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Regulation of Ins(3,4,5,6)P_4 Signaling by a Reversible Kinase/Phosphatase

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Summary

Regulation of Cl^- channel conductance by Ins(3,4,5,6)P_4 provides receptor-dependent control over salt and fluid secretion [1], cell volume homeostasis [2], and electrical excitability of neurones and smooth muscle [3]. Ignorance of how Ins(3,4,5,6)P_4 is synthesized has long hindered our understanding of this signaling pathway. We now show Ins(3,4,5,6)P_4 synthesis by Ins(1,3,4,5,6)P_5 1-phosphatase activity by an enzyme previously characterized [4] as an Ins(3,4,5,6)P_4 1-kinase. Rationalization of these phenomena with a ligand binding model unveils Ins(1,3,4)P_3 as not simply an alternative kinase substrate [4, 5], but also an activator of Ins(1,3,4,5,6)P_5 1-phosphatase. Stable overexpression of the enzyme in epithelial monolayers verifies its physiological role in elevating Ins(3,4,5,6)P_4 levels and inhibiting secretion. It is exceptional for a single enzyme to catalyze two opposing signaling reactions (1-kinase/1-phosphatase) under physiological conditions. Reciprocal coordination of these opposing reactions offers an alternative to general doctrine that intracellular signals are regulated by integrating multiple, distinct phosphatases and kinases [6].

Results and Discussion

Signaling entities are frequently controlled by quite delicate shifts in the dynamic balance of regulatory signals with competing impacts. Ion channels provide particularly impressive examples of the degree of signal amplification that can result; switching the conductance state of a single channel can influence the transmembrane movement of millions of ions per second [7]. Both stimulatory (Ca^{2+} and CaMKII) and inhibitory [Ins(3,4,5,6)P_4] signals converge on the family of so-called "Ca^{2+}-activated" Cl^- channels [3, 8, 9]. Thus, receptor-dependent changes in Ins(3,4,5,6)P_4 levels [10] is a topic of general biological significance, in that it impacts upon regulation of salt and fluid secretion from epithelial cells [1], cell volume homeostasis [2], and electrical excitability in neurones and smooth muscle [3, 11]. Unfortunately, understanding of the cellular control of Ins(3,4,5,6)P_4 signaling has been rudimentary, because the pathway of Ins(3,4,5,6)P_4 synthesis has not previously been characterized.

One possibility is that Ins(3,4,5,6)P_4 originates by phosphorylation of an InsP_3 precursor. We examined all potential InsP_3 precursors of Ins(3,4,5,6)P_4 by individually introducing them at 100 μM concentrations into human pancreatoma CFPAC-1 cells; the bioassay for Ins(3,4,5,6)P_4 synthesis by any endogenous kinase was the sensitivity of calcium-activated Cl^- channels to Ins(3,4,5,6)P_4 (IC_{50} = 3 μM) [3, 12]. Control whole-cell Cl^- current (54 ± 3 pA/pF, n = 17) was reduced to 24 ± 3 pA/pF (n = 7) by 10 μM Ins(3,4,5,6)P_4. In contrast, Ins(4,5,6)P_3 (56 ± 7 pA/pF, n = 10), Ins(3,4,6)P_3 (68 ± 5 pA/pF, n = 12), Ins(3,4,5)P_3 (61 ± 7 pA/pF, n = 9), and Ins(3,5,6)P_3 (60 ± 6 pA/pF, n = 13) showed no inhibitory effect. These results indicate that no substantial InsP_3 phosphorylation to Ins(3,4,5,6)P_4 occurred, while also demonstrating that all four phosphates of Ins(3,4,5,6)P_4 contribute to its exquisite specificity of action.

