Expert Opinion

1. Introduction
2. Inositol phosphate signalling and the control of calmodulin kinase II-regulated Cl- channels
3. Pharmacological intervention in inositol phosphate signalling
4 INO-4995 and its effect upon ENaC
5. Some consequences for drug development arising from Ins(3,4,5,6)P4 metabolism being regulated by a multifunctional enzyme
6. Expert opinion and conclusions

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General

Can intervention in inositol phosphate signalling pathways improve therapy for cystic fibrosis?

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Airway epithelial cells from cystic fibrosis (CF) individuals cannot secrete adequate Cl^- through cystic fibrosis transmembrane regulator, and their Na^+ channel (ENaC) activity is increased so that excessive Na^+ and water is absorbed from the lumen. These aberrant transport activities can, at least partly, be compensated by pharmacologically increasing the activities of Ca^{2+}-activated Cl^- channels (CaCCs). The therapeutic value of this approach is currently being examined in clinical trials of candidate CF drugs such as INS-37217 (Inspire Pharmaceuticals) and Moli1901 (Lantibio, Inc.). This review argues that these drug development programmes will be helped if one can fully understand how the CaCCs are inhibited by inositol 3,4,5,6-tetrakisphosphate (Ins(3,4,5,6)P_4), so that there can be pharmacological intervention in this process. Furthermore, genes that encode enzymes controlling Ins(3,4,5,6)P_4 metabolism should be viewed as impacting upon CaCC activity; this, in turn, may influence the severity of the CF condition. Expression profiling of genes that regulate inositol phosphate metabolism may also illuminate variability in patient response to treatment regimens that target CaCCs. Compounds have been developed that can activate CaCCs by antagonising their inhibition by Ins(3,4,5,6)P_4. One member of this drug family (INO-4995; Inologic) was recently shown to inhibit ENaC, thereby reducing fluid absorbtion by airway epithelial cells.

Keywords: Ca^{2+}-activated Cl^- channel (CaCC), cystic fibrosis, inositol phosphate

Expert Opin. Ther. Targets (2005) 9(6):xxx-xxx

1. Introduction

Individuals that suffer from cystic fibrosis (CF) have a median lifespan of just over 30 years, and almost all of them die from lung disease [1]. The pernicious nature of the disorder is primarily due to the mutations in the cystic fibrosis transmembrane regulator (CFTR) that lead to a reduction in Cl^- secretion from CF airway epithelia [1]. The loss of functional CFTR is also coupled to activation of an apical channel, ENaC, that accumulates Na^+ from the luminal milieu (**Figure 1**) [2-8]. This impairment of the secretory capacity of the CF airway cells also perturbs osmotic water movement (**Figure 1**), leading to a reduction in the airway surface liquid volume, thereby disturbing the height and the composition of the mucus and periciliary liquid layers [1]. The mucus layer contains a highly glycosylated and tangled network of mucin proteins that are secreted from surface goblet cells and submucosal glands [9]. The mucins normally engulf inhaled pathogens, but in the CF condition, water and salt imbalance has a strong negative impact on mucin structure and its defensive function [9]. Thickening of the mucus layer also impairs the ciliary beating which is required to expel pathogens from the host. Thus, in CF, bacterial colonisation is encouraged,

