Enzymes involved in the formation an degradation of inositol 1,4,5-triophosphate in Dictyostelium discoideum
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Introduction

It has been widely accepted that Ins(1,4,5)P$_3$ is an important intracellular messenger involved in the release of Ca$^{2+}$ ions from internal stores. Ins(1,4,5)P$_3$ can be formed by the action of the enzyme PLC on PIP$_2$. This reaction yields besides Ins(1,4,5)P$_3$ also the second messenger diacylglycerol. Termination of the Ins(1,4,5)P$_3$ signal can be achieved by dephosphorylation or phosphorylation of Ins(1,4,5)P$_3$, leading to biological inactive compounds. Thus the levels of Ins(1,4,5)P$_3$ are controlled by its formation and removal. In most resting cells the basal level of Ins(1,4,5)P$_3$ is between 0.1 and 0.2 μM, rising maximally to 1 μM during stimulation. In some cells, however, these values are 3 μM and 25 μM, respectively (Shears, 1992). In Dictyostelium basal and stimulated Ins(1,4,5)P$_3$ levels are 3.3 μM and 5.5 μM, respectively (Van Haastert, 1989).

The general theme throughout all of the chapters of this thesis has been: what determines the levels of Ins(1,4,5)P$_3$ in Dictyostelium, and what are the characteristics and functions of the enzyme activities involved.

The effect of lithium on Ins(1,4,5)P$_3$ levels

Lithium, an agent used in the treatment of manic-depressive patients, has been shown to have pronounced effects on the levels of Ins(1,4,5)P$_3$. Its site of action has been proposed to be the enzyme activity responsible for the degradation of inositol monophosphates to inositol. In mammalian cells this enzyme activity is inhibited by lithium and the hypothesis implies that this inhibition leads to a reduction of the inositol pool. This in turn could lead to changes in the levels of precursors for Ins(1,4,5)P$_3$ and thus to the observed reduction in the levels of (agonist-stimulated) Ins(1,4,5)P$_3$ (Berridge et al., 1989; Nahorski et al., 1992). Also in Dictyostelium, lithium has pronounced effects. It influences Dictyostelium development and decreases basal and receptor-stimulated Ins(1,4,5)P$_3$ levels (Peters et al., 1989). In chapter 2 the hypothesis that in Dictyostelium lithium changes Ins(1,4,5)P$_3$ levels and development by inhibition of inositol monophosphatase activity was tested by detailed analysis of these enzymes. Three different inositol monophosphatase activities were found of which only one is sensitive to lithium. The latter enzyme is mainly responsible for the degradation of Ins(1)P. If these three enzymes would be active in vivo, this should mean that lithium inhibits only effectively the degradation of Ins(1)P. Ins(4)P and Ins(3)P degradation would be more or less independent of lithium as these compounds are mainly degraded by the lithium insensitive enzymes (figure 1). Therefore the effect of lithium on in vivo levels of inositol
Figure 1 Effect of lithium on degradation of InsP isomers
The degradation of Ins(1)P, Ins(3)P and Ins(4)P by the three monophosphatase activities is indicated. The flow of substrate is indicated by the width of the arrows. Monophosphatase I, II and III are indicated with open, filled and hatched arrows, respectively. Short arrows indicate the activities that are inhibited by lithium. Long arrows indicate the resulting flow of substrate in the presence of lithium.

and inositol monophosphate isomers was determined. It turned out that lithium only induced a reduction of 20% in inositol. Lithium had different effects on the various inositol monophosphate isomers. Ins(1)P levels increased six-fold whereas Ins(4)P levels doubled and Ins(3)P levels were hardly affected. This strongly suggests that the enzymes that were identified in vitro are active in vivo. These results furthermore falsify the hypothesis that in Dictyostelium the effects of lithium on development and Ins(1,4,5)P₃ levels, are caused by depletion of the inositol pool due to inhibition of inositol monophosphatase activity.

Hokin and co-workers have demonstrated in slices of mammalian tissues that lithium increases rather than decreases agonist stimulated Ins(1,4,5)P₃ accumulation. This phenomenon was demonstrated both in cerebral cortex of guinea pig, rabbit and monkey, and in mouse pancreas (for references see Dixon and Hokin, 1994). In the mouse pancreas a decrease in inositol due to lithium was observed. However, addition of inositol did not alter the accumulation of Ins(1,4,5)P₃ indicating that enough inositol was present. Thus, a reduction in inositol is not correlated with changes in the level Ins(1,4,5)P₃. These data are in contrast with the data obtained in rat and mouse cerebral cortex slices, on the basis of which the inositol depletion theory was developed (Berridge et al., 1989;
Nahorski et al., 1992). These findings can, according to Hokin and co-workers, be explained by the experimental procedure used (high concentrations of stimulus), by the nature of the tissue used (rat cerebral cortex slices are uniquely deficient in inositol) and by effects of lithium on inositol uptake.

