisphosphate (PIP₂). PIP₂, comprising only 1% of PM phospholipids, stimulates activity of ATP-sensitive and insensitive Kir channels by increasing channel open probability (6). PIP₂ is an indispensable membrane phosphoinositide that participates in a wide variety of other cellular func-

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ions, including production of second messengers, endo- and exocytosis, and regulation of ion channels, transporters, and actin cytoskeleton (7–11). For $K_{\text{ATP}}$ channel regulation, a negatively charged inositol triphosphate headgroup of PIP$_2$ interacts electrostatically with positively charged amino acid residues in N- and C-terminal cytoplasmic domains of Kir6.2 (12, 13). However, several studies demonstrated that the SUR subunit plays an essential role in stabilizing PIP$_2$-Kir6.2 interaction. For instance, a brief application of PIP$_2$ shifted ATP inhibition of Kir6.1/SUR1 channels compared with Kir6.2 alone, and this PIP$_2$ recovery was more stable when SUR1 was present, indicating that SUR increases PIP$_2$ binding and stimulation on Kir6.2 (14, 15). A recent report showed that a mutation in SUR1-TMD0 induces spontaneous Kir6.2 current decay and was reversed with exogenous PIP$_2$ (16). PIP$_2$ therefore plays versatile roles in controlling β-cell $K_{\text{ATP}}$ channel activities and insulin exocytosis (12, 17). Lin et al. (18) showed that disrupting $K_{\text{ATP}}$ channel and PIP$_2$ interaction by overexpressing PIP$_2$-insensitive Kir6.2 mutants caused cellular depolarization and elevated basal insulin secretion. Conversely, up-regulation of PIP$_2$ expression causing activation of $K_{\text{ATP}}$ channels resulted in cellular hyperpolarization, which reduced insulin secretion despite the presence of high glucose (18).

Besides the aforementioned actions of PIP$_2$ on various ion channels, PIP$_2$ also interacts with various components of the exocytotic fusion machinery, including CAPS, synaptotagmins, rabphilin, and Syn-1A (19–23). Syn-1A is one of three SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins that form the ternary complex constituting the minimal membrane fusion machinery in neurons and neuroendocrine cells (24). Besides its role in membrane fusion machinery, Syn-1A appears to play additional roles in the secretory process, effectively regulating various calcium and potassium channels involved in both initiating and terminating exocytosis (25).

In a body of work, we have shown that Syn-1A could act as an endogenous regulator exhibiting potent inhibitory action on β-cell $K_{\text{ATP}}$ channels (25–28). We identified specific conserved motifs within the nucleotide binding domains of SUR1 that functionally interact with Syn-1A (28). Syn-1A contains highly charged, polybasic juxtamembrane motif in which neutralizing mutations abrogated Syn-1A-PIP$_2$ electrostatic interaction, causing a reduction in exocytosis by influencing the clustering of Syn-1A molecules on PM required for efficient membrane fusion (21, 22). In this study, we investigated the hypothesis that these actions of PIP$_2$ on Syn-1A could influence Syn-1A interactions with SUR1 to affect $K_{\text{ATP}}$ channel activities in β-cells.

**EXPERIMENTAL PROCEDURES**

**Material**—Syn-1A mutant in which highly conserved polybasic juxtamembrane residues (within the cytoplasmic domain) between positions 260 and 265 (Lys260, -264, and -265; Arg262 and -263) constituting the major lipid-binding domain were mutated to alanine, called the Syn-1A-5RK/A mutant (gift from Ed Stuenkel, University of Michigan, Ann Arbor, MI), which abrogates Syn-1A binding to PIP$_2$ (29). A polyphosphoinositide 5-phosphatase synaptojanin 1 (HA-IPI-CAAX tagged with RFP) construct in pcDNA3 (a gift from Geert van den Bogart and Reinhard Jahn, Max-Planck Institute for Biophysical Chemistry, Gottingen, Germany) was used to deplete endogenous P(4,5)P$_2$ from the PM (30, 31).

**Pancreatic Islet β-Cell Isolation**—Single male Wistar rat pancreatic β-cells were enzymatically dispersed from isolated islets as described (32), plated on coverslips, and cultured in RPMI 1640 (supplemented with 2.8 mM glucose, 7.5% FCS, 0.25% sodium, 100 μg/ml streptomycin) prior to recordings.

**HEK293 Cell Transfection**—To examine the differences between Syn-1A-WT and Syn-1A-5RK/A, HEK293 cells were co-transfected with rat Kir6.2 (gift from S. Seino, Chiba University, Chiba, Japan) and SUR1-EGFP (gift from C. G. Nichols, Washington University, St. Louis, MO) and then trypsinized and plated on glass coverslips for 17–18 h prior to electrophysiological experiments. For FRET imaging recording, HEK293 cells were co-transfected with full-length Syn-1A (aa 1–288)-mCherry or full-length Syn-1A-5RK/A-mCherry (acceptor) and SUR1-EGFP (donor) as reported (33), along with Kir6.2, using Lipofectamine 2000 (Invitrogen). FRET imaging was conducted 2 days after transfection.

**In Vitro Binding Assay and Western Blotting**—In vitro binding assays were performed as described (34). Briefly, 250 pmol of GST (control) and GST-Syn-1A (aa 1–265) or GST-Syn-1A-5RK/A (aa 1–265), both containing only the cytoplasmic domain bound to glutathione-agarose beads, were incubated with lysate extract of HEK293 cells (400 μg of protein) co-transfected with SUR1 and Kir6.2 in lysis buffer in the presence of increasing concentrations of PIP$_2$ or other indicated phospholipids (Echelon Biosciences Inc.) at 4°C for 2 h with constant agitation. Beads were washed three times, and samples were separated on 10% SDS-PAGE, transferred to nitrocellulose membrane, and identified with anti-SUR1 antibody (1:1,000; gift from J. Ferrer, Barcelona, Spain).