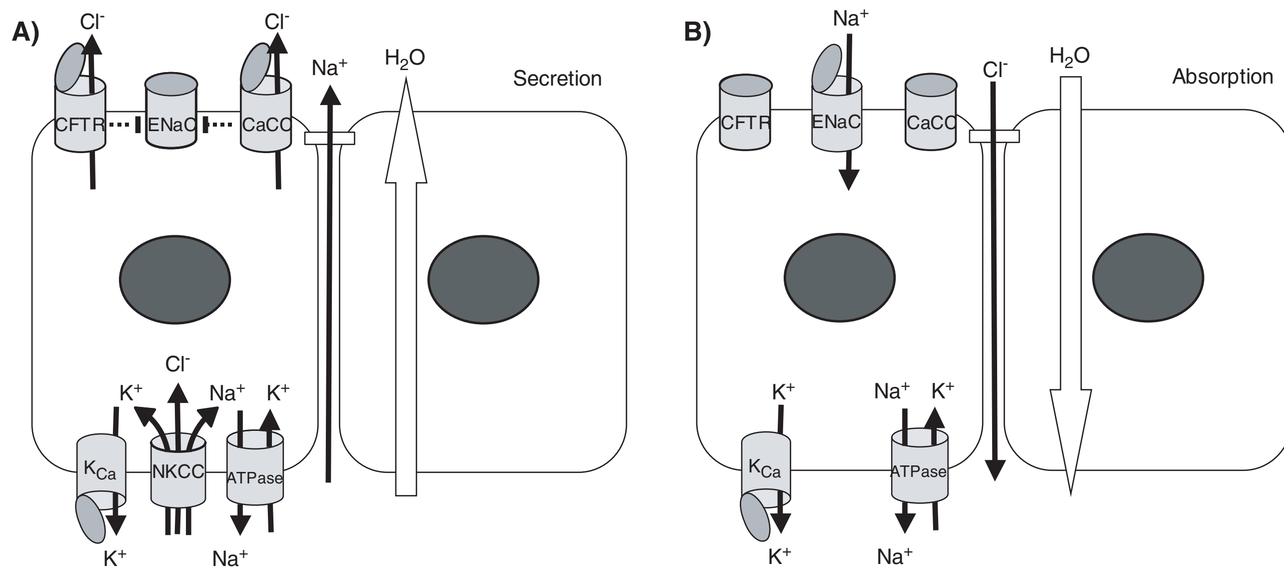

Figure 1. Na^+, Cl^- and water transport across airway epithelia. Airway epithelia coordinate opposing secretory and absorptive ion transport processes so as to regulate the quantity of salt and water in the periciliary liquid layer. In secretory mode (**A**), Cl^- enters the basolateral pole of the cell through the Na^+-K^+-$2Cl^-$ cotransporter (NKCC); Na^+ and K^+ is recycled through the Na^+/K^+-ATPase and Ca2+-activated K^+ channels (K_{Ca}). Both the CFTR and Ca^{2+}-activated Cl^- channels (CaCC) carry Cl^- out of the apical pole of the cell into the periciliary liquid layer. The epithelial Na^+ channel (ENaC) is inhibited by its electrochemical (and perhaps molecular) coupling with the Cl- transport proteins. Na^+ is secreted via the paracellular pathway, and is osmotically accompanied by secretion of water. In adsorptive mode (**B**) Na^+ is transported by ENaC into the apical pole of the cell. Na^+ exits the cell at the basolateral pole through the Na^+/K^+ ATPase and the Na^+-K^+-$2Cl^-$ cotransporter. Additionally, Cl^- now moves out of the periciliary liquid layer through the paracellular pathway. Although not shown in the figure, Cl^- may also enter the cell through CFTR and CaCC. The adsorption of ions is osmotically accompanied by water.
CaCC: Ca^{2+}-activated Cl^- channel; CFTR: Cystic fibrosis transmembrane regulator; ENaC: Epithelial Na^+ channel; K_{Ca}: Ca^{2+}-activated K^+ channels; NKCC: Na^+-K^+-$2Cl^-$ cotransporter;

and this, in turn, promotes an endobronchial inflammatory response that ultimately destroys the lung tissue [1].

Airway epithelia also posses Ca^{2+}- and calmodulin kinase II (CaMKII)-regulated Cl^- channels (CaCCs) in the plasma membrane (**Figures 1** and **2**) [10-12]. These channels can become activated following increases in extracellular levels of UTP and ATP. These nucleotides are released from the airway cells when they undergo shear-stress, following mechanical stimulation (after inhalation of foreign particles or bacteria) or upon increases in the rate of airflow (by tidal breathing or by coughing) [13,14]. Both UTP and ATP are purinergic receptor agonists which initiate phospholipase C (PLC)-dependent mobilisation of intracellular Ca^{2+}, thereby activating CaCC (**Figure 2**). This adaptive process promotes an increase in Cl^- secretion from airway cells, thereby aiding CFTR in maintaining an adequate periciliary liquid layer [13].

Pharmacological activation of CaCCs represents a candidate CF therapy. For example, Moli1901, a 19-residue cyclic peptide originally isolated from *Streptomyces cinnamoneum*, is currently being developed for clinical trials by Lantibio, Inc. (Chapel Hill, NC, USA) [15]. Moli1901 (which was originally known as duramycin) seems to have cell-specific ionophoretic properties that promotes both Ca^{2+} entry into cells, as well as the release of Ca^{2+} from intracellular stores [16]. The resulting elevation in cytosolic $[Ca^{2+}]$ activates CaCCs [15,17]. No adverse effects were described following a recent Phase I clinical trial [15]. However, it has been argued that receptor-independent elevations in $[Ca^{2+}]$ may affect a number of biological processes [18]. Furthermore, it has been suggested that elevations in $[Ca^{2+}]$ under the auspices of cell-surface, apical receptors could be a safer, more specific therapeutic approach; in this case, the $[Ca^{2+}]$ transients are more localised to the vicinity of CaCC, so it is less likely that there will be nonspecific effects of Ca^{2+} in other areas of the cell [18,19]. Thus, another candidate CF drug is the metabolically-stable purinergic agonist (INS-37217; P'-(uridine 5'-P^4-(2'-deoxycytidine 5')tetraphosphate) that has been developed by Inspire Pharmaceuticals (Research Triangle Park, NC) as a means of upregulating CaCC activity. This drug is currently undergoing Phase II clinical trials [20,101]. INS-37217 is delivered into the lungs by inhalation [1,20]. Moreover, there is some evidence that an increased CaCC activity is accompanied by inhibition of ENaC, thereby decreasing Na^+ absorption from airway fluid (**Figure 1**) [21-23]. Thus, drugs that act as purinergic agonists have the potential to increase secretion of both Cl^- and Na^+, which in concert could help to restore airway surface liquid volume in CF individuals.

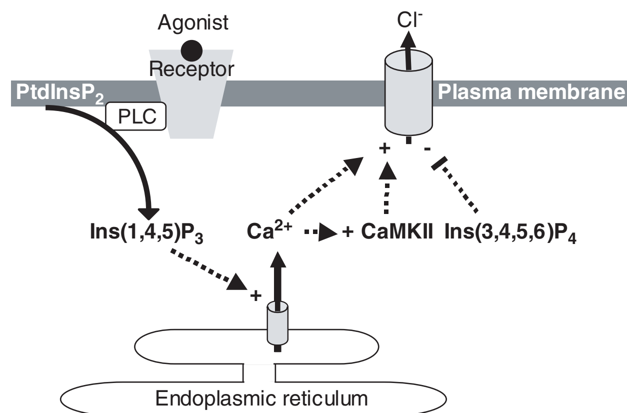

Figure 2. Receptor-dependent control over Ca^{2+}-dependent Cl^- channels. PLC activation is a receptor-coupled event. PLC hydrolyses the membrane-bound lipid, PtdIns(4,5)P_2, releasing the soluble inositol phosphate, Ins(1,4,5)P_3 [34]. The Ins(1,4,5)P_3 gates a Ca^{2+}-channel in the endoplasmic reticulum. The ensuing increase in cytosolic Ca^{2+}, which is augmented by a coupled influx of Ca^{2+} from outside the cell (not shown), activates the conductance of CaCCs in the plasma membrane; Ca^{2+} can interact directly with the channels, or indirectly through CaMKII, which phosphorylates some CaCCs [53] and perhaps some closely-associated, regulatory proteins. The net Cl^- conductance is carefully regulated by a dynamic balance between its activation by Ca^{2+}/CaMKII and its inhibition by Ins(3,4,5,6)P_4.

CaCC: Ca^{2+}-dependent Cl^- channel; CaMKII: Calmodulin-dependent protein kinase II; Ins: Inositol; PLC: Phospholipase C.

