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Phosphoinositide lipid second messengers: new paradigms for transepithelial signal transduction

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Abstract Multiple forms of phosphatidylinositol are generated by differential phosphorylation of the inositol headgroup. These phosphoinositides, specifically PI(4,5)P₂, have been implicated as modulators in a variety of transport processes. The data indicate that phosphoinositides can modulate transporters directly or via the activation of down-stream signaling components. The phosphoinositide pathway has been linked to changes in transporter kinetics, intracellular signaling, membrane targeting and membrane stability. Recent results obtained for several of the well-characterized transport systems suggest the need to reassess the role of PI(4,5)P₂ and question whether lower abundance forms of the phosphoinositides, notably PI(3,4,5)P₃ (PIP₃) and PI(3,4)P₂, are the pertinent transport regulators. In contrast to PI(4,5)P₂, these latter forms represent a dynamic, regulated pool, the characteristics of which are more compatible with the nature of signaling intermediates. A recently described, novel transepithelial signaling pathway has been demonstrated for PIP₃ in which a signal initiated on the basolateral membrane is transduced to the apical membrane entirely within the planar face of the inner leaflet of the plasma membrane. The new paradigms emerging from recent studies may be widely applicable to transporter regulation in other cell types and are particularly relevant for signaling in polarized cells.

transduction pathway whereby specific cleavage of the phosphatidylinositol head group produced soluble (inositol phosphates) as well as membrane-bound (diacylglycerols) intermediates [3, 8]. While this pathway is initiated at the plasma membrane, typically by extracellular stimuli, the products of the enzymatic cleavage enable the signal to be communicated to intracellular organelles, most often the endoplasmic reticulum, where the release of Ca²⁺ propagates and potentiates the stimulus.

More recently, a separate signal transduction mechanism, also originating with a modification of the phosphatidylinositol headgroup, has been elucidated. This mechanism, which has come to be described as the phosphoinositide (PI) pathway, originates with the enzymatic modification of the inositol headgroup by phosphorylation resulting in multiply phosphorylated forms of the lipid. In contrast to the previously mentioned pathway, this signal transduction mechanism is envisioned as being propagated by the intact lipid rather than by cleavage products. It is the latter pathway that is the topic of this review. However, it is important to point out that the two pathways need not be mutually exclusive—it is clear that they often co-exist in the same cells and utilize the same initial substrate. Thus, positive and negative effectors that change the level of the substrate phospholipid have the potential to modulate either pathway.

Phosphatidylinositol—a multifunctional substrate

Phosphatidylinositol is a low abundance membrane lipid that serves as a precursor for several signaling pathways. Research that culminated in the 1980s elucidated a

Modification of the phosphatidylinositol head group

In the intact phosphatidylinositol lipid, the myo-inositol head group can be enzymatically phosphorylated at three positions—3, 4, and 5—giving rise to mono-, bis-, and tris-phosphoinositides. Of the various possible combinations and permutations, three relatively well-studied forms have been identified as possible signaling intermediates. These are the bis-phosphoinositides, PI(3,4)P₂ and PI(4,5)P₂, and the tris-phosphoinositide, PI(3,4,5)P₃ (PIP₃). The enzymatic interconversions between these three forms are illustrated in Fig. 1.

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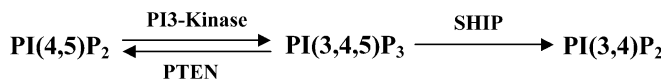


Fig. 1 Formation of low-abundance lipid signaling intermediates, PIP_3 and PI(3,4)P_2 from PI(4,5)P_2 . *PI3-kinase* phosphatidylinositol 3-kinase; *PTEN* phosphatase and tensin homologue; *SHIP* SH2-containing inositol 5'-phosphatase

Phosphatidylinositols (PI) represent less than 5% of the membrane phospholipid pool [27]. Within this phospholipid class, the various phosphorylated forms differ in relative amounts in a predictable manner in most cell types. Where the PI lipids have been isolated and quantitated, PI(4,5)P_2 has been found to comprise a large, relatively static pool, whereas PI(3,4)P_2 and PIP_3 are low abundance and dynamic, showing rapid changes in response to external stimuli. However, even in stimulated cells, PIP_3 and PI(3,4)P_2 represent only a small fraction of the total PI pool [6, 20, 27, 34, 37]. These characteristics implicate the latter two phospholipids as likely candidates for key players in signal transduction pathways.