**Electrophysiology**—$K_{\text{ATP}}$ channel recordings were performed on INS-1E cells using the inside-out patch clamp technique (35) and on rat β-cells and HEK293 cells using the whole-cell patch-clamp technique. Pipette resistance when filled with solution was 1.0–1.5 megaohms. GST, GST-Syn-1A, ATP (Sigma-Aldrich) and PIP$_2$ (Sigma-Aldrich) were perfused onto the cytoplasmic side of excised membrane patches. Membrane patches were held at −50 mV to evoke inward currents. For β-cell, HEK293, and INS-1 cell voltage-clamped whole-cell studies, membrane potential was held at −70 mV, and a pulse of −140 mV (500 ms) was given every 10 s to monitor $K_{\text{ATP}}$ current magnitude. Pipette resistance was 2–4 megaohms. Bath solution contained 140 mM NaCl, 4 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 10 mM HEPES, 2 mM glucose, pH 7.3. Pipette solution contained 140 mM KCl, 1 mM MgCl$_2$, 1 mM EGTA, 10 mM HEPES, pH 7.25. GST, GST-Syn-1A, and PIP$_2$ were added to intracellular solution for dialysis into cells via patch pipette. Tolbutamide (0.1 mM; Tolb) was perfused into bath solution after maximum current reached to completely inhibit and verify the $K_{\text{ATP}}$ current. All recordings were carried out at 22–24°C using an EPC10 amplifier with Pulse version 8.77 acquisition software (HEKA Electronik, Lambrecht, Germany). Data were sampled at 1 kHz.

**FRET Imaging**—As described previously (33), FRET study by total internal reflection fluorescence microscopy (TIRFM)
assesses molecular interactions on the surface of PM, avoiding contamination from intracellular FRET signals. HEK293 cells were transfected with different combinations of plasmids 2 days prior to the experiment, where EGFP fused with SUR1 was used as the FRET donor, and mCherry fused with full-length Syn-1A or full-length Syn-1A-5RK/A was used as the FRET acceptor; Kir6.2 co-infected to express functional K\textsubscript{ATP} channels localized correctly on PM. For FRET analysis, four images, including donor excitation/donor emission (DD), donor excitation/acceptor emission (DA), acceptor excitation/donor emission (AD), and acceptor excitation/acceptor emission (AA), were acquired under same conditions. Donor-only and acceptor-only samples were acquired at the beginning of each experiment for bleed-through calculation. FRET efficiency was used to indicate interaction of the two proteins, calculated with the equation, FRET efficiency \% = \left(\frac{((FRET_{raw} - (CoB \times Dd_{FRET})) - (CoA \times Ad_{FRET}))}{(Dd_{FRET})}\right) \times 100\%, where CoB is the amount of donor bleed-through in the absence of acceptor, and CoA is the amount of acceptor bleed-through in the absence of donor.

After baseline FRET images were taken, the cells were permeabilized with digitonin (10 \mu g/ml in intracellular buffer, 5 min, 37 °C). FRET images were then taken again, followed by perfusion with the indicated lipids for another 5 min, and then we waited for another 7–10 min before the final FRET images were captured. Intracellular buffer contained 20 mM HEPES, 5 mM NaCl, 140 mM potassium gluconate, and MgCl\textsubscript{2}, pre-equilibrated with O\textsubscript{2}/CO\textsubscript{2} = 95:5, pH 7.4, at 37 °C. PIP\textsubscript{2} powder dissolved in double-distilled H\textsubscript{2}O, was sonicated for 30–45 s to a stock concentration of 920 mM (per the manufacturer’s instructions). This PIP\textsubscript{2} stock was diluted to the indicated concentrations in intracellular buffer and sonicated again for 30 s before adding to the cells.

**Statistical Analysis**—For statistical analysis of FRET efficiency, regions of interest were drawn around entire areas of the PM surface expressing any FRET signal (blue, green, or red; see the pseudocolor bar in Figs. 5, 7, and 9) as indicated, not including the purple areas having no FRET signal. From these regions of interest, FRET efficiency was calculated as mean \pm S.E., and values were compared using the Mann-Whitney test by SigmaStat version 3.1 (Systat Software Inc., Chicago, IL). For electrophysiological experiments, data analysis was done using SigmaPlot version 11.0 (Systat Software Inc.). Data obtained from concentration-response curves were fitted to the drug responsiveness equation, \( Y = \left(\frac{(A1 - A2)/(1 + (X \times X0)p)}{A2}\right) + A2 \). Where \( Y \) is K\textsubscript{ATP} current at different [PIP\textsubscript{2}], \( A1 \) is K\textsubscript{ATP} current before PIP\textsubscript{2} application, \( A2 \) is maximal K\textsubscript{ATP} current activated by PIP\textsubscript{2}, \( X \) denotes [PIP\textsubscript{2}] applied to membrane patches, \( X0 \) denotes [PIP\textsubscript{2}] that produced half-maximal K\textsubscript{ATP} channel activation, and \( p \) is slope of the curve. Curve fitting was performed by Origin version 6.0. Inside-out electrophysiological data were analyzed using each cell as its own control. Whole-cell electrophysiological data were presented as mean \pm S.E., expressed as current normalized to cell capacitance (pA/pF). For multiple groups, channel activity was compared using one-way analysis of variance, followed by the Student–Newman–Keuls post hoc test. For the binding assay and Western blotting, blots were quantified by densitometry scanning followed by analysis with Scion Image (beta-4.0.2, Scion Corp.). Data were compared using Student’s \( t \) test. We considered \( p < 0.05 \) as a significant difference.

