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Regulation Of Inositol Biosynthesis And Cellular Consequences Of Inositol Depletion: Implications For The Mechanism Of Action Of Valproate

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REGULATION OF INOSITOL BIOSYNTHESIS AND CELLULAR CONSEQUENCES OF INOSITOL DEPLETION: IMPLICATIONS FOR THE MECHANISM OF ACTION OF VALPROATE

by

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DEDICATION

In memory of my beloved parents

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CHAPTER 1

INTRODUCTION

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Bipolar mood disorder (BD), previously referred to as folie circulaire (circular madness), is a major psychiatric illness that affects 2-5% of the worldwide population. It is characterized by intermittent cycles of severe mania and depression, with a high tendency for suicide. The underlying causes and the molecular basis of this debilitating disease are not known. Mood stabilizing drugs, including lithium, valproate (VPA), and carbamazepine, are used for the treatment of BD. However, they are not completely effective, and are associated with serious side effects, including teratogenicity and hepatotoxicity. Furthermore, the mechanism underlying the therapeutic effect of these three structurally dissimilar drugs is not well-understood. Intriguingly, all three of them lead to inositol depletion, albeit by different mechanisms, suggesting that depletion of inositol may underlie the therapeutic effect of these drugs, and that inositol, or inositol compounds may play a key role in the pathophysiology of BD.

The work presented in this thesis was carried out to elucidate cellular consequences of inositol depletion mediated by the anticonvulsant drug VPA. This study led to three major findings. First, I identified a novel regulatory mechanism of inositol

biosynthesis characterized by phosphorylation of the rate-limiting enzyme in inositol biosynthesis. Second, I demonstrated, for the first time, that the highly conserved vacuolar ATPase (V-ATPase) is a target of VPA. Third, my studies indicated that VPA-mediated inositol depletion perturbs endocytosis in both yeast and mammalian cells. These novel findings suggest new mechanisms that may underlie the therapeutic action of VPA, and suggest novel targets that may be used for the development of more effective and safer drugs.

A brief history of VPA

VPA is a simple, 8-carbon, branched chain carboxylic acid that was first synthesized in 1882 and used as an organic solvent (Burton, 1882). The anticonvulsant properties of VPA were fortuitously discovered eighty years later when it was used to solubilize khellinic compounds that were tested for potential anticonvulsant properties (Meunier et al., 1963). VPA was first used for the treatment of BD in 1966 (Lambert et al., 1966). Since then, it has become a widely prescribed drug for the treatment of epilepsy and BD (Bowden et al., 1994; Perucca, 2002), and more recently, for the treatment of migraine (Calabresi et al., 2007). Although several targets of VPA have been identified, the mechanism of action that underlies its therapeutic effect is not known. The decrease in inositol caused by VPA has many implications especially in light of the many roles played by inositol and inositol compounds. In the following sections, I highlight the significance of inositol and consequences of its depletion.

Inositol is an essential metabolite:

Inositol is a six-carbon cyclitol that is ubiquitous in biological systems. It is a precursor for the synthesis of numerous biologically important compounds, including inositol phosphates (IP_2-IP_9) and phosphoinositides such as phosphatidylinositol (PI) and glycosylphosphatidylinositol (GPI) (Breslow and Weissman, 2010; Pittet and Conzelmann, 2007). The inositol head group of PI can be reversibly phosphorylated at positions 3, 4, and 5 to yield a variety of biologically active phosphoinositides that can bind and recruit a wide range of proteins (Strahl and Thorner, 2007). At least four phosphoinositides have been identified in yeast, including PI3P, PI4P, $PI3,5P_2$, and $PI4,5P_2$ (Fig. 1.1). In higher eukaryotes, there are three additional phosphoinositides that include PI5P, $PI3,4P_2$ and $PI3,4,5P_3$ (Strahl and Thorner, 2007). Inositol-containing compounds are essential for various structural and functional processes important for cell function and viability. Some of these functions include membrane formation, gene regulation, signaling, regulation of ion channels, and membrane trafficking (Di Paolo et al., 2006; Falkenburger et al., 2010; Michell, 2013). Furthermore, inositol regulates hundreds of genes, including those involved in biosynthesis of inositol and phospholipids (Greenberg and Lopes, 1996; Carman and Henry, 1999), as well as genes involved in stress response pathways (Jesch et al., 2005). In the yeast *Saccharomyces cerevisiae,* inositol is derived from three sources: Import from the extracellular medium using two Na^+-driven *myo*-inositol transporters encoded by *ITR1* and *ITR2*; recycling of inositol 1,3-bisphosphate (IP_2), which is derived from the breakdown of $PI4,5P_2$ into
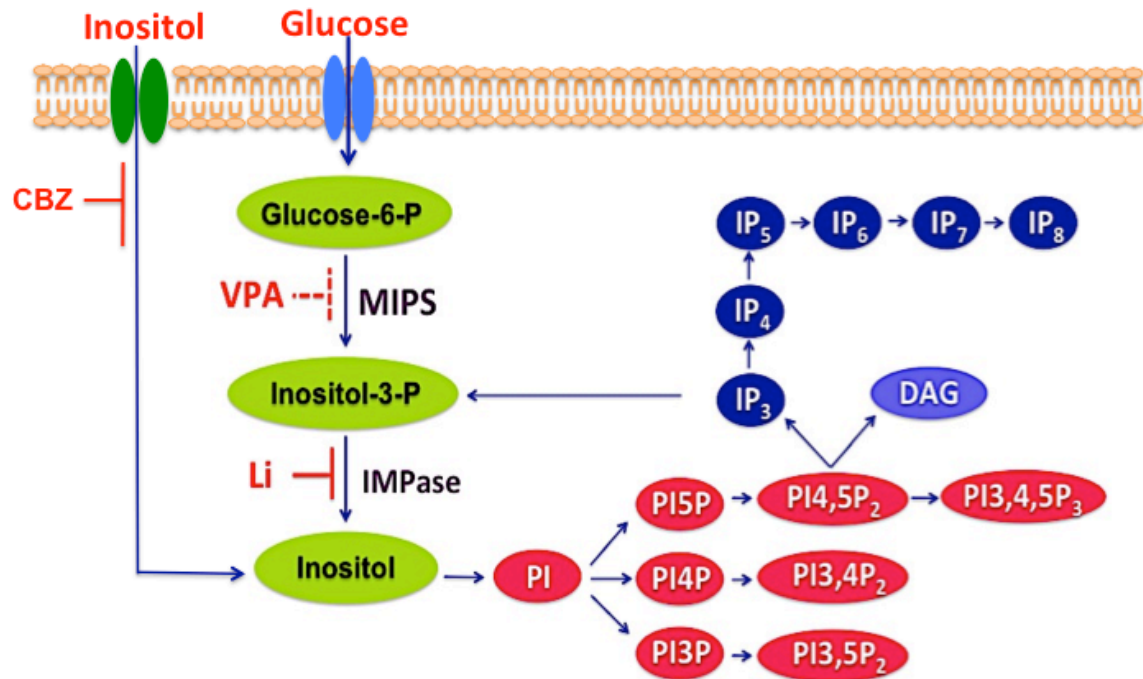
FIGURE 1.1. **The inositol biosynthetic pathway.** Inositol biosynthesis is regulated by two enzymes, *myo*-inositol-3-phosphate synthase (MIPS), which converts G-6-P to I-3-P, and inositol monophosphatase (IMPase), which dephosphorylates I-3-P to generate *myo*-inositol. Inositol is incorporated into phosphatidylinositol (PI), which is then phosphorylated to generate four types of phosphoinositides in yeast and three additional phosphoinositides in higher eukaryotes (PI5P, PI3,4P$_2$, and PI3,4,5P$_3$). The phosphoinositide PI4,5P$_2$ is hydrolyzed by phospholipase C (PLC) to generate the two signaling molecules DAG and IP$_3$. IP$_3$ is either dephosphoylated to produce IP$_2$ and I-3-P, or is further phosphorylated to produce a variety of inositol phosphates.

diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3); and *de novo* biosynthesis using glucose-6-phosphate (G-6-P) as a substrate. To maintain the homeostasis of this essential metabolite, a high level of regulation is required.

Regulation of inositol biosynthesis:

The enzyme responsible for the first and rate-limiting step in inositol biosynthesis is *myo*-inositol 3-phosphate synthase (MIPS) (Fig. 1.1), a highly conserved enzyme that was isolated for the first time from rat testes by Maeda et al. (1980), and from yeast by Donahue and Henry (1981). MIPS catalyzes the conversion of G-6-P to inositol-3-phosphate (I-3-P) in a four-step catalytic reaction using NAD^+ as a cofactor. Subsequently, I-3-P is dephosphorylated by the enzyme inositol monophosphatase (IMPase) to generate *myo*-inositol.

INO1 is the structural gene that encodes MIPS. It was identified and characterized for the first time in *S. cerevisiae* (Donahue and Henry, 1981). Since then, over 60 *INO1* genes have been identified across the kingdom of life, including bacteria, archaea, protozoa, fungi, plants, and animals. The *INO1* gene is tightly regulated by its product, inositol. Under inositol-limiting conditions, *INO1* is derepressed. The transcription activators Ino2 and Ino4 form a dimer that binds to the inositol-regulated upstream activating sequence (UAS_{INO}) leading to the transcription of *INO1*. However, when inositol is available, transcription of *INO1* is repressed. The negative regulator Opi1 translocates from the endoplasmic reticulum to the nucleus where it binds to Ino2,

preventing activation by the Ino2-Ino4 dimer and, thus, repressing transcription of *INO1* (Henry et al., 2012; White et al., 1991).

While transcriptional regulation of inositol biosynthesis has been extensively studied and well characterized (Carman et al., 1999; Greenberg et al., 1996; Nunez et al., 2006), regulation of inositol biosynthesis at the enzymatic level has not been addressed. The studies in Chapter 2 show that both yeast and human MIPS are phosphoproteins regulated by phosphorylation of at least three conserved residues. This identifies phosphorylation as a novel regulatory mechanism of inositol biosynthesis, suggesting that inositol homeostasis must be tightly controlled in eukaryotic cells.

Indeed, perturbation of inositol levels in brain has been associated with a variety of mental and mood disorders, including BD (Belmaker, 2004), Alzheimer's disease (McLaurin et al., 1998; Shimohama et al., 1998), and manic depressive psychosis (Nahorski et al., 1991). As mentioned earlier, inositol depletion is a common outcome of treatment with mood-stabilizing drugs, including lithium and VPA. Lithium inhibits two key inositol biosynthetic enzymes, IMPase (Fig. 1.1), and inositol polyphosphate phosphatase (IPPase), which dephosphorylates IP_3 and IP_2 to generate I-3-P (Hallcher and Sherman, 1980). VPA, in contrast, inhibits the enzyme MIPS leading to a decrease in I-3-P and *myo*-inositol (Shaltiel et al., 2004; Ju et al., 2004). The inositol depletion hypothesis proposed in the 1980s by Sir Michael Berridge and colleagues suggested that the therapeutic effect of Li derives from inositol depletion, which leads to attenuation of IP_3-mediated signaling (Berridge, 1984; Berridge et al., 1989). Since then, numerous studies have been done to identify the connection between decreased IP_3 signaling and

the anticonvulsant and antimanic effects. However, it has not been possible to directly correlate decreased IP_3 signaling with BD. This led many to argue against the inositol depletion hypothesis. A counter-argument is that Inositol depletion may have far more consequences than decreased IP_3 signaling. In the following sections, I address some of these possibilities.

What are the cellular consequences of inositol depletion?

The significance of inositol for cell physiology is dramatically exemplified by the inositol-less death phenomenon first observed in yeast, in which cells starved for inositol die rapidly within a few hours of starvation (Culbertson et al., 1975). Inositol depletion elicits profound changes in lipid metabolism, alters cell signaling, and activates numerous stress responses (Villa-Garcia et al., 2011; Henry et al., 2014).

Inositol depletion alters lipid metabolism:

Wild-type yeast cells grown in inositol-limiting conditions exhibit a 4-5 fold decrease in PI levels compared to cells grown in media supplemented with exogenous inositol (Gaspar et al., 2006). Subsequently, this leads to changes in levels of phosphoinositides, sphingolipids, and GPI (Jesch et al., 2010; Doering and Schekman, 1996). Changes in levels of ceramide have also been observed (Villa-Garcia et al., 2011). Furthermore, an increase in phosphatidic acid (PA), diacylglycerol (DAG), triacylglycerol (TAG), and phosphatidylcholine (PC) in response to inositol depletion has been reported (Gaspar et al., 2006).

A more drastic effect of inositol depletion is observed in *ino1Δ* cells upon deprivation of inositol. Starvation is induced in *ino1Δ* cells, which cannot make inositol, by preculturing them in I+ medium and then transferring them to I- medium. A study by Henry et al. (1977) showed that by the 5th hour of starvation, only 10% of cells survived. The rate of PI synthesis dropped to less than 10% within the first 2 hours after shifting the cells to inositol-free medium (Becker and Lester, 1977). The decrease was initiated as early as 2-25 minutes following removal of inositol (Hanson and Lester, 1980). A decrease in di- and tri-phosphoinositides, inositol-containing sphingolipids, and cell wall glycans was also observed in the same study. Thus, inositol starvation clearly has a drastic impact on cells. This may be caused by increased levels and accumulation of some lipids, such as PC and TAG, or by the decrease in inositol phospholipids such as PI and other phosphoinositides, which are indispensable mediators of signaling events, motility, and membrane trafficking (Michell et al., 2006; Sbrissa et al., 2007).

The significance of phosphoinositides is underscored by the variety of roles they play. PI4,5P$_2$, predominantly found in the plasma membrane, serves as the precursor for IP$_3$ and DAG and is involved in the regulation of actin cytoskeleton organization, and in the cell wall integrity and heat shock response pathways (Di Paolo and Di Camilli, 2000; Audhya and Emr, 2002; Levin, 2011). PI4P, found in the Golgi complex, plays a role in vesicular trafficking of secretory proteins (Hama et al., 1999; D'Angelo et al., 2008). PI3P, predominantly found in late endosomes and multivesicular bodies (MVB), is involved in endosomal and vesicular trafficking (Herman and Emr, 1990; Odorizzi et al.,

2000). PI3,5P$_2$, the most recently identified phosphoinositide, is associated predominantly with the vacuolar membrane (Yamamoto et al., 1995). Despite the low abundance of this phosphoinositide, it is involved in numerous functions in the cell, including control of endolysosomal vesicular trafficking, control of ion channels and pumps (Michell, 2013), and the most recently discovered role is the regulation of key genes involved in the metabolic switch from glycolysis to gluconeogenesis (Han and Emr, 2013).

The phosphoinositides PI3,4P$_2$ and PI3,4,5P$_3$ (PIP$_3$), found in higher eukaryotes, activate a wide range of effector proteins by targeting them to specific membrane locations, where they activate signal transduction pathways (Dowler et al., 2000; Mora et al., 2004). PIP$_3$ signaling plays a role in synaptic transmission (Xu et al., 2007), axonal guidance (Chadborn et al., 2006), and synaptogenesis (Martin-Pena et al., 2006). Thus, the wide range of roles played by phosphoinositides suggests that inositol depletion may have far reaching consequences. For example, decreased PIP$_3$ production in response to Li and VPA causes an increase in the size and spread of neuronal growth cones needed for the formation of synaptic trees (Williams et al., 2002). Reduced phosphoinositide synthesis in response to VPA is suggested to have a therapeutic role in treatment of epilepsy (Chang et al., 2011). This is consistent with the observation that phosphoinositide production is increased during seizures (Van Rooijen et al., 1986; Backman et al., 2001).

Inositol depletion alters signaling:

Inositol is essential for the production of several signaling molecules, including PIP_3, $PI4,5P_2$, IP_3, and DAG (King et al., 2009; Strahl and Thorner, 2007). In response to stimuli, phospholipase C (PLC) hydrolyzes $PI4,5P_2$ to generate IP_3 and DAG, which are important for neuronal activity. Binding of IP_3 to its receptors triggers the release of Ca^{+2} from the ER (or from the vacuole in yeast) (Mikoshiba et al., 1993). DAG activates PKC and, along with Ca^{+2}, plays a role in regulating vesicle fusion and recycling (De Camilli et al., 1996). Both VPA and Li lower the cellular concentration of IP_3 leading to attenuation of IP_3 signaling (King et al., 2009). Tokuoka et al. (2008) showed that levels of IP_3 and DAG were decreased in VPA-treated *Caenorhabditis elegans*, and that three activities regulated by IP_3 and DAG were inhibited in response to VPA treatment, including ovulation, defecation, and acetylcholine release.

Phosphorylation of IP_3 generates a variety of inositol polyphosphates (IPs), including IP_4, IP_5, IP_6, or inositol pyrophosphates IP_7 and IP_8. IPs are involved in regulation of gene expression at the level of transcription, chromatin remodeling, mRNA editing and mRNA export from the nucleus (Alcazar-Roman and Wente, 2008). IP_4 and IP_5 increase expression of *INO1* by activating the SWI/SNF complex, while IP_6 reduces expression of *INO1* by inhibiting nucleosome mobilization (Rando et al., 2003; Shen et al., 2003; Steger et al., 2003; Ye et al., 2013). Inositol pyrophosphates are distinguished by their high energy bonds. In addition, they are able to directly phosphorylate prephosphorylated proteins in an ATP-independent manner (Burton et al., 2009). It is very likely that a reduction in IP_3 levels caused by VPA would alter levels of other inositol

phosphates derived from IP$_3$. Thus a wide range of cellular processes may be affected by VPA treatment.

Inositol depletion activates stress response pathways:

Inositol limitation is a stress-inducing growth condition. The unfolded protein response (UPR) pathway is a highly conserved ER-associated stress response pathway that functions to protect cells from the deleterious accumulation of misfolded proteins (Cox and Walter, 1996; Cox et al., 1997). Jesch et al. (2006) showed that cells grown in inositol-limiting conditions induced the UPR pathway. However, unfolded proteins do not build up in the ER of inositol-starved cells (Jesch et al., 2006). This suggested that inositol starvation may trigger stress by causing ER-membrane lipid aberrations that, in turn, induce the UPR (Henry et al., 2014). An alternative possibility whereby inositol depletion may induce ER stress is through secretory stress caused by defective membrane trafficking (Chang et al., 2002). Several lines of evidence support the involvement of UPR dysfunction in the pathophysiology of BD. Hayashi et al. (2009) showed that the ER-stress response is impaired in lymphoblastoid cell lines derived from BD patients. The study by Konradi et al. (2004) indicated that expression of genes coding for proteins of the ubiquitin-proteasome system is greatly decreased in the hippocampus of BD patients. One interpretation of these findings is that the therapeutic benefits of inositol depletion induced by anti-bipolar drugs may derive from induction of the UPR and/or proteasome degradation.

Another stress response induced by inositol starvation is the highly conserved protein kinase C-MAP kinase cell wall integrity (PKC-CWI) pathway. Inositol starvation activates this pathway in actively dividing cells, leading to upregulation of genes involved in cell wall biogenesis (Nunez et al., 2008). The PKC-CWI pathway is essential for maintaining lipid homeostasis, especially when cell are grown in the absence of inositol, a condition that leads to accumulation of PC, TAG, and DAG (Gaspar et al., 2006). Mutants defective in the PKC-CWI pathway show much higher levels of these phospholipids, suggesting that activation of this pathway is essential for protecting the cell against damaging effects that may be caused by inositol depletion.