Dynamic changes in the abundance of PIP_3 and PI(3,4)P_2 are the result of specific kinases and phosphatases. Of these, phosphoinositide 3-kinase (PI 3-kinase) is the best characterized, in part because of the availability of specific non-toxic inhibitors that can be used in live cells. Wortmannin, a fungal metabolite, irreversibly blocks PI3-kinase activity [30], whereas LY294002 is a completely reversible inhibitor [44].

The enzymes responsible for the turnover of PIP_3 have been characterized more recently. PTEN (phosphatase and tensin homologue), the converse of PI3-kinase, removes the 3 position phosphate producing PI(4,5)P_2 [10, 12]. PTEN is a tumor suppressor which has been found to be mutated or deleted in a variety of tumors [11, 24]. The tumorigenic properties that result from PIP_3 accumulation have been ascribed to the kinases activated downstream of PIP_3 which are involved in cell growth and survival [11, 24].

The SHIP (SH2-containing inositol 5'-phosphatase) proteins are a family of enzymes initially identified in blood cells. These phosphatases remove the 5' phosphate from the respective PI forms, thereby resulting in heightened PI(3,4)P_2 production. SHIP knockout mice show myeloproliferation and growth factor hyper-responsiveness [35], whereas SHIP 2 knockout mice show increased sensitivity to insulin [36].

The physiological consequences resulting from a lack of these enzymes underscores the importance of the low abundance lipid products. Thus, despite their minute quantities, PIP_3 and PI(3,4)P_2 are important in cellular regulation.

PI pathway—building a scaffold

The PIP_2 s and PIP_3 are multifunctional protein binding entities capable of interacting with a variety of sub-

strates. In this regard, they can be envisioned as the nucleus of a signaling scaffold that recruits cytosolic signaling intermediates to the cell membrane [10, 12, 37]. Lipid binding pleckstrin homology (PH) domains in proteins show specificity in their affinity for PI(4,5)P_2 , PI(3,4)P_2 or PIP_3 [31, 32]. Therefore, the production of particular phosphorylated PIs can serve as targeting sites for signaling pathway intermediates. For example, the formation of PIP_3 and/or PI(3,4)P_2 by PI3-kinase, recruits soluble proteins such as protein kinase B (PKB) and serum glucocorticoid kinase (Sgk) to the membrane where they are activated and participate in downstream signaling events [38]. The kinase cascades set in motion by the formation of PIP_3 have been extensively reviewed [10, 12, 37].

Phosphoinositides can also bind to Src homology 2 (SH2) domains, including that of the p85 α subunit of PI3-kinase [14]. The ability of phosphoinositides to bind SH2 domains is not as well delineated but may play a crucial role in phosphotyrosine-dependent protein recruitment.

PI3-kinases are a family of homologous proteins usually consisting of a regulatory subunit and a catalytic subunit. The best characterized are the class 1a PI3-kinases, composed of an 85-kDa regulatory subunit and 110-kDa catalytic subunit. This is the type of PI3-kinase that is recruited to the plasma membrane in response to insulin receptor activation and the production of the tyrosine-phosphorylated insulin-receptor substrate (IRS). There are, of course, other signals that will recruit PI3-kinases to the membrane. Predominately these consist of cell surface receptors that, like insulin, can activate PI3-kinase directly or through adaptor proteins including the small GTP-binding proteins [37, 47].