**RESULTS**

**PIP\textsubscript{2}, Dose-dependently Inhibits Syntaxin-1A Binding to SUR1**—A substantial body of evidence indicates that highly negatively charged membrane phosphatidylinositol polyphosphates interact with positively charged residues on the N and C termini of the cytoplasmic domains of Kir channels (14, 35, 36). In addition, it is well established that highly charged, polybasic juxtamembrane regions of Syn-1A interact with PIP\textsubscript{2} (29); thus, the SNARE fusion machinery itself may be a target of regulation by phosphoinositides (22, 29, 37). Although there is strong evidence for the role of PIP\textsubscript{2} on Kir6.2 and SNARE protein interactions, we here explored the possibility that PIP\textsubscript{2} could activate K\textsubscript{ATP} channels in a manner contributed by its actions on Syn-1A, which, in turn, perturbs Syn-1A binding to SUR1.

We examined whether increasing PIP\textsubscript{2} concentrations can disrupt Syn-1A binding to SUR1 by employing in vitro binding assays. GST-Syn-1A-bound glutathione-agarose beads were used to pull down SUR1 from lysate extracts of HEK293 cells co-infected with SUR1 and Kir6.2. After adding increasing PIP\textsubscript{2} concentrations (Fig. 1A), SUR1 binding to GST-Syn-1A did not decrease at 0.5 \mu M but was reduced by 41% at a physiologic concentration of 10 \mu M (38) and by 65% at 20 \mu M (n = 3; Fig. 1A). GST, as negative control, did not pull down SUR1. Importantly, GST-Syn-1A contains the cytoplasmic domain (aa 1–265) and no transmembrane domain (aa 266–288), and the PIP\textsubscript{2}-binding domain is located at aa 260–265 (29) indicates that PIP\textsubscript{2} binding of this Syn-1A cytoplasmic domain is sufficient to sequester Syn-1A and disrupt Syn-1A-SUR1 interactions. To demonstrate that these effects are selective to PIP\textsubscript{2} (PI(4,5)P\textsubscript{2}, 38), we further assessed the effects of PI(3,5)P\textsubscript{2} (Fig. 1B), which has a negative charge similar to PI(4,5)P\textsubscript{2} and PIP\textsubscript{3} (PI(3,4,5)P\textsubscript{3}; Fig. 1C), which has a stronger negative charge. At a physiologic concentration of 10 \mu M, the other phospholipids had little (PI(3,5)P\textsubscript{2}; reduced by 17%, not significant) or no effect (PI(3,5)P\textsubscript{2}) on Syn-1A-SUR1 binding. At the highest concentration of 20 \mu M, these phospholipids disrupted Syn-1A-SUR1 binding but to a much lesser extent than PI(4,5)P\textsubscript{2} (at 65% reduction), for PI(3,5)P\textsubscript{2} (at 51% reduction) or PIP\textsubscript{3} (at 44% reduction). Importantly, PIP\textsubscript{3} is abundant in mammalian cells, whereas the other phospholipids are minor and not likely to reach these high concentrations (38). We then proceeded to dissect the functional implications PIP\textsubscript{2} disruption of Syn-1A-SUR1 interactions.

**PIP\textsubscript{2}, Activation of Kir6.2/SUR1 Channels Is Reduced by Syn-1A**—We examined PIP\textsubscript{2} dose-response activation of the K\textsubscript{ATP} channel on INS-1E cells in the absence and presence of 1 \mu M GST-Syn-1A using an inside-out patch clamp technique. Membrane patches were held at −50 mV to induce inward currents. Fig. 2, A and B, shows representative traces of the protocol utilized for PIP\textsubscript{3} in the absence and presence of Syn-1A. Membrane patches were initially exposed to 0, 1, and 3 mM ATP K\textsubscript{int} solution to characterize K\textsubscript{ATP} channels and verify the ATP sensitivity of recorded currents. In Fig. 2A, after patch excision, channels rapidly run down in the absence of ATP; however, subsequent exposure to 5, 10, and 20 \mu M PIP\textsubscript{2} gradu-
ally recovered the currents to the level observed immediately after patch excision. Consistent with previous reports, prior to PIP2 applications, both 1 and 3 mM ATP inhibited KATP channels; however, three subsequent applications of PIP2 decreased ATP sensitivity and completely abolished the inhibitory effect of 1 mM ATP, as reported previously (14). Of note, Fig. 2B shows that concomitant application of GST-Syn-1A and PIP2 did not completely recover the channels after rundown. In addition, in the presence of GST-Syn-1A, reapplication of ATP after PIP2 activation did not produce any less current inhibition. In Fig. 2C, adding GST-Syn-1A greatly reduced PIP2-mediated channel activation, causing a rightward shift of the dose response. EC50 values for PIP2 in the absence and presence of GST-Syn-1A are 2.38 ± 0.81 (n = 5) and 9.64 ± 0.14 (n = 3), respectively. Our results indicate that exogenously added GST-Syn-1A inhibits KATP channels through direct binding and interaction with SUR1 at its cytoplasmic nucleotide-binding folds (NBF-1 and NBF-2), as we reported previously (34), and that Syn-1A regulates KATP channels through PIP2 interactions. For the latter finding, our results suggest that the exoge-