We therefore turned to the alternate possibility that Ins(3,4,5,6)P_4 synthesis might involve dephosphorylation of InsP_5; the simplest, most direct route would be by direct 1-phosphatase attack on Ins(1,3,4,5,6)P_5. While cells possess active 3-phosphatase activity against this Ins(1,3,4,5,6)P_5 [13], a 1-phosphatase activity has never been observed, even in tissues taken from 3-phosphatase "knock-out" mice [13]. Thus, we speculated that if a 1-phosphatase were to exist, we might only observe it in vitro under specialized assay conditions. Since, in principle, a phosphokinase may be reversible to some degree if the ADP/ATP ratio is sufficiently high, we incubated the enzyme with 5 mM ADP, whereupon [^{3}H]Ins(1,3,4,5,6)P_5 was dephosphorylated to an [^{3}H]InsP_4 peak coeluting upon HPLC with a standard of Ins(3,4,5,6)P_4 (Figure 1A). Ins(1,4,5,6)P_4 would also elute at this point, but we eliminated this option, as the [^{3}H]InsP_4 that was formed was a 1-kinase substrate (data not shown), which Ins(1,4,5,6)P_4 is not [14]. Other potential products of InsP_5 dephosphorylation were all excluded by their earlier elution positions in this HPLC system (Figures 1B and 1C). In addition, we discovered (Figure 1B) that the Ins(1,3,4)P_3 6-kinase activity of this same enzyme [4] is also reversible. That is, Ins(1,3,4,6)P_4 was dephosphorylated to Ins(1,3,4)P_3 (Figure 1B).

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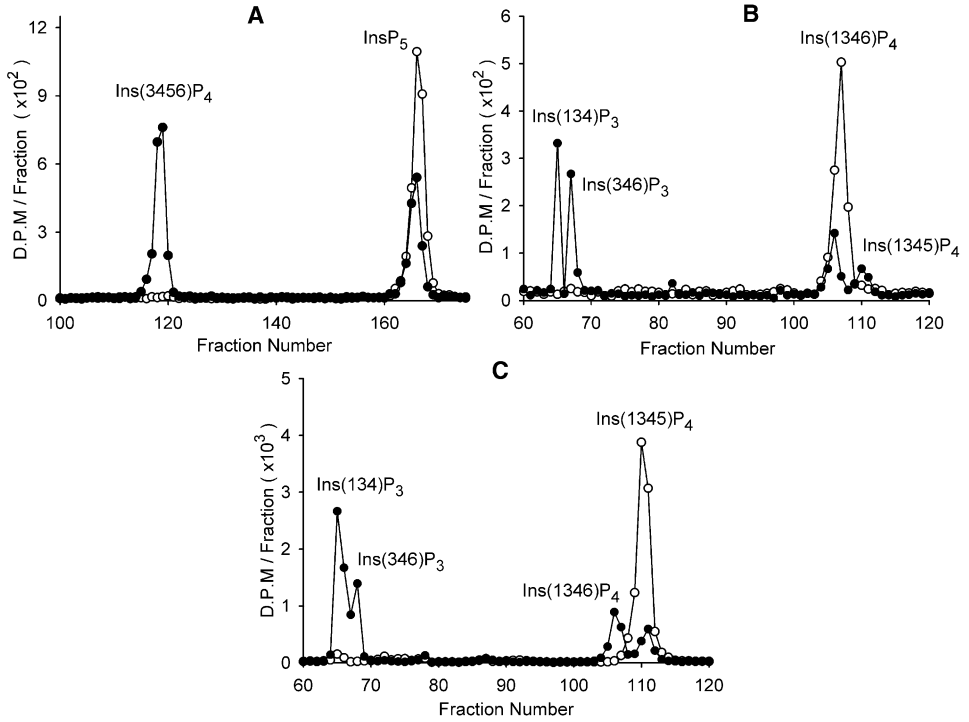

Figure 1. ADP-Dependent Dephosphorylation of Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, and Ins(1,3,4,5,6)P_5

Ins(3,4,5,6)P_4 kinase (0.4 μg) was incubated for 30 min in 100 μl phosphatase assay buffer (see Experimental Procedures) either with (closed circles) or without (open circles) 5 mM ADP plus trace amounts of either [^{3}H]Ins(1,3,4,5,6)P_5 (A) [^{3}H]Ins(1,3,4,6)P_4 (B), or [^{3}H]Ins(1,3,4,5)P_4 (C). Samples were analyzed by HPLC (see Experimental Procedures). The elution positions of Ins(1,3,4)P_3 and Ins(3,4,5,6)P_4 were verified with genuine standards. The elution positions of Ins(1,3,4,6)P_4 (106 min, [B]) and of Ins(1,3,4,5)P_4 (110 min, [C]) exclude these as products of InsP_5 dephosphorylation (A).