Inositol depletion and mitochondrial dysfunction

Mitochondrial dysfunction has been implicated in BD based on the findings of impaired oxidative phosphorylation, decreased intracellular pH, and increased level of lactate in the brains of BD patients (Stork and Renshaw, 2005; Hamakawa et al., 2004; Kato and Kato, 2000; Regenold et al., 2009). Neural mitochondria play an important role in apoptosis and in the regulation of intracellular calcium, which is ultimately needed for the release of neurotransmitters. Further evidence for mitochondrial dysfunction in BD comes from gene array analysis of expression of 12,558 nuclear genes in the hippocampus of healthy individuals, patients with BD, and patients with schizophrenia (Konardi et al., 2004). This study identified 43 genes that exhibited decreased expression in BD but not in schizophrenia or healthy controls. Of these, 18 genes coded for mitochondrial proteins. Stork and Renshaw (2005) suggested that impaired

phospholipid metabolism in BD, as perceived by alteration in levels of choline, *myo*-inositol, inositol monophosphates, and phosphomonoesters (PMEs), is the direct result of energy shortages caused by mitochondrial dysfunction (Stork and Renshaw, 2005). A study carried out by Ju and Greenberg (2004) indicated that inositol depletion in yeast caused by VPA led to an increase in the level of the mitochondrial phospholipid, cardiolipin. It is tempting to speculate that the therapeutic role of VPA may derive from improved mitochondrial function as a result of increased cardiolipin levels.

VPA causes inositol depletion:

The first demonstration that VPA causes inositol depletion was shown using rat brain (O'Donnell et al. 2000). A subsequent study showed that VPA causes a 20% reduction of inositol in mouse frontal cortex where the decrease was due to inhibition of the inositol-synthesizing enzyme, MIPS (Shaltiel et al., 2004). Decreased inositol and MIPS activity in response to VPA-treatment was also observed in the yeast *S. cerevisiae* (Vaden et al., 2001; Ju et al., 2004). *In vitro* studies showed that VPA does not affect MIPS directly, suggesting that other factors or regulators are involved in the inhibition (Ju et al., 2004). In Chapter 2, I show that VPA inhibits MIPS by increasing its phosphorylation. The implications of decreased inositol biosynthesis in response to VPA treatment are far reaching because of the numerous roles played by inositol-containing compounds. It is likely that more than one mechanism pertaining to inositol depletion may contribute to the therapeutic efficacy of this drug in the treatment of BD. In Chapter 3, I show that VPA perturbs the V-ATPase and alters the dynamics of $PI3,5P_2$, and in Chapter 4 I show

that VPA perturbs endocytic trafficking. This suggests that the effect of VPA may be multi-faceted, taking into consideration that factors other than inositol may contribute to the effects caused by VPA.

VPA potentiates an inhibitory GABA-dependent response:

Several studies have shown that VPA dampens neuronal excitability by potentiating GABA-mediated postsynaptic inhibition (Loscher, 1999), an effect that could mitigate the manic episodes. However, it is not clear how this effect is achieved. While some studies show that VPA increases the levels of the inhibitory neurotransmitter (Czapinski et al., 2005; Loscher and Schmidt, 1980; Patsalos et al., 1981), other studies show that VPA elicits its effect by modulating the response of the postsynaptic neurons (Cunningham et al. 2003). VPA has also been shown to modulate gene expression by inhibiting histone deacetylases (HDACs) (Phiel et al., 2001) and by activating AP-1 transcription factors (Chen et al., 1999). HDAC-mediated regulation of the GABA synthetic enzyme, glutamate decarboxylase, has been associated with the antiepileptic effect of VPA (Nalivaeva et al., 2009).

VPA inhibits glycogen synthase kinase (GSK3)

VPA has been implicated in the inhibition of GSK3, a ubiquitous kinase that regulates many processes and is involved in signaling, cell cycle progression, and cell survival (Grimes et al., 2001). Inhibition of GSK3-ß has been shown to protect neurons from programmed cell death (Hetman et al., 2000). This is consistent with the observed

neuroprotective effects of VPA. Furthermore, VPA was shown to protect cells from ER stress by inhibiting GSK3-ß (Kim et al., 2005). The reports regarding the effect of VPA on GSK3 are controversial. Several studies have shown that VPA directly inhibits GSK3-ß (Chen et al., 1999; De Sarno et al., 2002; Grimes et al., 2001; Kim et al., 2005). Others report no inhibition of GSK3-ß by VPA (Phiel et al., 2001). The studies in Chapter 3 show that one of the three phosphosites identified in MIPS, S296, is in a potential GSK3 recognition sequence. Mutating this residue caused a decrease in the activity of the enzyme. Furthermore, dephosphorylation of a mutated MIPS, with an intact S296, caused a decrease in activity, suggesting that this residue is part of an activation site, and that GSK3 may be the activator. If VPA indeed inhibits GSK3, this could explain the decrease in MIPS activity observed in cells that are treated with VPA.

VPA inhibits protein kinase C (PKC):

VPA induces alterations in PKC activity (Manji et al., 2001). In *S. cerevisiae*, there is only one PKC (Pkc1), while in humans, there are 15 different PKC isozymes comprising three groups based on their requirement for activation by DAG and Ca^{+2} (Mellor and Parker, 1998). PKCs are serine/threonine protein kinases that phosphorylate a variety of proteins, including neurotransmitter receptors, transcriptional factors, signaling molecules, and cytoskeletal proteins. In rat C6 glioma cells, chronic exposure to VPA was found to decrease PKC activity in membrane and cytosolic fractions, specifically

inhibiting the isozymes PKC-α and PKC-ϵ (Chen et al., 1994), suggesting that this inhibition may play a role in the antimanic effects of VPA.

Interestingly, PKC-ϵ regulates GABA$_A$ receptor trafficking (Chou et al., 2010). Phosphorylation of the receptors by PKC is a prerequisite for successful internalization and trafficking of those receptors (Brandon et al., 2000). Furthermore, gephyrin, a synaptogenic molecule that regulates GABAergic synaptic plasticity, is phosphorylated by GSK3-β (Tyagarajan et al., 2010). Phosphorylation of dynamin by GSK3-β is required for activity-dependent bulk endocytosis of synaptic vesicles (Clayton et al., 2010). Delayed trafficking of GABA$_A$ receptors may be an outcome of the effect of VPA on PKC and GSK3. In Chapter 4, I propose a new mechanism whereby VPA may elicit its GABAergic inhibitory effect. My finding that VPA slows the endocytic process suggests that VPA may be responsible for decreasing the internalization of the GABA$_A$ receptors, prolonging their exposure to the inhibitory effect of GABA.

VPA inhibits histone deacetylases (HDACs):

VPA is a histone deacetylase (HDAC) inhibitor that causes hyperacetylation of genes (Phiel et al., 2001; Yildirim et al., 2003). Histone acetylation is an important regulatory mechanism that controls about 2% of transcribed genes, many of which regulate the cell cycle and cellular differentiation (Van Lint et al., 1996). The finding that VPA is an HDAC inhibitor prompted many to attribute the therapeutic effects of VPA to changes in gene expression. For example, Akt activation by VPA has been associated with HDAC

inhibition (De Sarno et al., 2002). Many of the side effects caused by VPA, including teratogenicity and hepatotoxicity, have been linked to HDAC inhibition as well (Gurvich et al., 2004).

In studies to identify the molecular mechanism that underlies the therapeutic efficacy of VPA, many molecular targets and pathways affected by VPA have been identified. This prompted use of this drug for the treatment of other diseases. The anti-proliferative effects of VPA and its ability to inhibit angiogenesis (Michaelis et al., 2004) have led to its use as a chemotherapeutic agent in cancer treatment (Blaheta and Cinatl, 2002; Blaheta et al., 2005; Kuendgen and Gattermann, 2007). The neuroprotective effect of VPA suggests that it may be useful in treatment of Alzheimer's disease (Tariot et al., 2002), while its HDAC inhibitory effect prompted trials for HIV elimination from resting CD4^{+} T-cells (Lehrman et al., 2005).

Currently, VPA is one of the most commonly used drugs for the treatment of epilepsy, BD, and migraine (Calabresi et al., 2007; Macritchie et al., 2003), however, less than 50% of patients respond effectively to this drug. Furthermore, VPA causes severe side effects, including hepatotoxicity (Powell-Jackson et al., 1984) and teratogenicity (Brown et al., 1985). It also induces weight gain and alopecia (Terbach and Williams, 2009). For treatment to be effective, intake cannot be interrupted. Elucidating the therapeutic mechanism of action of VPA will not only enable the development of better drugs with fewer side effects and increased effectiveness, but will also shed light on the pathophysiology of BD.

Yeast as a model system:

The yeast *Saccharomyces cerevisiae* offers many advantages as a model to elucidate the mechanism of action of VPA. The relevance of the yeast model to neurological human disorders is underscored by the fact that at least 60% of yeast genes have human homologs, and more than 25% of cloned human disease genes have yeast homologs (Khurana and Lindquist, 2010). In addition, several cell biological processes, including membrane trafficking, cell signaling, and inositol and lipid metabolism, are highly conserved between yeast and mammalian cells. A great advantage to this study is the availability of viable yeast deletion mutants, especially for the inositol biosynthetic pathway. The *ino1Δ* mutant, for example, has been instrumental in elucidating the cellular consequences of inositol depletion and, furthermore, in determining the functional relevance of the phosphosite mutations for the activity of MIPS from yeast as well as human cells. Another advantage offered by yeast is the availability of viable V-ATPase deletion mutants (*vma* mutants), as inactivation of the ubiquitous V-ATPase is lethal in all organisms except fungi (Li and Kane, 2009). This makes yeast an invaluable model for the study of drugs or mechanisms that perturb the activity of the V-ATPase.

Although the use of yeast provides many advantages, including ease of handling, short generation time, and genetic tractability, there are some limitations to its use. For example, because of the unicellular nature of yeast, VPA-treatment effects that rely on multicellularity and cell-cell interactions may be obscured and may go undetected. Furthermore, the drug may perturb a cellular specialization in mammalian cells for

which there is no homology in yeast. Therefore, a complementary approach using more than one model system provides an ideal option for studies to elucidate the mechanism whereby VPA elicits its effect.

Project outline:

The two main objectives of the studies described in this thesis were to 1) identify the mechanism whereby VPA causes inositol depletion, and 2) identify molecular targets and cellular consequences of inositol depletion that arise from VPA-treatment.

The studies described in Chapter 2 identify phosphorylation of the rate-limiting enzyme of *de novo* inositol synthesis, MIPS, as a novel regulatory mechanism of inositol biosynthesis. This is the first demonstration that both yeast and human MIPS are regulated at the post-translational level. The finding that MIPS is a phosphoprotein was based on the observation that MIPS purified from VPA-treated cells shows a higher level of phosphorylation compared to MIPS purified from untreated cells. Three conserved phosphosites were identified, including two in the NAD^+ binding domain and one in the catalytic domain. Of the three phosphosites identified, two are inhibitory when phosphorylated, and one is a potential activation site. The finding that the elimination of the two inhibitory residues confers resistance to VPA suggests that VPA-mediated inositol depletion may result from phosphorylation of MIPS.

The studies in Chapter 3 show that VPA treatment and inositol depletion cause perturbation of the vacuolar ATPase. Both proton pumping carried out by the membrane embedded V_0 sector and ATP hydrolysis carried out by the cytosolic V_1 sector

are decreased. Inositol supplementation rescues the pumping defect of the V-ATPase but not the activity. Furthermore, VPA perturbs the synthesis of PI3,5P$_2$, the signature phosphoinositide of the vacuole, inhibits vacuole fission, and leads to vacuolar enlargement. These findings suggest that inositol depletion caused by VPA results in a decrease in PI3,5P$_2$, destabilizing the membrane embedded V$_0$ sector of the V-ATPase.

Chapter 4 describes a cDNA library screen to identify novel pathways and processes affected by VPA. The studies show that VPA decreases endocytosis in yeast and human cells, most likely due to perturbation of PI4,5P$_2$ in the plasma membrane. VPA also inhibits the PKC-CWI pathway, suggesting that the drug may inhibit the kinase PKC in yeast. This possibility is exciting because VPA has been shown to inhibit PKCα and ε in mammalian cells, in which PKC is required for the internalization of the GABA receptors.

Included in Chapter 5 are some questions that have emerged from this work and suggestions for future directions.

CHAPTER 2

PHOSPHORYLATION OF *MYO*-INOSITOL PHOSPHATE 3-SYNTHASE: A NOVEL

REGULATORY MECHANISM OF INOSITOL BIOSYNTHESIS

INTRODUCTION

Myo-inositol-3-phosphate synthase (MIPS) is the enzyme responsible for the first and rate-limiting step of *de novo* synthesis of inositol. It catalyzes the conversion of glucose-6-phosphate (G-6-P) to inositol 3-phosphate (I-3-P) in the cytosol in a four-step catalytic reaction using NAD^+ as a cofactor (Fig. 2.1*A*). MIPS has been isolated from bacteria (Bachhawat and Mande, 1999), archaea (Chen et al., 2000), protozoa (Lohia et al., 1999), plants (Loewus and Loewus, 1971), and animals (Adhikari and Majumder, 1988; Maeda and Eisenberg, 1980). In yeast, MIPS is encoded by the well characterized *INO1* gene (Dean-Johnson and Henry, 1989); in mammals, it is encoded by *ISYNA1* and exists as multiple isoforms (Guan et al., 2003; Seelan et al., 2009; Neelon et al., 2011).

A

| G-6-P | → (*INO1*) MIPS | I-3-P | → (*INM1,2*) IMPase | *myo*-inositol |

VPA
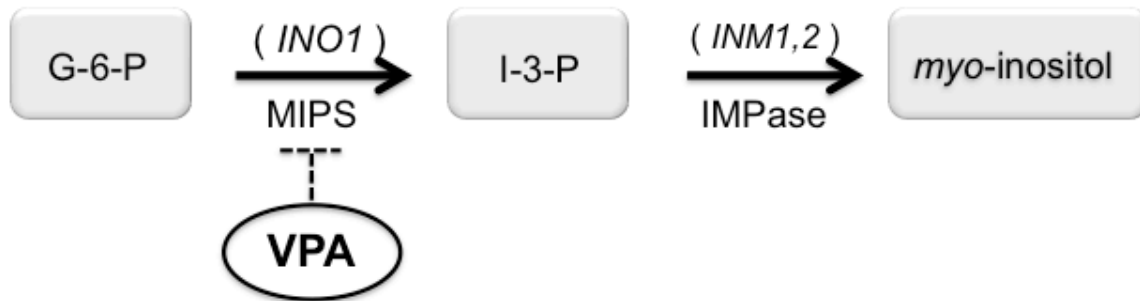
B
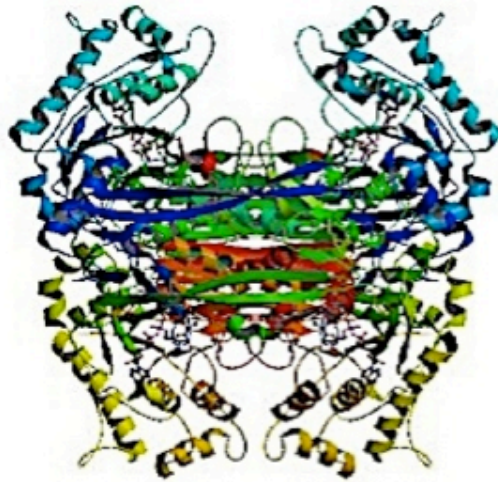
FIGURE 2.1. **MIPS catalyzes the rate limiting step in inositol biosynthesis.** *A*, MIPS, encoded by *INO1*, catalyzes the first and limiting step in the *de novo* biosynthesis of inositol. *B*, the three-dimensional structure of yeast MIPS (Protein Data Bank code 1P.1I) (Jin and Geiger, 2003). G-6-P, glucose 6-phosphate; I-3-P, inositol 3-phosphate.

The crystal structure of yeast MIPS shows that it is a homotetramer (Fig. 2.1*B*) (Geiger and Jin, 2006; Jin and Geoger, 2003) while mammalian MIPS exists as a trimer (Maeda and Eisenberg, 1980). Each monomer has three major domains: a catalytic domain that binds the substrate G-6-P, an NAD$^+$ binding domain, and a central domain, consisting of the N- and C-termini, which stabilizes the two other domains (Stein and Geiger, 2002). Sequence analysis showed that the enzyme is highly conserved, with remarkable conservation of the amino acid residues that are important for catalytic activity (Majumder et al., 2003).

Maintaining inositol homeostasis is essential for cell physiology and viability. This is clearly demonstrated by the inositol-less death phenomenon, in which cells starved for inositol die within a few hours of starvation (Culbertson and Henry, 1975; Henry et al., 1977; Becker and Lester, 1977). Inositol depletion leads to various consequences (Deranieh and Greenberg, 2009). Growth in the absence of inositol elicits profound changes in lipid metabolism, and activates numerous stress responses including the cell wall integrity (CWI), and unfolded protein response (UPR) pathways (Villa-Garcia et al., 2011; Henry et al., 2014).

Although the regulation of inositol biosynthesis at the level of *INO1* transcription has been extensively characterized (Greenberg and Lopes, 1996; Carman and Henry, 1999; Nunez et al., 2006), the regulation of MIPS activity has not been studied. The anticonvulsant drug valproate (VPA) induces depletion of inositol, and MIPS has been suggested as a possible target of the drug (Ju et al., 2004; Shaltiel et al., 2004; Vaden et al., 2001). The initial finding that VPA leads to a decrease in inositol-3-phosphate in

yeast (Vaden et al., 2001) suggested that VPA inhibits MIPS activity. The subsequent observation that VPA-mediated inhibition was indirect (Ju et al., 2004) suggested that MIPS is regulated post-translationally. Phosphorylation controls the regulation and localization of numerous enzymes, many of which, like MIPS, are transcriptionally regulated by UAS$_{INO}$ elements (Chang et al., 2007; Choi et al., 2010; Choi et al., 2011).

In this Chapter, I show that MIPS, from yeast and human cells, is regulated by phosphorylation of at least three residues, one in the catalytic domain and two in the NAD$^+$ binding domain, thus identifying a novel mechanism of regulation of inositol biosynthesis. These sites are conserved in yeast and human MIPS, suggesting that regulation by phosphorylation is a conserved regulatory mechanism of inositol biosynthesis. I also show that mutation of the two inhibitory phosphosites, S184 and S374, leads to decreased sensitivity to VPA, suggesting that VPA may inhibit MIPS activity as a consequence of phosphorylation of these two residues.

MATERIALS AND METHODS

Strains, media, and growth conditions*:*

The *Escherichia coli* and *Saccharomyces cerevisiae* strains used in this work are listed in

Table 2.1. Yeast cells were grown in synthetic minimal media containing 2% glucose and

lacking inositol unless otherwise stated. Media were supplemented with the amino

acids histidine, leucine, methionine, and lysine, and the nucleobase uracil using Difco®

standard concentrations. For selection of cells carrying specific plasmids, appropriate

amino acids were omitted from the media. For overexpression purposes, induction

media contained 2% galactose and 1% raffinose with no glucose added. Where

indicated, VPA was used at a concentration of 1 mM, and inositol at 75 μM. Yeast

strains were grown at 30°C. *E. coli* strain DH5α was used for plasmid maintenance and

amplifications. Bacteria were grown at 37°C in LB medium (0.5% yeast extract, 1%

tryptone, 1% NaCl), supplemented with ampicillin (100 μg/ml) for selection purposes.

For growth on plates, media were supplemented with 1.5% and 2% agar for *E. coli* and

yeast, respectively. Growth in liquid cultures was monitored spectrophotometrically by

measuring absorbance at 550 nm.

DNA manipulations, PCR, and DNA sequencing:

Standard methods were followed for isolation of genomic DNA, plasmid purification,

digestion with restriction endonucleases, and ligation. Transformation of bacterial and

yeast cells was carried out using electroporation (2.4 V for bacteria, and 1.4 V for yeast). All PCR reactions were optimized. DNA sequencing was carried out using ACGT Inc. sequencing facility (Illinois).