**FIGURE 1. Exogenously added PIP2 disruption of Syn-1A-SUR1 complex formation.** GST-Syn-1A-WT or GST-Syn-1A-5RK/A and GST (as control) were used to pull down SUR1 from HEK293 cells co-transfected with SUR1 and Kir6.2 in presence of indicated concentrations of the following inositol phospholipids: PIP2 (PI(4,5)P2 in A), PI(3,5)P2 (B), and PI(3,4,5)P3 (C). i (in A–C), representative blots showing the effects of increasing inositol phospholipid concentrations in disrupting Syn-1A binding to SUR1 (top) but not Syn-1A-5RK/A binding to SUR1 (bottom). ii (in A–C), summary of three separate experiments, with each band normalized as a percentage of the input (400-μg protein of HEK cell lysate extract; see “Experimental Procedures”). Data are expressed as mean ± S.E. (error bars); *, p < 0.05. NS, not significant. D, 20 μg of protein of GST-Syn-1A or GST-Syn-1A-5RK/A and control GST uniformly used in all of the samples of these experiments was assessed by Ponceau S staining. A representative sample in D shows equal amounts used. i, GST-Syn-1A WT; ii, GST-Syn-1A-5RK/A.
nous PIP2 bound GST-Syn-1A (as shown in the binding study in Fig. 1A) rather than becoming incorporated into the PM to affect endogenous PM-bound Syn-1A. From Fig. 2C, we selected 10 μM PIP2 to further study and analyze PIP2-Syn-1A interactions and their effects on KATP channel activities in the studies in Figs. 3 and 4.

We performed the converse experiment of inside-out recordings of INS-1E cells perfused with GST-Syn-1A (1 μM; indicated by black circles above the current traces) in the presence or absence of PIP2 (10 μM; indicated by solid bars). The summarized results are expressed as percentages of maximum current elicited at 0 mM ATP K_{inw} solution determined in each patch. Fig. 3Ai shows the representative K_{ATP} current tracing of GST with PIP2, and the corresponding summary data are shown in Fig. 3Aii (n = 5). Here, K_{ATP} currents underwent spontaneous decay in the absence of ATP; subsequent perfusion of PIP2 in the continuous presence of GST increased channel activities. When comparing K_{ATP} currents between GST alone (87.9 ± 3.62% of control maximal current) and subsequent perfusion of GST and PIP2, PIP2 exposure led to a pronounced increase in K_{ATP} current (108.06 ± 7.77%), which appeared larger but not significantly different from the initial current immediately after patch excision (0 mM ATP K_{inw}) (Fig. 2B). These results indicate that after patch excision, K_{ATP} channels undergo spontaneous rundown, and subsequent PIP2 exposure led to recovery of channel activity. In Fig. 3, Bi and Bii), administration of GST-Syn-1A alone reduced maximum control K_{ATP} currents by ~65% (n = 5). When comparing K_{ATP} currents between GST-Syn-1A alone and subsequent

FIGURE 2. Inside-out patch recording of INS-1 cells showing that PIP2 dose-dependent activation of K_{ATP} channels is reduced by Syn-1A. Shown are representative traces of the protocol utilized for PIP2 (as shown) in the absence (A) or presence (B) of 1 μM GST-Syn-1A. Membrane patches were initially exposed to 0, 1, and 3 mM ATP K_{inw} solution to characterize K_{ATP} channels and verify the ATP sensitivity of the currents recorded. C, PIP2 dose-dependent activation of K_{ATP} current in the absence (n = 5) and presence of Syn-1A (n = 3). Results are mean ± S.E. (error bars).

FIGURE 3. Inside-out patch recording of INS-1 cells showing PIP2 activation of K_{ATP} currents exceeds PIP2-mediated recovery of Syn-1A inhibition of K_{ATP} currents. Representative K_{ATP} current tracings of 1 μM GST with 10 μM PIP2 (A) and 1 μM GST-Syn-1A with 10 μM PIP2 (Bi) and their respective summary data (Aii and Bii; n = 5 cells each) of the maximum current in 0 mM ATP K_{inw} solution. Here, membrane patches were initially exposed to 0 and 1 mM ATP K_{inw} solution. Results are mean ± S.E. (error bars); *, p < 0.05; NS, not significant.

FIGURE 4. Whole-cell recording of rat islet β-cells showing PIP2 actions on Syn-1A to functionally disrupt Syn-1A-SUR1 interactions. A, representative K_{ATP} currents with 1 μM GST (i), 1 μM GST-Syn-1A (ii), or 1 μM GST-Syn-1A plus 10 μM PIP2 (iii) dialyzed into β-cells. Tolbutamide (Tolb; 0.1 mM) was added to verify the K_{ATP} current. B, summary data of A showing maximum current density with 1 μM GST (n = 6), 1 μM GST-Syn-1A (n = 8), or 1 μM GST-Syn-1A plus 10 μM PIP2 (n = 11). Results are mean ± S.E. (error bars); *, p < 0.05.
concomitant perfusion of GST-Syn-1A and PIP₂, PIP₂ recovered SYN-1A-inhibited currents to 89.6 ± 10.52% of maximal currents (n = 5), but the currents never reached that seen immediately after patch excision. These results taken together indicate that PIP₂ activation of K_{ATP} currents exceeds PIP₂-mediated recovery of SYN-1A inhibition of K_{ATP} currents. This is probably because of PIP₂ direct actions on the Kir6.2 subunit (12, 13).

