Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$) is required both as a substrate for the generation of lipid-derived second messengers as well as an intact lipid for many aspects of cell signaling, endo- and exocytosis, and reorganization of the cytoskeleton. ADP ribosylation factor (ARF) proteins regulate PI(4,5)P$_2$ synthesis, and here we have examined whether this is due to direct activation of Type I phosphatidylinositol 4-phosphate (PIP) 5-kinase or indirectly by phosphatidate (PA) derived from phospholipase D (PLD) in HL60 cells. ARF1 and ARF6 are both expressed in HL60 cells and can be depleted from the cells by permeabilization. Both ARFs increased the levels of PIP$_2$ (PI(4,5)P$_2$, PI(3,5)P$_2$, or PI(3,4)P$_2$ isomers) at the expense of PIP when added back to permeabilized cells. The PIP$_2$ could be hydrolyzed by phospholipase C, identifying it as PI(4,5)P$_2$. However, the ARF1-stimulated pool of PI(4,5)P$_2$ was accessible to the phospholipase C more efficiently in the presence of phosphatidylinositol transfer protein-α. To examine the role of PLD in the regulation of PI(4,5)P$_2$ synthesis, we used butanol to diminish the PLD-derived PA. PI(4,5)P$_2$ synthesis stimulated by ARF1 was not blocked by 0.5% butanol but could be blocked by 1.5% butanol. Although 0.5% butanol was optimal for maximal transphosphatidylation, PA production was still detectable. In contrast, 1.5% butanol was found to inhibit the activation of PLD by ARF1 and also decrease PIP levels by 50%. Thus the toxicity of 1.5% butanol prevented us from concluding whether PA was an important factor in raising PI(4,5)P$_2$ levels. To circumvent the use of alcohols, an ARF1 point mutant was identified (N52R-ARF1) that could selectively activate PIP 5-kinase α activity but not PLD activity. N52R-ARF1 was still able to increase PI(4,5)P$_2$ levels but at reduced efficiency. We therefore conclude that both PA derived from the PLD pathway and ARF proteins, by directly activating PIP 5-kinase, contribute to the regulation of PI(4,5)P$_2$ synthesis at the plasma membrane in HL60 cells.

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$) has a multitude of roles in cells ranging from cell signaling, membrane traffic to the reorganization of the actin cytoskeleton (1–4). PI(4,5)P$_2$ is a substrate for phospholipase Cs and phosphoinositide 3-kinases where its metabolism leads to the production of the second messengers, inositol 1,4,5-trisphosphate plus diacylglycerol and phosphatidylinositol 3,4,5-trisphosphate, respectively. PI(4,5)P$_2$ is now also recognized as an intracellular signaling molecule where it participates in exocytosis (5–8), endocytosis (9, 10), regulation of ion channels (11, 12), co-factor for phospholipase D activation (13), and for the regulation of the actin cytoskeleton (4). Many proteins have binding sites for PI(4,5)P$_2$, and these include a subset of PH domains, ENTH domains, and basic patches of sequence, which facilitates their association to PI(4,5)P$_2$-containing membranes. Signaling, endocytosis and exocytosis all occur at the plasma membrane, where the majority of the cellular PI(4,5)P$_2$ is localized (14, 15) as well as the PIP 5-kinase activity (16–20). Thus, the opposing requirement for PI(4,5)P$_2$ as a substrate for signaling purposes and as an intact lipid for cellular function at the plasma membrane demands that the availability of PI(4,5)P$_2$ is regulated.