Shepard and colleagues have suggested that the PI3-kinases serve as part of a larger scaffolding complex via the SH3 binding sites on the regulatory subunit [37]. Since this protein complex is recruited to the membrane, one can now consider the possibility of a rather large signaling complex, the strength and size of which is dependent on the number and affinity of the multiple binding partners.

PI lipids as modulators of transport proteins—regulation of exocytosis and endocytosis

One of the most widely studied roles of the PI family of lipids is the regulation of transport proteins. Demonstrated and hypothetical mechanisms include direct modulation of channel activity, alterations in channel membrane targeting and the formation of scaffolds which can recruit effectors as well as cytoskeletal elements. Interestingly, differentially phosphorylated forms of the phosphoinositides may regulate the same transport processes with different temporal phases.

The quantity of plasma membrane-expressed transporters represents a delicate balance between exocytic and endocytic events and both are regulated indepen-

dently. The prototypic example of phosphoinositide modulation of a transporter is the insulin mediated regulation of the glucose transporter, GLUT 4, in various tissues. PI(4,5)P₂ has well documented effects on actin polymerization and changes in PI(4,5)P₂ metabolism have been shown to affect the endocytic recycling pathway for GLUT4 resulting in a longer residence time for the transporter in the plasma membrane [22]. On the exocytic side of the recycling pathway, PIP₃ is necessary and sufficient for the insertion of GLUT4 into the plasma membrane, however, PIP₃-mediated insertion of the transporter is not accompanied by an increase in glucose uptake, suggesting the need for an additional, unknown step in the activation pathway [41]. Concurrent with these changes, the PI pathway also activates a number of downstream serine/threonine kinases such as PKB (also called Akt) which are also intimately involved in the regulation of GLUT4 trafficking and activity [1].

PI regulation of ion transport proteins

With regard to the modulation of ion transporters, the most abundant member of the PI lipids, PI(4,5)P₂ is the favored regulator [20]. PI(4,5)P₂ may be necessary for functional maintenance of ion channel activity in a manner analogous to the increased residency time of GLUT 4 in the plasma membrane. PI(4,5)P₂ has also been postulated to form a link between the cytoskeleton and the plasma membrane [21, 32]. An intact cytoskeleton is necessary for the function of some ion channels and, therefore, represents a further level of potential control. In addition, PI(4,5)P₂ has been demonstrated to reside at least partially in lipid rafts from which lateral diffusion is restricted [27]. In this regard, the “raft” could be envisioned as a confined area where specific intermediates are assembled. Finally, PI(4,5)P₂ has recently been shown to immobilize the inactivation domain of the delayed rectifier, voltage gated K⁺ channel thereby converting it into an A-type K⁺ channel [29].

Is PI(4,5)P₂ the lipid that regulates the majority of the PI-sensitive ion channels as is rather widely accepted? Upon careful consideration, PI(4,5)P₂ is not, a priori, the most logical candidate as a signal transduction intermediate or as a mediator that would regulate channel activity acutely in response to a stimulatory agent. The static nature of the PI(4,5)P₂ pool is not compatible with the usual characteristics of signaling intermediates. The literature also contains cautionary indications against uniformly assuming PI(4,5)P₂ is the pivotal regulator.

The elucidation of the patch clamp technique for studying transport through ion channels has afforded researchers with the unique ability to study the regulation and kinetics of these proteins at a single channel level [28]. Channels can be studied in their native environment, placed in an artificial lipid bilayer, or studied in a heterologous expression system. In addition, the formation of inside-out patches allows the investigator

to alter the composition of the fluid bathing the cytoplasmic face of the membrane in order to determine the effect of various intracellular mediators.