However, these experiments also yielded several unexpected results. First, an additional, later-eluting InsP_3 accumulated following Ins(1,3,4,6)P_4 dephosphorylation, which is presumably Ins(3,4,6)P_3 [not Ins(1,4,6)P_3, see below]. Second, the kinase yielded an identical pattern of InsP_3 products upon dephosphorylation of [^{3}H]Ins(1,3,4,5)P_4 (Figure 1C), despite the latter not possessing a 6-phosphate. Third, Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 were interconverted (Figures 1B and 1C). Such "phosphomutase" activity is unprecedented in the field of inositol phosphate metabolism.

To explain kinase reversibility and its phosphomutase activity, we noted earlier studies [15], which were more recently applied to an inositol phosphate kinase from yeast [16], showing that some inositol phosphates may interact with binding sites of receptors and enzymes in more than one orientation and that an inositol phosphate may mimic another by presenting key recognition features to the site in certain binding orientations. We propose a reaction pathway for the reversible kinase/phosphatase based on the idea that inositol phosphates bind in three different orientations (shown as modes 1, 2, and 3 in Figure 2), including two modes for Ins(1,3,4)P_3. This new model (Figure 2) predicts that Ins(3,4,6)P_3 is the later-eluting InsP_3 peak in Figures 1B and 1C and further provides a novel explanation for two previously puzzling observations. First, we recently showed but could not satisfactorily explain Ins(1,2,4)P_3 phosphorylation by this kinase at the 5 position [17]. Now we propose that Ins(1,2,4)P_3 is recognized as a mode 3 substrate. Second, the ability of the kinase to phosphorylate Ins(1,3,4)P_3 at the 5 and 6 positions [4, 5, 18] is rationalized as reflecting two Ins(1,3,4)P_3 binding modes (Figure 2), rather than a 5,6-cyclic intermediate [5]. Our model (Figure 2), which assumes a single active site, provides the simplest explanation for our data, but we do not exclude more complex scenarios in which inositol phosphates may bind to more than one site on the protein.

Assuming that our kinase/phosphatase utilizes a single active site, there are two phosphate groups on the inositol ring that are common to all binding modes and may therefore be structural determinants for substrate recognition (colored red in Figure 2). The position that is reversibly phosphorylated/dephosphorylated presumably also defines substrate recognition (also colored red in Figure 2). This model is consistent with other data showing the enzyme does not phosphorylate [^{3}H]Ins(3,4)P_2 (data not shown), nor Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4, and Ins(1,4,5,6)P_4 [14, 18]. While the groups colored red in Figure 2 are likely necessary for substrate recognition, they cannot be sufficient, because Ins(1,4)P_2 itself is not phosphorylated (data not shown). Therefore, other features of the inositol phosphate ligands (colored green in Figure 2) must contribute to recognition, and some of these additional interactions are, presumably, specific to certain binding modes.

The prediction that Ins(3,4,6)P_3 is a type 1 substrate (Figure 2) was verified (Figure 3) using an online mass