Synthesis of PI(4,5)P$_2$ at the plasma membrane requires both activation of the lipid kinases as well as a supply of PI, which is synthesized at the endoplasmic reticulum. During hydrolysis of PI(4,5)P$_2$ by phospholipase C, a soluble lipid transfer protein, phosphatidylinositol transfer protein (PITP) is required to make substrate available on demand for hydrolysis and spares the resident pool of PI(4,5)P$_2$ (21–23). The major pathway for PI(4,5)P$_2$ synthesis is by sequential phosphorylation of PI by a PI 4-kinase to PI(4)P followed by a PIP 5-kinase. Three PI 4-kinase enzymes (α, β, and γ) have been identified. PI 4-kinase α is a 230-kDa protein localized at the endoplasmic reticulum and PI 4-kinase β is a 92-kDa protein localized mainly at the Golgi and in the cytosol (24–26). Association and activation of PI 4-kinase β at the Golgi is regulated by ARF1 (27, 28). The most active PI 4-kinase is the Type II p55 isoform (PI 4-kinase γ), which is localized at the plasma membrane and secretory granules and accounts for the majority of global PI 4-kinase activity in mammalian cells (29–31). There are three isoforms of PIP 5-kinases, α, β, and γ, which are differentially expressed and can all be activated by PA in vitro (32). It had been reported that PIP 5-kinase purified from red blood cells could be activated more than a 100-fold by PA when PI(4)P alone was used as substrate (33). Subsequent studies report activation by PA of between 2- and 3-fold only (20, 28, 29).

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**Mechanism of ADP Ribosylation Factor-stimulated Phosphatidylinositol 4,5-Bisphosphate Synthesis in HL60 Cells**

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The reason for this discrepancy is not clear, but in the additional presence of ARF proteins, a further stimulation of the lipid kinase activity is observed (28, 34). ARF1 has also been found to directly stimulate PIP(5) kinase α in the absence of PA when the substrate PI(4)P was provided in the vesicle mixture as a minor component (PC-PI(4)P, 10:1). (28). Thus, from the *in vitro* analysis, it is clear that PIP(5) kinase activity can be regulated by inputs from either ARF proteins, PA alone, or by ARF proteins in synergy with PA, depending on the composition of the lipid vesicles used in the assay.

ARF1 has been shown to stimulate PI(4,5)P2 production in permeabilized cells (7, 8). Because ARF1 is also a direct activator of phospholipase D, it is not clear whether the effect of ARF1 on PI(4,5)P2 synthesis is a direct result of PIP(5) kinase activation or an indirect result of PA production or both. ARF1 also stimulates PI(4,5)P2 production in purified Golgi membranes, and in this case it was demonstrated that ARF1 sequentially activated PI 4-kinase β and PIP(5) kinase α directly and that PA derived from PLD was not required (27, 28). Alcohols were unable to inhibit PIP2 synthesis, and second, PLD activity was not enriched in this compartment. This was taken as evidence that PLD-derived PA was not required. In contrast, in two separate studies using purified lysosomes and Golgi membranes, it was reported that butanol blocked formation of PI(4,5)P2 with 375 µl of acidified chloroform, methanol, concentrated HCl (100:200:1.5), and the mixture was vortexed thoroughly to obtain a single phase. 10 µl of Folch extract was added (Folch extract (Sigma) is a brain lipid extract rich in phosphoinositides added to aid recovery of labeled phosphoinositides). 125 µl each of chloroform and of 0.1 M HCl was added to obtain two phases. The samples were vortexed thoroughly and centrifuged. The supernatants were used for assay with a "synthetic" top phase of chloroform, methanol, 0.1 M HCl (1:1:0.9). The top phase was replaced twice more to remove the majority of [32P]ATP not incorporated into lipid. Finally, all of the chloroform phase was transferred to a clean Eppendorf tube, dried under vacuum, and resuspended in 50 µl of chloroform. The polyphosphoinositides were separated by TLC on oxalate-treated plates using chloroform:methanol:acetic acid (15:3:2) and the labeled lipids were imaged using Fuji phosphorimaging, and the images were quantified using the AIDA software provided by the manufacturer.