Construction of plasmids and expression of yeast and human MIPS in *S. cerevisiae*:

All plasmids and primers used in this work are listed in Tables 2.2 and 2.3, respectively. The *INO1* gene was amplified from *S. cerevisiae* genomic DNA using the primer pair 5'-GTTGTCGGGTTCCTAATGTT-3' and 5'-CAACAATCTCTCTTCGAATCT-3', and tagged with a 6xHis and an Xpress epitope on the N-terminus using pRSETA as the source of the tag. The tagged *INO1* was then subcloned into pYES6 containing the blasticidin marker. *HindIII* and *Xho1* were used to transfer the tagged *INO1* into pRD015 to make the pRD*INO1* construct, a high copy *GAL1*-driven plasmid containing a *URA3* marker. All subsequent manipulations and mutagenesis experiments utilizing yeast MIPS were carried out using the pRD*INO1* vector. All mutations were confirmed by sequencing. The low copy set of all mutants was prepared by transferring the mutated genes to the centromeric low copy vector p415-ADH purchased from ATCC. For human MIPS, the human *INO1* gene (h*INO1*) was transferred from pRSETA-h*INO1* (Ju et al., 2004) and cloned into pRD015. All subsequent experiments for the human MIPS, including mutagenesis, growth, and enzyme purification were carried out using pRDh*INO1* and its derivatives.

Site-directed mutagenesis:

Site mutations were constructed using a two-step PCR protocol developed for this study. For each mutation, two overlapping, non-phosphorylated primers were designed. Two PCR reactions were run simultaneously, using one primer in each reaction. pRD*INO1* and pRDh*INO1* were used as templates for yeast MIPS and human MIPS, respectively. The first PCR reaction was run for 10 cycles. The two samples from the first PCR reaction were then pooled together, and the PCR reaction was resumed for another 20 cycles. After cooling, ligase was added to the reaction mix and incubated for 1 h at room temperature. Gel analysis was used to detect the presence of the amplified plasmid. The parent strands were digested using *Dpn1* treatment for 2 h at 37°C. Transformation of competent *E. coli* cells was carried out using electroporation. Selection was carried out on LB plates supplemented with ampicillin. Plasmids were purified and all mutations were verified by sequencing using the ACGT Inc. sequencing services. The constructs harboring the mutations were then used to transform yeast *ino1Δ* cells.

Purification of recombinant MIPS:

S. cerevisiae BY4741 *ino1Δ* mutant bearing the pRD*INO1* construct was used to express the recombinant 6xHis-tagged MIPS. Cells were grown at 30°C in synthetic minimal medium lacking uracil. Galactose (2%) was used to induce overexpression of the recombinant protein. Cell extracts were prepared by disrupting cells with glass beads

(0.5 mm diameter), vortexing for 30 min intermittently, keeping cells on ice. The disruption buffer contained 50 mM Tris-Cl (pH 7.4) and 300 mM NaCl. A cocktail of protease and phosphatase inhibitors was added to the disruption buffer before breaking the cells. MIPS was purified using ProBond nickel-chelating resin to bind the protein, gently mixing at 4°C for 1 h. The resin was washed twice with cold 20 mM then 60 mM imidazole in Tris buffer (50 mM Tris-Cl (pH 7.4) and 300 mM NaCl). The protein was eluted with 300 mM imidazole in Tris buffer, dialyzed, concentrated, and resuspended in (50 mM Tris-Cl, 50 mM NaCl, 10 mM DTT). Protein concentration was determined by the Bradford method using bovine serum albumin as the standard.

MIPS activity assay:

Purified MIPS enzyme activity was determined by the rapid colorimetric method of Barnett *et al.,* (1970) with minor modifications. Purified protein was suspended in the reaction buffer containing 100 mM Tris acetate (pH 8.0), 0.8 mM NAD, 2 mM DTT, and 14 mM NH_4Cl. After addition of 5 mM G-6-P to a final volume of 150 μl, the reaction mixture was incubated for 1 h at 37°C. The reaction was terminated by the addition of 50 μl of 20% (w/v) trichloroacetic acid and kept on ice for 10 min. The precipitated protein was removed by centrifugation. The supernatant (200 μl) was incubated with 200 μl of 0.2 M $NaIO_4$ for 1 h at 37°C. This was followed by the addition of 200 μl of 1 M Na_2SO_3 to remove excess $NaIO_4$. For the measurement of phosphate, a 600 μl reagent mixture consisting of 240 μl of H_2O, 120 μl of 2.5% ammonium molybdate, 120 μl of

10% ascorbic acid, and 120 µl of 6 N sulfuric acid was added to the reaction mix and incubated for 1 h at 37°C. Absorbance was measured at 820 nm, and activity was determined by the amount of inorganic phosphate liberated. A unit of MIPS activity is defined as the amount of enzyme that catalyzes the formation of 1 nmol of phosphate/min. Specific activity is defined as units per mg of protein.

Dephosphorylation of MIPS:

Dephosphorylation reactions were performed at 37°C in a total volume of 50 µl for 1 h. Ten µg of purified MIPS was gently mixed with Ni-coated magnetic beads for 1 h at 4°C. The supernatant was aspirated, and the beads were resuspended in alkaline phosphatase buffer consisting of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT). Alkaline phosphatase (10 units) was added and the mixture was incubated at 37°C for 45 min, after which orthovanadate was added to the reaction mix to a final concentration of 10 mM to inactivate the AP. The beads were then collected and washed 3 times with Tris purification buffer in preparation for the MIPS assay.

In vivo **phosphorylation of MIPS:**

Cells with recombinant MIPS were precultured in synthetic minimal medium, inoculated to A_{550} 0.03 in 20 ml phosphate-free medium to which 2 mCi $^{32}P_i$ was added, and grown to the early stationary phase with or without VPA. Cells were harvested by

centrifugation, washed with water, and disrupted with glass beads in 100 µl buffer containing 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 0.5% deoxycholate, 0.1% SDS, 1% NP40, 10 mM NaF, 5 mM β-glycerophosphate, 1 mM sodium vanadate, and protease inhibitor cocktail. The cell extracts were precleaned with 10 µl protein G plus protein A agarose for 1 h at 4°C and incubated with 2 µg anti-Xpress antibody, 50 µl protein G plus protein A agarose overnight at 4°C. After washing with the same buffer, the MIPS-Anti-Xpress antibody complex was dissociated by boiling for 5 min in protein sample buffer containing 2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-Cl (pH 6.8), and 0.001% bromophenol blue. After a brief low speed centrifugation, proteins were analyzed by SDS-PAGE. Bands were visualized by Coomassie Blue staining, and $^{32}P_i$-labeled proteins were identified by autoradiography.

Phosphoaminoacid analysis:

MIPS labeled with $^{32}P_i$ was digested with 6N HCl . The acid was evaporated and the sample was dissolved in 10 µl TLE buffer (pH 1.9) and mixed with 1 µg cold phosphoserine, phosphothreonine, and phosphotyrosine standards. The phosphoamino acids were separated in two dimensions, by electrophoresis followed by thin layer chromatography (TLC). Standard phosphoamino acids were visualized by Ninhydrin staining of the TLC plate. Labeled phosphoamino acids were visualized by autoradiography.

Mass spectrometry analysis of MIPS phosphorylation sites:

Purified protein samples were reduced with dithiothreitol, alkylated with iodoacetamide, and digested overnight with sequencing grade trypsin (Promega) or chymotrypsin (Roche) at 37°C. Peptides were desalted and separated by reverse phase chromatography before introduction into a linear ion trap mass spectrometer (LTQ-XL, Thermo Scientific). The top 5 peaks in the MS1 scan (400-1700 m/z) were sequentially selected for fragmentation by collision-induced dissociation (NCE=30, activation Q=0.25, activation time=30 ms). Dynamic exclusion was turned on (if 2 hits in 5 s then excluded for 20 s, list size=200). MS2 spectra were scored against a yeast FASTA protein database (NCBI, 6298 entries) using the SEQUEST algorithm (ver 27, rev 13). Search parameters included 1.6 Da/1.0 Da parent/fragment ion tolerances; +57 on Cys fixed modification; +16 on Met and +80 on Ser/Thr/Tyr variable modifications; and up to 2 missed cleavages. Results were imported into Scaffold (Proteome Software, ver 3.5) and MS2 spectra were reanalyzed against a subset database using X!Tandem (ver 2007.01.01.1). Peptide probabilities were scored using the Peptide Prophet algorithm and a ≥90% threshold was utilized. Localization probabilities of post-translational modifications were scored using Scaffold PTM (ver 2.1.1, Proteome Software).

TABLE 2.1. **Strains used this study:**

Strain	Genotype
E. coli:	
DH5α	*F⁻ φ80dlacZΔm15Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r_k⁻m_k⁺)phoA supE44l⁻thi-1gyrA96relA1*
S. cerevisiae:	
BY4741	***MATa*** *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*
BY4741*ino1Δ*	***MATa*** *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, INO1::KanMX*

TABLE 2.2. **Plasmids used in this study:**

Plasmid	Relevant characteristics	Source / Ref.
pRSET A	*E.coli* expression vector	Invitrogen
pYES6-CT	Multi-copy shuttle vector with *blasticidin* marker	Invitrogen
pYES2-CT	Multi-copy shuttle vector with *URA3* marker	Invitrogen
P415-ADH	Low copy shuttle vector containing *LEU2* marker	ATCC®
pYES6-*INO1*	Plasmid containing yeas*t INO1*	This study
pRD015	Dual tag shuttle vector constructed using pYES2-CT	This study
pRD*INO1*	*INO1*gene from pYES6-INO1 ligated into Hindlll/Xho1	This study
pRD-T48V	*INO1* ^{T48V} derivative of pRD*INO1*	This study
pRD-S177A	*INO1* ^{S177A} derivative of pRD*INO1*	This study
pRD-S184A	*INO1* ^{S184A} derivative of pRD*INO1*	This study
pRD-S296A	*INO1* ^{S296A} derivative of pRD*INO1*	This study
pRD-S374A	*INO1* ^{S374A} derivative of pRD*INO1*	This study
pRD-S177D	*INO1* ^{S177D} derivative of pRD*INO1*	This study
pRD-S184D	*INO1* ^{S184D}derivative of pRD*INO1*	This study
pRD-S296D	*INO1* ^{S296D}derivative of pRD*INO1*	This study
pRD-S374D	*INO1* ^{S374D}derivative of pRD*INO1*	This study
pRD-S184A/S374A	*INO1* $^{S184A/S374A}$derivative of pRD*INO1*	This study
pADH-*INO1*	*INO1* gene from pRD*INO1*	This study
pADH-T48V	*INO1* ^{T48V} derivative of pADH-*INO1*	This study
pADH-S177A	*INO1* ^{S177A} derivative of pADH-*INO1*	This study
pADH-S184A	*INO1* ^{S184A} derivative of pADH-*INO1*	This study
pADH-S296A	*INO1* ^{S296A} derivative of pADH-*INO1*	This study
pADH-S374A	*INO1* ^{S374A} derivative of pADH-*INO1*	This study
pADH-S177D	*INO1* ^{S177D} derivative of pADH-*INO1*	This study
pADH-S184D	*INO1* ^{S184D}derivative of pADH-*INO1*	This study
pADH-S296D	*INO1* ^{S296D}derivative of pADH-*INO1*	This study
pADH-S374D	*INO1* ^{S374D}derivative of pADH-*INO1*	This study
pADH-S184A/S374A	*INO1* $^{S184A/S374A}$derivative of pADH-*INO1*	This study
pRSETA/h*INO1*	Plasmid containing *Human MIP Synthase cDNA*	Ju et al.,2004
pRD-h*INO1*	Human *INO1(ISYNA1)*from pRSETA/h*INO1*	This study
pADH-h*INO1*	Human *INO1(ISYNA1)*from pRD-h*INO1*	This study
pRD –hS177A	h*INO1* ^{S177A} derivative of pRD-h*INO1*	This study
pRD –hS177D	h*INO1* ^{S177D} derivative of pRD-h*INO1*	This study
pRD –hS279A	h*INO1* ^{S279A} derivative of pRD-h*INO1*	This study
pRD –hS279D	h*INO1* ^{S279D} derivative of pRD-h*INO1*	This study
pRD –hS357A	h*INO1* ^{S357A} derivative of pRD-h*INO1*	This study
pRD –hS357D	h*INO1* ^{S357D} derivative of pRD-h*INO1*	This study

TABLE 2.3. **Primers used for site directed mutagenesis of yeast and human MIPS:**

Primer	Sequence (5' – 3')
Yeast MIPS	
S184A F S184A R	CCTTGGTGAAGCCTCTTCCTGCCATTTACTACCCT AGGGTAGTAAATGGCAGGAAGAGGCTTCACCAAGG
S184D F S184D R	CCTTGGTGAAGCCTCTTCCTGACATTTACTACCCTGATTTCA TGAAATCAGGGTAGTAAATGTCAGGAAGAGGCTTCACCAAGG
S296A F S296A R	GTCCCCTATATTAATGGTGCACCGCAGAATACTTTTG CAAAAGTATTCTGCGGTGCACCATTAATATAGGGGAC
S296D F S296D R	CTATCTTGGAAGGTGTCCCCTATATTAATGGTGATCCGCAGAATACTTTTGTT AACAAAAGTATTCTGCGGATCACCATTAATATAGGGGACACCTTCCAAGATAG
S374A F S374A R	GTCTAAGGAGATTTCCAAAGCTTCTGTCATAGATGACATC GATGTCATCTATGACAGAAGCTTTGGAAATCTCCTTAGAC
S374D F S374D R	ATTTAGGTCTAAGGAGATTTCCAAAGATTCTGTCATAGATGACATCATCGC GCGATGATGTCATCTATGACAGAATCTTTGGAAATCTCCTTAGACCTAAAT
Human MIPS	
hS177A F hS177A R	CGGCCCCGGCCTGCTGTTTACATCCC GGGATGTAAACAGCAGGCCGGGGCCG
hS177D F hS177D R	CCCTGCGGCCCCGGCCTGATGTTTACATCCC GGGATGTAAACATCAGGCCGGGGCCGCAGGG
hS279A F hS279A R	CCTTCCTCAATGGGGCTCCGCAGAACACC GGTGTTCTGCGGAGCCCCATTGAGGAAGG
hS279D F hS279D R	TGCCTTCCTCAATGGGGATCCGCAGAACACCCTG CAGGGTGTTCTGCGGATCCCCATTGAGGAAGGCA
hS357A F hS357A R	TAAGGAGGTGTCCAAGGCCAACGTGGTGGACGAC GTCGTCCACCACGTTGGCCTTGGACACCTCCTTA
hS357D F hS357D R	CTAAGGAGGTGTCCAAGGACAACGTGGTGGACGACA TGTCGTCCACCACGTTGTCCTTGGACACCTCCTTAG
Sequencing	
GAL1 ADH Seq INO1 Seq hINO1	TGCATAACCACTTTAACT TCAAGCTATACCAAGCATACAATCA TTAATGACACCATGGAAAACCTC CACCATTGAGCTCGGTCTG

RESULTS

VPA causes a decrease in MIPS activity:

The *INO1* coding sequence (1578 bp), tagged with 6xHis and an Xpress epitope, was cloned under the *GAL1* promoter in the expression vector pRD015 with *URA3* as the selection marker, herein, the construct pRD*INO1* (Fig. 2.2*A*). To determine if the tagged MIPS is functional, pRD*INO1* was transformed into *ino1Δ* cells that lack endogenous MIPS and therefore cannot grow independently in the absence of inositol. Transformants (*ino1Δ-INO1*) were selected on Ura- plates and then tested for growth in the absence of inositol (I-) in comparison with cells carrying the empty vector. The tagged MIPS rescued growth on I- media (Fig. 2.2*B*). It has previously been shown that cells grown in the presence of VPA exhibit a decrease in levels of inositol and inositol-3-phosphate (Vaden et al., 2001) and that VPA does not directly inhibit MIPS (Ju et al., 2004). To test the possibility that VPA indirectly inhibits MIPS activity by bringing about a post-translational change in the enzyme, MIPS was expressed and purified from *ino1Δ-INO1* cells grown to the mid-logarithmic phase and treated with 1 mM VPA for 3 hours. MIPS purified from the VPA-treated cells showed a decrease in activity of about 40% compared to enzyme purified from untreated cells suggesting that MIPS is regulated post-translationally (Fig. 2.3*A*).
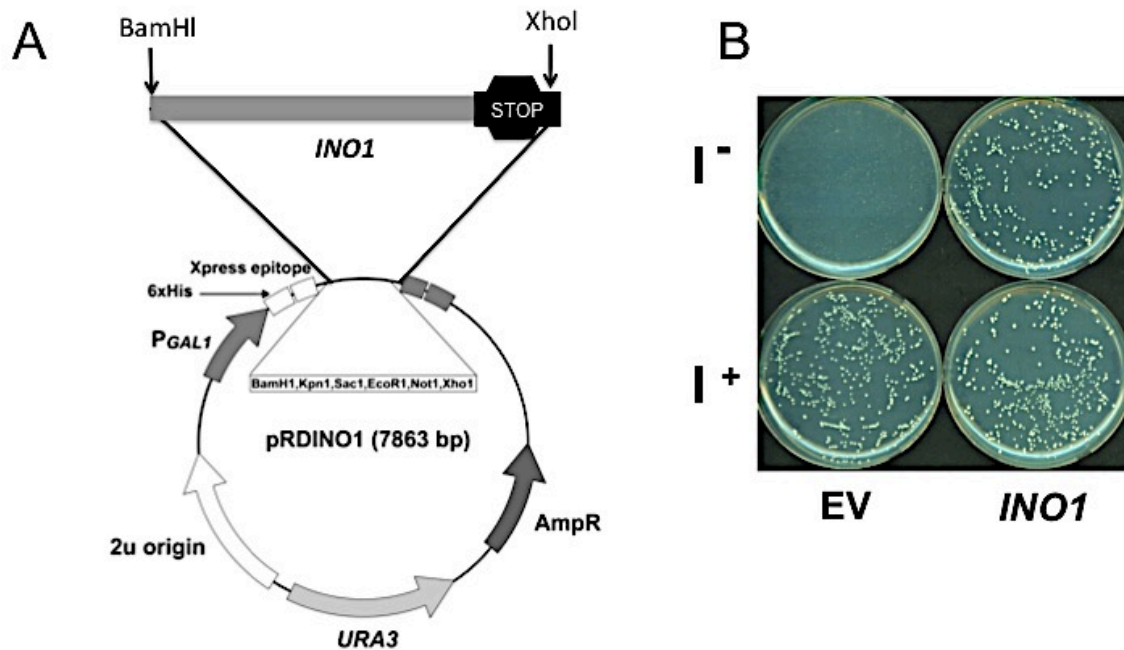
FIGURE 2.2. **The tagged MIPS is functional and rescues growth of *ino1Δ* in inositol-deficient media.** *A*, Map of the *INO1* construct used for expression and purification of MIPS. *B*, *ino1Δ* cells transformed with the empty vector (pRD015) or with the vector carrying the tagged *INO1* (pRD*INO1*) were precultured to the mid logarithmic phase in synthetic medium (SM) supplemented with 75 uM inositol . Cells were washed twice with dH$_2$O to remove residual inositol, diluted, and plated on selective SM with or without inositol supplementation.

MIPS is a phosphoprotein:

To determine if MIPS is post-translationally modified by phosphorylation, *ino1Δ* cells harboring the tagged *INO1* were grown in the presence of $^{32}P_i$ with or without VPA, and MIPS was precipitated with anti-Xpress antibody using protein A plus protein G conjugated sepharose. The ^{32}P- labeled MIPS protein was resolved using SDS-PAGE and visualized with Coomassie Blue and autoradiography. MIPS purified from VPA-treated cells showed a higher degree of phosphorylation compared to MIPS purified from untreated cells (Fig. 2.3*B*). Phosphoamino acid analysis of the ^{32}P-labeled protein indicated that MIPS was phosphorylated mostly at serine residues, although faint label of threonine residues was also detected (Fig. 2.3*C*).