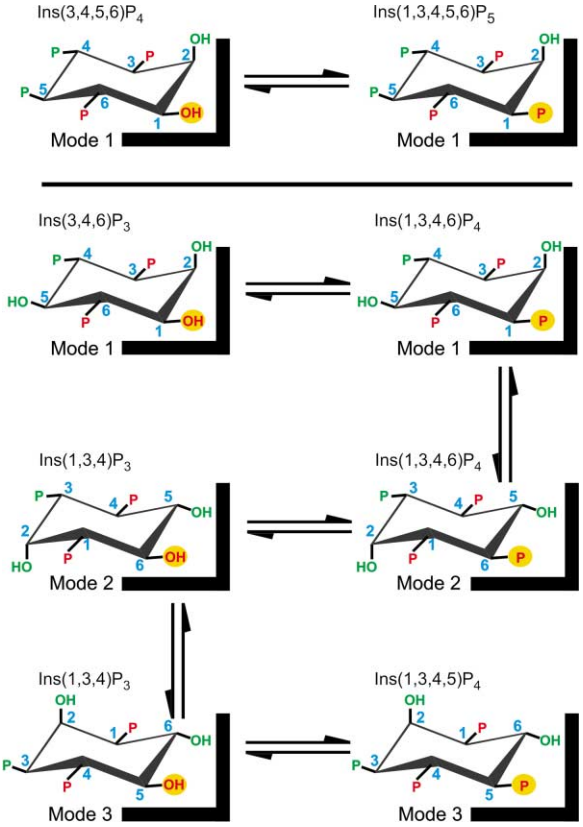

Figure 2. Proposed Kinase/Phosphatase Reaction Pathway

The schematic shows how three proposed substrate binding modes for the enzyme are employed to permit interconversion of $Ins(1,3,4)P_3$, $Ins(3,4,6)P_3$, $Ins(1,3,4,5)P_4$, and $Ins(1,3,4,6)P_4$; $Ins(3,4,5,6)P_4$/$Ins(1,3,4,5,6)P_5$ interconversion is also shown separately. All of these inositol phosphates are shown bound to the enzyme (depicted as a reverse "L"), although the switch from one binding mode to another may involve release and rebinding of an inositol phosphate by the enzyme. If a single inositolphosphate binding site is assumed, then the placement of certain hydroxyl and phosphate ("P") groups (colored red) is equivalent in all three binding modes. The group subject to reversible phosphorylation/dephosphorylation is highlighted in orange.

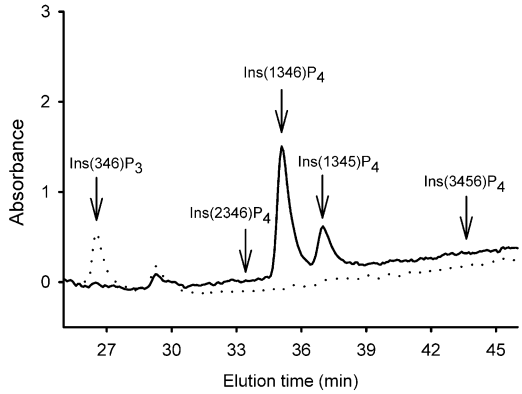

Figure 3. HPLC Analysis of the Products of $Ins(3,4,6)P_3$ Phosphorylation by the $Ins(3,4,5,6)P_4$ Kinase

$Ins(3,4,5,6)P_4$ kinase (3.4 μg) was incubated in 200 μl kinase assay buffer for 0 (dotted trace) or 4 hr (solid trace) with 50 μM $Ins(3,4,6)P_3$ as described in Experimental Procedures. Samples were analyzed by a mass detection HLPC technique (see Experimental Procedures). The absorbance at the 30 min elution time was arbitrarily set to zero. Elution positions of $Ins(2,3,4,6)P_4$ and $Ins(3,4,5,6)P_4$ are shown. Identical results were obtained with a second, independently synthesized source of $Ins(3,4,6)P_3$ (see Experimental Procedures).