**In vitro** Reconstitution of PIP2 Synthesis in Peroxidized HL60 Cells—For measurements of phospholipase C activation, [3H]inositol-labeled cells were permeabilized for 10 min as described above. The permeabilized cells were reconstituted with PTX and GTTPyS (10 µM at 1 µM Ca2+) in the presence of Li+ (10 mM) exactly as described previously (21). After 20 min, the samples were quenched with chloroform:methanol, and phase separation was achieved using chloroform and water exactly as above, except that no acid was present. Two methods were used to analyze the inositol phosphates. In the first case, a fraction of the top phase (100 µl) containing the soluble inositol-labeled compounds were loaded onto Dowex columns, and the total inositol phosphates were eluted using 3 ml of 1 M ammonium formate as described previously (21). Triplicate samples were monitored for each condition. The remainder of the samples (300 µl) was analyzed by HPLC, and in this case, the triplicate samples were combined. The combined top phases were dried down and resuspended in 70 µl of H2O. The inositol phosphates were analyzed by anion exchange HPLC on a Partisil 10 SAX column using a gradient of 1.4 m monobasic ammonium phosphate buffer adjusted to pH 3.7 with orthophosphoric acid as described previously (39). The peaks were identified with 1H NMR (405 MHz) and integrated with AIDA software provided by the manufacturer.

**In Vitro Assay for Measuring ARF1-stimulated PIP2 Synthesis**—Recombinant Type I PIP 5-kinase α was expressed in *E. coli* and purified as described previously (28). Activity was analyzed in 100 µl in buffer composed of 25 mM Hepes, 25 mM NaCl, 2.5 mM MgCl2, 0.2 mM succinate, 1 mM dithiothreitol, 1 mM MgATP, pH 7.2. Lipid vesicles were prepared by sonication to give final concentrations of 80 µg/mL phosphatidylincholine and 8 µg/mL cholesterol. The reaction mixture was incubated at 37°C for 30 min. At the end of this time samples were quenched with chloroform, methanol, concentrated HCl (100:200:1.5), and the production of PIP2 was analyzed by TLC as described above.
ATP was also present during the 20 min assay. PI(4,5)P₂ was reconstituted with 5 occasions. HL60 cells were permeabilized with streptolysin O for 5 min in the absence or presence of GTPγS (100 μM). The cytosol and membranes (10² cell equivalents) were probed with peptide-specific antibodies. Recombinant ARF1 (rARF₁) and ARF6 were used as standards to estimate the concentration of endogenous ARF1 and ARF6 proteins. Results are presented from a single experiment that was reproduced on three separate occasions. ARF proteins have been allowed to leak out of permeabilized HL60 cells, which has been previously shown to increase the formation of PI(4,5)P₂ isomer. This is in keeping with the levels of endogenous ARF proteins that we have estimated in HL60 cells. The ability of ARF proteins to stimulate PIP₂ synthesis was observed at both resting levels of Ca²⁺ (100 nM) and concentrations found in stimulated cells (10 μM) (Fig. 1A).

We next examined whether the incorporation of label into PIP₂ was due to increased turnover or due to an actual increase in PIP₂ levels. HL60 cells were grown in the presence of [³H]-inositol for 2 days to achieve equilibrium labeling of the entire pool of inositol-containing lipids. ARF1 in the presence of GTPγS caused an increase in PIP₂ levels at the expense of PIP with insignificant changes in PI (Fig. 2, A–C). We calculated that PI represents 81%, PIP as 13%, and PIP₂ as 6% of the total inositol lipids under steady state conditions, and ARF1 stimulated an increase in PIP₂ to 9%, and the percentage of PIP decreased to 10% (Fig. 2).