Using the technique of patch clamp, PI lipids have been perfused onto the cytoplasmic face of patches formed from various types of cells and the effect on a number of different channels has been documented. In many cases, PI(4,5)P₂ appears to directly modulate channels contained in excised patches [reviewed in 20]. For example, ATP-sensitive K⁺ channels (K_{ATP}) present in excised membrane patches from both native and heterologously expressing cells were found to be activated by PI(4,5)P₂ when the lipid was applied to the cytosolic face [2, 19, 38]. Following these experiments, it was assumed for several years that PI(4,5)P₂ was the pertinent channel activator. However, an elegant series of studies by Harvey and co-workers have subsequently demonstrated that both PI(4,5)P₂ and PIP₃ can activate the channel [17, 18]. Furthermore, the effect of PI(4,5)P₂ is sensitive to LY294002 and wortmannin suggesting that the formation of PIP₃ is required for channel function. These studies indicate that the excised patches contain the PI3-kinase necessary to convert PI(4,5)P₂ to PIP₃ and that the latter is most likely the pivotal regulator. Is this an isolated finding or is it likely that PI(4,5)P₂ is not the key PI in the regulation of other transporters?

The epithelial sodium channel, ENaC, has also been shown to be regulated by PIs. The evolution of the understanding of which phosphoinositides are key in the modulation of ENaC is, in some ways, similar to that of K_{ATP}. However, in the case of ENaC, PI lipids may exert a bewildering array of effects on the Na⁺ transport processes by affecting both acute and chronic ENaC channel activity.

The multiplicity of effects of PI lipids on ENaC—questions and controversies

Regulation of ENaC by phosphoinositides has been examined from a functional standpoint in confluent high resistance epithelia, at a single channel level, in heterologous systems as well as in cells natively expressing the channel. The techniques include electrophysiological assays (whole tissue and patch clamp), biochemical quantitation, and visualization of lipid production in live, responsive cells.

The apparent controversies that emerge from a compilation of these studies are: (1) a discrepancy as to the nature of the key phosphoinositides which regulate the channel, and (2) the mode of action of the lipid in regulating the channel, often expressed as a stimulation of ENaC insertion into the apical membrane versus a change in the kinetic parameters of membrane-resident channels.

The first controversial issue, that of which phosphoinositide lipid functionally interacts with ENaC, may be analogous to the apparent discrepancies reported for

regulation of the K_{ATP} channel. We will review some of the findings below. The second controversy, the question of whether the phosphoinositides regulate ENaC trafficking or channel kinetics, may not be a controversy at all—in fact, PIs may regulate channel exocytosis, endocytosis and open probability albeit with different time frames.

Initial studies indicating PI regulation of ENaC arose from the observation that LY294002, a reversible inhibitor of PI3-kinase, blocked insulin-stimulated Na^+ transport in the A6 cell line, a well-characterized model of the principal cells of the distal nephron [34]. Within 4 min, insulin causes a measurable increase in transepithelial Na^+ flux in intact monolayers [9, 34]. The natriuretic (Na^+ retaining) effect is preceded by a rapid (~ 1 min) increase in PIP_3 in the cells. LY294002 blocked the formation of the PIP_3 as well as the insulin-induced Na^+ transport. Thus, the correlation between function and biochemistry suggests that PIP_3 may be a key regulator of ENaC-mediated Na^+ transport.

In addition to cell surface receptors like insulin, the PI pathway can also be activated by steroid hormones in absorptive epithelial cells. For example, aldosterone causes an increase in the production of PIP_3 in A6 cells. Consistent with the long-term, protein synthesis dependent effects of the steroid hormone, the increase in PIP_3 formation requires 20–60 min to reach a measurable level. This is in agreement with the increase in transcellular Na^+ flux which begins within 45–60 min after hormone addition, also in a protein synthesis dependent fashion. Both the production of PIP_3 and the functional effects of aldosterone in this cell type are blocked by LY294002 [6].