From our work and the work of Hokin’s laboratory it can be concluded that effects of lithium can not be explained by depletion of the inositol pool. It thus remains unclear what the target of lithium is. In Dictyostelium lithium inhibits activation of G-proteins, suggesting that G-proteins are the target of lithium (Peter et al., 1992). As it has been shown that in Dictyostelium PLC is under control of G-proteins (Bominaar and Van Haastert, 1994), the observed effects of lithium on Ins(1,4,5)P₃ might be due to reduced G-protein mediated PLC activation. This hypothesis can be tested by investigation of the effects of lithium on Dictyostelium cells defective in the different G-proteins. Also PLC itself or other PLC-independent Ins(1,4,5)P₃ formation (see below) could be a target for lithium, leading to the observed effects on Ins(1,4,5)P₃ levels. This however, remains to be investigated.

The source of Ins(1,4,5)P₃ in plc Dictyostelium cells

In order to study the role of PLC and Ins(1,4,5)P₃ in Dictyostelium, a cell-line with a disrupted gene for PLC was constructed (Drayer et al., 1994). These cells had no PLC activity, but surprisingly, the cells had no abnormal phenotype under standard laboratory conditions and the levels of Ins(1,4,5)P₃ were hardly different from wild-type cells (Drayer et al., 1994). This result means that in Dictyostelium basal Ins(1,4,5)P₃ can be maintained in a PLC-independent manner. This implies that a PLC-independent source of Ins(1,4,5)P₃ is present and this source had to be found.

Chapter 3 describes the use of techniques that were instrumental for finding the source of Ins(1,4,5)P₃ in the absence of PLC. The technique of HPLC purification, and conversion of inositol phosphates by specific enzymes was employed. These techniques were later used in chapter 4 and 5 to identify specific inositol phosphate isomers. Furthermore, chapter 3 describes the synthesis of ³²P-labeled Ins(1,3,4,5)P₄ and a ³²P-labeled Ins(1,3,4,5)P₄ analogue that were used in chapter 4, 5 and 6. Finally, chapter 3 provides information about the structural requirements of the mammalian enzymes Ins(1,4,5)P₃ 3-kinase and Ins(1,4,5)P₃/Ins(1,3,4,5,5)P₄ 5-phosphatase.

In chapter 4 the degradation of Ins(1,3,4,5)P₄ by Dictyostelium homogenates was investigated. As it was shown in mammalian cells that Ins(1,3,4,5)P₄ can be degraded to Ins(1,4,5)P₃, it was hypothesized that also in Dictyostelium Ins(1,3,4,5)P₄ could be a
PLC-independent source for Ins(1,4,5)P\(_3\). The results showed that in Dictyostelium, Ins(1,3,4,5)P\(_4\) was degraded by two enzymes. A Mg\(^{2+}\) sensitive 1-phosphatase, yielded Ins(3,4,5)P\(_3\). This enzyme activity could not degrade \([3-\text{32P}]\text{Ins(3,4,5)P}_3(1)\text{PS}\). Furthermore, Ins(1,3,4,5)P\(_4\) was degraded by a Mg\(^{2+}\)-independent 3-phosphatase yielding Ins(1,4,5)P\(_3\). The degradation of Ins(1,3,4,5)P\(_4\) has been studied in a number of other tissues. In porcine brain (Höer et al., 1988), rat basophilic leukemia cells (Cunha-Melo et al., 1988), human erythrocytes (Doughney et al., 1988) and in human platelets (Oberdisse et al., 1990) Ins(1,3,4,5)P\(_4\) was degraded by a Mg\(^{2+}\)-dependent 5-phosphatase and a Mg\(^{2+}\)-independent 3-phosphatase. Thus Dictyostelium is unique in having a Mg\(^{2+}\)-dependent Ins(1,3,4,5)P\(_4\) 1-phosphatase. Table 1 lists the Ins(1,3,4,5)P\(_4\) phosphatases identified so far in Dictyostelium and mammalian cells.