The inositol-containing lipids were separated by TLC, which separates PI, the mono- and di-phosphorylated species without resolving the individual isomers. Thus PIPs will include PI(3)P, PI(4)P, and PI(5)P, and PIP₂ will include PI(4,5)P₂, PI(3,5)P₂, and PI(3,4)P₂ isomers. PI(4)P and PI(4,5)P₂ constitute the major phosphoinositides, and the magnitude of changes induced by ARF1 would suggest changes in these two lipids. To verify that the PIP₂ pool synthesized under the influence of ARF1 was indeed PI(4,5)P₂, lipid samples from control and from ARF plus GTPγS-stimulated HL60 cells were deacylated and analyzed by HPLC. Using appropriate radiolabeled standards, the increase in PIP₂ was exclusively in PI(4,5)P₂ and was accompanied by a decrease in PI(4)P (data not shown). Additionally, we examined whether this pool of PIP₂ could be hydrolyzed by the G-protein-regulated phospholipase Cβ that is present in these cells (41). Phospholipase Cβ is present at the plasma membranes (16) and can only utilize PI(4,5)P₂ as substrate but not the 3-phosphorylated phosphoinositides. [³H]-inositol-labeled permeabilized cells were stimulated with GTPγS in the presence of ARF1, and as an additional control we included PITPα, which has been previously shown to increase the formation of inositol phosphates (41). We initially analyzed a fraction of the samples by chromatography on anion exchange resin (Dowex), which does not resolve the individual inositol phosphates (Fig. 3). ARF1 only showed a marginal increase in total inositol phosphate production in comparison to PITPα. Because PITPα influences the availability of the PI(4,5)P₂ for the PLC, we included ARF1 and PITPα together and observed that PITPα was able to allow PLC access to the pool of PI(4,5)P₂ that was synthesized by ARF1 (Fig. 3).

The initial products of PI(4,5)P₂ hydrolysis by PLC were I(1,4)P₂ and diacylglycerol. I(1,4,5)P₃ was rapidly removed by sequential degradation to I(1,4)P₂ and I(4)P, and finally, inositol. To identify the inositol phosphates as products of PLC hydrolysis, the samples were analyzed by HPLC, and Fig. 4 shows a selection of the traces with the quantification of the results in Table I. GTPγS caused an increase in both I(4)P and I(1,4)P₂ that were marginally increased in the presence of ARF1. In the combined presence of PITPα and GTPγS, a substantial increase in I(4)P and I(1,4)P₂ occurred that was further increased when ARF1 was also present. I(1,4,5)P₃ was detected at a very low level, making its quantification unreliable, and this was attributed to the highly active membrane-localized inositol polyphosphate 5-phosphatase (42, 43). This was demonstrated by incubating radiolabeled I(1,4,5)P₃ with permeabilized HL60 cells (data not shown). These data further confirm that the PIP₂ formed by ARF1 is the PI(4,5)P₂ isomer.

**RESULTS**

Both ARF1 and ARF6 Stimulate the Synthesis of PI(4,5)P₂ in Permeabilized HL60 Cells—HL60 cells were first analyzed for the presence of endogenous ARF1 and ARF6 proteins using isoform-specific antibodies. Both ARF1 and ARF6 proteins were detected and found to leak out of the cells after permeabilization with streptolysin O for 5 min (Fig. 1A, lane 3). The level of ARF6 expressed in HL60 cells is 10–20-fold less than that of ARF1 calculated using recombinant ARF proteins as standards. 20 ng of ARF1 was detected compared with 1–2 ng of ARF6 in 3 × 10⁵ HL60 cells. We estimate the concentration of ARF1 as 10 μM and that of ARF6 as 0.5–1 μM. ARF proteins cycle between the membrane and cytosol depending on their activation state. Thus the presence of GTPγS during the permeabilization led to the recruitment of ARF proteins to membranes (Fig. 1A, lane 2) and were thus no longer found in the leaked cytosol (Fig. 1A, lane 4).

