Alterations in an inositol phosphate code through synergistic activation of a G protein and inositol phosphate kinases

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In mammals, many cellular stimuli evoke a response through G protein activation of phospholipase C, which results in the lipid-derived production of inositol 1,4,5-trisphosphate (IP_3). Although it is well established that IP_3 is converted to numerous inositol phosphates (IPs) and pyrophosphates (PP-IPs) through the action of up to six classes of inositol phosphate kinases (IPKs), it is not clear that these metabolites are influenced by G protein signaling. Here we report that activation of $G\alpha_q$ leads to robust stimulation of IP_3 to IP_8 metabolism. To expose flux through these pathways, genetic perturbation was used to alter IP homeostasis. Coupled expression of a constitutively active $G\alpha_q$QL and one or more IPK gene products synergistically generated dramatic changes in the patterns of intracellular IP messengers. Many distinct IP profiles were observed through the expression of different combinations of IPKs, including changes in previously unappreciated pools of IP_5 and IP_6, two molecules widely viewed as stable metabolites. Our data link the activation of a trimeric G protein to a plethora of metabolites downstream of IP_3 and provide a framework for suggesting that cells possess the machinery to produce an IPK-dependent IP code. We imply, but do not prove, that agonist-induced alterations in such a code would theoretically be capable of enhancing signaling complexity and specificity. The essential roles for IPKs in organism development and cellular adaptation are consistent with our hypothesis that such an IP code may be relevant to signaling pathways.

G_q | metabolomics | phospholipase C | signal transduction | G protein-coupled receptor

In mammals, hundreds of G protein-coupled receptors (GPCR) and receptor tyrosine kinases (RTKs) are present and link to numerous intracellular signaling modules that, in combination, enable selective cellular responses. One of the most commonly activated signaling modules is the generation of the second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol by the action of phospholipase C isozymes (PLCs) on phosphatidylinositol 4,5-bisphosphate (PIP_2) (1–5). The ubiquity of PLC activation in response to a wide range of stimuli supports its central role in signaling, but also emphasizes that the production of two messengers alone, IP_3 and diacylglycerol, is insufficient to encode the breadth of specific cellular responses necessary for survival and adaptation. Therefore, it has been postulated that further signaling diversity could theoretically arise from the conversion of IP_3 to numerous inositol tetrakisphosphate (IP_4), inositol pentakisphosphate (IP_5), inositol hexakisphosphate (IP_6), and inositol pyrophosphate (PP-IP) molecules (2, 6–9). The production of these regulators occurs through the action of up to six classes of evolutionarily conserved IP kinase (IPK) gene products that include: IPMK/IPK2 (10–14), IPK1 (15–17), IP3K (18–20), ITPK/IP56K (21, 22), IP6K/IHPK (23–25), and VIP1 (25–27) (Fig. 1). Genetic and biochemical studies of IPKs have provided valuable insights into the essential regulatory roles and biology of their IP and PP-IP products (7–9, 28–30).

In contrast to the wealth of evidence demonstrating that activation of GPCR and RTK pathways leads to the stimulation of PKC and calcium release through DAG and IP_3 second messengers, links of receptor activation to alterations in the levels of IP_4, IP_5, IP_6, and PP-IPs have been limited. This result has impeded acceptance that these putative messengers lie downstream of GPCR or RTK signaling. However, recent studies have shown that, in a variety of organisms, IPK gene products are required for proper development and cellular adaptation, examples of highly orchestrated receptor-mediated signaling processes, providing support that these pathways are in fact used by the cell for encoding selective responses.

In light of this apparent paradox, we considered an alternative explanation that receptor activation may indeed result in flux through these pathways, but such changes have not been observed because of rapid reequilibration, masking, or an inability to trap transient bursts of messenger synthesis and breakdown. Precedent for the latter explanation comes from monitoring agonist-induced IP_3 changes in which cellular studies frequently use lithium treatment to inhibit IP phosphatases, thereby enabling accumulation of signaling intermediates that are stable and easily quantified (31, 32). Because pharmacological traps are not readily available for the IPK-mediated pathways, we examined whether genetic perturbation in combination with metabolic labeling would enable visualization of flux through these pathways. We report that coupling the overexpression of a constitutively active G protein $G\alpha_q$ and the IPKs exposed dramatic changes in IP_3, IP_4, IP_5, IP_6, IP_7, and IP_8 metabolism. Our data reveal that constitutively elevated molecules, such as IP_5 and IP_6, appear to have multiple pools, including those that are low at resting state and undergo dramatic elevation in response to the activation of G protein. Collectively, these data indicate that G protein activation works in concert with the six classes of IPKs to regulate flux through a complex number of IP molecules, thereby enhancing signaling complexity downstream of PLC. The ability to observe alterations in the patterns and flux of these messengers provides a framework for suggesting that individual IP species represent the building blocks of a potentially dynamic IP code.