<table>
<thead>
<tr>
<th>substrate</th>
<th>enzyme</th>
<th>Ins(1,4,5)P(_3)</th>
<th>Ins(1,3,4,5)P(_4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mammalian 5-phosphatase I</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>mammalian 5-phosphatase II</td>
<td>+</td>
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<td></td>
<td>mammalian 5-phosphatase III</td>
<td>+</td>
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<tr>
<td></td>
<td>mammalian 3-phosphatase</td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>Dictyostelium 1-phosphatase I</td>
<td>+</td>
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</tr>
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<td>Dictyostelium 1-phosphatase II</td>
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<td>Dictyostelium 3-phosphatase</td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>Dictyostelium 5-phosphatase II</td>
<td>+</td>
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</tr>
</tbody>
</table>

Table 1 Ins(1,4,5)P\(_3\) and Ins(1,3,4,5)P\(_4\) phosphatases identified in mammalian and Dictyostelium cells

Also in the case of Ins(1,4,5)P\(_3\) degradation Dictyostelium is unique in having a 1-phosphatase (Van Lookeren Campagne et al., 1988). Table 1 summarizes also the Ins(1,4,5)P\(_3\) phosphatase activities identified. In mammalian cells Ins(1,4,5)P\(_3\) is not degraded by a 1-phosphatase but by a 5-phosphatase activity. Three types of 5-phosphatase activity exist of which type I and III also degrade Ins(1,3,4,5)P\(_4\) (Verjans et al., 1994). In addition to degradation by a 1-phosphatase, in Dictyostelium Ins(1,4,5)P\(_3\) is also degraded by a 5-phosphatase (Van Lookeren Campagne et al., 1988). This enzyme is
a type II 5-phosphatase as it does not degrade Ins(1,3,4,5)P4. It is unclear whether Ins(1,4,5)-1-phosphatase activity and Ins(1,3,4,5)P4 1-phosphatase activity are carried out by one enzyme. In that case this 1-phosphatase shares with mammalian type I and III 5-phosphatase the characteristic that it uses both Ins(1,4,5)P3 and Ins(1,3,4,5)P4 as substrate.

The results obtained in chapter 4, showing that Ins(1,4,5)P3 can be derived from Ins(1,3,4,5)P4, gave an answer to the question about the origin of Ins(1,4,5)P3 in plc- Dictyostelium cells, but also raised a new question, namely about the origin of Ins(1,3,4,5)P4 in plc- cells. An answer to this question is given in chapter 5; Ins(1,3,4,5)P4 can be derived from Ins(1,3,4,5,6)P5. Stephens and Irvine (1990) have already shown previously that this InsP5 isomer is one of the intermediates in the phosphorylation of inositol to InsP6. This finding closes a PLC-independent route of Ins(1,4,5)P3 formation composed of de novo synthesis of inositol from glucose 6-phosphate, phosphorylation of inositol to Ins(1,3,4,5,6)P5 and dephosphorylation of Ins(1,3,4,5,6)P5 to Ins(1,4,5)P3.

**Characteristics of PLC-independent Ins(1,4,5)P3 formation**

Ins(1,3,4,5,6)P5 is degraded not only to Ins(1,3,4,5)P4, but also to Ins(1,4,5,6)P4. Both these compounds can be degraded to Ins(1,4,5)P3, and all reaction-steps require Ca2+ (chapter 5). There are several indications that all reaction steps are carried out by a single enzyme. First, the formation of Ins(1,4,5)P3 from both Ins(1,3,4,5,6)P5 and Ins(1,3,4,5)P4 has the same Ca2+-dependency and both are located in the particulate cell fraction (chapter 5). Furthermore, Ins(1,4,5)P3 formation from Ins(1,3,4,5,6)P5 remains intact after diverse procedures such as solubilization, partial purification and subcellular fractionation (chapter 6). The enzyme is called inositol 1,3,4,5,6-pentakisphosphate 3,6-bisphosphatase, and is a peripheral membrane enzyme with its enzymic activity localized at the inner side of the plasma membrane (chapter 6). The activity of this enzyme is probably not under control of a G-protein-linked pathway, but under control of receptor stimulated Ca2+-influx.

In cells from rat liver the same type of reactions are carried out by an enzyme called Multiple Inositol Polyphosphate Phosphatase. But, as mentioned in chapter 6, the localization of the enzyme inside the endoplasmic reticulum where it has no apparent access to its substrates makes it unlikely that it will contribute to Ins(1,4,5)P3 formation in the cell. A function for this enzyme has yet to be found. It could be that under certain conditions this enzyme is transported to the outside of the cell. It is known that transport
of proteins occurs from the endoplasmic reticulum to the golgi apparatus and from this organelle to the exterior of the cell (Stryer, 1988). Furthermore, extracellular degradation of \( \text{Ins}(1,3,4,5,6)P_5 \) has been demonstrated before (Carpenter et al., 1989). As \( \text{Ins}(1,3,4,5,6)P_5 \) functions sometimes as some kind of hormone (Perney and Kaczmarek, 1992), it is possible that MIPP serves as a terminator of hormone action.