GTPγS-stimulated PIP₂ synthesis is compromised when proteolytic digestion has been allowed to leak out of permeabilized HL60 cells (7). Re-addition of ARF1 or ARF6 proteins to permeabilized cells was sufficient to restore the synthesis of PIP₂ (Fig. 1B). Synthesis of PIP₂ monitored by incorporation of label from γ-labeled [³²P]ATP, was maximally stimulated at 2–5 μM ARF proteins, and was concentration-dependent (data not shown). Although the maximal concentration of ARFs required was between 2 and 5 μM, the effective concentration of active ARF required is between 0.4 and 1 μM due to partial myristoylation of the recombinant proteins. This is in keeping with the levels of endogenous ARF proteins that we have estimated in HL60 cells.
In addition to the inositol phosphates, glycerophosphoinositol was also present but did not alter in the presence of GTPγS, PITPα, or ARF1. We noted that in the presence of GTPγS and PITPα, an additional minor peak (labeled as × in Fig. 4, C and D) eluted at 31 min. This peak was not glycerophosphoinositol 4-phosphate, since its elution time was 26.5 min. Because extraction of the inositol phosphates was performed under neutral conditions, cyclic derivatives of inositol phosphates would still be present. To check this possibility, we treated the samples with acid and observed the complete disappearance of the peak, suggesting that it is cyclic-inositol diphosphate. The presence of the cyclic inositol phosphates is not surprising because they are normal products of all PLCs (44).
tion of PBut (Fig. 8). The optimal concentration for maximal transphosphatidylation was 0.5% butanol (Fig. 8).

From the data presented above, it is clear that the conclusions drawn from the studies with butanol can lead to problems of interpretation. At concentrations of butanol when optimal transphosphatidylation occurs, PA can still be available. To avoid these problems of interpretation, we investigated the possibility of identifying ARF point mutants that are selective for either PLD activation or PIP 5-kinase activation. We had previously reported that a point mutant, N52R-ARF1, made as a non-myristoylated protein, was unable to activate PLD1 both in vitro and in permeabilized HL60 cells (46). We have used myristoylated N52R-ARF1 in these experiments and first examined that the myristoylated mutant was defective in the activation of PLD. In two independent assays for PLD activity, release of \[^{3}H\]choline and formation of \[^{3}H\]PBut using \[^{3}H\]-alkyl-lyso-PC-labeled cells, N52R-ARF1 was ineffective in stimulating PLD activity compared with wild-type ARF1 (Fig. 9, A and B). In contrast, N52R-ARF1 fully retains the ability to activate PIP 5-kinase in vitro (Fig. 10A). Having identified a mutant that was defective in PLD activation but was still able to activate PIP 5-kinase fully, we were able to test whether ARF1 activation of PIP\(_2\) synthesis in permeabilized cells was

![Fig. 4. HPLC analysis identifies inositol 4-phosphate and inositol 1,4-phosphate as the major inositol phosphates formed in the presence of ARF1 and PITP\(_\alpha\). \[^{3}H\]inositol-labeled HL60 cells were reconstituted with ARF1, PITP\(_\alpha\), and GTP\(_\gamma\)S as in Fig. 3, and the inositol phosphates were analyzed by HPLC. Fractions were collected every 0.5 min. Elution times were 19 min for glycerophosphoinositol 4-phosphate, 21 min for I(4)P, 26.5 min for glycerophosphoinositol 4-phosphate, 35 min for I(1,4)P\(_2\), and 62 min for I(1,4,5)P\(_3\). An identified peak eluted at 31.5 min (labeled \(\times\)), which was tentatively identified as cIP\(_2\). A representative set of HPLC runs from an experiment is shown. The data for the entire experiment is shown in Table I. Similar results were obtained in three independent experiments. A, no additions; B, GTP\(_\gamma\)S (10 \(\mu\)M); C, GTP\(_\gamma\)S (10 \(\mu\)M) and PITP\(_\alpha\) (5 \(\mu\)M); D, GTP\(_\gamma\)S (10 \(\mu\)M), PITP\(_\alpha\) (5 \(\mu\)M), and ARF1 (5 \(\mu\)M).