To identify the putative phosphorylated residues, MIPS was overexpressed and purified from *ino1Δ-INO1* cells grown to the mid-logarithmic phase. Duplicate samples of control (no treatment) and VPA (1mM) - treated cells were utilized. The protein was purified using ProBond resin, dialyzed, concentrated, and digested with either trypsin or chymotrypsin. The resulting peptides were separated by reverse phase chromatography and analyzed by tandem electrospray mass spectrometry. MIPS peptides and phosphopeptides were identified using an algorithm that compares MS/MS spectra against database sequences. A total sequence coverage of 88.4% was obtained with both enzymes (Fig. 2.4*A*).

Five phosphosites were identified in 4 independent samples: pT-48 in the N-terminal domain, pS-177, pS-184, and pS-296 in the NAD$^+$ binding domain, and pS-374 in
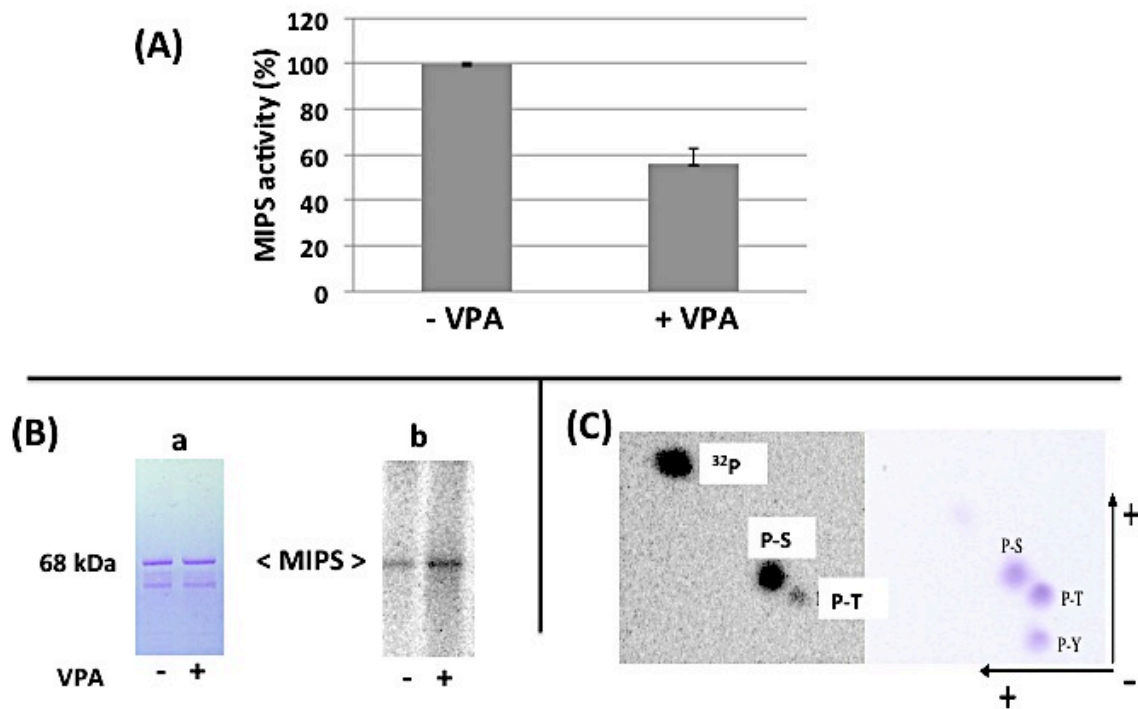
FIGURE 2.3. **MIPS is a phosphoprotein**. *A*, MIPS purified from VPA-treated *ino1Δ* cells harboring the pRD*INO1* vector showed decreased activity. *B*, MIPS purified from VPA-treated cells shows an increased level of phosphorylation in comparison to MIPS from untreated cells. *C*, Phosphoamino acid analysis of MIPS protein using $^{32}P_i$ labeling showed that phosphorylation is mostly at serine residues. The data are representative of 2 independent experiments. Parts B and C were carried out by Quan He (Deranieh et al., 2013).
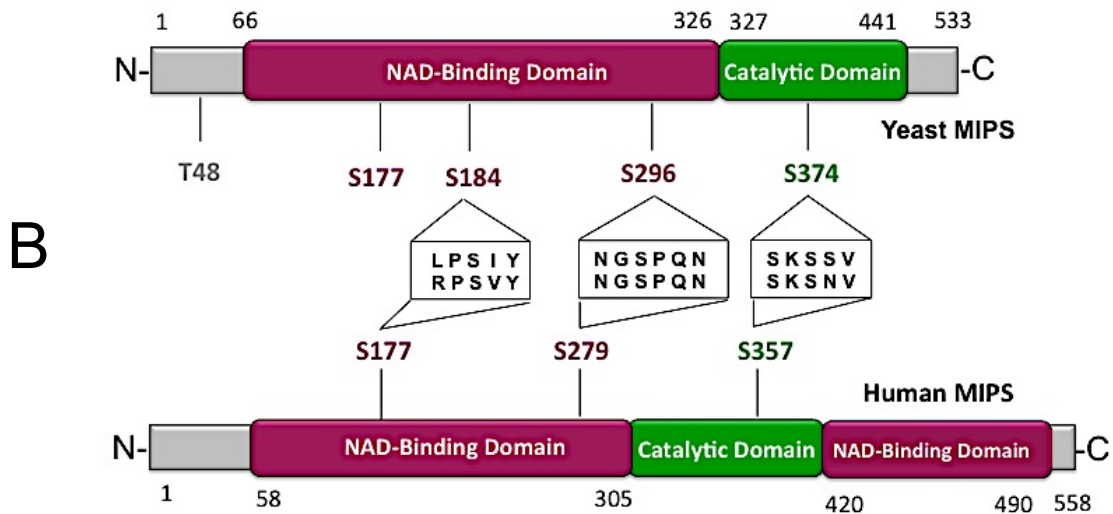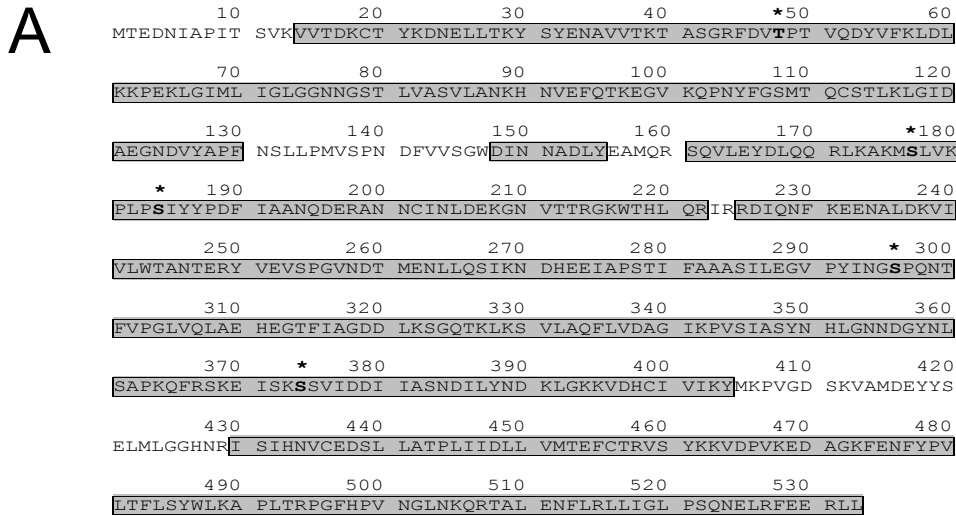
FIGURE 2.4. **Domains and phosphosites of MIPS.** *A*, Protein sequence of yeast MIPS (NCBI accession NP_012382.2). Sequenced residues are highlighted (471/533 = 88.4% sequence coverage). Phosphosites sequenced in at least 2 of 4 samples analyzed are marked with an asterisk (*). *B*, A schematic diagram illustrating the domain structure of yeast and human MIPS, and the position of the phosphorylation sites identified by mass spectrometry.

the catalytic domain (Fig. 2.4*B*). For pS-48, pS-177, pS-184 and pS-296, unambiguous localization of the phosphosites was made on the basis of the corresponding MS/MS spectra. A representative fragmentation spectrum for the identification of pS-296 is shown in Fig. 2.5*A*.

The termini of peptides do not yield fragmentation information as robustly as the central region; therefore, phosphosite determination for the [374]SSVIDDIIASNDILYNDK[392] phosphopeptide was not as direct. The b_4-b_9 ion series indicated that the phosphosite was either at S-374 or S-375 and not S-383 or Y-388 (Fig. 2.5*B*). A doubly charged y_{17} ion was consistently seen in MS/MS spectra that matched this phosphopeptide. Although it has relatively low abundance, this fragment ion indicated that S-375 was not phosphorylated (Fig. 2.5*B*). Finally, the serine residue at position 374 of yeast MIPS is conserved across disparate species (Fig. 2.5*C*). This correlative evidence suggests that S-374 has a critical role for protein function.

Three phosphosites modulate activity of yeast MIPS:

To determine which of the identified phosphorylation residues are functionally important for the activity of MIPS, two site mutants were constructed for each of the residues: a phosphorylation-deficient mutant in which serine (S) was changed to the unphosphorylatable alanine (A), or threonine (T) to valine (V); and a phosphomimetic mutant, in which serine (S) was changed to the phosphorylation-mimicking aspartate (D) (Fig. 2.6*A*). All the mutations were confirmed by sequencing. Each mutation was
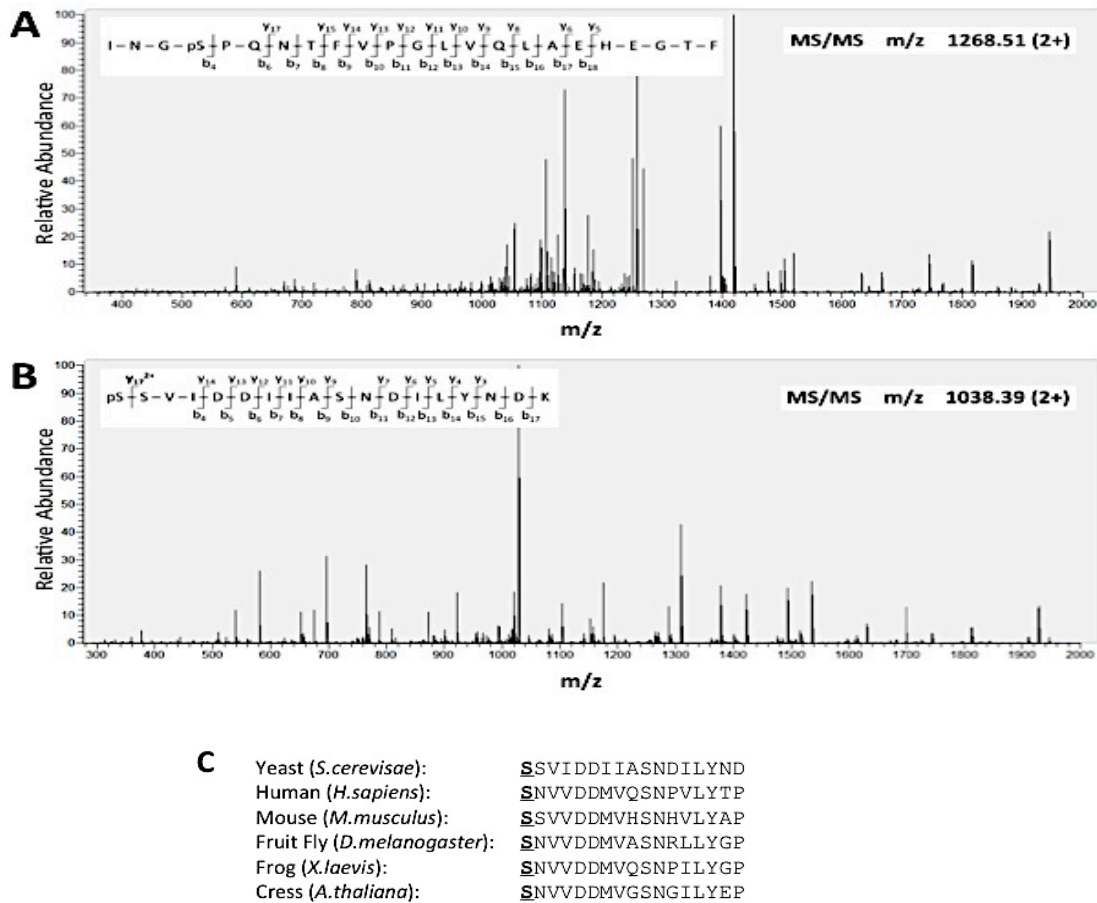
FIGURE 2.5. **MIPS is phosphorylated at 5 serine and threonine residues.** *A*, Representative MS/MS spectrum of the doubly-charged MIPS phosphopeptide [293]INGpSPQNTFVPGLVQLAEHEGTF[315]. The phosphorylation site was unambiguously assigned to pS-296 based on the mass assignment of b_4. Not all peaks are annotated. *B*, the b4 fragment ion within the MS/MS spectrum of [374]SSVIDDIIASNDILYNDK[391] indicates that one of the N-terminal serine residues is phosphorylated. Two lines of evidence suggest that the phosphorylated residue is pS-374: the y_{17}^{2+} fragment ion indicates that S-375 is not phosphorylated; and the S-374 residue within yeast MIPS is highly conserved across several species (shown in *C*).

constructed in two vectors: a centromeric low expression vector, p415-ADH driven by

the *ADH* promoter, used for growth experiments; and a high expression vector, pRD015

driven by the *GAL1* promoter, used for overexpression and purification of the enzyme.

All constructs were transformed into *ino1Δ* cells. To assess the physiological effect of

the mutations on growth, cells transformed with the empty vector, wild-type *INO1*

gene, or mutated genes were grown on synthetic media with or without inositol. When

MIPS was expressed from the high copy vector, all the site-mutants supported growth of

ino1Δ cells (data not shown). However, clear differences in growth were observed in

ino1Δ cells transformed with the MIPS mutants on the low copy vector (Fig. 2.6*B*). Cells

carrying the plasmid with wild-type *INO1* grew normally on inositol-deficient medium.

Mutations in residues in the NAD-binding domain, S184 and S296, affected growth. Cells

carrying S184A grew well, but S184D did not support growth, suggesting that

phosphorylation of this residue inhibits the activity of MIPS, and hence, the synthesis of

inositol. Both mutants S296A and S296D did not support growth on inositol deficient

medium, suggesting that a serine residue is essential at that position. Mutants of

residue S374 in the catalytic domain were also assessed. Cells carrying S374A grew well,

but S374D did not support growth, suggesting that phosphorylation of this residue

inhibits MIPS activity. Cells carrying mutations of T48 and S177 showed the same growth

pattern as that of WT (data not shown), indicating that these sites are not critical or

regulated by phosphorylation.

To determine if the altered growth patterns seen in the mutants was due to altered MIPS activity, wild-type and mutant MIPS were overexpressed and purified from *ino1Δ* cells grown to the late log phase in selective medium supplemented with inositol. Using equivalent amounts of purified protein from all mutants, MIPS activity was assayed using the method of Barnett et al., (1970). The activity of MIPS from phosphorylation deficient mutants S184A and S374A was similar to or slightly greater than that of the wild type MIPS (Fig. 2.6*C*). In contrast, MIPS from the phosphomimetic mutants S184D and S374D showed a decrease in activity to about 30% and 60% of wild-type levels, respectively. This suggests that phosphorylation of residues S184 and S374 has an inhibitory effect on the activity of MIPS.

In contrast to S184 and S374, both mutations of residue S296 (S296A and S296D), led to a decrease in MIPS activity (Fig. 2.6*C*), suggesting that a serine residue at this site is important for maintaining catalytic activity.

The effect of phosphorylation on MIPS activity was also addressed by dephosphorylating the protein using alkaline phosphatase (AP). Dephosphorylation increased the activity of wild type MIPS by about 130% (Fig. 2.7). Dephosphorylation of the S184A and S374A mutants showed a smaller increase in activity (50% and 83% for S184A and S374A, respectively), suggesting that each of these putative inhibitory sites partially contributes to the overall inhibitory effect. Dephosphorylation of S296A caused more of an increase in activity than either S184A or S374A, suggesting that the two phosphosites S184 and S374 have a greater inhibitory effect than either site alone.

FIGURE 2.6. **Effect of site mutations of yeast MIPS on growth and enzyme activity.** *A*, Schematic representation of the mutated phosphorylation sites in the yeast MIPS protein. (*) Phospho-deficient mutation, (D) phosphomimetic mutation. *B*, *ino1Δ* cells transformed with the empty vector pADH, vector carrying wild-type yeast *INO1* or vector carrying the indicated mutated *INO1* gene were spotted in a 10-fold dilution series on Leu⁻ medium in the presence or absence of 75 µM inositol (I). *C*, Effect of site mutations on activity of yeast MIPS**.** The results are representative of three experiments. Values are mean ± S.E.

To examine the effect of a phosphorylated S296, a double mutant S184A/S374A was constructed. Dephosphorylation of the double mutant did not significantly alter MIPS activity (Fig. 2.7), suggesting that under the physiological conditions in which the protein was purified, phosphorylation of S296 was not significant. Taken together, these results indicate that yeast MIPS is regulated by phosphorylation of at least three residues, two of which are inhibitory when phosphorylated (S184 and S374). The third residue, S296, is strictly dependent on the presence of a serine at that position.

The phosphorylation sites are conserved in human MIPS :

Ju et al. (2004) have previously cloned and expressed a human cDNA encoding MIPS and showed that human MIPS (hMIPS) is functional in yeast (Ju et al., 2004). Amino acid sequence alignment of yeast and hMIPS showed that the three residues I have found to modulate yeast MIPS are conserved in the human enzyme (Fig. 2.4*B*). These hMIPS residues are S177 and S279, both of which are in the NAD$^+$-binding domain, and S357, which lies in the catalytic domain (Fig. 2.4*B*). To determine if hMIPS is regulated by phosphorylation, the *hINO1* gene was transferred from pRSETA-h*INO1* (Ju et al., 2004) and cloned into the overexpression vector pRD015. Two independent mutations were created for each residue: a phosphodeficient S to A, and a phosphomimetic S to D (Fig. 2.8*A*). All the mutations were confirmed by sequencing.

To determine if any of the three conserved residues are important for the function of hMIPS, all constructs along with the controls were transformed into *ino1Δ*

cells. Transformants were grown on selective medium with or without inositol. The wild type *hINO1* gene rescued growth on I- medium, indicating that hMIPS expressed from the pRD015 vector is functional in yeast (Fig. 2.8*B*). Mutations S177A and S177D did not support growth in the absence of inositol (Fig. 2.8*B*). Consistent with this observation, hMIPS purified from these mutants showed decreased activity (Fig. 2.8*C*), suggesting that a serine residue is required at this site for the activity of the enzyme. For residue S279, the S279A mutation supported growth on I- media although enzyme activity was lower than wild type. The S279D mutation did not support growth on I-media, and caused a big decrease in activity, suggesting that phosphorylation of this residue inhibits hMIPS activity, and hence, the synthesis of inositol. The S357A mutation of the catalytic domain supported growth, but S357D did not. Consistent with this, S357A caused a slight increase in activity while S357D caused a decrease in activity, suggesting that phosphorylation of S357 inhibits hMIPS activity.