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Abbreviations: DIPP, diphosphoinositol phosphate phosphatase; GPCR, G protein-coupled receptor; IP, inositol phosphate; IP_3, inositol 1,4,5-triphosphate; IP_4, inositol tetrakisphosphate; IP_5, inositol pentakisphosphate; IP_6, inositol hexakisphosphate; IPK, inositol phosphate kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C isozymes; PP-IP, inositol pyrophosphate; RTK, receptor tyrosine kinase.

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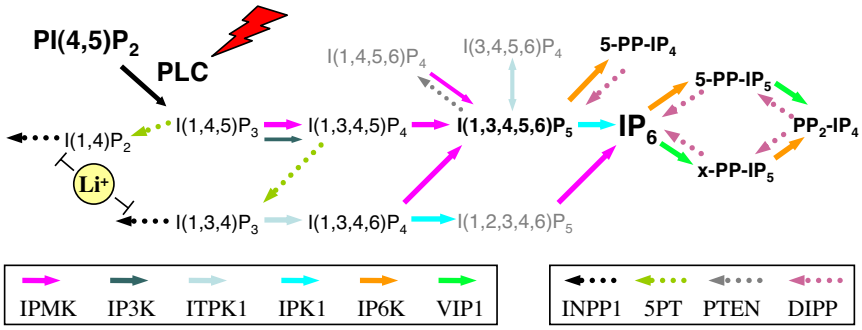

Fig. 1. Six classes of evolutionarily conserved IPKs generate an IP code in mammalian cells: (*i*) IPMK (also known as IPK2 or ARG82), a multikinase capable of producing $I(1,3,4,5,6)P_5$ through the dual phosphorylation $I(1,4,5)P_3$ at the 3- and 6-kinase positions, as well as through a 5-kinase activity toward $I(1,3,4,6)P_4$; (*ii*) IPK1, an IP_5 2-kinase responsible for the generation of IP_6; (*iii*) IP3K, a selective IP_3 3-kinase that produces $I(1,3,4,5)P_4$ from $I(1,4,5)P_3$; (*iv*) ITPK1 (also known as IP56K), an $I(1,3,4)P_3$ 5- and 6-kinase capable of producing $I(1,3,4,6)P_4$ and $I(1,3,4,5)P_4$, and a reversible 1-kinase/phosphatase that regulates $I(3,4,5,6)P_4$ levels; (*v*) IP6K/IHPK, an $IP_5/IP_6/IP_7$ 5-kinase that generates diphosphoinositol phosphates (also known as PP-IPs), including 5-PP-IP_4 and 5-PP-IP_5; and (*vi*) VIP1, an IP_6/IP_7 kinase that generates a unique species of PP-IP_5 (*x*-PP-IP_5) and $(PP)_2$-IP_4 (IP_8). In addition, the action of four IPs are relevant to this pathway: (*i*) INPP1, a lithium-inhibited $I(1,4)P_2/I(1,3,4)P_3$ 1-phosphatase; (*ii*) 5PT, an $I(1,4,5)P_3/I(1,3,4,5)P_4$ 5-phosphatase; (*iii*) PTEN, a phosphoinositide 3-phosphatase that also has been shown to dephosphorylate IP_5; and (*iv*) DIPP, which degrades PP-IPs. The lightning bolt represents GPCR and RTK pathways that activate PLC isoforms; the yellow sphere represents lithium, a pharmacological agent used to inhibit INPP1. Legend for colored arrows indicates the six kinase (solid) and four phosphatase (dotted) enzymes.

Results

Enhancing Detection of G Protein-Activated IP_3 Release by Trapping Rapidly Fluxing Metabolites. The premise of our study was that detecting signaling-dependent changes in IP metabolites would require a robust activation of IP_3 production or a pharmacological or genetic mechanism to trap rapidly equilibrating, masked, or fluxing IPs or both. As a means to increase IP_3 production through PLC stimulation, we overexpressed an activated $G\alpha_q$ mutant. The $G\alpha_q$ family of heterotrimeric G proteins, comprised of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$, specifically activate PLCβ isoforms (33). A mutation of a glutamine residue at position 209 to leucine in $G\alpha_q$ ($G\alpha_q$QL) inactivates the GTPase of the G protein, locking $G\alpha_q$QL in a constitutively active state (34). Adenoviral introduction of $G\alpha_q$QL into Rat-1 cells radiolabeled with [^{3}H]inositol indeed led to substantial increases in the levels of IP_2, IP_3, and IP_4 (Fig. 2 *Right Upper*). To examine the second component of our hypothesis, we combined the treatment of cells expressing $G\alpha_q$QL with the addition of 10 mM lithium chloride in the growth medium for 1 h before harvest. Although lithium treatment alone did not result in alterations in any of the IP metabolites (Fig. 2 *Left Upper*), the combination of lithium plus $G\alpha_q$QL expression resulted in an additional increase in the levels of IP_2 and $I(1,3,4)P_3$, consistent with an inhibition of INPP1 (see Fig. 1), but did not further alter levels of $I(1,4,5)P_3$, IP_4, IP_5, or IP_6 (Fig. 2 *Right Lower*). Most notably, the levels of $I(1,3,4)P_3$ increased >20-fold by using a combined $G\alpha_q$QL plus lithium as compared with control cells. These data confirm that (*i*) $G\alpha_q$ activates IP metabolism, and (*ii*) the stimulus-dependent increases in $I(1,4)P_2$ and $I(1,3,4)P_3$ are examples of lithium-trapped metabolites that serve as reporters for signaling activated flux through $I(1,4,5)P_3$.