The metabolism of inositol in *Dictyostelium*

From the data presented in this thesis and data from literature, it is now possible to give an overview of the metabolism of inositol in *Dictyostelium*. Figure 2 summarizes all the reaction-steps identified so far. From the figure it can be seen that two independent links between inositol and \( \text{Ins}(1,4,5)P_3 \) exist. One pathways runs through the phospholipids yielding \( \text{Ins}(1,4,5)P_3 \) by the action of PLC. The other pathway is composed of the sequential phosphorylation of inositol to \( \text{Ins}(1,3,4,5,6)P_5 \), yielding \( \text{Ins}(1,4,5)P_3 \) by the action of \( \text{Ins}(1,3,4,5,6)P_5 \) 3,6-bisphosphatase.

The existence of these two pathways raises the question about the function and importance of both pathways and the relationship between them. It is possible that these two pathways are redundant, but it also possible that both pathways have different functions. The function of a PLC specific pathway was studied in *plc* cells (see below), the role of the \( \text{Ins}(1,3,4,5,6)P_5 \) 3,6-bisphosphatase can be studied in a cell-line lacking \( \text{Ins}(1,3,4,5,6)P_5 \) 3,6-bisphosphatase activity. Whether the two pathways are redundant can be investigated in a double knock-out mutant lacking both PLC and \( \text{Ins}(1,3,4,5,6)P_5 \) 3,6-bisphosphatase activity.

**Ins(1,4,5)P_3 function**

It has been hypothesized that in *Dictyostelium* \( \text{Ins}(1,4,5)P_3 \) is involved in chemotaxis and/or development. This was inferred from experiments described in permeabilized cells as discussed in the "Introduction" chapter of this thesis. But more clearly this can be deduced from experiments described by Bominaar and Van Haastert (1993); antagonists of chemotaxis caused a reduction in PLC activity and a reduction of the level of \( \text{Ins}(1,4,5)P_3 \). Furthermore, in a mutant defective in PLC inhibition, no decrease of \( \text{Ins}(1,4,5)P_3 \) levels was observed and also no antagonism of chemotaxis could be detected anymore in this mutant. Also the observed relationship between lower basal and receptor-stimulated \( \text{Ins}(1,4,5)P_3 \) levels and disturbed development caused by lithium
Figure 2 Inositol metabolism in *Dictyostelium discoideum*

Summary of the reaction steps involved in the metabolism of inositol in *Dictyostelium* cells. For abbreviations see the list of abbreviations used.

pointed in the same direction (Peters et al., 1989). Thus, all these described experiments suggest a correlation between the level of Ins(1,4,5)P₃ and chemotaxis and/or development. In an attempt to clearly demonstrate a function for Ins(1,4,5)P₃, *Dictyostelium* PLC was cloned (Drayer and Van Haastert, 1992) and a plc⁻ cell-line was constructed. However, this cell-line showed no abnormal phenotype under optimal laboratory conditions and had nearly normal Ins(1,4,5)P₃ levels. Thus no definite function can be assigned to Ins(1,4,5)P₃ or PLC in chemotaxis and development. However, it seems that basal Ins(1,4,5)P₃ levels are important in *Dictyostelium* because in the absence of PLC, Ins(1,4,5)P₃ levels are near-normal whereas Ins(1,3,4,5,6)P₅ is reduced 70%. This suggests that the pool of Ins(1,3,4,5,6)P₅ is emptied in order to keep the basal pool of Ins(1,4,5)P₃ full. It is very well possible that the decrease in Ins(1,4,5)P₃ observed after stimulation with a chemotactic antagonist (Bominaar and Van Haastert, 1993), on a longer time (> 30 seconds) scale would be followed by a return to normal levels due to formation of Ins(1,4,5)P₃ from Ins(1,3,4,5,6)P₅. This in turn may suggest that PLC generated Ins(1,4,5)P₃ and Ins(1,3,4,5,6)P₅ derived Ins(1,4,5)P₃ flow into one pool. The localization of both enzymes at the (inner side of) plasma membrane implies that this pool...
is the cytosol, in which both pathways can deliver their product.

As mentioned above, basal Ins(1,4,5)P$_3$ levels are probably important in Dictyostelium. It is unknown what the function is of stimulated Ins(1,4,5)P$_3$ levels. The rise in Ins(1,4,5)P$_3$ after stimulation in Dictyostelium is small as compared to other cell-types. In Dictyostelium less than a doubling in Ins(1,4,5)P$_3$ whereas in other cells much larger effects are observed (Shears, 1992). It could be possible, however, that the rise in Ins(1,4,5)P$_3$ locally is much larger than in the cell as a whole leading to the small totally observed increase.