Similar to the yeast enzyme, dephosphorylation of hMIPS led to increased activity (data not shown). Taken together, hMIPS, similar to its yeast counterpart is regulated by phosphorylation of at least three residues, all of which are inhibitory when phosphorylated.
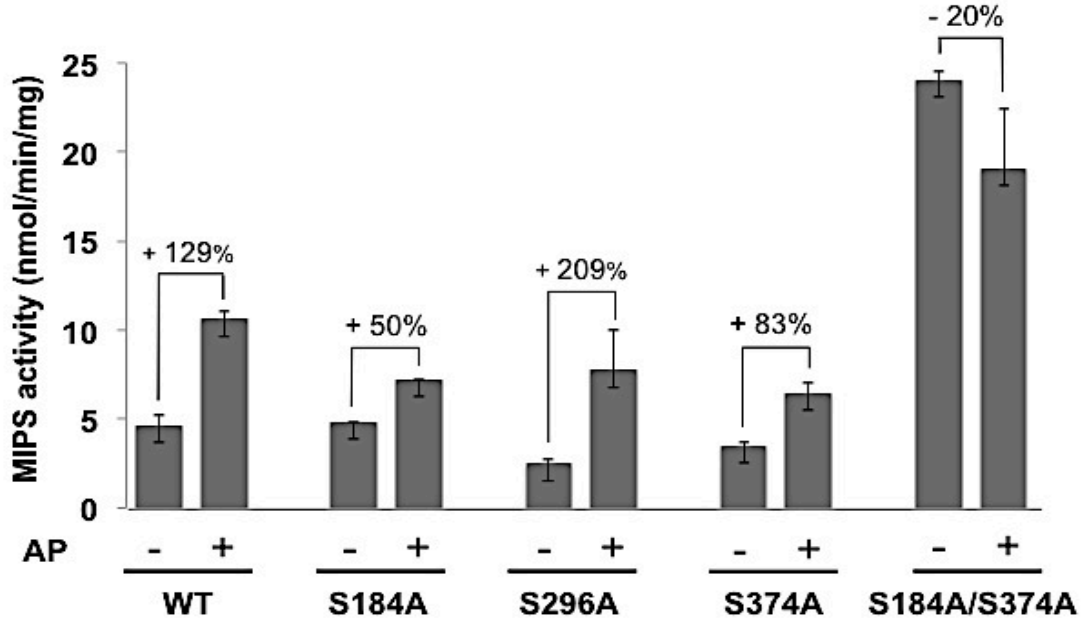
FIGURE 2.7. **Effect of dephosphorylation on MIPS activity.** *ino1Δ* cells expressing wild-type (WT) or mutated yeast MIPS were grown to the exponential phase in SM I[+]. MIPS was purified and mixed with Ni-coated magnetic beads and gently mixed for about one h at 4°C. The beads were collected, rinsed, and resuspended in alkaline phosphatase (AP) buffer with or without AP, and incubated at 37°C for 1 hr. The beads were then washed thrice with buffer to get remove AP and free phosphate. Activity of the bound MIPS was assayed as described under "Materials and Methods." The % change in activity after dephosphorylation is indicated. Values are mean of at least two experiments ± S.E.
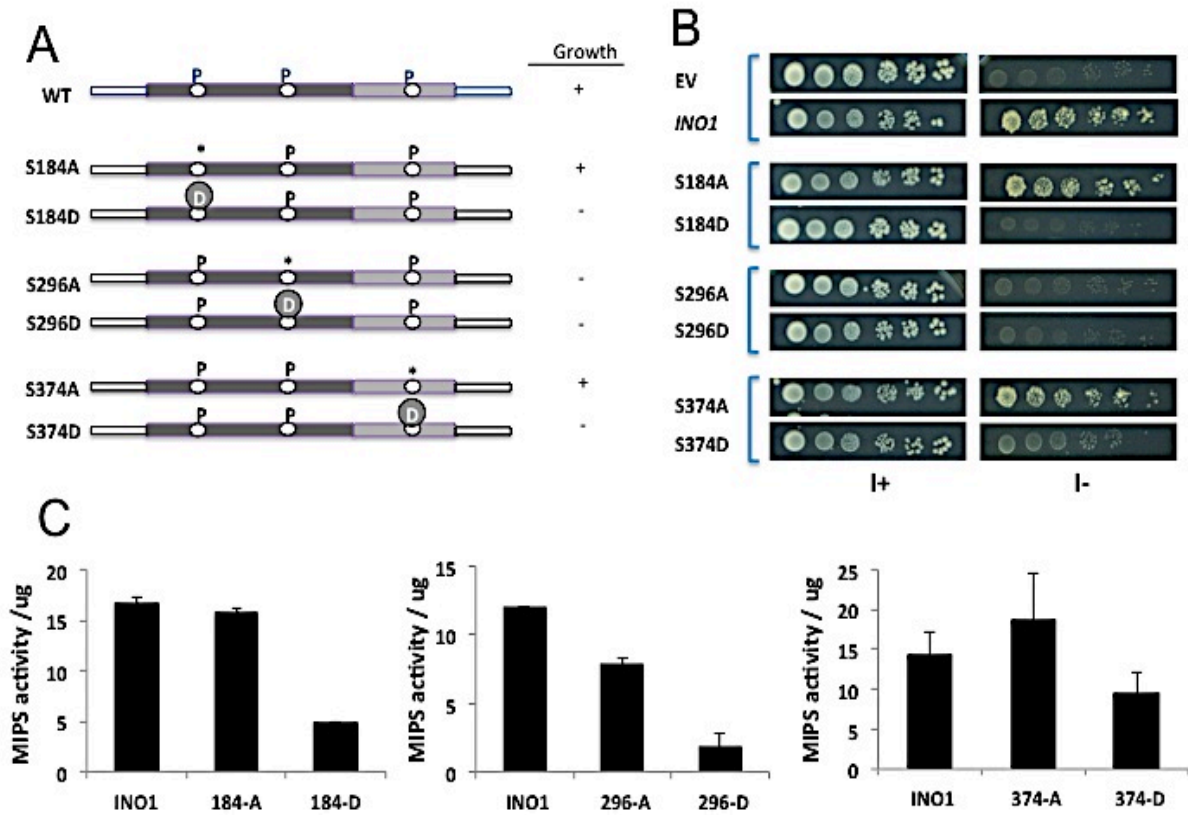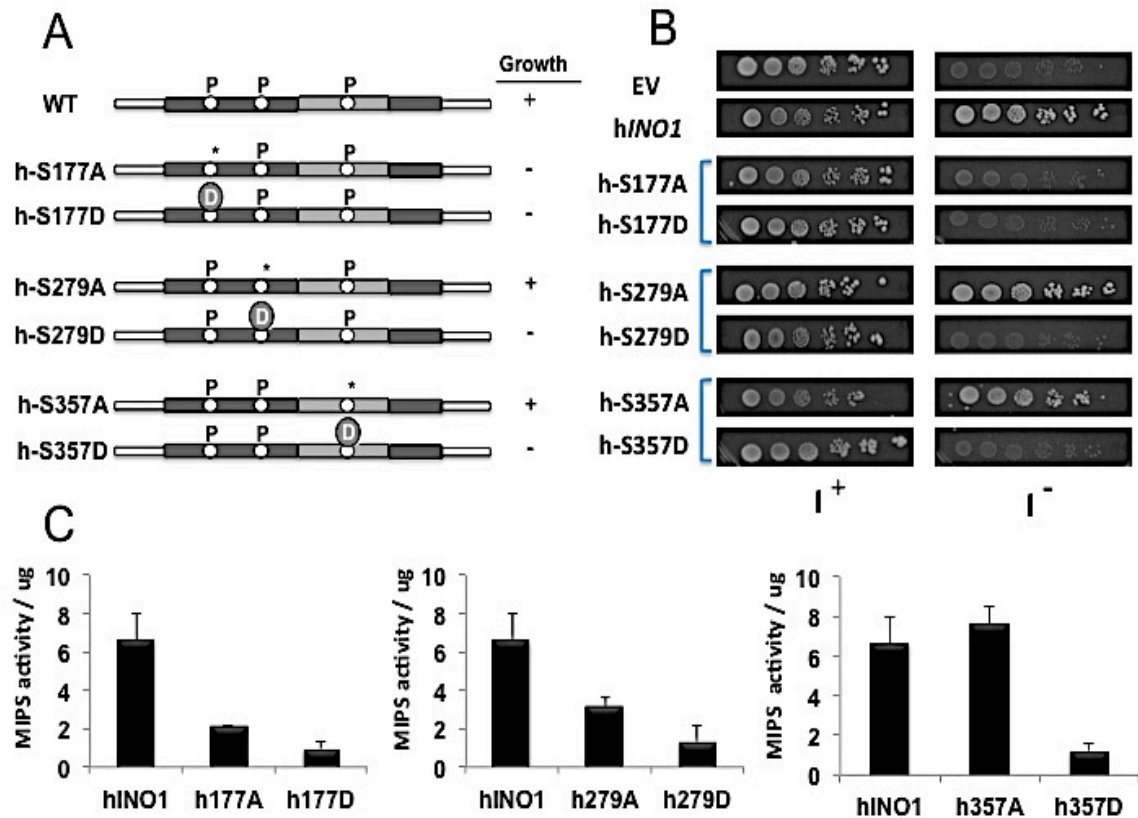
FIGURE 2.8. **Effect of site mutations of human MIPS on growth and enzyme activity.**

A, Schematic representation of the mutated phosphorylation sites in the human MIPS

protein. (*) Phospho-deficient mutation, (D) phosphomimetic mutation. *B*, *ino1Δ* cells

transformed with the empty vector pRD015, vector carrying wild-type human *INO1* or

vector carrying the indicated site mutations were spotted in a 10-fold dilution series on

Ura⁻ medium in the presence or absence of 75 µM inositol, and grown for 3 days at

30°C. *C*, Effect of site mutations on the activity of human MIPS. Values are mean of at

least two experiments ± S.E.

The S184A/S374A double mutation in yeast MIPS confers growth advantage and resistance to VPA:

To determine the effect of loss of both S184 and S374 phosphosites, *ino1*Δ cells were transformed with a vector containing the yeast *INO1* gene with both S184A and S374A mutations. As shown in Fig. 2.9*A*, cells carrying the S184A/S374A mutated *INO1* (DM) exhibited a greatly increased growth rate and reduced lag phase compared to cells carrying the wild-type *INO1* gene (WT). Importantly, the double mutant exhibited significantly better growth in the presence of VPA (Fig. 2.9*A,B*).

Consistent with increased growth conferred by the double mutation, the activity of the double mutant MIPS was almost twice that of wild-type MIPS (Fig. 2.9*C*). Importantly, the decrease in activity in response to VPA was twice as much in wild-type MIPS (50%) as in the double mutated MIPS (25%). Together, the data show that loss of the two phosphosites S184 and S374 leads to increased MIPS activity and decreased sensitivity to VPA.
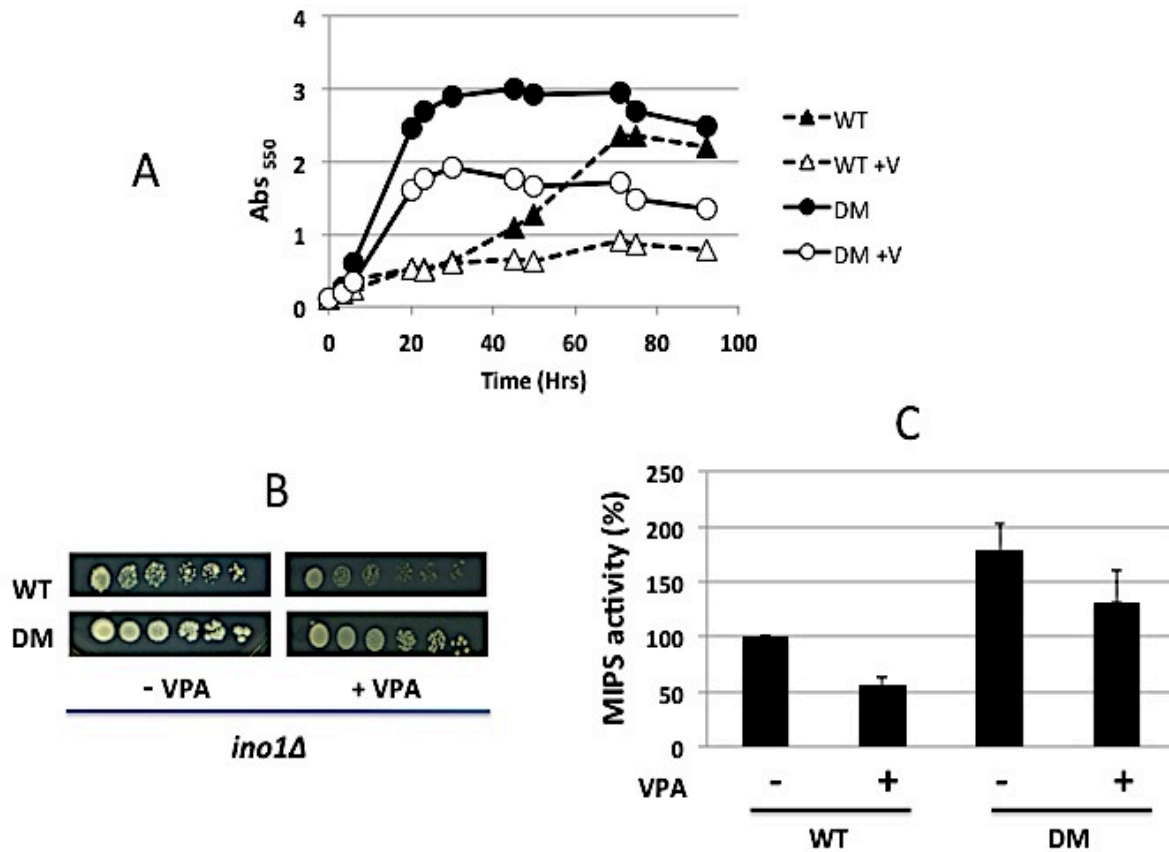
FIGURE 2.9. **The double mutation S184A/S374A (DM) confers a growth advantage, increases MIPS activity, and partially rescues sensitivity to VPA**. *A*, *ino1Δ* cells carrying the double mutation S184A/S374A (DM) grow better than cells carrying the wild type *INO1* (WT). *B*, The double mutant grows better than WT on plates. *C*, The double mutation causes an increase in MIPS activity, and partially rescues sensitivity to VPA. Values are mean of at least two experiments ± S.E.

DISCUSSION

The current study shows for the first time that MIPS is regulated at the post-translational level by phosphorylation. The findings are: (1) Yeast MIPS activity is regulated by phosphorylation of at least three residues. (2) Phosphorylation of the corresponding residues affects activity of human MIPS. (3) Eliminating the two inhibitory phosphosites confers resistance to VPA. These findings identify phosphorylation as a novel mechanism of regulation of inositol synthesis, and suggest that VPA-mediated inositol depletion may result from phosphorylation of MIPS.

MIPS was identified as a phosphoprotein by phospholabeling and phosphoamino acid analysis (Fig. 2.3). Consistent with this finding, mass spectrometry identified five phosphosites in MIPS isolated from VPA-treated cells, (T48, S177, S184, S296, and S374). Mutation of three of the five sites modulated MIPS activity, including S184, S296, and S374. Phosphomimetic mutations of these three sites decreased enzymatic activity. Among the three residues, only one phosphorylation-deficient mutation, S296A, decreased the activity of MIPS, indicating that this site is crucial for function.

The decreased activity of MIPS carrying either the S184D or S374D mutation suggests that phosphorylation of these two residues promotes conformational changes that alter the activity of the enzyme, or block access of the substrate to its catalytic domain. These residues lie in two functionally critical domains. Residue S184 is in the middle of the NAD^{+}-binding domain, which encompasses residues 66-326, and is one of

fourteen residues that directly interact with NAD^+ (Stein and Geiger, 2002). The adenine portion of NAD^+ specifically forms tight hydrogen bonds between N1 and S184 (Jin and Geiger, 2003). Structural studies suggest that the binding of NAD^+ to the *apo*-enzyme is a prerequisite for the orderly binding of the substrate G-6-P to the active site (Jin et al., 2004 ;Jin and Geiger, 2003). Therefore, it is likely that NAD^+ binding is disrupted when S184 is phosphorylated, resulting in decreased activity of MIPS, which is dependent on NAD^+ as a cofactor.

Residue S374 lies within the catalytic domain of MIPS. For MIPS to complete its catalytic cycle, the active site folds and completely encapsulates the substrate in an extreme example of induced fit (Jin et al., 2004; Jin and Geiger, 2003; Stein and Geiger, 2002). We have previously shown that multi-substrate adducts that carry a phosphate group attached at the C_6 position of the 2-deoxyglucitol end are more potent inhibitors of MIPS, suggesting that the presence of a phosphate group in the catalytic domain inhibits activity (Deranieh et al., 2012). Phosphorylation of S374 may perturb access of the substrate to the catalytic domain, or may destabilize the induced fit by creating steric hindrance, thus decreasing catalytic activity. Additionally, the catalytic domain is populated with hydrophobic residues (Geiger and Jin, 2006; Stein and Geiger, 2002). The negative charge of the phosphate group may alter the electrostatic balance of these residues and thus cause further perturbation of enzyme activity.

While the S184A and S374A mutations did not significantly affect activity, both S296A and S296D mutations decreased activity of the enzyme, suggesting that the presence of a serine residue at this location is critical. This residue is also in the NAD$^+$-binding domain. Interestingly, S296 is within a stretch of seven residues, all of which are completely conserved. It is possible that any distortion in this region destabilizes the protein structure, or disrupts the interaction between monomers that is needed to form the tetrameric structure of the enzyme, leading to loss of activity.

Alignment of the amino acid sequences of hMIPS and yeast MIPS revealed a consensus of 49.3%. Of the 533 amino acids of yeast MIPS, 263 residues are conserved. The highest level of conservation is in the catalytic domain (68%). The observation that the three regulatory sites identified in yeast MIPS are conserved in the human homolog was intriguing. The inability of hMIPS carrying the S357D mutation to rescue inositol auxotrophy of yeast *ino1Δ* cells (Fig. 2.8*B*), and the decreased activity of the enzyme (Fig. 2.8*C*), suggest that phosphorylation perturbs the active site, or obstructs binding of the substrate. Similarly, phosphorylation of the human S279 may perturb the activity of the enzyme as a consequence of altering its structure, or obstructing the binding of NAD$^+$. The decreased activity of both S177A and S177D of hMIPS suggest that a serine residue is critical at that position. The crystal structure of MIPS has been solved for *S. cerevisiae* (Kniewel et al., 2002; Stein and Geiger, 2002), *Mycobacterium tuberculosis* (Norman et al., 2002), and *Archaeoglobus fulgidus* (Neelon et al., 2011). To our knowledge, the crystal structure of hMIPS has not been reported. The structural

knowledge pertaining to the catalytic mechanism of MIPS is based largely on the *S. cerevisiae* and *A. fulgidus* models (Neelon et al., 2011; Stein and Geiger, 2002). The catalytic and NAD$^+$ -binding domains of hMIPS are recognized based on sequence similarity with the yeast homolog. The current study is the first to shed light on the mechanism of regulation of hMIPS by characterizing the human protein.

Based on the prediction of NetPhos Yeast, the three identified residues S184, S296, and S374 are potential phosphorylation sites for PKA, GSK3, and PKC, respectively. With regard to the human MIPS, *h*S177 is predicted to be a PKA recognition site. In preliminary experiments, MIPS activity decreased when treated with PKC, suggesting that PKC may be one of the regulators of MIPS activity. GSK3 treatment increased MIPS activity. This is consistent with the slight decrease in activity I observed in the dephosphorylated double-mutant, in which, out of the three phosphosites, only S296 is available for phosphorylation. It also supports the earlier finding of Azab et al. (2007) that the GSK-3 mutant shows decreased MIPS activity and increased sensitivity to VPA.

A previous study has shown that VPA causes a decrease in I-3-P and *myo*-inositol (Vaden et al., 2001), suggesting that VPA inhibits MIPS. Inhibition of MIPS by VPA was not observed *in vitro* (Ju et al., 2004), suggesting that VPA inhibition is indirect. The current findings suggest that at least one mechanism whereby VPA causes inositol depletion may involve the phosphorylation of S184 and S374. First, phosphorylation of yeast MIPS is increased in cells grown in VPA (Fig. 2.3*B*). Second, the double mutation S184A/S374A partially rescues sensitivity of yeast cells to VPA (Fig. 2.9*A*,*B*), and

increases activity of the enzyme (Fig. 2.9*C*). I speculate that VPA triggers a signal(s) that culminates in the phosphorylation of MIPS.