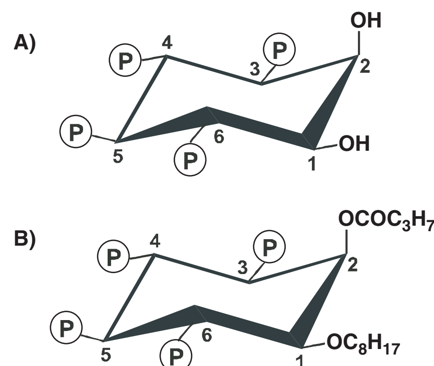

Figure 3. Structures of myo-Inositol 3,4,5,6-tetrakisphosphate and 2-*O*-butyryl-1-*O*-octyl-myoinositol-3,4,5,6-tetrakisphosphate. Structure A is the naturally-occurring inositol 3,4,5,6-tetrakisphosphate (Ins(3,4,5,6)P_4). Note the anticlockwise numbering system that labels each of the six carbon atoms around the inositol ring. This nomenclature enables us to classify which carbons have phosphate groups attached to them (carbons 3,4,5 and 6 in structure A). In this way, the many different inositol phosphate molecules can be distinguished [34]. Structure B is 2-*O*-butyryl-1-*O*-octyl-myo-inositol 3,4,5,6-tetrakisphosphate. The latter has also been designated INO-4913 by Inologic, Inc. [21]. A modified, cell-permeant version of structure B is designated INO-4995 [21]; this molecule is slated to enter clinical trials in the near future.

The issue of concern in this review is that cells also synthesise an inhibitor of CaCCs; this inhibitor, inositol tetrakisphosphate (Ins(3,4,5,6)P_4; **Figure 3A**), is a member of the inositol phosphate signalling family [24-28]. Cellular levels of Ins(3,4,5,6)P_4 are always elevated whenever PLC is activated (Section 2.2.2) [26,28]. In addition to responding to purinergic agonists, PLC in airway epithelial cells is also activated by histamine (released during allergic responses [29,30]) and inflammatory mediators [31]. The accompanying elevation in Ins(3,4,5,6)P_4 levels will be exacerbated following PLC stimulation with INS-37217, potentially restricting the extent to which this agent can activate CaCC. It is this situation which initially suggested the idea that perhaps a drug could be developed that antagonised the action of endogenous Ins(3,4,5,6)P_4; such a molecule might improve the efficacy of drugs such as INS-37217 or Moli1901, and thereby bring more relief to the CF condition [32,33]. The current status of this idea is discussed in this review. In addition, this review shows how efforts to develop an Ins(3,4,5,6)P_4 antagonist led to the discovery of a drug which significantly restores airway fluid balance through its actions upon ENaC.

2. Inositol phosphate signalling and the control of calmodulin kinase II-regulated Cl^- channels

2.1 Receptor-dependent activation of CaCCs by inositol phosphate signalling

A full understanding of the roles that inositol phosphates play in regulating CaCC does require an appreciation of the terminology that distinguishes the multitude of the isomeric variants of these polyphosphorylated compounds. This nomenclature, which is mentioned briefly in the legend to **Figure 3**, provides a ready means to describe the combinatorial manner in which a varied number of phosphate groups are placed around the six-carbon inositol ring, through the actions of an array of kinases and phosphatases. For those readers requiring a more thorough description, this is given in references [34,35]. A number of the inositol phosphates that are present inside cells are (or at least are proposed to be) 'diffusible' second messengers. In fact, there are few aspects of cell biology that have not been accused of being regulated in some way or another by one or more inositol phosphates. Many of these polyphosphates are not discussed here, or are only noted in passing, because they are not directly relevant to the focus of this review. However, it is worth reiterating that the biology of inositol phosphates extends well beyond the topics covered in this review.

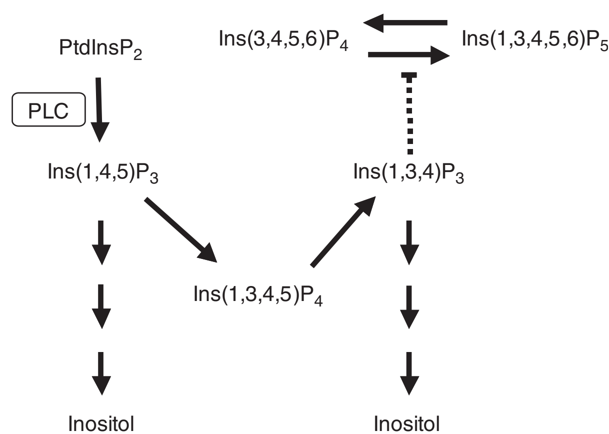

Figure 4. Cellular levels of Ins(3,4,5,6)P_4 are linked to PLC activation. Some of the Ins(1,4,5)P_3 (formed by PLC-dependent breakdown of PtdIns(4,5)P_2, is metabolised to Ins(1,3,4)P_3 through the sequential actions of 3-kinases and 5-phosphatases [34]. As the concentration of Ins(1,3,4)P_3 increases, it progressively inhibits the 1-kinase that phosphorylates Ins(3,4,5,6)P_4 to Ins(1,3,4,5,6)P_5. There is also evidence that Ins(1,3,4)P_3 simultaneously activates the Ins(1,3,4,5,6)P_5 1-phosphatase [42]. These effects alter the poise of the dynamic equilibrium of Ins(3,4,5,6)P_4/Ins(1,3,4,5,6)P_5 interconversion, leading to increases in Ins(3,4,5,6)P_4 levels. This is the process by which cellular Ins(3,4,5,6)P_4 levels are regulated by changes in PLC activity.
Ins: Inositol; PLC: Phospholipase C.

Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) was the first member of this family of polyphosphates that was shown to be an intracellular signal, when it was revealed that it released Ca^{2+} from endoplasmic reticulum [36]. The cellular levels of Ins(1,4,5)P_3 are increased following the activation of PtdIns(4,5)P_2 hydrolysis by PLC (**Figure 2**). The occupation of purinergic receptors that is illustrated in **Figure 2** represents only one means by which PLC can be activated but it is this process that is particularly pertinent here (see Section 1). The Ins(1,4,5)P_3-mediated depletion of the Ca^{2+} stores in the endoplasmic reticulum activates a Ca^{2+} influx across the plasma membrane by a process that was originally termed 'capacitative Ca^{2+} entry' [37]; in the more recent literature, the reader will frequently see this phenomenon described as 'store-operated calcium entry' [38]. Both of these different aspects of Ca^{2+} mobilisation – release from endoplasmic reticulum and influx across the plasma membrane – work together to increase cytosolic [Ca^{2+}]. Activation of CaCCs is a key consequence of the elevation of [Ca^{2+}] inside airway epithelial cells.

2.2 Receptor-dependent inhibition of CaCC activity by inositol phosphate signalling

2.2.1 The integration of stimulatory and inhibitory signals

Section 2.1 describes how an inositol phosphate signal is responsible for activating CaCCs. However, cellular activities are not typically regulated just by stimulatory input. Instead, regulatory models are usually much more complex, frequently utilising biological 'logic gates' that selectively assimilate and transduce inputs from competing stimulatory and inhibitory signals. For example, there are situations where substrate flux between two metabolic intermediates is controlled by opposing enzyme activities; this phenomenon is known as a 'substrate cycle' [39] (in less enlightened times, it would have been called a 'futile cycle'). Another example of the complexity of regulatory processes is the coordination of the competing actions of protein kinases and protein phosphatases on a target protein [40]. In both of these cases, the strength and timing of the output response is very finely tuned by the interpretation of both the stimulatory and inhibitory inputs.

Ion channels, the most rapidly acting of all signalling entities, provide an especially striking example of this paradigm. In the specific case of certain CaCCs, it is Ca^{2+} and CaMKII (supervised initially by Ins(1,4,5)P_3, **Figure 2**) that provide the inputs which activate the Cl^- channels. Another inositol phosphate, Ins(3,4,5,6)P_4 (**Figure 3A**), is an inhibitory signal. Subtle alterations in the strength of one or more of these competing signals can have a considerable impact upon Cl^- ion movements. This is because a small shift in the cellular concentrations of either Ca^{2+} or Ins(3,4,5,6)P_4 can yield an 'all-or-nothing' response: a switch in the conductance state of a single CaCC between its 'on' and 'off' states. This can influence the transmembrane movement of millions of Cl^- ions per second [41]. Such a process represents a particularly impressive example of signal amplification.

2.2.2 Regulation of Ins(3,4,5,6)P_4 levels

If strategies to intervene in the Ins(3,4,5,6)P_4 signalling system are to be designed, it is first helpful to understand the mechanisms that the cell uses to control the steady-state levels of Ins(3,4,5,6)P_4. The metabolism of Ins(3,4,5,6)P_4 is the responsibility of two enzyme reactions: Ins(3,4,5,6)P_4 is formed in cells by removal of the 1-phosphate from Ins(1,3,4,5,6)P_5 [42]; the 1-phosphate is replaced when Ins(3,4,5,6)P_4 is phosphorylated by an ATP-dependent kinase [43,44]. That is, Ins(3,4,5,6)P_4 is interconverted with Ins(1,3,4,5,6)P_5 in a closed 1-kinase/1-phosphatase substrate cycle (**Figure 4**). As already mentioned above (Section 2.2.1), substrate cycles offer opportunities for careful control over a metabolic process. Importantly, the dynamic equilibrium of this substrate cycle is affected by PLC activation in the following manner: the Ins(1,4,5)P_3 product of PLC activity is converted to Ins(1,3,4)P_3 (**Figure 4**), which inhibits the Ins(3,4,5,6)P_4 1-kinase activity (see Section 5 for a mechanistic explanation). Ins(1,3,4)P_3 also has the complementary ability to stimulate the Ins(1,3,4,5,6)P_5 1-phosphatase activity [42]. These two effects of Ins(1,3,4)P_3 lead to increases in cellular Ins(3,4,5,6)P_4 levels whenever PLC is activated. Moreover, Ins(3,4,5,6)P_4 1-kinase activity *in vivo* is a relatively slow reaction, so that Ins(3,4,5,6)P_4 levels can remain elevated for a substantial time after the PLC-activating stimulus is

removed [45]. A particularly fascinating aspect of this field is that both the 1-kinase and 1-phosphatase reactions are performed by the same enzyme, inositol triphosphate kinase type-1 (ITPK1, [this is the HUGO-approved acronym; see Section 5 for more explanation]). The mechanistic basis for the molecular gymnastics that are performed by ITPK1 are still being investigated.

In previous experiments with several different cell types, the total cellular levels of the Ins(3,4,5,6)P_4 signal were determined to fluctuate from a baseline of ~ 1 µM in 'resting' cells to between 4 – 10 µM when PLC is activated [33]. The level of Ins(3,4,5,6)P_4 that prevails in airway cells *in vivo* will depend upon the nature and the duration of any stimuli of PLC activity to which the cells are exposed. Airway epithelial PLC can be activated by a number of factors, including purinergic agonists (released by tidal air flow, by coughing or by turbulence in the mucus layer created by either particulate matter or bacteria; see Section 1), histamine (released during allergic responses [29,30]) and inflammatory mediators [31]. Thus, the 'resting' airway epithelial cell is probably a rare beast *in vivo.* Instead, the cellular level of Ins(3,4,5,6)P_4 can be anticipated to be sufficient to elicit some inhibition of CaCC activity under most physiological conditions.