detection HPLC technique [19, 20]. Kinetic data indicate a Km of 0.2 μM, a value close to that for $Ins(1,3,4)P_3$ [4]. We detected an $InsP_4$ product eluting in the position expected of $Ins(1,3,4,6)P_4$ (Figure 3). $Ins(1,3,4,5)P_4$ was also produced (Figure 3), although we cannot accurately quantify the $Ins(1,3,4,6)P_4$/$Ins(1,3,4,5)P_4$ ratio; the signal strength of this HPLC technique is not proportional to the number of phosphate groups [19] and even varies considerably between isomers containing the same number of phosphates [20]. Nevertheless, $Ins(3,4,6)P_3$ is clearly metabolized to $Ins(1,3,4,5)P_4$ (Figure 3), consistent with the reaction pathway shown in Figure 2. This involves dephosphorylation of $Ins(1,3,4,6)P_4$ to $Ins(1,3,4)P_3$ even though ATP (and not ADP) was added to these assays (Figures 2 and 3). Equally, the phosphorylation of $Ins(1,3,4)P_3$ to $Ins(1,3,4,6)P_4$ occurs when ADP (and not ATP) was added to the assays (Figures 1C and 2). Thus, the ability of the enzyme to act as both a kinase and a phosphatase is not entirely dictated by the ATP/ADP ratio added to the incubation media. It is possible that $InsP_4$ participates more directly in the phosphotransferase reactions. One way in which $Ins(1,3,4)P_3$ could be converted into $Ins(1,3,4,6)P_4$ when $Ins(1,3,4,5)P_4$ is the original substrate (Figure 1C) would be for $Ins(1,3,4,5)P_4$ to donate its 5-phosphate group to the enzyme, forming a phosphorylenzyme intermediate, which then could transfer the phosphate to the 6-hydroxyl of $Ins(1,3,4)P_3$, albeit in a manner apparently dependent upon some adenine nucleotide being present. Furthermore, in incubations containing 5 μM $Ins(1,3,4,5,6)P_5$ and 5 mM ADP, net accumulation of $Ins(3,4,5,6)P_4$ was increased by up to 5-fold upon addition of 1–5 μM $Ins(1,3,4)P_3$ (Figure 4A). One explanation for this result is that the newly formed $Ins(3,4,5,6)P_4$ can be rephosphorylated, but less effectively in the presence of $Ins(1,3,4)P_3$, a competing kinase substrate [4]. Indeed, in these experiments, there was net phosphorylation of $Ins(1,3,4)P_3$, but in a manner dependent upon $Ins(1,3,4,5,6)P_5$ (Figure 4B), which presumably donates the phosphate group, via a phosphorylenzyme intermediate, to either $Ins(1,3,4)P_3$ or $Ins(3,4,5,6)P_4$. Whatever the explanation, our exploration of several unusual features of this enzyme have led us to uncover assay conditions that optimize $Ins(3,4,5,6)P_4$ synthesis (Figure 4A). With $Ins(1,3,4)P_3$ present, the V_{max} for $Ins(1,3,4,5,6)P_5$ dephosphorylation was 82 pmol/μg protein/min, ~10-fold less than the V_{max} for $Ins(3,4,5,6)P_4$ phosphorylation by these same preparations of recombinant enzyme (data not shown and see [4]), although the latter reaction was previously estimated to operate at only 5%–10% of its capacity in receptor-activated cells [21].

Can this $InsP_5$ 1-phosphatase operate in vivo? Human colonic epithelial T_{84} cells were stably transfected with FLAG-tagged enzyme (theoretical size, 46.9 kDa). Expression was verified using anti-FLAG antibodies (Figure 5A, 49 ± 0.3 kDa, n = 3). Despite the enzyme act-

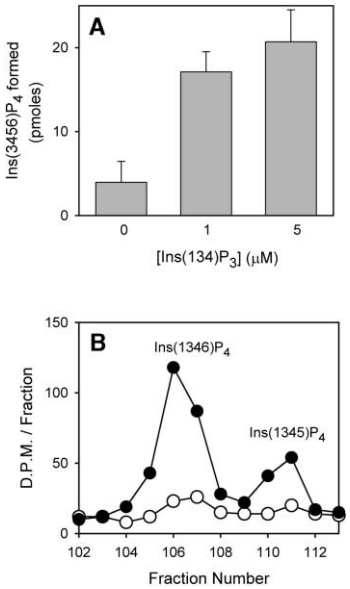

Figure 4. Ins(1,3,4)P_3 Activates Ins(1,3,4,5,6)P_5 1-Phosphatase Activity

Panel (A) shows the degree of 1-phosphatase activity toward 5 μM [^{3}H]Ins(1,3,4,5,6)P_5 in 15 min assays performed as described in the legend to Figure 1; reactions were supplemented with either 0, 1, or 5 μM Ins(1,3,4)P_3 plus 0.06 μg enzyme (means ± SE, n = 5). Additional incubations were performed with 5 μM [^{3}H]Ins(1,3,4)P_3 (~1200 d.p.m.) plus either no Ins(1,3,4,5,6)P_5 (open circles) or 5 μM nonradiolabeled Ins(1,3,4,5,6)P_5 (closed circles); these reactions were assayed by HPLC (Synchropak Q100 column), and the InsP_4 region of the chromatogram is shown in Panel (B).

ing in vitro as both an Ins(3,4,5,6)P_4 1-kinase and Ins(1,3,4,5,6)P_5 1-phosphatase, the elevated levels of [^{3}H]Ins(3,4,5,6)P_4 in enzyme-transfected cells indicate that phosphatase activity can predominate, specifically upon receptor activation (Figure 5B). Levels of [^{3}H]InsP_5 were not significantly affected by transfection (Figure 5C).