![TABLE I](http://www.jbc.org/)

Quantification of the inositol phosphates formed from the HPLC analysis

The experimental data from Fig. 4 are tabulated below. Because the two major inositol phosphate peaks observed upon GTP\(_\gamma\)S stimulation were I(4)P and I(1,4)P\(_2\), only these data are presented.

| Additions made | DPM in I(4)P | DPM in I(1,4)P\(_2\) |
|----------------|--------------|---------------------|
| Control        | 390          | 498                 |
| GTP\(_\gamma\)S| 4,479        | 8,387               |
| PITP\(_\alpha\) alone | 553          | 1,156               |
| ARF1 alone     | 334          | 768                 |
| PITP\(_\alpha\) + ARF1 | 606          | 876                 |
| ARF1 + GTP\(_\gamma\)S | 4,904        | 11,583              |
| PITP\(_\alpha\) + GTP\(_\gamma\)S | 13,530       | 36,397              |
| ARF1 + PITP\(_\alpha\) + GTP\(_\gamma\)S | 14,452       | 48,998              |
analyzed the role of ARF proteins in regulating PIP2 levels in organization of the actin cytoskeleton. In this study we have signaling molecule for endocytosis, exocytosis, and for the re-
strate for phospholipase C and PI 3-kinase as well as an intact 

5 were –

hamster ovary cells, it was demonstrated that ARF1

Fig. 5. Butanol does not inhibit ARF1-stimulated PIP2 synthesis. HL60 cells were reconstituted with ARF1 in the presence and absence of 0.5% butanol or butan-2-ol in the presence of 100 nM Ca²⁺, 1 mM MgATP, and 1 μCi of [³²P]ATP. Results are an average of duplicate values (± range) from a single representative experiment repeated on eight separate occasions.

in the absence of 0.5% butanol or butan-2-ol in the presence of 100 nM Ca²⁺, 1 mM MgATP, and 1 μCi of [³²P]ATP. Results are an average of duplicate values (± range) from a single representative experiment repeated on eight separate occasions.

dependent on prior activation of PLD. N52R-ARF1 was still capable of increasing PIP₂ synthesis but had reduced activity when compared with wild-type ARF1 (Fig. 10B). From four independent experiments we calculated that the response to N52R-ARF1 was 55 ± 7% of the wild-type ARF1 response.

DISCUSSION

PIP₂ functions at the plasma membrane serving as a substrate for phospholipase C and PI 3-kinase as well as an intact signaling molecule for endocytosis, exocytosis, and for the reorganization of the actin cytoskeleton. In this study we have analyzed the role of ARF proteins in regulating PIP₂ levels in HL60 cells. HL60 cells contain ARF1 and ARF6 proteins, which are freely soluble, as demonstrated by their ability to exit from the permeabilized cells. Binding of GTP or GTPγS catalyzed by guanine nucleotide exchange factors is required for activation of the ARF proteins, and this stabilizes its association with target membranes. Thus, when GTPγS was present during the permeabilization, both ARF1 and ARF6 became membrane-associated (Fig. 1A). In a previous study conducted in Chinese hamster ovary cells, it was demonstrated that ARF1–5 were cytosolic and could be recruited to membranes with GTPγS but that ARF6 was stably membrane-bound and, moreover, restricted to the plasma membrane (47). Subsequent studies using overexpression identify that ARF6 localizes to and regulates a plasma membrane-endoosome-trafficking pathway (48–50). In some cells, ARF6 is both cytosolic and membrane-bound, and like ARF1, its distribution is regulated by its GTPase cycle (51–53). Thus, depending on the cell type, the distribution of the membrane-bound form versus the cytosolic form must be variable to account for the differences observed, and in the case of the HL60 cells, the membrane-bound pool is insignificant.