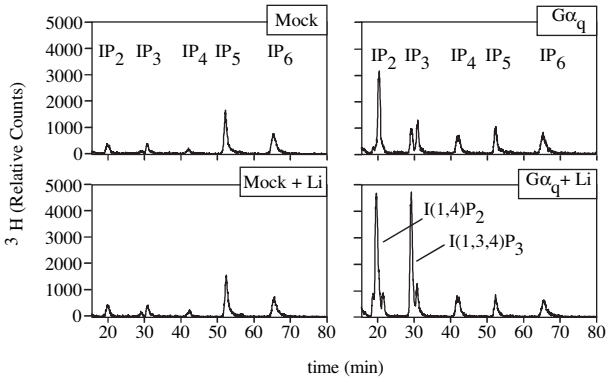

Fig. 2. Enhanced detection of $G\alpha_q$QL-induced IP_3 production by the trapping of metabolites. Rat1 cells were plated at densities of 25,000 cells per milliliter and labeled metabolically for 72 h with *myo*-[^{3}H]inositol as described in *Methods*. Twenty-four hours before harvest, cells were infected with control adenovirus (labeled Mock) or $G\alpha_q$QL adenovirus (labeled $G\alpha_q$). Lithium treatment was performed by adding LiCl to the cells at a final concentration of 10 mM 1 h before harvest. Soluble IPs were extracted by HCl and resolved by HPLC. Peaks were normalized to the total inositol counts present in the samples.

It is noteworthy that changes in $I(1,4,5)P_3$ levels in G protein-activated cells, even in the presence of lithium, were relatively minimal compared with the 20-fold elevation observed for $I(1,3,4)P_3$. Because the only known route of $I(1,3,4)P_3$ synthesis occurs through the metabolism of $I(1,4,5)P_3$ and $I(1,3,4,5)P_4$ (2), we infer that the synthesis of $I(1,4,5)P_3$ and $I(1,3,4,5)P_4$ also was up-regulated 20-fold, but such changes were not observed because of their rapid equilibration. Our data highlight that indeed $G\alpha_q$QL overexpression induces a robust stimulation of $I(1,4,5)P_3$ and $I(1,3,4,5)P_4$ production, and for these untrapped molecules analysis of steady-state metabolic labeling may not accurately reflect their flux. Furthermore, the data provided evidence consistent with the notion that the homeostatic mechanisms aimed at maintaining IP levels in cells were tuned to rapidly reequilibrate to prevent inappropriate fluctuations. Thus, we next sought to perturb IP_4, IP_5, and IP_6 homeostasis alongside PLC activation to further test our initial hypothesis.

Coupled Activation of $G\alpha_q$ and Distinct IP_3 Kinases Generate Unique IP Signatures. Because pharmacological regulators of IP_4 or IP_5 metabolism are not readily available, we examined the effects of genetic perturbation of the three classes of IP_3 kinase, IPMK, IP3KA, or ITPK1, in the context of activated $G\alpha_q$. To perform these studies, we switched to a human embryonic kidney cell, HEK293T, and used transient transfection methods to facilitate the introduction of multiple gene products and the rapid assessment of metabolic changes. In the absence of $G\alpha_q$QL, expression of any one of the three individual IP_3 kinases had minimal effect on soluble IP levels (Fig. 3 *Left*). Similar to Rat-1 cells, the expression of $G\alpha_q$QL alone in HEK293T cells resulted in small changes in IP_3 and IP_4 levels, but did not significantly alter IP_5 or IP_6 (Fig. 3 *Right Upper*). In contrast, when the different IP_3