The enhancement of the receptor-initiated Ins(3,4,5,6)P_4 response in enzyme-transfected cells was accompanied by a 40% reduction in Ca^{2+}-activated Cl^- secretion (Figure 5D) from an intact cell monolayer. This provides a unique validation of the signaling importance of Ins(3,4,5,6)P_4 in a physiological context. As well as regulating salt and fluid secretion, these Ca^{2+}-activated Cl^- channels mediate cell volume homeostasis and electrical excitability in neurones and smooth muscle [3, 11], which testifies to the wide-ranging biological impact of our observations. Reciprocal coordination of the opposing 1-kinase/1-phosphatase reactions, catalyzed by a single enzyme, offers an alternative to general doctrine that intracellular signals are regulated by integrating multiple phosphatases and kinases [6]. Finally, this demonstration that the InsP_5 1-phosphatase regulates secretion (Figure 5) may also be of therapeutic interest. Upregulation of InsP_5 1-phosphatase in airway epithelia could inhibit the gob-5 chloride channel that drives mucus secretion, which, when hyperresponsive, contributes to the asthmatic condition [22]. Downregulation of InsP_5 1-phosphatase could be used as a strategy for enhancing Cl^- secretion in the therapy of cystic fibrosis [3].

Experimental Procedures

Enzyme Assays

Inositol phosphate phosphatase activity was assayed in buffer containing 100 mM KCl, 20 mM HEPES (pH 7.2), 5 mM ADP, 6 mM $MgSO_4$, 0.3 mg/ml bovine serum albumin. Assays were acid quenched, neutralized, and analyzed by HPLC using a Synchropak Q100 column [14]; 1 ml fractions were collected for 70 min, followed by 0.5 ml fractions. Inositol phosphate kinase activity was assayed in buffer containing 100 mM KCl, 20 mM HEPES (pH 7.2), 5 mM ATP, 10 mM phosphocreatine, 6 mM $MgSO_4$, 10 μg/ml creatine phosphokinase (Calbiochem), 0.3 mg/ml bovine serum albumin. Some assays were acid quenched and analyzed by HPLC using a Synchropak Q100 column. Other reactions were heat inactivated (95°C, 3 min) and analyzed by a metal dye detection, HPLC method [19, 20].