The values for the range of cellular Ins(3,4,5,6)P_4 concentrations that are quoted above, assume that the polyphosphate is distributed evenly throughout the cell. There is some evidence that the situation may actually be more complex. In the mouse tracheal epithelial cell, for example, ITPK1 was recently shown by confocal immunofluorescence to be concentrated at the apical domain of the cell, where, presumably, Ins(3,4,5,6)P_4 synthesis and metabolism is also focused [28]. Of course, the extent to which this situation leads to a localised increase in the Ins(3,4,5,6)P_4 concentration will depend upon the rate of cellular diffusion of Ins(3,4,5,6)P_4. Using as an example another soluble and supposedly freely-diffusible intracellular signal – cAMP – it has been noted that, in certain cells, it exhibits a restricted rate of diffusion away from its site of synthesis at the plasma membrane [46]. This phenomenon leads to locally elevated levels of cAMP [46]. One proposed explanation for this phenomenon is that the architecture of the cell interior may sometimes present structural barriers that restrict the diffusion of cytosolic constituents [46]. It has been suggested that such barricades may be formed by invaginations of caveolae that protrude into the cell, or by the placement of cell organelles in close proximity to the plasma membrane [46]. At least in the case of human airway epithelia, there is a considerable enrichment of endoplasmic reticulum and mitochondria near the apical domain [47]; perhaps this may also act as a barrier that slows down the movement of Ins(3,4,5,6)P_4 away from the plasma membrane at which it is synthesised. In other words, Ins(3,4,5,6)P_4 metabolism might be focused in subcellular domains near to the apical plasma membrane of airway epithelial cells. Such compartmentalisation would not only extend the dynamic range within which cellular levels of Ins(3,4,5,6)P_4 may vary, but it also offers the cell finer temporal control over the actions of Ins(3,4,5,6)P_4 by manipulating its levels in close proximity to the channels that it regulates. Of course, the more important the role of Ins(3,4,5,6)P_4 in regulating CaCC, the greater the therapeutic potential that might arise from intervening in this signalling cascade.

2.2.3 *Inhibition of CaCC activity by Ins(3,4,5,6)P_4*

The most direct evidence that physiologically-relevant changes in Ins(3,4,5,6)P_4 levels inhibit the conductance of CaCCs comes from whole-cell, patch clamp experiments, in which Ins(3,4,5,6)P_4 was perfused into a single cell through the patch pipette [25,27]. One of the key results to emerge from these particular studies was that Ins(3,4,5,6)P_4 was effective within the concentration range that prevails *in vivo*. In the electrophysiological experiments, the perfusion into the cell of 1 µM Ins(3,4,5,6)P_4 (the 'basal' level, see above), did not affect CaCC activity [25,27]. Ins(3,4,5,6)P_4 only inhibited CaCCs when its concentration was increased to a level that would normally be seen *in vivo* after PLC activation (4 – 10 µM). This was a highly specific inhibitory effect that could not be imitated by a wide range of other inositol phosphates [24,25,27,45,48]. Once it was apparent what are the structural determinants of specificity within the Ins(3,4,5,6)P_4 molecule, it was possible to develop an appropriate strategy for the design of Ins(3,4,5,6)P_4 antagonists [49].

In order to pharmacologically intervene in the actions of Ins(3,4,5,6)P_4, it might help if the molecular nature of the CaCCs that are inhibited by this inositol phosphate are better understood. Then, one might be able to design drugs that interact with the ion channels themselves. Unfortunately, the issue of the molecular identity of CaCCs continues to be steeped in controversy [11,18,50]. Only one recombinant channel protein, a bovine, tracheal member of the so-called CLCA family, has been reconstituted into an experimental situation in which inhibition by Ins(3,4,5,6)P_4 has been demonstrated [24,51]. However, one feature that emerged from the latter experiments is rather curious, namely, the very low K_i of Ins(3,4,5,6)P_4 (low nanomolar; [24]). This is orders of magnitude different from the 4 – 10 µM Ins(3,4,5,6)P_4 that is required before Cl^- channels are inhibited *in situ* (see above). As such, it is difficult to envisage that this bovine CLCA channel could be responsible for the Ins(3,4,5,6)P_4-regulated Cl^- current in the whole-cell experiments. Instead, this CLCA would be anticipated to be constitutively blocked by physiological (low micromolar; [33]) levels of Ins(3,4,5,6)P_4 that prevail even in resting cells. If this is an example of inhibition of an ion channel by default, it could still form the basis for a regulatory process – if the cell had a signalling mechanism to reverse this constitutive inhibition. However, no such process has been found, so the significance of the high potency with which Ins(3,4,5,6)P_4 inhibits the bovine CLCA protein remains entirely speculative.

There are also strong arguments that, in fact, the CLCA proteins are not even Ca^{2+}-dependent Cl^- channels in their

own right *in vivo* [11,12,18,52]. This is one of the reasons that other molecular candidates have been sought that might provide a more physiologically acceptable explanation for CaCC activity. The varied electrophysiological properties of CaCCs that have been recorded from experiments in intact cells make it likely that multiple CaCC isoforms are in existence [11,18,50]. For example, at least one member of the ClC family, namely, ClC3, has been proposed to act as a CaMKII-activated Cl^- channel [53]. More recently, the bestropins [54] have emerged as a new family of CaCCs. There is a need to investigate if these CaCC candidates might be regulated by Ins(3,4,5,6)P_4.

Uncertainties over the molecular identity and tissue-distribution of the Cl^- channels that are regulated by Ins(3,4,5,6)P_4 make it problematic to predict in which cell types Ins(3,4,5,6)P_4 might regulate CaCC activity. Currently, this question can only be determined empirically. At least it has been demonstrated that Ins(3,4,5,6)P_4 inhibits CaCC activity in immortalised wild type and CF murine tracheal epithelial cells [28]. It can, therefore, be anticipated with some confidence that human CF airways are a reasonable target for therapeutic intervention in the Ins(3,4,5,6)P_4 signalling pathway.

The mechanism of action of Ins(3,4,5,6)P_4 is not yet known; it does not seem to act as a channel blocker [27]. Instead, accessory proteins, such as protein phosphatases, contribute to the actions of Ins(3,4,5,6)P_4 in a manner that is also not yet understood [27]. Once again, the molecular identities of these other proteins have yet to be established. It is worth noting the scale of this problem: one of these protein phosphatases might be the type 2A form (PP2A) [48]. However, PP2A functions as a heterotrimeric complex containing over 75 different possible combinations of varied catalytic and regulatory subunits [55]. Insight into the nature of the proteins that modulate the actions of Ins(3,4,5,6)P_4 is clearly a challenging task, but if successful, it could reveal additional strategies for therapeutic intervention in this signalling pathway.