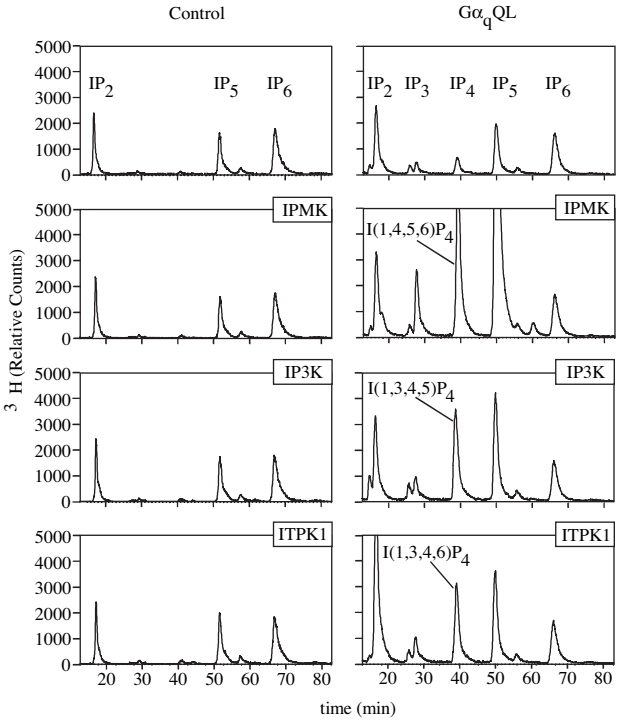

Fig. 3. Coincident activation of IP_3 kinases along with $G\alpha_qQL$ synergistically enhances inositol phosphate metabolism and distinct species of IP_4. HEK293T cells were plated at a density of 100,000 cells per milliliter and labeled metabolically for 72 h with *myo*-[^{3}H]inositol. Twenty-four hours before harvest, cells were transfected by the addition of FuGENE6/DNA complexes directly into the labeling media. DNA complexes were prepared by mixing pcDNA3.1 plasmids expressing IPMK, IP3K, or ITPK1 with either pcDNA3.1 (*Left*) or pcDNA3.1-$G\alpha_q$QL (*Right*); 100 ng of each cDNA was used for the transfection, and empty pcDNA3.1 was used to bring the total DNA in each transfection to 500 ng per well. Soluble IPs were extracted from the cells by using HCl and resolved by HPLC.

kinases were coexpressed with $G\alpha_q$QL, tremendous changes in the profiles of individual IP metabolites were observed (Fig. 3 *Right Lower*). Coexpression of $G\alpha_q$QL + IPMK led to massive up-regulation of IP_3, IP_4, and IP_5, whereas coexpression of $G\alpha_q$QL + IP3KA and $G\alpha_q$QL + ITPK1 led to large increases in IP_4 and smaller but significant increases in IP_5. Significantly, the IP_4 species produced as a result of the expression of each IPK were unique (Table 1). IPMK expression resulted in an increased production of $I(1,4,5,6)P_4$, IP3KA expression yielded $I(1,3,4,5)P_4$, and ITPK1 produced a mixture of IP_4 composed of 60% $I(1,3,4,6)P_4$ and 30% $I(1,3,4,5)P_4$. The IP_4 species that accumulate in response to IP3KA and ITPK1 expression are the expected products of these enzymes, whereas the $I(1,4,5,6)P_4$ that accumulates in response to IPMK may be a mass action increase through metabolism of IP_5 (see Fig. 1) because mammalian IPMK has been shown to function as a dual-specificity $I(1,4,5)P_3$ 3-kinase and $I(1,3,4,5)P_4$ 6-kinase. These results demonstrate three important findings: (*i*) expression of $G\alpha_q$QL generates a release of IP_3 that is accessible to IP_3 kinases, (*ii*) there is a synergistic activation of IP metabolism between G protein and IPKs, and (*iii*) the different IP_3 kinases enable the generation of distinct IP profiles or patterns in response to G protein activation, thereby enhancing the complexity of signals downstream of PLC.

Table 1. Identification of IP_4 species in cell extracts by enzymatic analysis

Cell extract	Enzymatic treatment, percent conversion				Primary IP_4 species
	IPMK	IPK1	5-Ptase	ITPK1	
Gaq + IPMK	82	22	11	18	$I(1,4,5,6)P_4$
Gaq + IP3K	92	14	86	0	$I(1,3,4,5)P_4$
Gaq + ITPK1	94	60	32	0	$I(1,3,4,6)P_4$

Cell extracts were prepared from [^{3}H]inositol-labeled 293T cells and were treated enzymatically with IPMK, Ipk1, 5-Ptase, or ITPK1 to identify the major IP_4 species present. Assignments were based on the expected substrates of each enzyme. $I(1,4,5,6)P_4$ is a substrate for IPMK, $I(1,3,4,5)P_4$ is a substrate for IPMK and 5-Ptase, $I(3,4,5,6)P_4$ is a substrate for ITPK1, and $I(1,3,4,6)P_4$ is a substrate for IMPK and IPK1. Results are expressed as the percentage of the IP_4 peak that was converted by the enzymatic treatment.

$G\alpha_q$ Induces Robust IP_5 Synthesis. To ascertain whether similar genetic perturbation strategies could be used to expose changes in IP_5 and IP_6 metabolism, two molecules widely viewed as stable and whose levels are relatively high at resting state in mammalian cells (Fig. 3 *Right Upper*), we overexpressed IP6K1 and IPK1 in activated cells. We surmised that these experiments also would enable monitoring the effects of G protein activation at endogenous levels of the IP_3 and IP_4 kinases. Similar to what was observed for the IP_3 kinases, overexpression of IPK1 or IP6K, or both, in the absence of $G\alpha_q$QL had minimal effects on IP profiles, with the exception of a decrease in IP_5 levels observed in IPK1-expressing cells (Fig. 4*A Left*). When $G\alpha_q$QL was coexpressed along with IP6K1, significant increases in the levels of PP-IP_4 and, to a lesser extent, two PP-IP species more polar than IP_6 were observed (Fig. 4*A Right Top*). As a means to determine whether these pyrophosphate species emerged from flux through IP_5 or IP_6, or both, we treated the extracts with recombinant diphosphoinositol phosphate phosphatase (DIPP) and found that all PP-IPs were quantitatively hydrolyzed to IP_5 (Fig. 4*B Left*), indicating that they emerged from IP_5 metabolism and not from IP_6. Our data provide evidence that IP_5 synthesis increases significantly in response to $G\alpha_q$ and that IP6K1 is a useful sensor for exposing this metabolism. Importantly, we find that this flux does not require overexpression of IP_3 and IP_4 kinases and occurs at endogenous levels of these enzymes.