In order to get a more definite answer on Ins(1,4,5)P$_3$ function, a few options are open and worth trying. First, the recent availability of antibodies against Ins(1,4,5)P$_3$ offers an opportunity to deplete cells of Ins(1,4,5)P$_3$ by injection of the antibodies inside the cell (Shieh and Chen, 1995). Secondly, a PLC/Ins(1,3,4,5,6)P$_5$ 3,6-bisphosphatase double null Dictyostelium mutant could be constructed. If no other route of Ins(1,4,5)P$_3$ formation exists, this mutant will be devoid of Ins(1,4,5)P$_3$. Purification of the Ins(1,3,4,5,6)P$_5$ 3,6-bisphosphatase is essential for this purpose. Cloning and expression of the gene for this enzyme would also definitively resolve the question whether all reaction steps in the formation of Ins(1,4,5)P$_3$ from Ins(1,3,4,5,6)P$_5$ are due to one enzyme.

Another approach would be to find the target for Ins(1,4,5)P$_3$. Although it has been shown that in Dictyostelium Ins(1,4,5)P$_3$ releases Ca$^{2+}$ from internal stores (Europe-Finner and Newell, 1986; Flaadt et al., 1993), the target of Ins(1,4,5)P$_3$ carrying out this function has never been found. A rigorous study into downstream target and disruption of these pathways could also yield valuable information about Ins(1,4,5)P$_3$ function.

**The role of phospholipase C**

PLC is not essential for chemotaxis and development, as *plc* cells are still capable of chemotaxis and development. Thus, it was still a question what the physiological function of PLC is. Apparently, under optimal laboratory conditions PLC is dispensable.

At several stages in the Dictyostelium life cycle cells respond to non-optimal environmental conditions. For instance, spore germination is arrested when the activated spore detects harsh environmental conditions. In chapter 7 it is described that under non-optimal conditions *plc* cells do not proper sense or respond to these conditions leading to fate decisions that could negatively influence the change of survival. As PLC changes the levels of PIP$_2$, diacylglycerol and Ins(1,4,5)P$_3$, it is possible that one or more of these compounds is involved in this process. It is even possible that a yet unidentified activity
of PLC is exerting this function. This suggestion is not without precedent because it has been shown that antibodies against PLC-γ, that do not inhibit PLC activity, inhibit PLC-γ induced mitosis (reviewed in Noh et al., 1995).

*Dictyostelium* contains a PLC-δ activity, that is regulated by Ca^{2+} and by a receptor-G-protein pathway, and a Ins(1,3,4,5,6)P₃ 3,6-bisphosphatase activity that is regulated by receptor mediated Ca^{2+} influx. Both activities can give rise to the formation of Ins(1,4,5)P₃. Mammalian cells do have different types of PLC. PLC-δ is regulated by the Ca^{2+} concentration only, whereas PLC-β is regulated by Ca^{2+} and G-proteins and PLC-γ is regulated by receptor protein tyrosine kinases. Thus on the level of regulation, *Dictyostelium* Ins(1,3,4,5,6)P₃ 3,6-bisphosphatase and mammalian PLC-δ are similar (Ca^{2+}) and *Dictyostelium* PLC-δ and mammalian PLC-β are similar (G-proteins). It is tempting to suggest that different enzymes perform similar functions in different organisms. If this is true, it will not be surprising to find a third route of Ins(1,4,5)P₃ formation in *Dictyostelium* that is regulated by protein tyrosine kinases.

**Finally**

In this thesis several enzymes have been discussed that take part in the determination of Ins(1,4,5)P₃ levels in *Dictyostelium*. Some characteristics and functions of these enzymes have been revealed. However, many aspects have not been resolved yet. For example the function of the Ins(1,3,4,5,6)P₃ 3,6-bisphosphatase and its relationship to PLC are still unclear (see above). Furthermore, the exact role of Ins(1,4,5)P₃ itself in *Dictyostelium* is still not resolved, yet. Further identification of the components involved in Ins(1,4,5)P₃ functioning should be performed, although this alone might not lead to a complete understanding. The relationships and interactions of the various components should be investigated as well. This can be done by a biochemical approach leading to purification of the components and subsequent reconstitution of the system. Furthermore, a genetic approach can be chosen to make mutants, as has been done in the case of the plc *Dictyostelium* mutant. Finally, modelling can be used to get a better understanding of the role of each component in relationship to all other components. All these approaches might be performed using *Dictyostelium* as the subject of investigations.