3. Pharmacological intervention in inositol phosphate signalling

3.1 The development of cell-permeant Ins(3,4,5,6)P4 analogues

It was early studies into the effects of inositol phosphates upon Cl^- secretion from epithelial monolayers that first led to the development of an analogue of Ins(3,4,5,6)P_4 that could diffuse into cells in a monolayer [45,56]. This technological achievement was motivated by the following experiments: it had been established that intracellular levels of Ins(3,4,5,6)P_4 correlated with the time course by which receptor activation and subsequent deactivation, respectively, switched on and off a process by which Ca^{2+} was prevented from stimulating Cl^- secretion from cells [45]. However, at this point, this evidence for a biological role for Ins(3,4,5,6)P_4 was still circumstantial. To further pursue this idea, it was still necessary to test if there were a direct effect of Ins(3,4,5,6)P_4 upon Cl^- secretion. Intracellular levels of Ins(3,4,5,6)P_4 cannot be raised simply by adding the inositolphosphate to the medium bathing the cells. The high negative charge density that results from the placement of multiple phosphate groups around the inositol ring prevents these molecules from crossing the lipid bilayer that encloses the cell. Fortunately, chemists have developed a number of strategies to mask the negative charges on phosphate groups [57]. Following such modifications, the resulting uncharged derivatives can now cross the plasma membrane. Once inside the cell, the masking groups are removed by either chemical or enzymatic hydrolysis, liberating the native phosphate groups.

With regards to the specific case of facilitating Ins(3,4,5,6)P_4 entry into cells (see above), an analogue was synthesised in which the hydroxyl groups were masked by the addition of butyrate, and the charges on each of the phosphate groups were hidden by the addition of two acetoxymethylesters [45]. This molecule, Bt_2Ins(3,4,5,6)P_4/AM, could now cross the plasma membrane; once inside the cell, the protecting groups were removed by intracellular esterases, liberating Ins(3,4,5,6)P_4. Moreover, once the highly polar phosphate groups of the molecule were unveiled, this prevented the compound from escaping across the plasma membrane and back into the medium. Using this approach, it was clearly demonstrated that the entry of Ins(3,4,5,6)P_4 into cells resulted in inhibition of Ca^{2+}-dependent Cl^- secretion in the T84 colonic epithelial cell line [45]. Similar effects of cell-permeant Ins(3,4,5,6)P_4 upon Ca^{2+}-dependent Cl^- secretion have also been observed in experiments with a human CFPAC-1 pancreatic ductal cell line [26] and immortalised mouse airway cells [28]. This development of a technique for empowering inositol phosphates with the ability to enter cells was key to the discovery that Ins(3,4,5,6)P_4 is biologically active. In addition, it can now be anticipated that an aerosolised, cell-permeant inositol phosphate analogue that can easily be delivered into the lungs by inhalation would be expected to readily pass into airway epithelial cells to exert its actions.

3.2 The development of Ins(3,4,5,6)P_4 antagonists

The practical demonstration that a cell-permeant Ins(3,4,5,6)P_4 analogue could be introduced into cells and inhibit Cl^- secretion (Section 3.1), led to the idea that it might be possible to also develop a cell-permeant antagonist of endogenous Ins(3,4,5,6)P_4 [32,49]. As mentioned in Section 2.2.2, frequent activation of PLC and, therefore, elevated levels of Ins(3,4,5,6)P_4, should be considered as being physiologically-relevant in airway cells *in vivo* (**Figure 4**). When also taking into account the relatively long half-life of Ins(3,4,5,6)P_4 *in vivo* [45], some constitutive inhibition of CaCC by this inositol phosphate is likely a physiologically normal occurrence. Thus, it seems that any drug that is deployed to activate CaCC by elevating [Ca^{2+}] (including Moli1901 and INS-37217; see Section 1), would stand to benefit from the co-administration of an Ins(3,4,5,6)P_4 antagonist.

The route that was chosen in the design of Ins(3,4,5,6)P_4 antagonists arose from an appreciation that it would probably not be productive to make any alterations to the four phosphate groups, because their placement around the inositol ring has to be precisely maintained in order to preserve ligand recognition [24,25,32,42,48,49]. Instead, it seemed that modifications to the hydroxyl groups at carbons 1 and/or 2 might be more appropriate [48,49]. The 1-*O*-butyl-2-*O*-butyryl derivative of Ins(3,4,5,6)P_4 was one of the first compounds that was shown to enhance Cl^- secretion by antagonising the actions of endogenous Ins(3,4,5,6)P_4 [32,49]. The latter work verifies that, in principle, pharmacological intervention in the Ins(3,4,5,6)P_4 signalling pathway is an appropriate approach to increasing CaCC activity. However, this concept still awaits further development. Instead, the one company that is promoting this particular drug development programme has switched its focus away from CaCC and has instead concentrated upon ENaC as a more fruitful target.

4 INO-4995 and its effect upon ENaC

The initial screen for the efficacy of candidate Ins(3,4,5,6)P_4 antagonists was the measurement of Cl^- secretion from epithelial monolayers (Section 3). However, it became apparent that some of the Ins(3,4,5,6)P_4 analogues that were synthesised were especially effective at inhibiting Na^+ uptake through ENaC in CF airway monolayers [21]. The cell-permeant form of 1-*O*-octyl-2-*O*-butyryl-myo-inositol 3,4,5,6-tetrakisphosphate (INO-4995; Inologic, Seattle, WA) emerged as the most potent analogue in this screen. This was an especially startling development, as it enables INO-4995 to substantially correct one of the persistent consequences of the CF condition – enhanced Na^+ uptake by ENaC [7]. The effect of INO-4995 upon Na^+ uptake has also been observed in MDCK cells in which ENaC was overexpressed, consistent with the Na channel being the likely target of the drug [21]. Presumably as a consequence of altering ion transport, INO-4995 was also shown to inhibit fluid uptake by human nasal epithelial cells [21] and is, therefore, a candidate for restoring airway surface liquid volume. In epithelial monolayers, the inhibition of ENaC persisted for at least 24 h after only a single dose of only 5 µM INO-4995 [21]. The longevity of the effects of the drug is probably owed to the poor metabolism of the bioactivated, INO-4913 derivative (**Figure 3**) of INO-4995. The modification at the 1-carbon of INO-4913 (compare **Figures 3A** and **3B**) prevents it from being phosphorylated by the Ins(3,4,5,6)P_4 1-kinase [21], which is the major route by which Ins(3,4,5,6)P_4 is metabolised.