Interestingly, aldosterone also causes an increase in the synthesis of Sgk—a downstream kinase recruited to the membrane and activated in response to increased PI3-kinase activity [13, 26]. Sgk phosphorylates and inactivates NEDD4, a ubiquitin ligase which is thought to stimulate channel endocytosis and degradation via ubiquitination of ENaC [39, 40]. Thus, aldosterone has at least two effects on the PI pathway—the formation of PIP_3 which may have immediate effects on the channel, and the increased synthesis of Sgk which is known to function in the regulation of channel endocytosis after activation by the PI3-kinase pathway.

The results obtained in the intact cell studies are in contrast to patch clamp studies also performed in the A6 cell line. Yue et al. found an increase in NP_o (N denotes the number of channels in the membrane; P_o represents the open probability) in excised patches perfused with 30 μM $PI(4,5)P_2$ in the presence of 100 μM GTP or 10 nM αG_{i-3} [47]. Curiously, neither PIP_3 nor $PI(3,4)P_2$ were effective in increasing activity even in the presence of GTP.

In a simultaneous publication, Ma et al. found that either 5 μM $PI(4,5)P_2$ or PIP_3 prevented ENaC channel rundown (loss of activity) in excised patches from A6 cells. Since phosphatidylserine was also effective in preventing channel rundown, the authors suggested that the

action was mediated mainly by anionic lipids [23]. In contrast to the Yue study, these effects were not dependent on GTP.

The differences between the findings of the Yue and Ma studies and those performed in intact A6 cells remain unexplained—particularly the discrepancies between the requirement for GTP and the structure of the PI which modulates channel activity. It is important to note that both of the patch clamp studies were performed on A6 cells that had been chronically treated with aldosterone. Based on the previous studies in intact A6 cells, aldosterone causes the activation of PI3-kinase [6]. Activation of this enzyme is associated with a translocation to the plasma membrane. If the cells had been chronically treated with the steroid hormone, it is likely that a substantial amount of PI3-kinase may have been associated with the plasma membrane and, therefore, could have been part of the patch used to examine ENaC activity.

The requirement for GTP in the Yue study is intriguing particularly given the finding that the effects of insulin and aldosterone are additive in the A6 cell line [5, 33]. It is somewhat enigmatic that two hormones which stimulate the same intracellular pathway could have additive effects. The patch clamp finding may provide some clues to this puzzle. Several types of PI3-kinases are found in cells. Class 1b PI3-kinases are G-protein regulated while class 1a are independent of G-proteins but are stimulated in response to insulin [37]. Thus, the formation of PIP_3 via two different mechanisms could explain the additive functional effects of the two hormones, particularly if a step at or before the formation of PIP_3 were rate limiting.

Tong et al. also used patch clamp to examine the effects of the PI lipids on ENaC in Chinese hamster ovary (CHO) cells heterologously expressing the Na^+ channel [42]. In the CHO cells, co-expression of ENaC and constitutively active PI3-kinase increased ENaC-specific inward currents. When applied to an inside-out patch containing ENaC, $PI(3,4)P_2$ and PIP_3 caused increases in the open probability, P_o . This effect was not dependent on GTP. The authors concluded that the products of PI3-kinase can regulate the channel directly.

Recently Markadieu and colleagues examined the effects of phosphoinositide lipids in intact A6 cells by delivering the physiologically relevant long carbon chain derivatives to the native cells via histone carriers [25]. It is important to note that, unlike the patch clamp studies, the A6 cells were not chronically treated with aldosterone. Within 60 min of complex addition to the apical membrane, PIP_3 and $PI(3,4)P_2$ increased transepithelial Na^+ transport while $PI(4,5)P_2$ was completely ineffective. Expression of PTEN in excess over endogenous levels, decreased both insulin-stimulated and PIP_3 -stimulated Na^+ transport. Since PTEN decreases the amount of PIP_3 in cells (Fig. 1), simultaneously adding to the $PI(4,5)P_2$ pool, these findings argue against a stimulatory effect of $PI(4,5)P_2$ in the intact A6 cells. The authors also demonstrated that the p85 regulatory

subunit of PI3-kinase was recruited to a tyrosine-phosphorylated complex (presumably containing the insulin-receptor substrate) within minutes after insulin stimulation. As expected, PTEN over-expression did not affect this recruitment but did decrease the downstream activation of PKB in the cells [25]. These results indicate that the initial steps of the PI pathway proceed normally in the transfected cells but decreases in cellular PIP₃ inhibit downstream portions of the pathway resulting in decreased insulin-stimulated Na⁺ transport.