It is possible that MIPS is regulated by phosphorylation of additional sites, which may not have been detected in the current study. The use of the *GAL1*-driven vector was essential for obtaining sufficient amounts of the enzyme for characterizing MIPS activity and for the MS analysis. However, overexpression may have perturbed the stoichiometric balance of phosphorylation, thus underestimating the importance of phosphorylation of some residues. It is also possible that overexpression may have mistargeted the protein and exposed it to non-physiological phosphorylation. Phosphorylation of some amino acids may be transient (rapidly reversible). It is also possible that phosphorylation may occur in a sequential manner, in which a phosphate at one residue may be required to activate a kinase that phosphorylates another residue(s). For example, phosphorylation of S374 may facilitate the phosphorylation of S184 because the increase in activity following dephosphorylation of a single mutant is almost half of that observed for the WT. Alternatively, phosphorylation may occur at different growth stages in response to different cues or signals.

While the replacement of serine with alanine or aspartate creates phosphodeficient or phosphomimetic mutations, respectively, these mutations may not recapitulate the phosphorylated residues' functions. The mutations may alter the structural conformation of the enzyme, triggering changes in activity that are not necessarily reflective of a phosphosite. However, my findings are strongly supported by

MS data, which unambiguously show that the residues identified in MIPS are phosphosites. The correlation between the growth experiments and enzyme activity demonstrates that MIPS activity is regulated by phosphorylation.

The work reported here identifies for the first time a novel mechanism of regulation of inositol biosynthesis. The knowledge that MIPS is regulated by phosphorylation will facilitate studies to identify signals that play a role in regulating this enzyme, which is crucial for maintaining inositol homeostasis.

CHAPTER 3

INOSITOL DEPLETION PERTURBS THE VACUOLAR-ATPase: A NOVEL MECHANISM OF ACTION OF VALPROATE

INTRODUCTION

The "inositol depletion hypothesis" proposed by Sir Michael Berridge in the 1980s suggested that inositol depletion caused by lithium leads to a decrease in IP_3-mediated signaling (Berridge, 1988). However, the mechanism underlying the therapeutic effect of decreased signaling is not obvious, suggesting that alternate consequences of inositol depletion may underlie the therapeutic mechanism. Valproate (VPA), an anticonvulsant drug that for decades has been used successfully for the treatment of epilepsy and bipolar disorder, also causes inositol depletion (Vaden et al., 2001). The mechanism that underlies its therapeutic efficacy is not known. In humans, perturbation of inositol and phosphoinositide metabolism is associated with several disorders, including bipolar disorder (Belmaker, 2004), Alzheimer's disease (McLaurin et al., 1998; Shimohama et al., 1998), Lowe's syndrome (Lowe, 2005), X-linked myotubular

myopathy (Laporte et al., 1996; Taylor et al., 2000), and Type 2 diabetes (Clement et al., 2001; Marion et al., 2002). The genetic disorders Charcot-Marie-Tooth disease (Chow et al., 2009), and fleck corneal dystrophy (Kotoulas et al., 2011) are specifically associated with dysfunction of the phosphoinositide $PI3,5P_2$.

$PI3,5P_2$ is the signature phosphoinositide of the vacuole (the yeast lysosome) (Strahl and Thorner, 2007) . It is generated from PI3P by the sole conserved kinase Fab1p (the homolog of PIKfyve) (Cooke et al., 1998; Gary et al., 2002; Sbrissa et al., 1999), which is positively regulated by Vac7p and Vac14p. Together, Fab1p and its regulators form part of a protein complex that localizes to the vacuolar membrane during $PI3,5P_2$ synthesis (Duex et al, 2006; Botelho et al., 2008). The regulated synthesis and turnover of $PI3,5P_2$ is proposed to underlie many aspects of vacuole function (Weisman, 2003), including regulation of vacuolar ion transport, membrane efflux, membrane turnover, and signaling (Baars et al., 2007; Weisman, 2003; Efe et al., 2005; Odorizzi et al., 2000). Although $PI3,5P_2$ makes up less than 0.1% of all phosphoinositides (Michell et al., 2006), its absence from the cell has a profound effect. The *fab1Δ* mutant (which cannot synthesize $PI3,5P_2$) exhibits enlarged vacuoles, reduced vacuolar acidification, and perturbed trafficking. The mechanism that leads to these phenotypes is not known. It was suggested that $PI3,5P_2$ may modulate V-ATPase activity, thus explaining the decreased acidification (Weisman, 2003). In the same context, Baars *et al*., (2007) showed that acidification of the vacuole is crucial for vacuolar fission, suggesting that $PI3,5P_2$ supports vacuolar fission by regulating V-ATPase activity.

The V-ATPase is an ATP-driven proton pump (Fig. 3.1). It plays a significant role in maintaining the pH homeostasis necessary not only for the function of vacuoles, but also for other organelles (Kane, 2006). It mediates ATP-driven proton transport across membranes to acidify intracellular organelles and regulate the ionic balance within cells (Kane, 2007). Present in plasma membranes of specialized cells, the V-ATPase regulates extracellular pH (Breton and Brown, 2007). In osteoclasts, the V-ATPase facilitates bone resorption (Blair et al., 1989; Kartner et al., 2010). In kidney cells, it is involved in the regulation of systemic acid-base balance (Brown et al., 2009). Neuronal cells require the V-ATPase for uptake and storage of neurotransmitters in synaptic vesicles (El Far and Seagar, 2011; El Far and Seagar, 2011), and recent evidence indicates that the V-ATPase is required for endocytosis (Zhang et al., 2010) as well as for exocytosis and neurotransmitter release (Di Giovanni et al., 2010; Poea-Guyon et al., 2013).

The mechanism that links PI3,5P$_2$ to V-ATPase was not well understood until recently, when a report by Li et al. (2014) showed that PI3,5P$_2$ stabilizes the V$_1$-V$_0$ sector interactions and activates the V-ATPase. In this study, I examined the cellular consequences of inositol depletion caused by VPA. As mentioned previously, the therapeutic mechanism of this drug is not understood. I show that inositol depletion, induced genetically (by starving inositol auxotrophs) and by VPA treatment, causes a decrease in V-ATPase activity and a disruption in PI3,5P$_2$ synthesis. This is the first report to demonstrate a role of inositol in the activity of the V-ATPase, and suggests a possible new therapeutic mechanism of VPA.
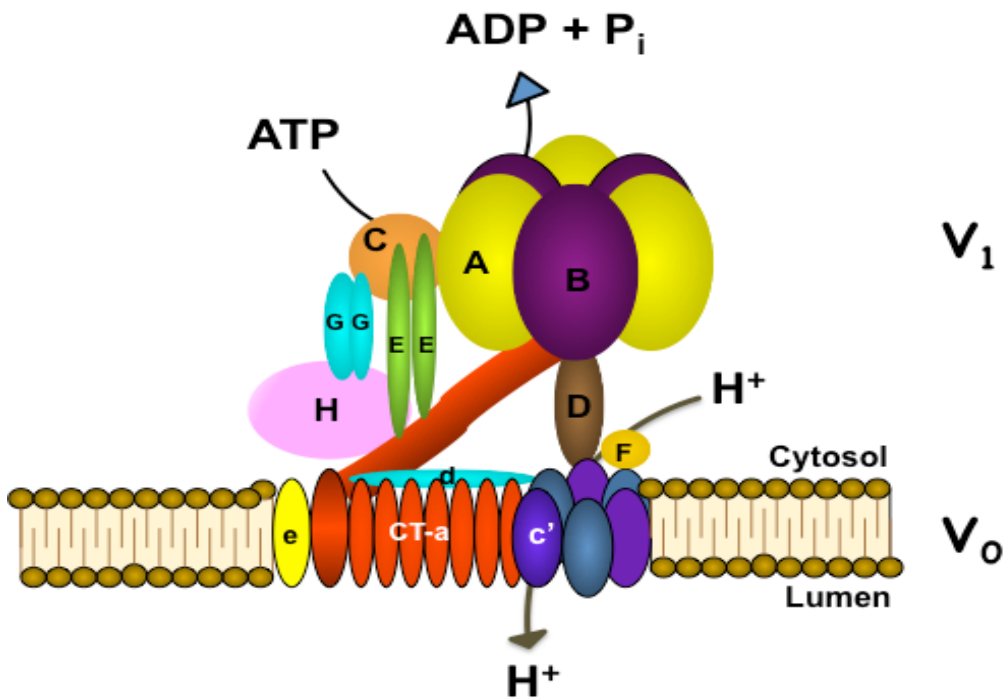
FIGURE 3.1. **The vacuolar ATPase**. The vacuolar ATPase (V-ATPase) is an ATP-driven proton pump consisting of a membrane-embedded V_0 domain (subunits a, c, c', c", d, and e), and a peripheral V_1 domain (subunits A-H). The energy generated from ATP hydrolysis by the V_1 domain is coupled to the transport of protons across the V_0 domain in the membrane.

MATERIALS AND METHODS

Strains, media and growth conditions:

The *Saccharomyces cerevisiae* strains used in this work are listed in Table 3.1. Cells were maintained on YPD medium (2% bactopeptone, 1% yeast extract, and 2% glucose). For growth of the deletion mutants, the medium was supplemented with G418 (200 μg/ml). Synthetic minimal medium (SM) contained all the essential components of Difco® yeast nitrogen base (minus inositol), 2% glucose, 0.2% ammonium sulfate, vitamins, and the four amino acids histidine (20 mg/L), leucine (60 mg/L), methionine (20 mg/L), and lysine (20 mg/L), and the nucleobase uracil (40 mg/L). Where indicated, inositol was used at a concentration of 75 μM (I+). For selection of cells carrying plasmids, appropriate amino acids were omitted. For solid media, 2% agar was added. To starve cells for inositol, *ino1Δ* cells were grown overnight in SM supplemented with inositol, harvested, washed twice in dH_2O, and resuspended in inositol-free medium (I-). For osmotic stress experiments, media were supplemented with 0.9 M NaCl. Growth in liquid cultures was monitored spectrophotometrically by measuring absorbance at 550 nm. All incubations were at 30°C unless otherwise stated.

Cytosolic pH measurement :

Cytosolic pH was measured using the yeast pHluorin, a pH-sensitive, ratiometric green fluorescent protein (Orij et al., 2009). Wild-type cells were transformed with the

construct pPGK-pHluorin, a generous gift from Dr. Rajini Rao (Johns Hopkins University), in which the yeast pHluorin is under control of a phosphoglycerate kinase promoter. Transformants were selected on SM lacking uracil, and checked for fluorescence before every experiment. Cells were grown to the mid-log phase in selective SM with or without 75 μM inositol, in the presence of different concentrations of VPA. Cells were harvested by centrifugation, washed, and resuspended in 50 mM MES buffer (50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 0.2 M NH_4 acetate, 10 mM NaN_3, 10 mM 2-deoxyglucose). Fluorescence intensity was measured at excitation wavelengths 405 and 485 nm, and emission wavelength 508 nm. A calibration curve was constructed using buffers titrated to pH 4, 5, 6, 7, and 8. Cells were incubated for one hour at 30°C in the presence of 75 μM monensin and 10 μM nigericin.   A standard curve of fluorescence was generated and used to calculate the cytosolic pH from the observed fluorescence ratios.

FM4-64 staining:

To visualize vacuolar membranes *in vivo*, cells were stained with the lipophilic dye FM4-64 (Molecular Probes) as described (Vida and Emr, 1995) with slight modifications. Cells were grown overnight at 30°C with constant shaking. One ml culture was pelleted and resuspended in fresh medium containing 10 μM FM4-64. Cells were incubated for 30 min, harvested, washed twice and chased in fresh medium for about 2 h. For microscopy, cells were pelleted and resuspended in a small volume of fresh medium

with 50 mM PIPES. To immobilize cells, coverslips were coated with a thin layer of 1%

agarose dissolved in SM. Cells were examined using a fluorescence microscope.

Vacuole isolation, V-ATPase activity and coupling assays:

For isolation of vacuoles from VPA-treated wild-type cells, the cells were grown

overnight in I- medium to the mid-log phase. The cells were diluted two-fold in the

same medium, then divided into three separate samples: control (no additions), VPA

(0.6 mM VPA added), and VPA + inositol (0.6 mM VPA + 100 μM inositol).   The

incubation was continued for 3.5 hours at 30°C with shaking.   For experiments with

ino1Δ, cells were grown overnight in I+ medium to the mid-log phase, washed, and

resuspended in I- medium buffered to pH 5 with 50 mM 2-(*N*-

morpholino)ethanesulfonic acid.   For the I+ samples, cells were resuspended to a

density of 0.3 OD_{600}/ml (to allow for growth during the incubation) and 75 μM inositol

was added. The I- samples were resuspended to 1.0 OD_{600}/ml and showed little growth

during the incubation. Both samples were incubated for 4 hours at 30°C with shaking.

After incubation, cells from both types of experiments were converted to spheroplasts

and washed and vacuolar vesicles were isolated through two Ficoll density gradient

centrifugation steps as described (Roberts et al., 1991).

ATPase activity was assessed by a coupled enzyme assay (Lotscher et al., 1984)

and V-ATPase activity was determined by measuring ATPase activity in the presence and

absence of the V-ATPase specific inhibitor concanamycin A (VWR).   Total vacuolar

protein was measured by Lowry assay (Lowry et al., 1951). Proton pumping was measured by the 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence quenching assay (Liu et al., 2005). Pumping was initiated by addition of ATP and Mg^{2+} to final concentrations of 0.5 mM and 1 mM, and was inhibited by the addition of 100 nM concanamycin A. Coupling ratios were calculated by determining the rate of fluorescence quenching per mg of vacuolar protein during the first 10 sec after MgATP addition and dividing that rate by the rate of concanamycin-sensitive ATP hydrolysis per mg protein.

Fluorescence microscopy:

Imaging of yeast cells was performed using an Olympus BX41 epifluorescence microscope. Images were acquired using an Olympus Q-Color3, a digital charge-coupled device (CCD) camera operated by QCapture2 software. The plasmids pRS415-FYVE-GFP used to localize PI3P, and pRS416-Atg18-GFP used to localize PI3,5P$_2$ were a kind gift from Dr. Scott Emr (Cornell University).

Electron microscopy :

Cells were grown in synthetic minimal medium to the mid-log phase. Cells were harvested, and fixed in 3% glutaraldehyde, 0.1 M Na-Cacodylate pH 7.4, 5 mM CaCl$_2$, 5 mM MgCl$_2$, 2.5% sucrose, stained with osmium-thiocarbohydrazide, and subjected to EM analysis at the Integrated Imaging Center at Johns Hopkins University.

TABLE 3.1. **Strains used in this study**

Strains	Genotype	Reference
BY4741	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0*	Invitrogen
ino1Δ	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, ino1Δ::KanMX4*	Invitrogen
vma3Δ	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, vma3Δ::KanMX4*	Invitrogen
vma9Δ	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, vma9Δ::KanMX4*	Invitrogen
vma11Δ	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, vma11Δ::KanMX4*	Invitrogen
vma16Δ	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, vma16Δ::KanMX4*	Invitrogen
vph1Δ	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, vph1Δ::KanMX4*	Invitrogen
stv1Δ	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, stv1Δ::KanMX4*	Invitrogen
fab1Δ	*MATα, his 3Δ1, leu 2Δ0 lys 2Δ0, ura 3Δ0, fab1Δ::KanMX4*	Invitrogen

RESULTS

VPA perturbs the vacuolar structure in a manner similar to that caused by inositol starvation:

The vacuolar structure is dependent on the delicate balance of solutes between the vacuolar and cytosolic compartments. This balance is largely determined by the vacuolar membrane, which has a distinctive lipid composition (Li and Kane, 2009) and a variety of protein pumps that transport ions and protons across the membrane (Cagnac et al., 2010). The proton gradient is maintained by the V-ATPase, which acidifies the vacuole. In yeast, loss of function of the V-ATPase results in a vma^- phenotype, partly characterized by poorly acidified vacuoles, and sensitivity to elevated extracellular pH and high concentrations of calcium (Kane, 2006).

A screen of the yeast deletion collection was previously carried out by Yihui Shi (Ph.D. Dissertation, 2005). Several vma mutants were found to be sensitive to VPA. This suggested that VPA may perturb the vacuolar structure. To test this possibility, WT cells were grown overnight in synthetic medium in the presence or absence of VPA. VPA-treated cells exhibited decreased incorporation of FM4-64 (Fig. 3.2), which enters the cells by endocytosis and labels the vacuolar membrane. In contrast, cells grown in the absence of VPA exhibited clear, well-defined vacuoles. Inositol supplementation rescued the vacuolar defect of VPA-treated cells, suggesting that inositol depletion caused by VPA is responsible for perturbing the vacuolar membrane. To determine if vacuolar
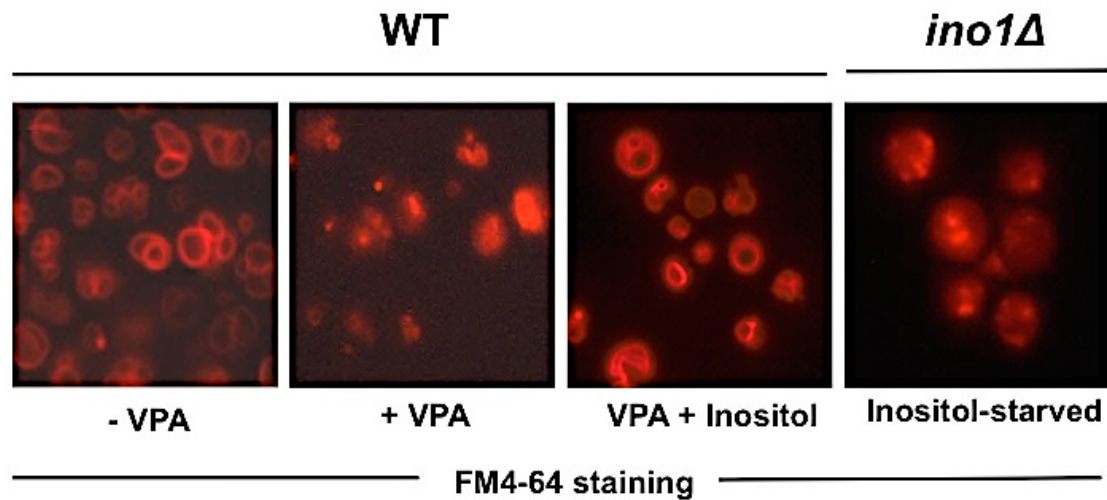
FIGURE 3.2. **Vacuolar defects in VPA-treated cells are rescued by supplementation with inositol.** To determine the effects of VPA on wild-type cells, cells were grown to the mid-log phase in SM with or without 1 mM VPA and 75 μM inositol. The effects of inositol starvation were determined in *ino1Δ* cells precultured in SM I+, washed 2x to remove inositol, and resuspended in SM I- . To visualize vacuoles, cells were stained with FM4-64 as described in Materials and Methods.

perturbation is inositol dependent, *ino1Δ* cells, which cannot synthesize inositol, were grown overnight in the presence of inositol (I+) and then switched to I- medium to induce inositol starvation. FM4-64 staining showed a pattern of vacuolar perturbation similar to that observed in the VPA-treated cells (Fig. 3.2). Taken together, these findings suggest that VPA perturbs vacuolar structure as a consequence of inositol depletion.