INO-4995 is a member of a second generation of cell-permeant inositol phosphates, in which propionoxymethyl esters have replaced the original acetoxymethylesters; the newer compounds have an improved ability to penetrate cells [57]. INO-4995 is now poised to be evaluated in Phase I clinical trials [101].

How does INO-4995 inhibit ENaC? As INO-4995 was originally developed with the goal of antagonising the actions of endogenous Ins(3,4,5,6)P_4, is it possible that Ins(3,4,5,6)P_4 normally activates ENaC? Thus, is INO-4995 further verification that intervention in inositol phosphate signalling is a valid approach to CF therapy? Unfortunately, these questions cannot yet be answered because the mechanism of action of INO-4995 upon ENaC activity has not been established; it is not known whether or not INO-4995 is distorting an inositol phosphate-regulated physiological process.

One possible explanation for the effects of INO-4995 that should be considered, is based on the presence of CaCC and ENaC in the same membrane, which means that they can influence each others function due to their electrical coupling (**Figure 1**) [8]. Thus, increased Cl^- secretion following activation of CaCCs will inevitably cause a reduction in Na^+ uptake by ENaC [8]. In other words, if INO-4995 were an antagonist of Ins(3,4,5,6)P_4 (which it was originally designed to be), this could increase CaCC activity, and then ENaC activity would also be expected to be inhibited (**Figure 1**). However, a key experiment speaks against this idea: ENaC was overexpressed in 3T3 cells, and channel activity was measured in electrophysiological experiments that used excised, outside-out patches [21]. Pretreatment with INO-4995 reduced ENaC activity [21]. Yet, in these isolated patches there would not be any electrical coupling between CaCC and ENaC because the membrane voltage is fully controlled by the voltage clamp. Even if there were an effect of INO-4995 treatment upon the conductance properties on CaCC, this would not affect ENaC by a mechanism that relied on electrical coupling alone. So at present, the mechanism of action of INO-4995 upon ENaC remains a mystery.

5. Some consequences for drug development arising from Ins(3,4,5,6)P_4 metabolism being regulated by a multifunctional enzyme

As mentioned above (Section 2.2.2), the ITPK1 protein acts *in vivo* as both an Ins(3,4,5,6)P_4 1-kinase and an Ins(1,3,4,5,6)P_5 1-phosphatase [42]. This dual functionality gives changes in the degree of *ITPK1* expression the capacity to alter the dynamic range within which Ins(3,4,5,6)P_4 concentrations will fluctuate [28,42]. This is possible because even a moderate change in *ITPK1* expression can affect levels of Ins(3,4,5,6)P_4 in receptor-activated cells, without affecting the baseline level of Ins(3,4,5,6)P_4 in resting cells [28,42]. In the latter scenario (i.e., resting cells) an increase in the degree of *ITPK1* expression would alter the 1-kinase and 1-phosphatase activities to the same extent, and so these effects would be self-cancelling. This has been empirically verified in T_{84} colonic epithelial cells, for example, in which a 2-fold stable overexpression of ITPK1 did not alter resting levels of Ins(3,4,5,6)P_4 [42]. This same conclusion also came from experiments with a different model cell: resting levels of Ins(3,4,5,6)P_4 were unaffected by a

2-fold difference in *ITPK1* expression between two immortalised tracheal epithelial cell lines [28]. On the other hand, once PLC is activated, then the degree of expression of *ITPK1* does have consequences for Ins(3,4,5,6)P_4 levels. This is because the receptor-dependent, PLC-mediated increases in Ins(1,3,4)P_3 levels (**Figure** 4) specifically augment the Ins(1,3,4,5,6)P_5 1-phosphatase activity at the expense of the Ins(3,4,5,6)P_4 1-kinase [42]. Thus, the degree to which a purinergic (or other) PLC-agonist can elevate Ins(3,4,5,6)P_4 levels can be controlled by the level of *ITPK1* expression [28,42]. Indeed, these considerations suggest that genetic downregulation of *ITPK1* expression might even be a viable strategy for improving CaCC activity in the CF condition.

There are important consequences to arise from this hypothesis that if there is variability in the degree of airway epithelial *ITPK1* expression, this will affect the extent to which CaCCs can be activated by extracellular stimuli. As the extent of CaCC activity may contribute to the severity of the CF disease, it may be considered that *ITPK1* is a candidate for having an epistatic influence over CF pathogenesis – or, in modern parlance [1,58], *ITPK1* may be a 'modifier gene' for *CFTR.* The search for genuine CF modifier genes is driving one of the newer approaches to improving CF therapy [1]. This strategy has been brought about by the recognition that even if those patients in whom the most common CFTR mutation, F508, is homozygous, there is considerable variability in the severity of CF-based lung disease that cannot be predicted purely from the CFTR genotype of an individual [1,58]. An improved ability to predict phenotype from genotype, by expression analysis of genuine modifier genes ('molecular profiling' [59]), could aid an appropriate choice of CF therapy [1].