We report here that the addition of either ARF1 or ARF6 to permeabilized HL60 cells is able to reconstitute the synthesis of PIP₂ (because ARF1 or ARF6 are equally effective at stimulating PIP₂ synthesis, we have routinely used ARF1 rather than ARF6 because ARF6 is more difficult to purify, unstable, and has a tendency to aggregate). That the PIP₂ synthesized by the addition of ARF1 is available for hydrolysis by a plasma membrane-located PLCβ2 would suggest that not only ARF6 but also ARF1 could function at the plasma membrane at least in the permeabilized cells used here. Although ARF6 has an established function in the endosomal recycling to the plasma membrane as discussed above, ARF1 is involved in trafficking in the endoplasmic reticulum-Golgi and endosomal systems. At the Golgi, ARF1 functions to recruit a number of coat proteins including GGAs, coatomer, and adaptor protein 2/clathrin (54), PI 4-kinase β, and PIP 5-kinase α (27, 28). Thus, when purified Golgi are primed with ARF1, synthesis of both PIP and PIP₅ is observed due to ARF1 activation of PI 4-kinase β and PIP 5-kinase α (27, 28). In the permeabilized cells, ARF1 decreases PIP levels and causes an increase in PIP₅ levels, a phenomenon that is different from what ARF1 has been shown to do in purified Golgi fractions. This would suggest that in the permeabilized cells, the changes monitored in PIP₂ levels by ARF1 are exclusively due to activation of PIP 5-kinase α at the plasma membrane, where a pre-existing pool of PIP is used as a substrate.

This conclusion is further supported by the use of brefeldin A. At the Golgi, activation of ARF1 is dependent on the high molecular weight ARF-guanine nucleotide exchange factors, which are sensitive to brefeldin A (54, 55). At the plasma membranes the low molecular weight family of ARF-guanine nucleotide exchange factors, which include ARNO, cytohesin, and EFA6, are resistant to brefeldin A (55). Brefeldin A, however, has no effect on ARF1-stimulated PIP₂ synthesis (data

Fig. 6. Production of PA in the absence and presence of butanol. [³H]Alkyl-lyso-PC-labeled HL60 cells were reconstituted with ARF1 and GTPγS in the absence (A) and presence (B) of 0.5% butanol at a range of Ca²⁺ concentrations. PA and PBut levels were analyzed by TLC. Results are the average of duplicate values (± range) from a single representative experiment repeated on two separate occasions.
not shown), again supporting the notion that ARF1 very likely stimulates PIP2 synthesis at the plasma membrane. Finally, if ARF1 was functioning to activate PIP2 synthesis at the Golgi in permeabilized cells, it would be necessary for the PIP2 to be transported to the plasma membrane where it can be utilized as a substrate for the PLC. However, GTP\(^{\gamma}\)S inhibits constitutive vesicular transport out of the Golgi (56). Collectively, these results support the conclusion that the major effect of ARF1 on the PIP2 levels is at the plasma membrane rather than the Golgi in permeabilized cells. It is highly possible that ARF1 also influences phosphoinositide levels at the Golgi, but this effect may be masked by the more extensive changes taking place at the plasma membrane. Thus ARF1 can function at the plasma membrane as an activator of lipid-metabolizing enzymes in permeabilized cells. It is possible that this may not be its normal function and that in permeabilized cells, ARF1 compensates for ARF6 at the plasma membrane.

How Does ARF Increase PIP\(_2\) Levels—Two possible regulators of PIP 5-kinase activity at the plasma membrane are PA and ARF or a combination of both. PA is produced via ARF regulation of PLD activity at the plasma membrane (16). Thus, the question can be restated as to whether ARF activates PIP 5-kinase directly or indirectly via PA-derived PLD. We used butanol to reduce the amount of PA produced by the PLD pathway and observe that under conditions of maximal transphosphatidylation, PIP2 synthesis is unaffected. We also observed that 0.5% butanol did not completely stop PA production (see Fig. 6 and Ref. 40). Thus, these data were not conclusive to exclude PA as an activator of PIP 5-kinase activity.