**PIP₂ Acts on SYN-1A to Functionally Disrupt SYN-1A-SUR1 Interactions in Rat β-Cells**—To delineate the physiological relevance of our findings in INS-1E, we employed rat islet β-cells using whole-cell patch clamp recordings. 1 μM GST, 1 μM GST-SYN-1A, or a combination of 1 μM GST-SYN-1A and 10 μM PIP₂ was dialyzed into β-cells via a patch pipette. In the presence of 1 μM GST only (Fig. 4, Aii and B), K_{ATP} currents gradually developed in β-cells, reaching a maximum current density of 118.97 ± 13.79 pA/pF (n = 6). In contrast, dialyzing 1 μM GST-SYN-1A (Fig. 4, Aii and B) reduced K_{ATP} channel density (65.16 ± 8.62 pA/pF, n = 8). In accordance with the aforementioned INS-1E studies, concomitant dialysis of 1 μM GST-SYN-1A and 10 μM PIP₂ (Fig. 4, Aiii and B) gave a maximum current density of 133.48 ± 13.41 pA/pF (n = 11). These results, taken together with the results in Figs. 1–3, suggest that exogenous PIP₂ and GST-SYN-1A seemed to bind and sequester each other from acting on the K_{ATP} channel, with PIP₂ sequestration of SYN-1A-SUR1 interactions and GST-SYN-1A sequestration of PIP₂ reducing PIP₂ actions on Kir6.2.

**PIP₂-insensitive SYN-1A-5RK/A Mutant Reduces PIP₂ Opening of K_{ATP} Channels**—Mutations at the SYN-1A juxtamembrane five basic residues (Lys-260, -264, and -265 and Arg-262 and -263), called SYN-1A-5RK/A, rendered SYN-1A PIP₂-insensitive (29). The postulate is that if SYN-1A-5RK/A mutant inhibitory actions on K_{ATP} channels would not be disrupted by PIP₂, then PIP₂ activation of K_{ATP} channels may be reduced. Furthermore, the contribution of SYN-1A inhibitory action on SUR1 may be able to oppose the direct actions of PIP₂ on Kir6.2 in opening the channel.

We first examined the effects of PIP₂ on SYN-1A-5RK/A binding to SUR1 employing two assays, an *in vitro* binding assay (Fig. 1A) and *in vivo* FRET assay (Fig. 5). In contrast to PIP₂ dose-dependent disruption of GST-SYN-1A-WT protein binding to SUR1 (in SUR1/Kir6.2-expressing HEK cells), GST-SYN-1A-5RK/A (containing only cytoplasmic domain aa 1–265) binding to SUR1 remained intact (i.e. unperturbed by PI(4,5)P₂ (Fig. 1A), PI(3,5)P₂ (Fig. 1B), or PI(3,4,5)P₃ (Fig. 1C)). When the amount of GST-SYN-1A binding to SUR1 was assessed as a percentage of total HEK cell SUR1 input content, it appeared that GST-SYN-1A-5RK/A-SUR1 complexes (16.28 ± 1.86, n = 3) were 27% higher than GST-SYN-1A-SUR1 complexes (12.86 ± 1.49; Fig. 1A). These results indicate that SYN-1A is able to bind SUR1 tightly in *in vitro* in the absence of PIP₂ and that SYN-1A binds SUR1 at other H3 domains (27) outside the PIP₂-binding domain.

The previous studies in Figs. 1–4 examined the effects of exogenously added GST-SYN-1A (containing only the cytoplasmic domain) and PIP₂ on K_{ATP} channels. Live cell FRET imaging analysis enables the examination of full-length SYN-1A-mCherry versus full-length SYN-1A-5RK/A-mCherry and SUR1-EGFP expressed in HEK293 cells. Here, we assessed whether PIP₂ can disrupt their *in vivo* (thus physiological) interactions on PM by TIRF imaging (Fig. 5), which optically isolates the PM surface (see “Experimental Procedures”). HEK293 cells were permeabilized with digitonin to permit entry of PIP₂ (10 μM). Of note, the addition of this physiologic PIP₂ concentration seemed to increase the fluorescence intensity of the SYN-1A-mCherry hotspots (indicated by arrows), suggesting an increase in SYN-1A-mCherry cluster formation in these PM areas (Fig. 5A, top images). Remarkably, in the same experiment (Fig. 5A, bottom images), the 0 μM PIP₂ disrupted SYN-1A-SUR1 complexes (indicated by arrowheads), shoving a reduction of FRET efficiency from 30.69 ± 2.2 to 15.38 ± 1.3 (Fig. 5, A and C). This was a ~50% reduction, which, when taken with the similar disruption of GST-SYN-1A-SUR1 complexes in the protein-binding study (Fig. 1A), suggests that PIP₂ disruption of SYN-1A-SUR1 complexes seems to “free” more SYN-1A molecules to participate in the formation of SYN-1A clusters, the latter also promoted by PIP₂. Remarkably, PIP₂ did not disrupt SYN-1A-5RK/A-SUR1 complexes (indicated by arrowheads in Fig. 5B; no PIP₂, 37.14 ± 3.1; with PIP₂, 35.42 ± 1.7 (Fig. 5C)). We noted larger areas of SYN-1A-5RK/A-SUR1 FRET fluorescence (Fig. 5B) than SYN-1A-SUR1 fluorescence (Fig. 5A, bottom). Thus, we calculated the fluorescence area against total PM area (Fig. 5D) and found that the SYN-1A-RK/A-SUR1 FRET area occupied 31.9 ± 4.9%, which is 198% of the SYN-1A-SUR1 FRET area of 16.1 ± 3.7%. Along with the binding studies in Fig. 1A, it seems that the SYN-1A-5RK/A mutation increased its abundance on the PM from increased formation of SYN-1A-5RK/A-SUR1 complexes. Last, we noticed that the locations of SYN-1A-SUR1 FRET signals (Fig. 5A, bottom; indicated by arrowheads) were mostly not colocalized with the areas with abundant SYN-1A-mCherry (Fig. 5A, top and bottom; indicated by arrows), an important point that we discuss further below along with the results in Fig. 9. In these studies, we were a little surprised by these strongly positive results because we had initially expected that exogenously added PIP₂ would not incorporate substantially into the PM (30), thus exhibiting lesser effects on the SYN-1A-SUR1 interactions in the PM. The most likely explanation is that PIP₂ incorporates into PM through lipid tails over time, which would occur with greater frequency the longer duration PIP₂ in solubilized solution is exposed to the interior surface of the cell. Another explanation is that digitonin (used for permeabilization) affects PM cholesterol in a manner that could influence PIP₂ incorporation into PM or increase the sensitivity to PIP₂ promotion of SYN-1A cluster formation in the PM (21, 22). Digitonin permeabilization (prior to the addition of PIP₂), however, did not independently affect SYN-1A cluster formation or SYN-1A-SUR1 FRET interactions (Fig. 5A). Alternatively, PM permeabilization might have led to inadvertent depletion of some cytosolic factors that can influence the state of SYN-1A (free versus complexed) or the sensitivity of SYN-1A-SUR1 complex disruption by PIP₂.