VPA decreases V-ATPase proton-pumping:

V-ATPases catalyze ATP-driven proton transport. To determine if perturbation of the vacuole is a consequence of altered V-ATPase function, the activity of ATPase and proton pumping was measured in vacuolar vesicles isolated from wild-type cells deprived of inositol and treated with 0.6 mM VPA, in the presence and absence of inositol (100 mM). As shown in Fig. 3.3*A*, VPA reduced ATPase activity, and this reduction was not rescued by the presence of inositol. VPA had an even more striking effect on proton pumping, as shown in Fig. 3.3*B*. Addition of MgATP to untreated vacuolar vesicles results in rapid quenching of ACMA fluorescence, which is reversible upon addition of the V-ATPase inhibitor concanamycin A. In contrast, VPA-treated vacuoles exhibited no MgATP-driven proton pumping. (In some preparations, reduced levels of proton pumping were observed.) In vacuolar vesicles treated with inositol and VPA, a small amount of proton pumping was observed.
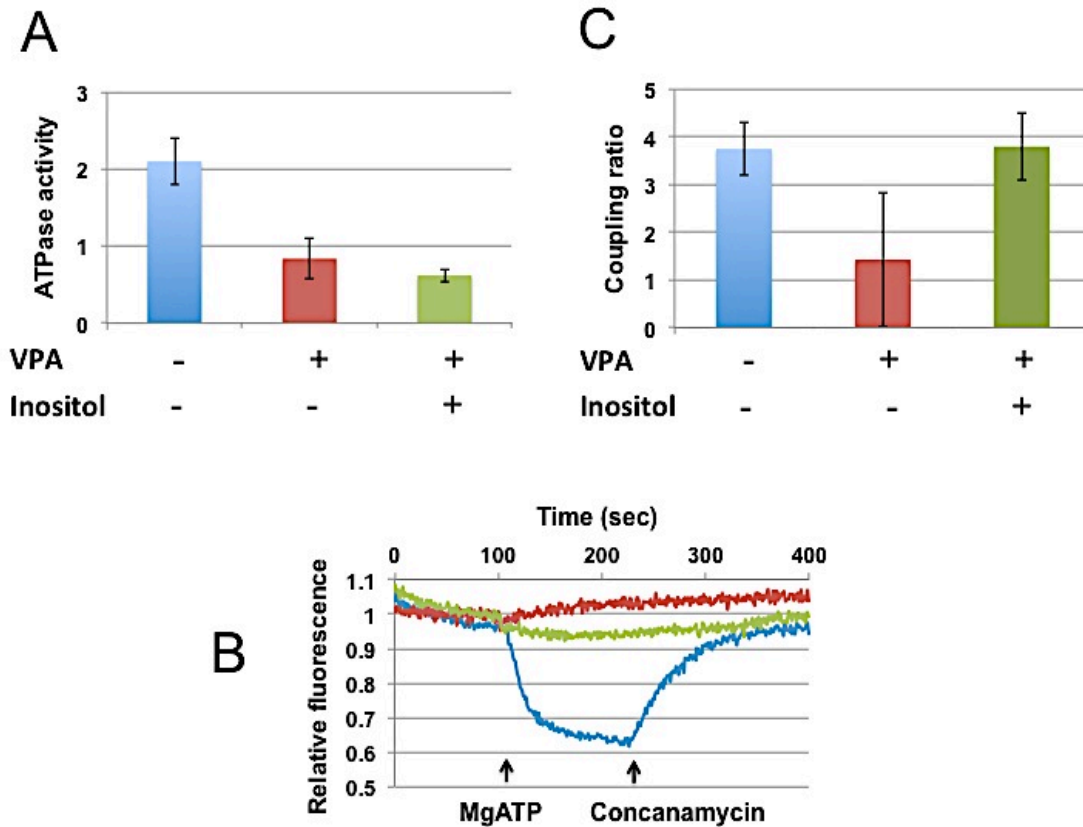
FIGURE 3.3. **VPA inhibits V-ATPase activity and reduces coupling in WT cells.** *A,* Mean concanamycin A-sensitive ATPase activity (mmol ATP hydrolyzed/min/mg protein) for two independent vacuolar preparations is shown; error bars represent the range of the two measurements. *B,* Proton pumping assay for one of the two vacuole preparations is shown. At the indicated times, MgATP was added to initiate proton pumping, and concanamycin A was added to inhibit the V-ATPase. *C,* Coupling ratios were calculated for each sample as described under "Materials and Methods." The average ratio of two independent measurements is shown, with error bars corresponding to the range.

The coupling ratio represents a ratio of the initial rates of proton pumping and ATP hydrolysis; if ATPase activity and proton pumping are compromised to the same degree by valproate treatment, the coupling ratio will be the same as in the control. The average coupling ratio for the two independent vacuole preparations is shown in Fig. 3.3*C*. VPA treatment reduced the coupling ratio, consistent with a greater inhibition of proton pumping than ATPase activity, but the vesicles from inositol and valproate-treated cells had a wild-type coupling ratio. These results suggest that valproate partially uncouples ATP hydrolysis from proton pumping, but loss of coupling can be prevented by the presence of inositol. Although the inositol dependence of the VPA effect on coupling was striking, inositol did not rescue the reduced activity of the enzyme in the presence of valproate (Fig. 3.3*A*).

In order to further explore the possibility that inositol deprivation could account for uncoupling of the V-ATPase by VPA, vacuolar vesicles were isolated from *ino1Δ* mutants incubated in the presence or absence of added inositol. As shown in Fig. 3.4*A*, inositol deprivation reduced ATP hydrolysis by an average of 59%, but had a much greater effect on proton pumping and coupling ratio (Figure 3.4*B* and *C*). Figure 3.4*B* shows a complete loss of proton pumping; some preps showed a low level of pumping after inositol deprivation, but in all cases, the coupling ratio was reduced. These results are similar to those observed for vesicles from VPA-treated cells, and indicate that VPA may uncouple the V-ATPase by inositol depletion, even though inositol supplementation does not fully reverse the effects of VPA.
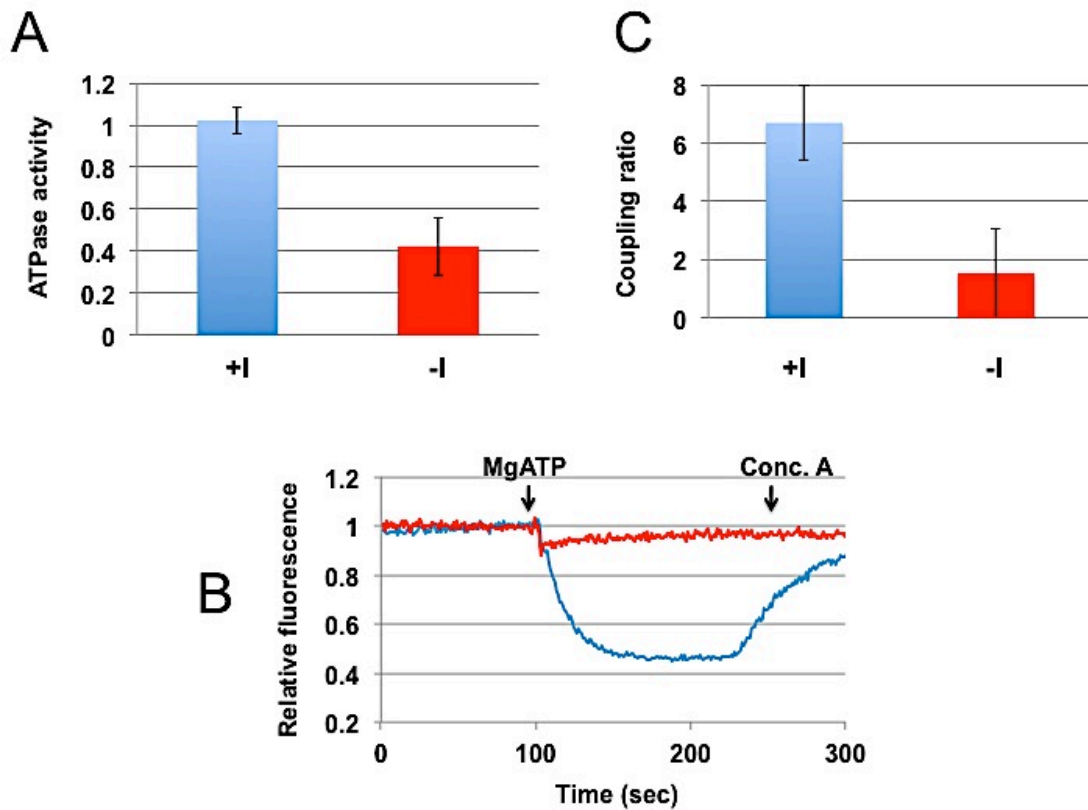
FIGURE 3.4. **Inositol depletion inhibits V-ATPase activity and reduces coupling.** *ino1Δ*

cells were grown in SM I+ overnight, then washed twice and resuspended in either SM

I+ or SM I- and incubated for 4 hrs at 30°C. Vacuolar vesicles were isolated as

described under "Materials and Methods." *A,* Mean concanamycin A-sensitive ATPase

activity (in mmol ATP hydrolyzed/min/mg protein) is shown for three independent

vacuole preparations. Error bars represent S.E. *B,* Proton pumping assay for one of the

three preparations is shown. *C,* The coupling ratio was calculated for each sample as

described under "Materials and Methods". The mean is shown, with error bars

corresponding to S.E.

To determine if VPA causes growth defects associated with loss of V-ATPase function, I examined wild-type (WT) cells for their ability to grow at pH 7.5 and 100 mM $CaCl_2$ in the presence or absence of VPA (Fig. 3.5). The $vma3\Delta$ cells (used as a control) showed a clear vma^- phenotype, including inability to grow at elevated pH and in high calcium. In contrast, WT cells grown in the presence of VPA were able to grow in these conditions. Thus, although VPA causes a decrease in V-ATPase activity, the effect is not sufficient to cause a vma^- phenotype.

Because only a subset of the vma mutants were identified in the screen for VPA sensitivity, I further explored the sensitivity of the vma mutants, and particularly those disrupting subunits of the membrane bound V_0 sector, to VPA. All of the $vma\Delta$ mutants tested showed sensitivity to VPA, and this sensitivity was rescued by inositol (Fig. 3.6). Vph1 and Stv1 encode two isoforms of the largest membrane subunit of the V-ATPase. Single deletion mutants of these subunits exhibit modest VPA sensitivity that is rescued by inositol. These findings suggest that VPA triggers an inositol-dependent mechanism that exacerbates the vma^- phenotype. At least three possible mechanisms may explain this outcome:

First, VPA may alter cytosolic pH, which could be detrimental to the vma^- mutants already defective in cellular pH homeostasis. To test this, I used a pHluorin-based assay (Orij et al., 2009) to measure the effect of different VPA concentrations on cytosolic pH. Within the range of concentrations of VPA used therapeutically, VPA did not alter the cytosolic pH (Fig. 3.7). A second possibility is that VPA triggers a mechanism
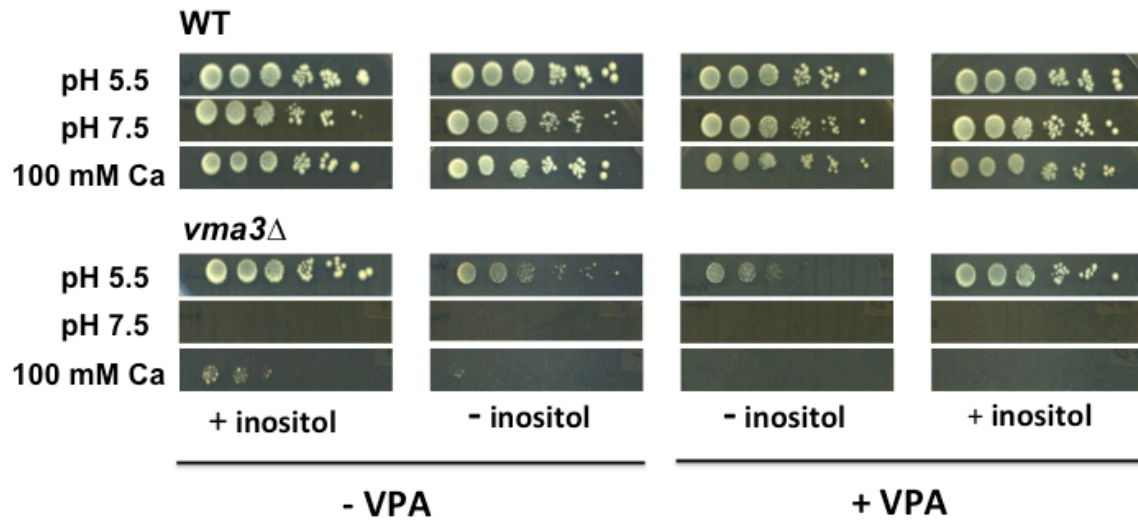
FIGURE 3.5. **VPA does not cause a *vma*- phenotype.** Wild-type (WT) BY4741 and isogenic *vma3Δ* mutant cells were precultured in SM I+, washed twice to remove inositol, and spotted in a 10-fold dilution series on SM in the presence or absence of 75 μM inositol and 2 mM VPA. Media were buffered to either pH 5.5 or 7.5, or unbuffered medium supplemented with 100 mM $CaCl_2$. Cells were grown for 3-4 days at 30°C.
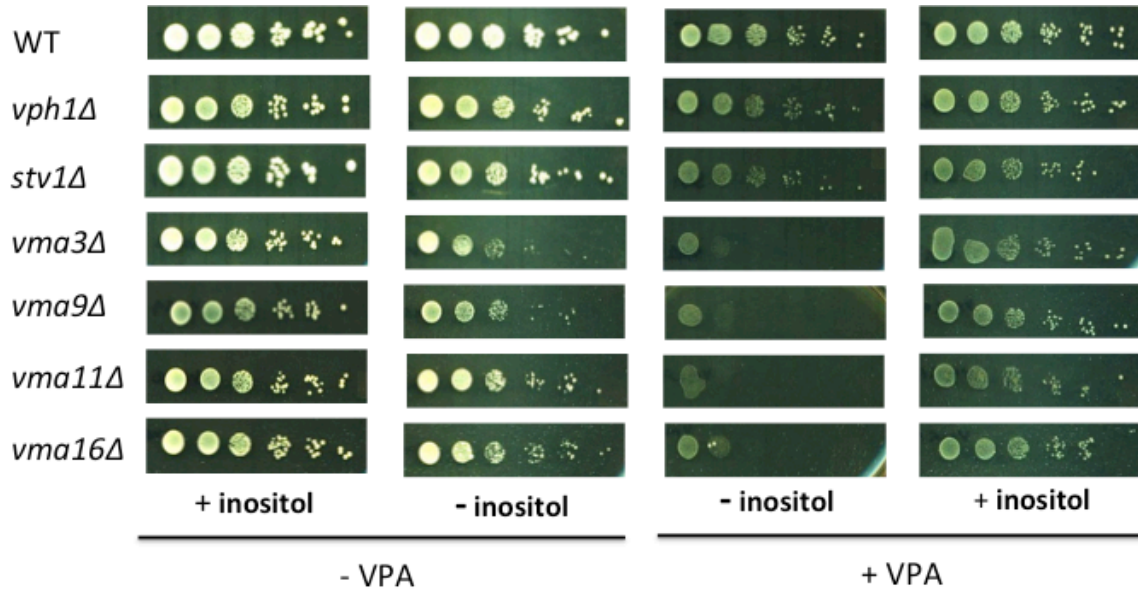
FIGURE 3.6. **Inositol rescues sensitivity of V-ATPase V$_0$ deletion mutants to VPA.** Wild-type (WT) BY4741 and isogenic *vmaΔ* mutant cells were precultured in SM I+, washed twice to remove inositol, and spotted in a 10-fold dilution series on SM in the presence or absence of 75 μM inositol and 2 mM VPA. Cells were grown for 3-4 days at 30°.
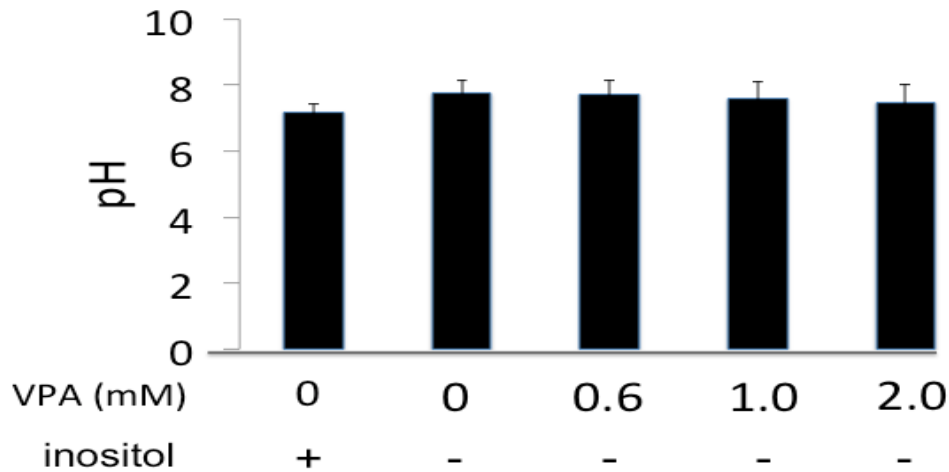
FIGURE 3.7. **Cytosolic pH is not altered by VPA treatment.** Cytosolic pH was measured

in wild-type cells containing the construct pPGK pHluorin harboring a yeast pHluorin.

Cells were grown to the mid log phase in SM (Ura-) with or without 75 μM inositol and

the indicated concentrations of VPA. Cells were harvested, washed, and resuspended in

neutral buffer. Fluorescence intensity was measured at excitation wavelengths 405 and

485 nm, and emission wavelength of 508 nm. A calibration curve constructed using

buffers titrated to pH 4, 5, 6, 7, and 8, was used to convert the fluorescence values to

cytosolic pH. Values represent the mean of three independent experiments ± S.E. (*error*

bars).

that increases intracellular calcium levels, which inhibit growth of *vma⁻* mutants. This is not likely as VPA does not appear to inhibit growth of wild-type cells in the presence of high Ca^{+2} (Fig. 3.5). A third possibility is that VPA disrupts the synthesis of phosphoinositides, and that this deleteriously affects the V-ATPase. This possibility was investigated further.

VPA alters the dynamics of vacuolar PI3,5P$_2$:

I addressed the possibility that VPA-induced inositol depletion affects PI3,5P$_2$, the key phosphoinositide of the vacuolar membrane. It was previously shown that VPA causes inositol depletion within the first few hours after addition to growing cells (Ju and Greenberg (2003); Vaden et al., 2001). To monitor the effect of VPA treatment on the dynamics of PI3,5P$_2$, I visualized this phosphoinositide in the vacuolar membrane over a period of four hours using a GFP-tagged FLARE containing an atg18 domain (Efe et al., 2007) that specifically recognizes PI3,5P$_2$ (Fig. 3.8). Within the first two hours, striking differences were observed between the treated and untreated cells (Fig. 3.8). VPA-treated cells showed punctate fluorescent structures on the vacuolar membrane, indicative of localized accumulation of PI3,5P$_2$ in those structures. These punctae persisted for a short time (less than an hour) and then gradually diminished as the vacuole grew in size. In the untreated cells, smaller punctae formed. Interestingly, however, these punctae rapidly developed into fluorescent, membranous, bubble-like structures (arrows in Fig. 3.8), which protruded into the lumen of the original large

vacuole. These protrusions formed septae that resulted in the fragmentation of the original large vacuole into multiple smaller ones. The intense fluorescence indicates that these membranes are enriched with $PI3,5P_2$, which suggests that vacuolar fission is preceded by a phase of increased $PI3,5P_2$ synthesis. This phenomenon was not observed in the VPA-treated cells, in which fluorescent membranes were not observed to emerge out of punctae. The vacuoles continued to increase in size for a few hours, after which the single enlarged vacuole started to shrink in size, rather than divide into multiple small vacuoles. Starvation for inositol in *ino1Δ* cells resulted is a similar phenotype (Fig. 3.8). Taken together, these observations suggest that VPA perturbs the synthesis of $PI3,5P_2$-enriched membranes, possibly by decreasing the synthesis of $PI3,5P_2$ during the first two hours. As a consequence, the vacuoles do not undergo fission but remain enlarged, a phenotype similar to that of the *fab1Δ* mutant, which cannot synthesize $PI3,5P_2$ (Yamamoto et al., 1995).