In two recent studies, a requirement for PA-derived PLD was demonstrated for PIP\(_2\) synthesis based on the use of 1.5% butanol (35, 36). We confirmed that 1.5% butanol was indeed...
inhibitory for PIP$_2$ synthesis. However, this concentration of butanol is far in excess of what is required for maximal transphosphatidylation, which is 0.5%. We also note that PIP levels are reduced substantially by 1.5% butanol. Phosphoinositides play an important role in maintaining Golgi structure and function, and thus, the effects of 1.5% butanol could be attributed to depletion of PIP levels rather than PA as suggested previously (36). More importantly, we show here that 1.5% butanol actually inhibits the activation of PLD. Thus the observed inhibition of PIP$_2$ synthesis reported earlier and also shown in this paper cannot be attributed to a requirement for PA-derived from the PLD. Instead, the inhibition of PIP$_2$ synthesis observed could be due to effects on other lipid-metabolizing enzymes.

To circumvent the use of alcohols in HL60 cells and obtain a more definitive answer, we identified N52R-ARF1 mutant that could selectively activate PIP 5-kinase activity in vitro but was unable to activate PLD. This mutant was still active in the synthesis of PIP$_2$ in permeabilized HL60 despite being unable to activate PLD. However, PIP$_2$ synthesis was reduced by 45%, illustrating that PA-derived from PLD pathway can make an important contribution to the activation of PIP 5-kinase.

From the in vitro studies of PIP 5-kinase activation, ARF proteins could either activate PIP 5-kinase directly (28) or require PA as a co-stimulus (94). The in vitro studies used vesicle preparations that do not reflect the lipid composition that PIP 5-kinase would encounter in cells. The studies using permeabilized cells circumvent this problem. Since the activation of the enzyme is studied in its "native" membrane environment. These studies allow us to conclude that ARF proteins can activate PIP 5-kinase without being entirely dependent on PA generated from the PLD pathway, and second, PA can further enhance the ARF effect and, therefore, make a contribution to the activity of PIP 5-kinase. Whether PA is capable of activating PIP 5-kinase $\alpha$ independently of ARF in cells awaits the identification of ARF mutants, which can selectively activate PLD but not PIP 5-kinase activity.

Fig. 11. P(4,5)P$_2$ synthesis; the relative contribution made by the direct activation of PIP 5-kinase and indirect activation via PA. The contribution made by ARF directly stimulating PIP 5-kinase activity is 55 ± 7%, calculated from 4 independent experiments using N52R-ARF1 as the activator (denoted by the solid black line). PA derived from ARF-stimulated phospholipase D pathway can further enhance P(4,5)P$_2$ synthesis (route denoted by dotted black line). The P(4,5)P$_2$ is only accessible to degradation by the plasma membrane phospholipase C provided that PITP is also present. DG, diacylglycerol.
insulin-stimulated PLD activity and mitogen-activated protein kinase (59), receptor-mediated endocytosis (60), Ca\(^{2+}\)-dependent exocytosis (61), and constitutive protein transport from the trans Golgi network to the plasma membrane (62). However, these tools can only be applied to cell types that can be transfected and is not an easy option for many hematopoietic cells including HL60 cells, which have transfection efficiencies of less than 1%.

In this study we have focused on ARF proteins and PA as regulators of PIP 5-kinase. Rho and Rac proteins have also been shown to interact with and regulate Type I PIP 5-kinase (63). The addition of Rac into permeabilized platelets or over-expression of Rho or Rho kinase in human embryonic kidney cells increased PIP\(_2\) synthesis (64) (65). Because Rho proteins or Rho kinase are also effective in regulating PLD activity, the possibility that these proteins regulate PIP 5-kinase in concert with PA cannot be excluded (66). Analysis of PIP 5-kinase expression of Rho or Rho kinase in human embryonic kidney 293 cells showed that over-expression of Rho or Rho kinase resulted in less than 1%.

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