We then determined the functional implications of these binding studies by examining whether PIP₂ activation of K_{ATP} channels in INS-1 cells would be perturbed by the PIP₂-insensitive mutation of SYN-1A. As shown in Fig. 6A (analysis in Fig.
(48), dialyzing INS-1 cells with standard pipette solution displayed a $K_{\text{ATP}}$ channel current density of $100.3 \pm 18.7 \, \text{pA/pF}$ ($-140 \, \text{mV}$ stimulation, used as control in Fig. 6C). Under identical conditions, overexpression of full-length Syn-1A-WT plasmid in INS-1 cells (Fig. 6A, left traces) reduced $K_{\text{ATP}}$ channel current density to $29\%$ ($29.1 \pm 4.2 \, \text{pA/pF}$; Fig. 6C) of control. Application of $1 \, \mu\text{M} \, \text{PIP}_2$ (a physiologically lower concentration than $10 \, \mu\text{M} \, \text{PIP}_2$ used in previous binding and functional studies) blunted the overexpressed Syn-1A inhibition to $60\%$ of control ($60.2 \pm 5.4 \, \text{pA/pF}$). In contrast, overexpression of Syn-
assembly in Fig. 1A), we found no differences in \( K_{\text{ATP}} \) channel activities between control, Syn-1A-WT, and Syn-1A-5RK/A (Fig. 6B; analysis in Fig. 6D). These results indicate that at higher PIP2 concentration, direct effects of PIP2, on the Kir6.2 subunit predominate over the effects of PIP2 on Syn-1A-5RK/A (and Syn-1A-WT) and SUR1 interaction to open \( K_{\text{ATP}} \) channels, although this higher PIP2 dosage remained unable to disrupt the abundant Syn-1A-5RK/A/SUR1 complexes (Figs. 1A and 5, C–E). These results modified our original thinking to suggest several modes by which PIP2 activates \( K_{\text{ATP}} \) channels in insulin-secreting \( \beta \)-cells: one at physiologic low PIP2 concentration acting on Syn-1A at its PIP2-binding site that finely modulates Syn-1A/SUR1 interactions and one at higher PIP2 concentrations, which act on \( K_{\text{ATP}} \) channels by two mechanisms, first by directly binding Kir6.2 and second by sequestering Syn-1A molecules into Syn-1A clusters, which reduces the availability of free Syn-1A molecules to bind SUR1 and could disrupt Syn-1A/SUR1 complexes.

Specificity of Cellular Inositol Phospholipids in Modulating Syn-1A/SUR1 Complex Disassembly and \( K_{\text{ATP}} \) Channel Activity—We next assessed whether other abundant cellular phospholipids might similarly affect Syn-1A/SUR1 complexes (Fig. 7) to modify \( K_{\text{ATP}} \) channel activity (Fig. 8) and whether this is mainly attributable to the negative charge of phospholipids purported to bind positively charged juxtamembrane polybasic residues of Syn-1A (14, 35, 36). FRET imaging assessment showed that 10 \( \mu \)M phosphatidylcholine (0 net charge) and phosphatidyl-L-serine (less negative charge than PIP2) did not affect Syn-1A/SUR1 complex formation (Fig. 7, A, B, and D) or HEK293 \( K_{\text{ATP}} \) channel activities (Fig. 8, A, B, C, and E). Inositol trisphosphate (IP3), which has a larger negative charge compared with PIP2 (38), caused only a minor disruption (24%) in Syn-1A/SUR1 complexes (Fig. 7, C and D; without IP3, 33.05 ± 2.9; with IP3, 24.91 ± 3.9) but did not significantly affect \( K_{\text{ATP}} \) channel activity (Fig. 8, D and E). These results indicate that the PIP2 effects on Syn-1A/SUR1 interactions that modulate \( K_{\text{ATP}} \) channel activity are specific.

Effects of PIP2 Depletion from PM on Syn-1A/SUR1 Complex Formation and \( K_{\text{ATP}} \) Channels—Our experiments above so far have used exogenous PIP2 to alter Syn-1A/SUR1 interactions. Synaptotagmin-1 is a polyphosphoinositide 5-phosphatase, which, when overexpressed using the construct HA-IPPC1-CAXX, was reported to deplete endogenous PIP2 from the PM, causing disruption of the Syn-1A clusters on the PM (30, 31). Consistently, expressing this synaptotagmin-1 construct to deplete endogenous PIP2 (in Fig. 9A), we saw a reduction of the larger hotspots (suggesting clusters, indicated by arrowheads in the top images) of Syn-1A-mCherry fluorescence (Syn-1A-mCherry expressed in HEK cells) to small fluorescence spots (indicated by arrowheads in the bottom images), indicating a dispersion of Syn-1A molecules from Syn-1A clusters; this is similar to the previous report using this construct (31). Further intensity profile analysis of cross-sections of the indicated regions of these images shows that in the absence of synaptotagmin-1 (Fig. 9B, pink line), there was sustained high intensity Syn-1A-mCherry fluorescence suggesting Syn-1A clusters, whereas in the cell treated with synaptotagmin-1, Syn-1A-mCherry fluorescence appeared as narrow spikes, indicating either dispersed Syn-1A molecules