Thus, a consensus of the studies in the A6 cells seems to indicate that the products of PI3-kinase can regulate ENaC-mediated Na⁺ transport acutely, which is consistent with insulin's immediate action in this model of the principal cell type. Long-term regulation of the channel, consistent with aldosterone's natriuretic effects, may be mediated by a slower, protein synthesis dependent formation of PIP₃ and/or by activation of downstream elements of the pathway such as Sgk. Is this the whole story or do the phosphoinositide lipids have effects on channel function other than increasing open probability and inhibiting channel turnover via endocytosis?

Blocker induced noise analysis experiments have been performed on confluent monolayers of A6 cells stimulated with both insulin and aldosterone [5, 8]. Noise analysis is a technique that can independently measure N_T (channel density) and P_o in a non-invasive manner. These studies found that both insulin and aldosterone increase the density of active ENaC channels in the apical membrane with no increase in P_o . Inhibition of PI3-kinase with LY294002 reduces the number of open, functional channels in the membrane. These studies indicate that the formation of PIP₃ may also be a factor needed for channel insertion.

PIP₃ as a transcellular transduction element: a novel role for lipids in polarized cells

In addition to the functions summarized above, we have recently elucidated another, rather unique role of PIP₃—that of the element in the pathway that conveys the insulin signal from its origin on the basolateral membrane to the final effectors on the apical membrane (Fig. 2). Insulin binding to its receptor on the basolateral membrane recruits PI3-kinase to the basolateral membrane resulting in the production PIP₃ [8]. Lipids, such as PIP₃, formed in the inner leaflet of the bilayer can cross the tight junction of polarized epithelial cells [15, 43]. This is a property not shared by integral membrane proteins, confined to either the basolateral or apical membrane by their inability to cross the junctional complex. In this regard, lipids of the inner leaflet are uniquely positioned to act as signal transducers in polarized cells. However, until recently there was scant evidence that intact lipids played a role in signal transduction. Clearly, such a function requires the formation of a low-abundance molecule whose synthesis and deg-

radation are precisely controlled—a molecule such as PIP₃.

An A6 cell model expressing low, physiologically relevant levels of ENaC-GFP subunits has been produced and used to examine the natriuretic effects of insulin [7, 8]. Insulin stimulates the rapid recruitment of ENaC from a diffuse intracellular localization and stimulates its insertion into the apical membrane in a PI3-kinase-dependent manner [8, 9]. The route followed by the ENaC is somewhat atypical in that the channel appears to move first to the area of the lateral membrane where a co-localization with PI3-kinase is observed. This association is not preserved as ENaC crosses the tight junction and inserts into the apical membrane. Inhibition of PI3-kinase activity by LY294002 does not inhibit the co-localization but does alter the intracellular site of the interaction. In the presence of the inhibitor, the ENaC-PI3-kinase complex is not found along the lateral membrane but, rather, is present in a diffuse intracellular dispersion [8].

The production and localization of PIP₃ was examined in the A6 cell model. These recent studies used confocal microscopy with rapid image acquisition to visualize live cells in three dimensions. The live-cell studies confirmed the previous studies (done in fixed cellular monolayers) showing that ENaC is inserted into the apical membrane in response to insulin [9]. In addition, the most recent studies also confirm that ENaC is first recruited to the lateral membrane but extend the previous experiments to show that the channel does not enter the lateral membrane but seems to track along the cell periphery before entering the apical membrane.