We next examined the coexpression of IPK1 with $G\alpha_q$QL and observed only a slight increase in IP_6 levels compared with cells transfected with IPK1 alone (Fig. 4*A Middle*). Although these data did not corroborate a G protein-induced flux through IP_5, we reasoned that if increases in IP_6 were rapidly metabolized, such effects could be masked. Therefore, we coexpressed both IPK1 and IP6K1 along with $G\alpha_q$QL and observed a large increase in the levels of PP-IP_5 (Fig. 4*A Bottom*). The identity of this peak was confirmed to be PP-IP_5 by treatment with DIPP, which hydrolyzed the molecule to form IP_6 (Fig. 4*B Right*). These data provide further support of a model in which activated $G\alpha_q$QL induces a flux of IP_5 synthesis and indicate that coincidently coupling IPK1 and IP6K1 activation shifts production from PP-IP_4 to PP-IP_5, providing yet another layer of signaling complexity.

Another intriguing aspect of these results was that overexpression of IP6K alone resulted in minimal increases in PP-IP_4 and PP-IP_5 despite the presence of significant amounts of IP_5 and IP_6. In contrast, when IP6K1 expression was coupled to $G\alpha_q$QL or $G\alpha_q$QL + IPK1, there were relatively large elevations in the PP-IPs. These data are consistent with a model in which IP_5 and IP_6 are present in multiple pools, including those that are accessible to IP6K only in response to the activation of G protein. Furthermore, these data highlight that the endogenous cellular machinery has a tremendous capacity to maintain the levels of both IP_5 and IP_6 even in the presence of what appeared to be significant up-regulation of their synthesis in response to G protein activation.