**Syn-1A-PIP2 Interaction in SUR1/\( K_{\text{ATP}} \) Regulation**
or much smaller clusters of Syn-1A molecules (Fig. 9C, blue line). These results are consistent with Fig. 5A (top images), where the addition of exogenous PIP$_2$ appeared to increase Syn-1A-mCherry clustering on PM. Synaptojanin-1-induced depletion of endogenous PIP$_2$ resulted in increased FRET signals (59.3 ± 6.2%; Fig. 9D) compared with the absence of synaptojanin-1 (35.6 ± 7.1%; note more green to red in +synaptojanin-1 versus blue to green in −synaptojanin-1 in Fig. 9A), indi-
cating increased Syn-1A-SUR1 complex formation in the PM. Of note, in −synaptojanin-1 cells, the FRET signals (indicated by arrows) were mostly located away from the Syn-1A-mCherry clusters (indicated by arrowheads; Fig. 9A), as was similarly observed in Fig. 5A. In contrast, in +synaptojanin-1 cells, the FRET signals were mostly in small Syn-1A-mCherry hotspots (arrows and arrowheads point to the same hotspots). These results led us to further strengthen our thinking that PIP2 depletion releases Syn-1A molecules from the large Syn-1A clusters to migrate away from the cluster to other PM sites where SUR1 molecules are located to then form Syn-1A-SUR1 complexes.

We then examined the functional implications of the endogenous PIP2 depletion that we showed to increase Syn-1A-SUR1 complex formation. In Fig. 10, A and B, INS-1 cells transfected with synaptojanin-1 caused a 63% reduction of KATP current, from 148.35 ± 23.2 pA/pF (control, n = 7) to 55.38 ± 11.2 pA/pF (synaptojanin-1, n = 9; p < 0.01). The application of exogenous GST-Syn-1A (1 μM) to the synaptojanin-1-transfected cells showed a KATP current of 51.54 ± 9.3 pA/pF (n = 7; p < 0.05), which is a 65% reduction compared with control. This is very similar to the effects of synaptojanin-1 treatment alone, indicating that exogenously added GST-Syn-1A could not further reduce the KATP current that was already inhibited by the PIP2 depletion caused by synaptojanin-1 treatment. Taken together, these results suggest that the enhanced “endogenous” Syn-1A-SUR1 complex formation caused by PIP2...
Syn-1A-PIP₂ Interaction in SUR1/KₐₐₜP Regulation

**A**

![Control](image1.png)

**B**

![GST-Syn-1A](image2.png)

FIGURE 10. PIP₂ depletion from PM disables exogenous Syn-1A from further inhibiting KₐₐₜP channels. A, representative KₐₐₜP channel current tracings of control, GST-Syn-1A (1 µM infused into cells via patch pipette), synaptotagmin-1, and synaptotagmin + GST-Syn-1A in INS-1 cells. B, summary results showing that exogenous GST-Syn-1A could not cause further inhibition of KₐₐₜP channels when endogenous PIP₂ levels were depleted by synaptotagmin-1. Results are mean ± S.E.; *, p < 0.05; **, p < 0.01. NS, not significant.

depletion must have had all of the SUR1 sites occupied by endogenous Syn-1A to have resulted in optimal inhibition of KₐₐₜP channels, leaving no available SUR1 sites for the exogenous GST-Syn-1A to bind and further inhibit KₐₐₜP channels.

**DISCUSSION**

In this work, we showed that the actions of PIP₂ on activating pancreatic islet β-cell KₐₐₜP channel are contributed by alteration of Syn-1A interactions with SUR1, in addition to the known actions of PIP₂ on Kir6.2 (14–18, 35, 36). Specifically, we showed that in vitro binding of recombinant GST-Syn-1A (containing only the cytoplasmic domain) and SUR1 was dose-dependently disrupted by increasing PIP₂ concentrations (Fig. 1A). Although these results suggest that exogenous GST-Syn-1A and PIP₂ could sequester each other from acting on KₐₐₜP channels, our FRET study showed that PIP₂ could also disrupt in vivo FRET interactions of SUR1 (~EGFP) and full-length Syn-1A (~mCherry) (Fig. 5). PIP₂ effects on Syn-1A-SUR1 interactions were relatively specific at physiologic concentrations, with similar charged PI(3,5)P₂ having reduced effects and more (PIP₃) or less negatively charged phospholipids having little to no effect on Syn-1A-SUR1 complex assembly or KₐₐₜP channel activity (Figs. 1B and C, 7, and 8). The functional implication of GST-Syn-1A-SUR1 sequestration was demonstrated by electrophysiological studies on rat islet β-cells, INS-1E cells, and SUR1/Kir6.2-expressing HEK293 cells, which uniformly showed that efficacy of PIP₂ activation of KₐₐₜP channels could be reduced by the addition of GST-Syn-1A (Figs. 2–4). All of these effects of PIP₂ on Syn-1A-SUR1 complex formation and consequent KₐₐₜP channel activity could be abrogated by the PIP₂-insensitive Syn-1A mutant, Syn-1A-5RK/A. Importantly, we demonstrated multiple modes by which PIP₂ activates β-cell KₐₐₜP channel, whereby modulation of Syn-1A-SUR1 complex formation by physiologic low PIP₂ concentration sufficient to alter KₐₐₜP channel activity seemed to be more sensitive than the direct actions of PIP₂ (at higher concentration) on Kir6.2 (Fig. 6). These results suggest three mechanisms for the actions of the exogenous PIP₂ in opening KₐₐₜP channels. First, PIP₂ binds and sequesters exogenous GST-Syn-1A from binding SUR1. Second, some PIP₂ is incorporated into the PM to disrupt Syn-1A-SUR1 complexes in the PM. Third, PIP₂ acts on Kir6.2. The release of PM-bound Syn-1A molecules from PIP₂-induced disruption of Syn-1A-SUR1 complexes seemed to contribute to the availability of Syn-1A to participate in the increase in Syn-1A clustering on the PM (Fig. 5A). This was assessed more critically with experiments whereby we depleted endogenous PIP₂ from the PM with synaptotagmin-1, reported to reduce Syn-1A clustering on PM (31). Here, endogenous PIP₂ depletion appeared to release Syn-1A molecules from the PM clusters (Fig. 9). The “free” Syn-1A molecules could then migrate to, find, and bind SUR1 molecules to form tight Syn-1A-SUR1 complexes (Fig. 9), which effected optimal inhibition of KₐₐₜP channels, leaving no available SUR1 molecules for additional exogenous GST-Syn-1A to bind and further inhibit KₐₐₜP channels (Fig. 10).