To follow the route of the PIP₃ taken after insulin stimulation, cells were transiently transfected with the PIP₃ biosensor probe, EGFP-PH/Grp which specifically binds the tris-phosphate form of phosphatidylinositol [9, 46]. Using this probe, it is possible to track the production and movement of the lipid in real time. In response to insulin, PIP₃ is formed in the basolateral membrane, diffuses along the lateral membrane, crossing the tight junction and entering the apical membrane. ENaC also enters the apical membrane with a similar rapid time-course to that of PIP₃ [9].

Many questions are raised by these studies. What effectors are responsible for the recruitment and tracking of ENaC along the lateral membrane? What are the specific subcellular environmental conditions that enable ENaC to track along the inner leaflet of the basolateral membrane but to be inserted in the apical membrane? The reader can, no doubt, envision many other issues that remain unsolved. However, this unique signaling pathway has many satisfying aspects. It is difficult to imagine how the communication of a basolateral signal can be delivered to the apical membrane by diffusion of intracellular signaling components across the entire 3-dimensional volume of the cell. This is particularly difficult to comprehend when many of the components of the pathway are found to be associated with cellular

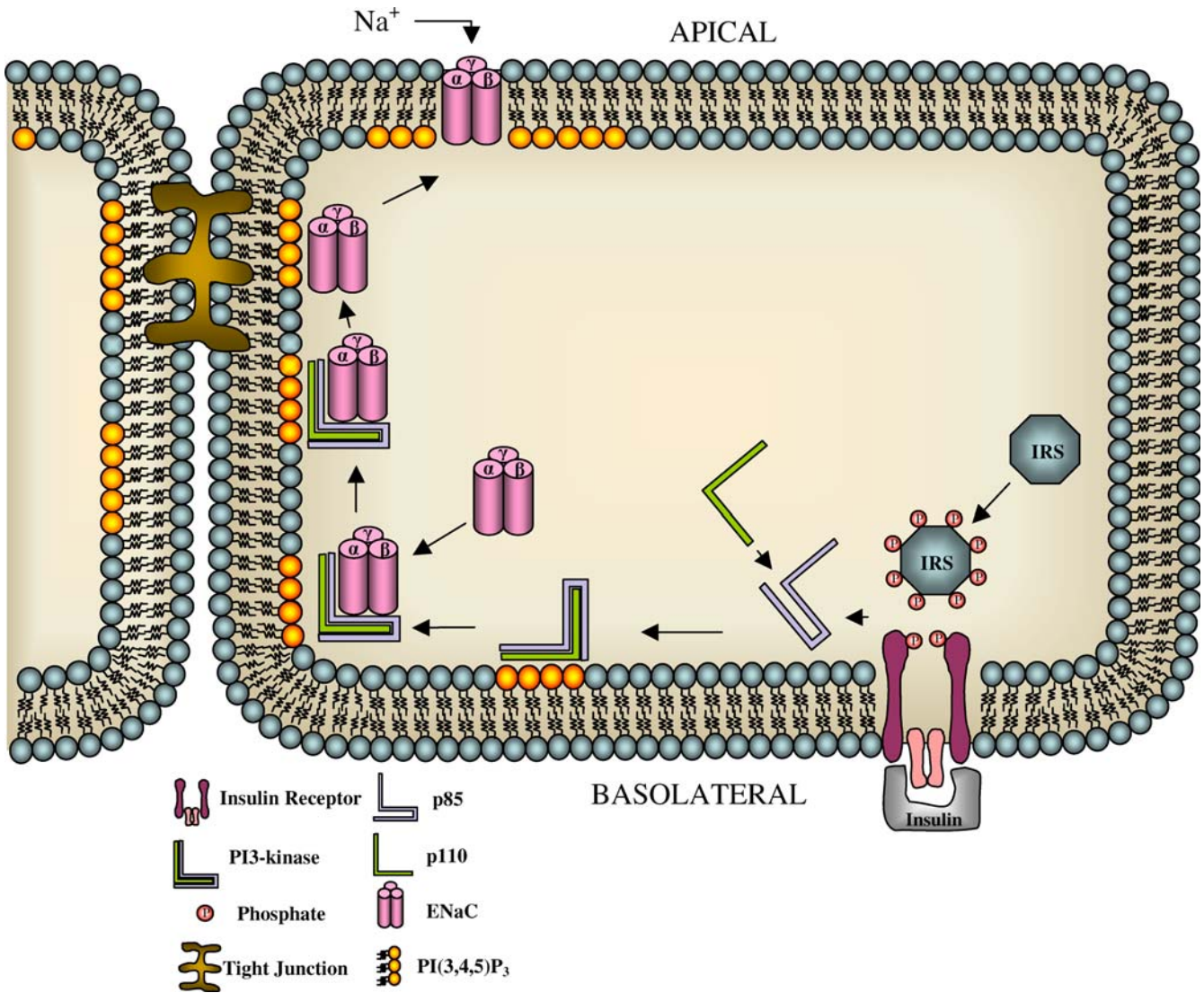


Fig. 2 Model of PIP_3 transepithelial signal transduction. Insulin binds to its receptor on the basolateral membrane activating receptor autophosphorylation which leads to the subsequent phosphorylation of the insulin receptor substrate (*IRS*). Phosphorylated *IRS* stimulates the activation of PI3-kinase and its translocation to the membrane. PI3-kinase catalyzes the formation of PIP_3 in the inner leaflet of the plasma membrane and this lipid diffuses within the plane of the membrane, crossing the tight junction to enter the apical membrane. Experimental results show

that immediately after insulin stimulation, the epithelial Na^+ channel (*ENaC*) colocalizes with PI3-kinase along the lateral membrane. The confocal methods used to characterize this colocalization do not allow a determination of whether *ENaC* and PI3-kinase are simply in close proximity or whether they physically interact. The colocalization is not seen when *ENaC* enters the apical membrane. The diffusion of PIP_3 into the apical membrane, appears to create a favorable environment for the insertion of *ENaC*.