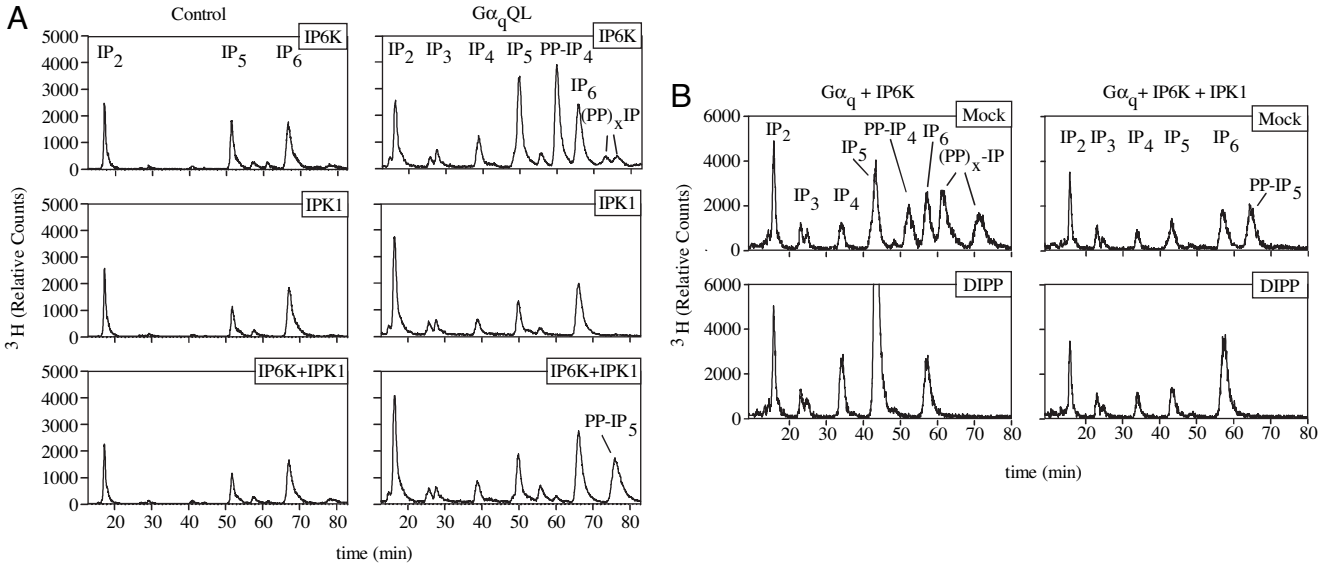

Fig. 4. Detection of a flux of IP_5 emanating from activated $G\alpha_q$ through the coexpression of IP_5/IP_6 kinases. (*A*) Capture of a flux of IP_5 synthesis by IP_5 kinases. *myo*-[^{3}H]inositol-labeled cells were transfected with plasmids expressing IP6K1, IPK1, or IP6K1 and IPK1 with either pcDNA3.1 (*Left*) or pcDNA3.1-$G\alpha_qQL$ (*Right*). Soluble IPs were extracted from the cells by using HCl and resolved by HPLC. (*B*) Enzymatic analysis of IP6K generated IP pyrophosphates. HEK293T cells were metabolically labeled and transfected with $G\alpha_qQL$ and IP6K1 (*Left*) or $G\alpha_qQL$, IP6K1, and IPK1 (*Right*). Cells were harvested in boiling hot 50 mM Tris, and tubes containing collected cell debris were further heated for 5 min in boiling water to minimize phosphatase activity against soluble IPs. Extracts were incubated in reaction buffer for 30 min at 37°C in the absence or presence of 1 μg of human DIPP. Reactions were halted by the addition of 0.5 M HCl, and samples were resolved by HPLC. Slight changes in the elution times of IPs in *A* and *B* are due to the use of different HPLC columns between the experiments.

G Protein Activation, Along with Expression of the Four Evolutionarily Conserved IPKs, Increases IP_3 to IP_8 Metabolism. Four of the six classes of IPK gene products, IPMK/IPK2, IPK1, IP6K/KCS1, and VIP1, are present in genomes from fungi to man. To test whether all four kinases were linked to G protein-activated IP_3 metabolism, we coexpressed them in combinations in activated HEK293T cells. Expression of any one individual or combinations of two, three, or all four IPKs in the absence of coexpression with $G\alpha_qQL$ resulted in minimal observable changes in the IP profiles (data not shown). In contrast, when $G\alpha_qQL$ was concomitantly expressed, dramatic changes were observed (Fig. 5). As reported in Fig. 3, G protein-mediated induction of IP_5 synthesis was massively up-regulated by the coexpression with IPMK, which can be fully appreciated by extending the *y* axis of the HPLC analysis (Fig. 5 *Top*). The up-regulation of IP_5 synthesis was quantitatively shifted to IP_6 by the coexpression of IPK1, along with $G\alpha_qQL$ and IPMK (Fig. 5 *Top Middle*). This result differed from $G\alpha_qQL$ + IPK1 (Fig. 4*A Right Middle*), suggesting that concomitant up-regulation of both IPMK and IPK1 cooperate to boost IP_6 synthesis in response to G protein activation. An increase in PP-IP_5 also was observed. However, its level was 5% of the IP_6 peak, similar to the relative ratio of IP_6 and PP-IP_5 in untreated cells, indicating that this increase is likely a mass-action effect. Remarkably, the coexpression of $G\alpha_qQL$, IPMK, IPK1, and IP6K1 resulted in yet another pattern of IP/PP-IP synthesis, in which the IP_6 levels were comparable to those seen in $G\alpha_qQL$ + IPMK + IPK1 cells, but the PP-IP_4 and PP-IP_5 levels were now dramatically elevated (Fig. 5 *Bottom Middle*). Last, overexpression of all four evolutionarily conserved IPKs and $G\alpha_qQL$ resulted in the sequential conversion of IP_3 to IP_8 as measured by the accumulation of PP_2-IP_4 (Fig. 5 *Bottom*). Collectively, our data suggest that these four gene products link to G protein activation and indicate that an evolutionarily conserved minimum kinase requirement for conversion of IP_3 to IP_8 is encoded by IPMK, IPK1, IP6K, and VIP1.

Discussion

Our results identify that coupled activation of a G protein and one or more of six classes of evolutionarily conserved IP kinases synergistically produce many distinct patterns of IP and PP-IP messengers. The ability to manipulate the concentrations and profiles of each IP species through differential activation of IPKs

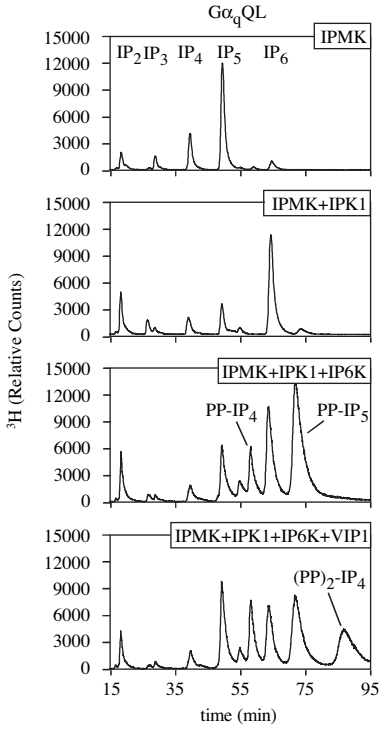

Fig. 5. Coexpression of activated $G\alpha_q$ with four of the six classes of IP kinases stimulates production of IP_8. $G\alpha_qQL$ and IPMK were coexpressed with IPK1, IPK1 + IP6K1, or IPK1 + IP6K + VIP1. Soluble IPs were extracted from the cells by using HCl and resolved by HPLC. Note the changes in the scale of the *x* and *y* axes from previous figures.