In our in vitro binding studies employing GST-Syn-1A binding to expressed SUR1, the GST-Syn-1A (without the transmembrane domain) would not be expected to form physiologic Syn-1A clusters, but nonetheless the PIP₂-binding site at the cytoplasmic aa 260–265 domain directly binds SUR1, and the resulting GST-Syn-1A-SUR1 complex could be disrupted by exogenously added physiologic PIP₂ concentrations. These results raise the question of how PIP₂ modulation of Syn-1A interactions with SUR1 could influence KₐₐₜP channel opening kinetics. We recently reported that Syn-1A binds SUR1 domains at the W₈ motif of NBF-1 and W₉ and W₁₀ motifs of NBF-2 (33). This suggests that Syn-1A might be binding the NBF-1/2 dimer rather intimately, perhaps as a scaffolding protein, and this configuration and as yet undefined sites within these binding interfaces of SUR1 with Syn-1A might be putative sites for PIP₂ disruption. Although we do not know what the PIP₂-sensitive sites are in SUR1, the PIP₂-sensitive site in Syn-1A is known (29). We directly tested the latter by employing PIP₂-insensitive mutations of the juxtamembrane basic residues in Syn-1A (Syn-1A-5RK/A) (29), and indeed, PIP₂ could not disrupt Syn-1A-5RK/A-SUR1 complexes. This was demonstrated by in vitro and in vivo (FRET) binding studies. The fact that abrogating mutations of the PIP₂-binding sites actually increased Syn-1A-SUR1 binding indicates that the Syn-1A can bind SUR1 in a PIP₂-independent manner via other H3 domains (27). Consistently, Syn-1A-5RK/A inhibition of SUR1/Kir6.2 KₐₐₜP channels was rendered more resistant to PIP₂ activation, indicating that the actions of PIP₂ on SUR1 via Syn-1A binding have an important contribution to channel opening kinetics. Moreover, we found that PIP₂ at 1 µM, which did not disrupt Syn-1A-SNARE complexes could already modulate Syn-1A-SUR1 complex actions on β-cell KₐₐₜP channel activity. This was lower than the PIP₂ concentrations required to disrupt Syn-1A-SUR1 complexes, and PIP₂ with this lower concentration could also directly act on Kir6.2 to induce channel activation. Interestingly, a recent study showed that the N-terminal TM0 domain of SUR1 profoundly influenced Kir6.2 channel sensitivity to PIP₂ (16), which, however, is a domain...
that does not bind Syn-1A\(^5\) but which nonetheless could still affect the more distant NBF domains’ interactions with Syn-1A.

Hence, we conclude that the PIP\(_2\) effects on SUR1 proteins that influence \(K_{\text{ATP}}\) channel gating are both direct and indirect. The direct effects we showed in this work are PIP\(_2\) actions on Syn-1A binding to SUR1, probably at the NBF domains (26, 28). The indirect effects would be PIP\(_2\) actions on Syn-1A clusters on the PM, whereby a physiologic increase or reduction of PM PIP\(_2\) levels will determine how many Syn-1A molecules will be released from the Syn-1A clusters to bind adjacent SUR1 molecules on the PM.

\(K_{\text{ATP}}\) Channels play a most important role in regulating insulin secretion from islet \(\beta\)-cells (4, 5). Thus, any factor that acts directly or indirectly on \(\beta\)-cell \(K_{\text{ATP}}\) channels could have consequential effects on insulin secretion, which we now show to include PIP\(_2\) actions on Syn-1A binding to SUR1. PM and cytosolic PIP\(_2\) levels are likely to be perturbed in diabetes, metabolic syndrome, and lipid disorders. Syn-1A levels are severely reduced in islets of type-2 diabetic patients (39). Because the electrostatic binding between PIP\(_2\) and Syn-1A might enable each to sequester the other (21, 22), the excess or deficiency of either one in these pathologic conditions could profoundly influence the availability of Syn-1A to bind SUR1 or the availability of PIP\(_2\) to bind Kir6.2. Thus, this combined perturbation of \(\beta\)-cellular lipids and Syn-1A levels would probably contribute to perturbation of \(K_{\text{ATP}}\) channels that in turn could partly account for the deficient biphasic insulin secretion in diabetes.

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