membranes. Diffusion within the cytosol of the cell is also hard to regulate, especially if the intermediates modulate multiple cellular processes. Diffusion within the plane of the membrane, specifically the inner leaflet, provides for interaction with cytosolic elements but confines the signal to a more manageable planar surface and allows for the very rapid diffusional transit of a small quantity of signal.

The elucidation of the membrane de-limited pathway was made possible by several recent advances including the production of specific biosensor probes and the availability of confocal microscopes capable of rapid imaging of live cells. Perhaps the application of these

techniques to other cellular systems will indicate that this novel signaling pathway is not confined to *ENaC* but is a component of multiple types of polarized cell signaling.

Summary

The PI pathway is an integral part of the regulation of many different transport proteins. The role of the lower abundance members of this class of phospholipids remains largely unappreciated because of the difficulty in detecting and quantitating the very low quantities found

in both quiescent and stimulated cells. However, with the advent of specific inhibitors and sensitive bioprobes comes a new appreciation for the full range of potential mediators which can result from modification of phosphatidylinositol.

The formation of several different effector molecules raises the possibility of modulating multiple steps in a biochemical pathway. Taking as an example the amiloride-sensitive Na^+ transporter, ENaC, where the pathway has been studied both functionally and biochemically, it is clear that there are, indeed, multiple levels of regulation. Transepithelial Na^+ transport represents a beautifully orchestrated system in which components of the PI pathway have effects on channel insertion, change in open probability and membrane stabilization all initiated by the same signal(s) and all controlled as separate entities within different timeframes. This provides not only failsafe mechanisms but also precisely regulated control of ion transport and integration with multiple hormonal signals. As our knowledge of the nuances of the PI pathway intermediates evolves, it is likely that similar complexities will be elucidated in the regulation of other transport proteins in a variety of cell types.

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