indicates that collectively these molecules could comprise an IP code capable of enhancing intracellular messenger complexity in mammalian cells. In this case, each species represents a building block of the code. By transiently assembling different combinations of IP and PP-IPs, the cell could, in theory, rapidly remodel the signaling landscape downstream of PLC activation. At this point, we have shown that the IPK machinery exists to alter IP profiles under engineered conditions. However, proof that agonist stimulation induces changes in a putative IP code remains to be established. Nonetheless, our data linking changes in this putative IP code to G protein activation in mammals provide an impetus to explore whether agonist stimulation of any of the hundreds of GPCR or possibly even the RTK pathways alters the code and whether one or more of the IPK gene products is required to mediate signaling processes. Of particular relevance, during the revision of this manuscript, Gao *et al.* (35) found that Wnt3a activation of $G\alpha_q$ (through the Frizzled-1 receptor) causes a transient increase in IP_5, and indicated that siRNA knockdown of IPMK and IP3KB inhibited the ability of Wnt3a to stimulate the canonical β-catenin/lymphoid enhancer factor/T cell factor pathway.

Is there evidence that other signaling processes depend on a putative IPK-dependent IP code? Yes. Recent studies of several IPKs have demonstrated their essential involvement in organism development and function (17, 36–41). Given the highly orchestrated nature of these processes, it is reasonable to conclude that the production of an IP code that emerges from IP_3 is involved in the execution of these instructions. Studies of the IPKs in yeast and *Dictyostelium* demonstrate their roles in cellular adaptation and survival under conditions of nutritional change and stress and have provided important insights into a wide range of cellular processes that are regulated by the IP and PP-IP messengers (10, 15, 24, 25, 42–51). On this basis, it has been proposed that a primordial role for activation of PLC is to up-regulate IPK-dependent signaling (9, 52). This notion is further supported by our current studies in mammalian cells. Additionally, data from both uni- and multicellular organisms have provided clues into the intracellular receptors that participate in decoding the IPK-dependent IP_4, IP_5, IP_6, and PP-IP messengers, in particular with respect to mRNA export (49, 50), chromatin remodeling (46, 47), RNA editing (53), protein phosphorylation (54), phosphate signaling (25, 51), auxin biology (41), and immune function (40). Collectively, these data provide exciting evidence supporting a role for an IPK-dependent IP code in signal-transduction pathways.

Do IPK-dependent products IP_4, IP_5, IP_6, and PP-IPs function as classical second messengers in a manner analogous to IP_3 or do they regulate signaling through a novel mechanism? Evidence exists that supports each model. For molecules such as IP_4 and some of the PP-IPs, there is convincing evidence that, upon stimulation, their concentrations are transiently induced and quickly returned to low levels, consistent with classic second-messenger behavior. In contrast, for molecules whose levels appear elevated even under resting conditions, such as IP_6 and, in some cell types, IP_5, other modes of regulation have been proposed (55). Our data support a model in which IP_5 and IP_6 are present in cells in multiple pools, such that there may be both stable reservoirs as well as those that are signal-induced (low at resting state and that, upon stimulation of G protein, are rapidly elevated, as highlighted by our IP6K1 overexpression studies). In this way, IP_6 may serve as both a second messenger and a precursor to other signaling reactions, analogous to the lipid phosphatidylinositol 4,5-bisphosphate. Evidence for a mechanism of action for IP_6 comes from elegant structural studies of an RNA editing enzyme, ADAR2, and from the auxin receptor in plants, TIR1 (41, 53). The crystal structure of ADAR2 revealed that IP_6 was a structural cofactor buried within the protein whose presence was critical for deaminase activity (53). In the case of TIR1, the hormone auxin and IP_6 were bound within the structure at distinct, but adjacent, sites consistent with TIR1 functioning as a coincidence detection sensor (41). IP_6 appeared necessary for the stabilization of the superhelical structure of the leucine-rich repeats and is likely critical for the function of TIR1. Given the nonexchangeable nature of IP_6 binding in both structures, it is unlikely that acute changes in a messenger would be rapidly transduced. In this scenario, IP_6 would function as a regulatory cofactor that could alter the stability or folding of the receptor over longer periods of time.

As our studies demonstrate, cells have a tremendous capacity to maintain IP homeostasis, none more obvious than the control of IP_5 and IP_6. We suspect that this finding may provide a plausible explanation for why IPK-dependent changes in IP metabolism in response to agonist stimulation have been difficult to detect. Our use of genetic perturbation to trap or alter this homeostasis provides new tools to approach these types of studies. Because metabolic labeling has limitations with respect to detecting large fluxes in IP metabolism, measurements of IP metabolism in response to agonists would be greatly aided by cell-based sensors of the individual IP or PP-IP molecules or the processes they regulate. Regardless of the exact mechanisms of regulation, our work provides a compelling link between the activation of G proteins and the production of IP_4, IP_5, IP_6, and several PP-IPs. We now have important reagents and information that enable further examination of the involvement of the IPKs as transducers of the myriad of potential signaling pathways downstream of GPCRs.