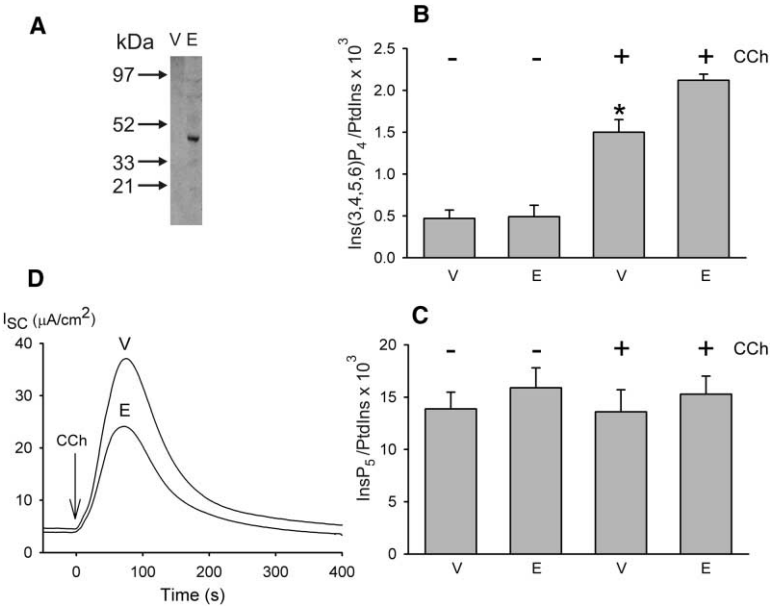

Figure 5. Overexpression of InsP_5 1-Phosphatase Activity in T_{84} Cells

(A) Western blotting of the FLAG epitope in aliquots of cell lysates from vector (V, 20 μl) and enzyme (E, 10 μl) transfected T_{84} cells. Molecular weight markers are given. Panels (B) and (C), respectively, show levels of [^{3}H]Ins(3,4,5,6)P_4 and [^{3}H]InsP_5 in vector- and enzyme-transfected T_{84} cells, prelabeled with 50 μCi [^{3}H]inositol/ml for 4 days, and incubated for 15 min with either vehicle or 100 μM carbachol (CCh). Data were normalized to the level of [^{3}H]PtdIns. The asterisk denotes that levels in vector-transfected cells were significantly ($p < 0.02$, n = 4, paired t test) lower than those in enzyme-transfected cells. Panel (D) shows Cl^- secretion (assayed as short-circuit current, I_{SC}) across a T_{84} monolayer in response to 100 μM carbachol added to the basolateral surface. Data are composite curves from 12 experiments with vector- and enzyme-transfected cells.

Transfection of the 1-Kinase/1-Phosphatase Gene into T_{84} Cells

T_{84} cells were cultured at 37°C (5% CO_2:95% air) in Iscove's Modified Dulbecco's medium (Hyclone) supplemented with 5% (w/v) FBS (Hyclone, Logan, UT), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.8 mg/ml G418 selection agent. The gene previously denoted a Ins(3,4,5,6)P_4 1-kinase [4] was cloned into PCMV-Tag4 expression vector between the BamHI and XhoI sites after the stop codon was removed. The transfection was carried out in a 60 mm dish, using LipoTAXI Mammalian Transfection Kit from Stratagene (La Jolla, CA), according to the manufacturer's instructions. Control transfections were performed at the same time using PCMV-Tag4 vector alone. Data shown are from one pair of cell lines stably transfected with either enzyme or vector; similar data were obtained with two additional, independently and stably transfected pairs of cell lines.

Electrophysiology

T_{84} cells were detached by incubation with 0.25% (w/v) trypsin/0.02% (w/v) EDTA for 5–6 min. After trypsin removal, 200 μl aliquots (1.5×10^6/ml) were seeded into the circular wells (area = 0.45 cm^2) of permeable supports, made by gluing (Silastic® sealant, Dow Corning, Midland, MI) a Sylgard® ring to filters composed of mixed cellulose esters (Millipore Corp., Bedford, MA). Seeded supports were floated on culture medium and incubated for 11–14 days before being mounted in modified Snapwell holders in Ussing chambers (EasyMount System, Physiologics Instruments, San Diego, CA). Transcellular Cl^- flux was then recorded as the short-circuit current as previously described [1]. The resistance of the monolayers were (vector) 346 ± 44 ohms/cm^2 (mean ± SE, n = 12) and (kinase) 354 ± 62 ohms/cm^2 (mean ± SE, n = 12). Whole-cell Cl^- currents in CFPAC-1 cells were measured at +40mV as previously described, using an intracellular Ca^{2+}-BAPTA buffer to clamp free [Ca^{2+}] to 0.5 μM [8].

Materials

American Type Cell Culture (Manassas, VA) supplied the CFPAC-1 cells and T_{84} cells. Recombinant human Ins(3,4,5,6)P_4 1-kinase was expressed in *E. coli* and purified [4]. Nonradiolabeled Ins(1,3,4)P_3 and Ins(1,3,4,5,6)P_5 were purchased from CellSignals Inc (Lexington, KY). The details of the synthesis of nonradiolabeled Ins(3,4,5)P_3, Ins(3,5,6)P_3, Ins(4,5,6)P_3, and Ins(3,4,6)P_3 (as sodium salts) will be published separately by S.-K.C. Ins(3,4,6)P_3 was also prepared by a different synthetic route [23]. [^{3}H]Ins(1,3,4,5,6)P_5 was prepared as previously described [24]. [^{3}H]Ins(1,3,4)P_3 was prepared from [^{3}H]Ins(1,3,4,5)P_4 (New England Nuclear), using recombinant Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase [25]. [^{3}H]Ins(1,3,4)P_3 was converted to [^{3}H]Ins(1,3,4,6)P_4, using Ins(1,3,4)P_3 6-kinase activity [4]. [^{3}H]Ins(3,4)P_2 was prepared by alkaline phosphatase attack on [^{3}H]Ins(1,3,4)P_3 in 20 mM glycine (pH 9.0 with KOH). [^{3}H]Ins(1,4)P_2 was purchased from New England Nuclear.

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