In Section 3.2 it was noted that a drug that acted as an Ins(3,4,5,6)P_4 antagonist could be therapeutically useful in the CF condition by stimulating CaCC activity. The degree of improvement in CaCC activity that can be elicited by this approach would be expected to be influenced by the extent to which endogenous Ins(3,4,5,6)P_4 inhibits the Cl^- channels, which, in turn, depends upon the Ins(3,4,5,6)P_4 concentration [27]. Those CF individuals with the higher levels of *ITPK1* expression, and thus the higher levels of Ins(3,4,5,6)P_4 after receptor activation, potentially stand to gain the most from therapy based on Ins(3,4,5,6)P_4 antagonists. Conversely, those CF individuals with the lower levels of ITPK1 in airway cells would synthesise less Ins(3,4,5,6)P_4 following activation of PLC; in these patients, airway CaCC would be expected to have higher capacity, so these particular individuals might receive greater benefit from the inhalation of purinergic agonists, such as INS-37217 (Section 1). These considerations argue that clinical trials of these pharmacological approaches to CF that depend upon activation of CaCCs should be correlated with *ITPK1* expression profiling in order to detect responsive patient subgroups. Such profiling might be especially useful if performed early in life, prior to the development of persistent inflammatory lung disease that itself affects the expression of many genes [60]. It is, perhaps, gratifying to reflect that this proposal that *ITPK1* expression profiling might help evaluate clinical trials of INS37217, arises out of a recently-acquired, increased understanding of the role of inositol phosphate signalling in regulating Cl^- transport.

There is another facet to the versatility of ITPK1 that is also relevant here. As was mentioned above (Section 2.2), the mechanism by which cellular Ins(3,4,5,6)P_4 levels are coupled to changes in PLC activity involves inhibition of Ins(3,4,5,6)P_4 1-kinase activity by Ins(1,3,4)P_3 (**Figure 4**). The reason that Ins(1,3,4)P_3 is an inhibitor is because it is actually an alternate substrate of this kinase. A rationale for both Ins(3,4,5,6)P_4 and Ins(1,3,4)P_3 being phosphorylated by the same kinase has been given previously [35,42], and these ideas were recently vindicated when the 3D structure of this protein was solved [61]. To digress for a moment, it is worth noting that it was recognition of the enzyme's ability to phosphorylate Ins(1,3,4)P_3, which predates the discovery of its other catalytic activities, which led to *ITPK1* becoming the HUGO-approved name for this gene.

To return to the topic of the biological impact of the Ins(1,3,4)P_3 kinase activity of ITPK1 [62], this is one of the anabolic reactions that participates in the cells' pathways for *de novo* synthesis of Ins(1,3,4,5,6)P_5, InsP_6 and the inositol pyrophosphates, which are all functionally important molecules [34,35]. Because INO-4913 (the biologically active derivative of INO-4995; **Figure 3**) is an analogue of another ITPK1 substrate – Ins(3,4,5,6)P_4 – it is possible that INO-4913 might bind to ITPK1 and inhibit its kinase activity. A reduced synthesis of Ins(1,3,4,5,6)P_5, InsP_6 and the inositol pyrophosphates could result if the Ins(1,3,4)P_3 kinase activity of ITPK1 were inhibited, and this could have deleterious effects upon cells. Luckily, there may be an escape clause: another inositol phosphate kinase (known as either IPMK or IPK2) provides an alternate route of inositol phosphate synthesis that bypasses ITPK1 [34,35]. Indeed, Ins(1,3,4,5,6)P_5 and InsP_6 pool size is under tight homeostatic control through the actions of several enzymes. Moderate changes in the expression of one of these enzymes can be countered by compensatory alterations in the activities of others [63]. However, it is not known if, in airway cells, the IPMK pathway might have the capacity to circumvent ITPK1 and synthesise adequate quantities of the more highly phosphorylated inositol derivatives so as to sustain normal cell function. It will, therefore, be important to check whether or not the cellular synthesis of higher inositol phosphates is affected by prolonged INO-4995 treatment.

6. Expert opinion and conclusions

INO-4995, a candidate CF drug [101], initially arose from a programme that was designed to develop Ins(3,4,5,6)P_4 antagonists (Section 4). It is not yet known whether or not interference in inositol phosphate signalling by INO-4995 explains why, *in vitro* at least, it inhibits ENaC and restores salt and fluid secretion to CF airway monolayers. As such, the therapeutic

promise of INO-4995 does not strictly answer the question posed by the title of this review. However, one can reasonably expect that the very nature of INO-4995 – an inositol phosphate analogue – means that some intervention in normal inositol phosphate signalling is somehow being achieved. The resolution of this question is not just academic. Knowledge of the molecular mechanism of action of a drug is important in assessing the likelihood of potential off-target effects. At present, therefore, the potential for toxicity of INO-4995 is difficult to predict. Detailed pharmacokinetic data are also lacking, as this drug is in very early stages of development.

Whatever the mode of action of INO-4995, it has also been shown that Cl^- secretion from CF cells can be increased by treatment with Ins(3,4,5,6)P_4 antagonists (Section 3.2). This review has also put forward the suggestion that genetic intervention to downregulate *ITPK1* expression might also increase Cl^- secretion (Section 5). This may even be possible without nonspecifically perturbing other inositol phosphate metabolic pools, as there is an alternate pathway to synthesise higher inositol phosphates (Section 5). These other strategies, which certainly involve intervention in inositol phosphate signalling, remain as potential additional approaches to treating the CF condition.

This review has also noted that the degree of *ITPK1* expression, through its impact on endogenous Ins(3,4,5,6)P_4 levels and hence CaCC activity, may contribute to the severity of the CF condition, and this may impact on the efficacy of therapy based upon pharmacological activation of CaCC by drugs such as INS-37217 and Moli1901. Expression profiling of *ITPK1* in CF individuals may be a useful parameter to obtain in the ongoing clinical trials with CaCC activators. Thus, insight into the regulation of Cl^- channels by inositol phosphates has certainly opened up several opportunities for possibly improving CF therapy.

Acknowledgements

The author is indebted to Dr Alexis Traynor-Kaplan and Dr Sherif Gabriel for their many helpful comments during the writing of this manuscript. The author also notes that he is not professionally affiliated with any of the companies mentioned in this review (Inspire, Lantibio and Inologic).

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