Methods

Materials and Cell Culture. Tissue culture reagents were purchased from Sigma–Aldrich (St. Louis, MO) and Invitrogen (Carlsbad, CA). Inositol free-labeling DMEM was from Specialty Media (Phillipsburg, NJ). Molecular biology reagents used for subcloning and PCR were purchased from New England Biolabs (Ipswich, MA) and Stratagene (La Jolla, CA). Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). HEK293T and Rat1 cells were obtained from the Duke University Medical Center Cell Culture Facility. All cell lines were maintained in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Adenovirus and Plasmid Constructs. Construction of recombinant control adenovirus and adenovirus expressing human $G\alpha_qQL$ were described previously (56). The cDNA for human $G\alpha_qQL$ in pcDNA was from UMR cDNA Resource Center (Rolla, MO). The construction of the rat IPMK cDNA construct pBabePuro-GFP-rIPMK was described previously (57). The human Vip1 kinase domain (residues 1–387) CFP fusion construct pmCFP-*hs*VIP1-KD has been described (27). The cDNA for human IP6K1 (pCMV-SPORT6-hIP6K1), human IPK1 (pBluescriptR-hIPK1), human IP56K (pCMV-SPORT6-hIP56K), and human ITPKA (pBluescriptR-hITPKA) were from Open Biosystems (Huntsville, AL). The cDNA for hIPK1 was subcloned from pBluescriptR-hIPK1 into pcDNA3.1(−) by using 5′-XhoI and 3′-BamHI restriction sites that flank the ORF to generate pcDNA3.1-hIPK1. The cDNA of hITPKA from residues 185–461 was amplified by PCR by using pBluescriptR-hITPKA as a template and inserted into a pcDNA3.1 construct containing the cDNA for CFP, generating pcDNA3.1-CFP-hITPKA.

Metabolic Labeling in Cells and HPLC Analysis of Inositol Phosphates. Cells were seeded on tissue culture plates at densities of 25,000–100,000 cells per ml. Twelve-well plates (1-ml media) were used for direct HPLC analysis of soluble IPs, and 6-well plates (2-ml media) were used to generate extracts for enzymatic analysis. After incubation overnight, cells were washed one time with

inositol-free DMEM and then labeled for 72 h with 37.5 μCi/ml *myo*-[^{3}H]inositol in inositol-free DMEM supplemented with 10% dialyzed FBS. Cells were infected with adenovirus or transfected with FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) after 48 h of labeling directly into the labeling media. Cell extracts destined for direct HPLC analysis were prepared by aspirating the labeling media, washing the cells with 1 ml of PBS, and adding 0.4 ml of 0.5 M HCl. HCl was left on the cells for 5 min to extract the soluble IPs and was then removed from the cells and filtered through a 0.45-μm nylon filter to remove cell debris. Samples were stored at −80°C.

Cell extracts for enzymatic analysis were prepared by aspirating the labeling media from cells, washing the cells with 2 ml of PBS, and adding 0.8 ml of boiling hot 50 mM Tris·HCl (pH 8.0). Cells were scraped from the plate, transferred to microfuge tubes, and boiled for an additional 5 min. Extracts were passed through a 22-gauge needle several times, cell debris was pelleted by centrifugation, and samples were filtered through 0.45-μm nylon filters. Samples were stored at −80°C before enzymatic analysis.

Before HPLC analysis, samples were diluted with four volumes of 10 mM $NH_4H_2PO_4$ (pH 3.5). Soluble IPs were separated by HPLC on a Partisphere SAX column (4.6 × 125 mm; Whatman, Clifton, NJ) by using the following buffer profile: 10 mM $NH_4H_2PO_4$ (pH 3.5) for 5 min, a linear gradient of 10 mM $NH_4H_2PO_4$ to 1.7 M $NH_4H_2PO_4$ for 65 min, and 1.7 M $NH_4H_2PO_4$ for 30 min. Radiolabeled IPs eluting from the column were quantified by using an inline radiation detector.

Determination of IP Molecule Identity by Using Enzymatic Analysis. The expression and purification of *Arabidopsis thaliana* IPK1, *A. thaliana* IPK2/IPMK, human I(1,4,5)P_3 type I 5-phosphatase (5-Ptase), and human DIPP have been described (17, 58). Recombinant human ITPK1 was generously provided by S. Shears (National Institute of Environmental Health Sciences, Research Triangle Park, NC) and used under published conditions. Metabolically labeled cell extracts that were harvested in boiling 50 mM Tris·HCl (pH 8.0) were incubated with 1 μg of enzyme in 50 mM Hepes (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, and 1 mM ATP at 37°C for 20 min. Reactions were halted by the addition of 0.5 M HCl, and the samples were processed for HPLC analysis as described above.

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