Electron microscopic (EM) analysis of cellular changes associated with VPA treatment showed that VPA enhances vacuole fusion within the first hour of exposure to the drug (Fig. 3.9*A,B*). In this analysis, 80% of VPA-treated cells showed fused vacuoles compared to about 20% in the untreated cells (Fig. 3.9*A,B*). These results are consistent with the finding shown in Fig. 3.8, suggesting that VPA-induced vacuolar fusion may be a consequence of decreased synthesis of $PI3,5P_2$.
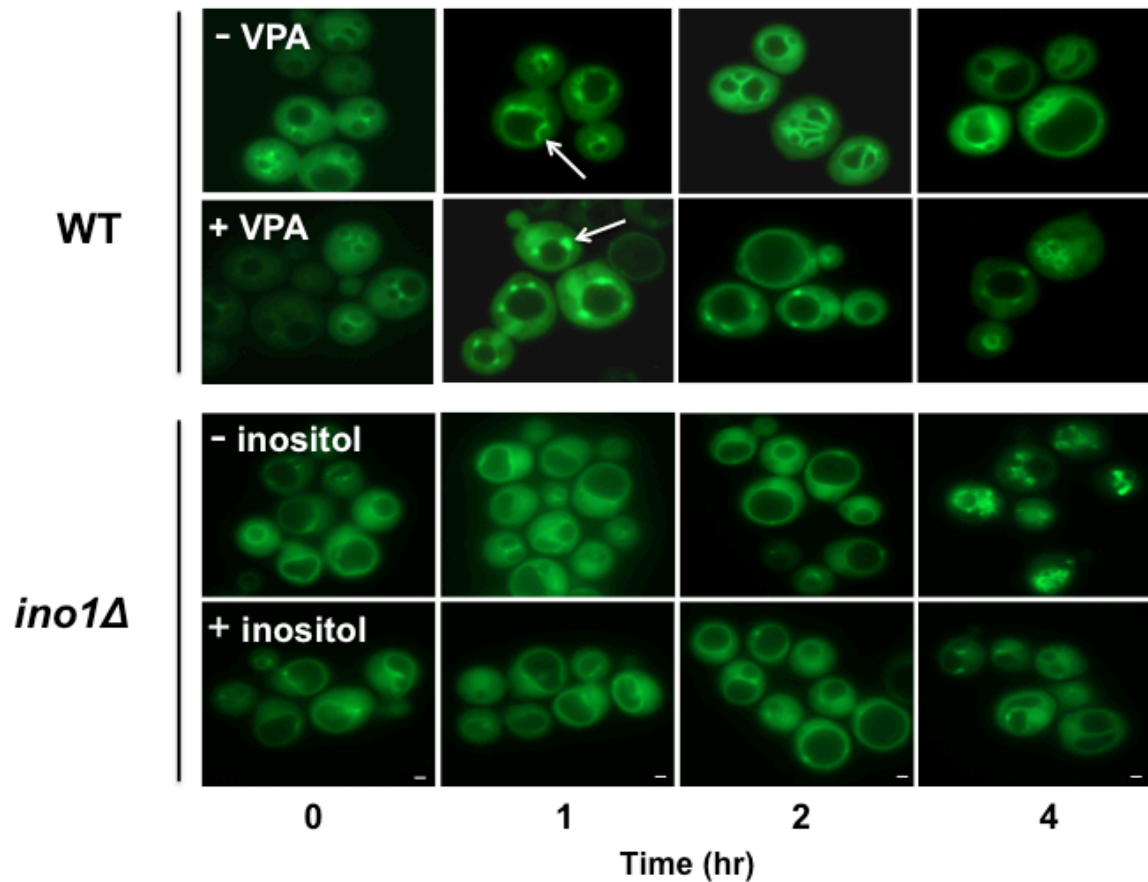
FIGURE 3.8. **VPA alters the distribution of vacuolar PI3,5P$_2$.** Wild type cells constitutively expressing a GFP-tagged PI3,5P$_2$-binding probe (Atg18-GFP) were grown in SM at 30°C to the mid-log phase. VPA was added to a final concentration of 2 mM. Cells were harvested at the indicated times following addition of the drug, and visualized by fluorescence microscopy. *Ino1Δ* cells expressing the GFP-tagged PI3,5P$_2$-binding probe were initially grown in I+ medium and then transferred to inositol deficient medium to initiate starvation. Cells were harvested at the indicated times following starvation for inositol. *Scale bar*, 1 μm.
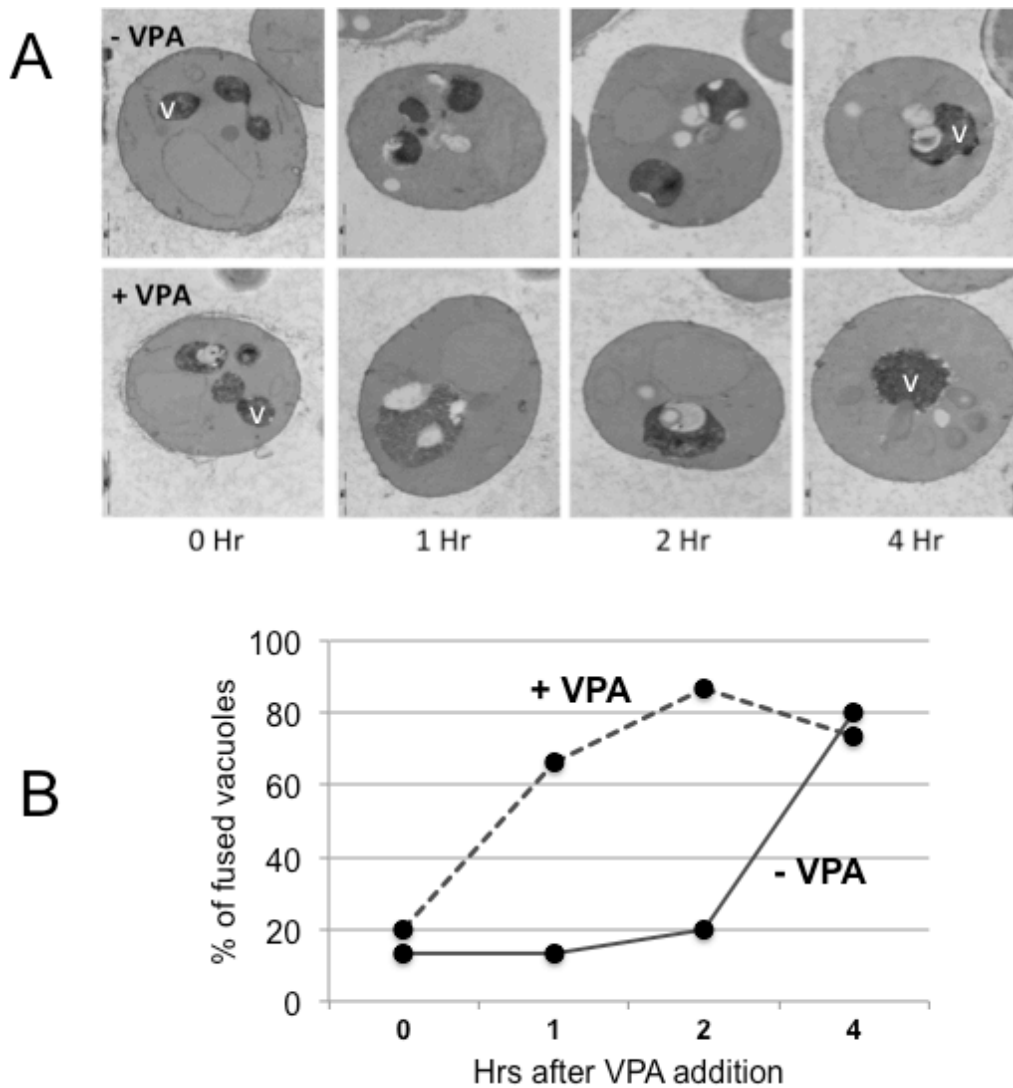
FIGURE 3.9. **VPA enhances vacuolar fusion.** *A*, Electron micrographs of wild-type cells grown in SM to the mid log phase and treated with VPA for the indicated times. Upper panel, untreated cells (-VPA); bottom panel, cells treated with 1 mM VPA (+VPA). The label V indicates the vacuole. *B*, The percentage of VPA-treated cells with single enlarged vacuoles was compared to that of untreated cells.

The increase in PI3,5P$_2$ in response to osmotic stress is limited in the presence of VPA:

PI3,5P$_2$ is the least abundant of the phosphoinositides in eukaryotic cells, comprising less than 0.1% of all phosphoinositides (Michell et al., 2006). The loss of PI3,5P$_2$ is associated with an increase in vacuolar size (Yamamoto et al, 1995), while an increase in levels of PI3,5P$_2$ is associated with vacuolar fragmentation (Bonangelino et al., 2002). Osmotic stress leads to a 20-fold increase in levels of PI3,5P$_2$ (Bonangelino et al., 2002) and an increase in vacuolar fission. To determine if VPA interrupts the synthesis of PI3,5P$_2$, I exposed WT cells harboring the GFP-tagged PI3,5P$_2$ FLARE to osmotic stress and monitored the cells for accumulation of fluorescence. While the untreated cells showed an increase in fluorescence within minutes of exposure to 0.9 M NaCl, significantly less fluorescence was observed in VPA-treated cells (Fig. 3.10*A*). The finding that osmotic stress, which normally causes an increase in PI3,5P$_2$ levels in cells, does not cause a similar increase in VPA-treated cells, suggests that VPA compromises the synthesis of PI3,5P$_2$.

Consistent with the association between vacuole size and PI3,5P$_2$ levels, osmotic stress did not induce vacuolar fission in VPA-treated cells (Fig. 3.10*B*). WT cells were exposed to 0.9 M NaCl in the presence or absence of VPA, and the number of vacuoles per cell was monitored (Fig. 3.10*C*). For this purpose, about 100 cells were counted for each condition, and the percentage of cells with 1, 2-3, 4-5, or more than 5 vacuoles were calculated. In the absence of VPA, osmotic stress caused a shift towards a higher number of vacuoles per cell (Fig. 3.10*C*). In contrast, about 85% of VPA-treated cells had
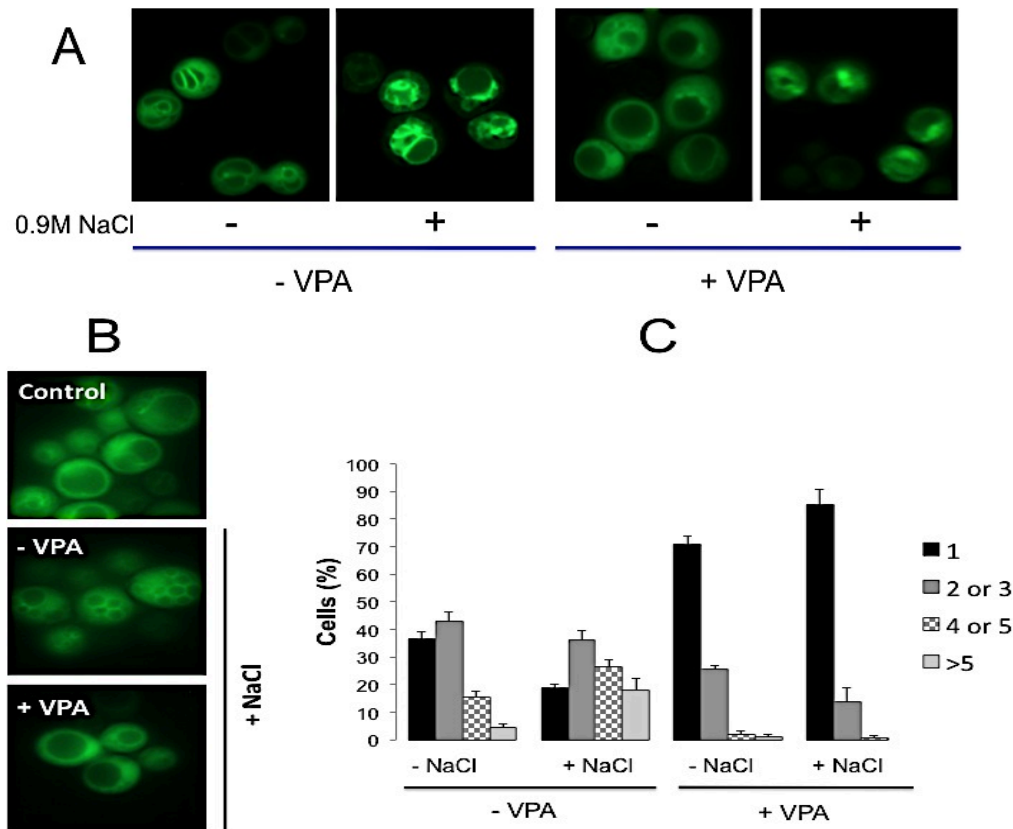
FIGURE 3.10. **Synthesis of PI3,5P$_2$ is perturbed in VPA-treated cells.** *A,* Levels of PI3,5P$_2$ are higher in untreated cells (-VPA) following exposure to osmotic shock. Wild type cells constitutively expressing the GFP-tagged PI3,5P$_2$-binding probe were grown in SM at 30°C to the mid-log phase and treated with 2 mM VPA for one hour. Cells were harvested and exposed to hyperosmotic stress for 5 min in SM containing 0.9 M NaCl. *B,* Vacuolar fragmentation in response to hyperosmotic stress. *C,* Vacuolar morphology of VPA-treated and untreated cells was analyzed and quantified. The number of cells containing 1, 2-3, 4-5, or >5 vacuoles was determined. For each condition, about 100 cells were analyzed. The results are the mean of three independent experiments ± S.E. (*error bars*).

single vacuoles (Fig. 3.10C). This suggested that the inhibition of PI3,5P$_2$ synthesis persists even under osmotic stress. Interestingly, when VPA-treated cells were kept under osmotic stress for about 2 hours, total lysis of cells was observed. This suggests that VPA may cause cell wall defects or perturbation of the cell wall integrity (CWI) pathway, rendering cells sensitive to the increased turgor pressure caused by the osmotic stress response. Alternatively, VPA may inhibit the osmotic stress response pathway. Taken together, these findings suggest that VPA causes a constitutive decrease in PI3,5P$_2$,which cannot be rescued by increased osmotic stress.

To determine if decreased PI3,5P$_2$ could be explained by decreased levels of PI3P (the precursor of PI3,5P$_2$), I visualized the changes in PI3P levels using $fab1\Delta$ cells transformed with a PI3P-specific GFP-tagged FLARE (Burd and Emr, 1998). The $fab1\Delta$ mutant was used because PI3P localizes to the vacuole in the absence of $FAB1$, and changes in levels of PI3P are thus easier to detect. VPA treatment did not cause a decrease in PI3P levels (Fig. 3.11). This observation suggests that the decrease in PI3,5P$_2$ levels may be due to altered regulation of Fab1p or its regulators Vac7p and Vac14p.
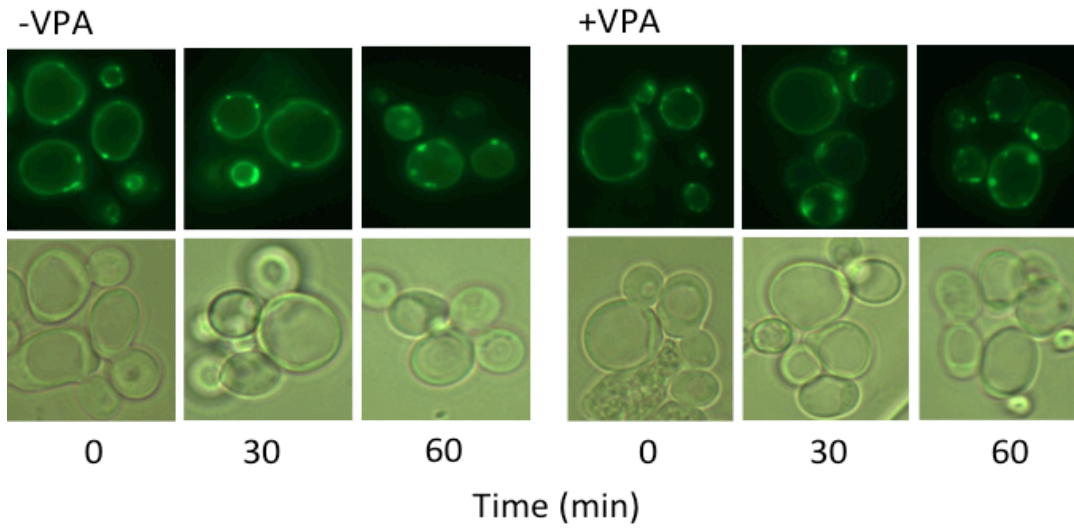
FIGURE 3.11**. Levels of PI3P are not altered by VPA**. Wild type cells constitutively expressing a GFP-tagged PI3P-binding probe (FYVE-GFP) were grown in SM at 30°C to the mid-log phase. VPA was added to a final concentration of 2 mM. Cells were harvested at the indicated times following addition of the drug, and visualized by fluorescence microscopy.

DISCUSSION

The current study shows for the first time that inositol depletion mediated by VPA causes perturbation of the V-ATPase and that VPA compromises $PI3,5P_2$ synthesis. I report the following novel findings: (1) Inositol depletion by VPA or starvation of $ino1\Delta$ cells leads to perturbation of vacuolar structure. (2) Inositol is required for V-ATPase function and coupling. (3) VPA causes partial uncoupling of the V-ATPase. Inositol depletion mediated by VPA causes a decrease in V-ATPase activity and pumping. The pumping defect, but not activity, is rescued by inositol. (4) VPA perturbs $PI3,5P_2$ synthesis, inhibits vacuole fission and leads to vacuolar enlargement. These perturbations are not rescued by osmotic stress which upregulates $PI3,5P_2$ when inositol is present. These findings identify novel consequences of inositol depletion and provide evidence for a previously unidentified link between inositol levels and the V-ATPase.

The findings that inositol starvation perturbs the vacuole (Fig. 3.2), and that inositol supplementation rescues the decreased V-ATPase activity and the decreased coupling observed in inositol-starved cells, indicate that inositol is required for V-ATPase function and coupling. V-ATPase responds to environmental cues. The V_0/V_1 complex disassembles in response to glucose deprivation (Kane, 1995). V-ATPase activity is also modulated by ATP (Arai et al., 1989). This work is the first to show that inositol is capable of modulating V-ATPase activity. One mechanism to explain this is based on the availability of glucose-6-phosphate (G-6-P), a substrate that can drive either the inositol

biosynthesis pathway or the glycolytic pathway. In the absence of exogenous inositol, *de novo* biosynthesis of inositol is activated, using G-6-P as the key substrate for *myo-*inositol 3-phosphate synthase to generate inositol 3-P, which is subsequently dephosphorylated to produce inositol. However, when inositol is supplied in the medium, *de novo* biosynthesis of inositol is repressed, thus more G-6-P is available for the glycolytic pathway. ATP has been shown to modulate the tightness of coupling of V-ATPase activity and proton pumping (Arai et al., 1989). Furthermore, glucose deprivation results in the rapid disassembly of the V-ATPase complex (Kane, 1995). Therefore, it is possible that the availability of inositol ultimately increases V-ATPase coupling as more G-6-P is available for glycolysis and, consequently, more ATP is generated. This may account for the increased coupling in the presence of inositol. The finding that VPA uncouples V-ATPase, and that inositol rescues coupling but not activity (ATP hydrolysis) suggests that the effect of VPA as a consequence of inositol depletion is more pertinent to proton pumping. The observation that activity is not rescued by inositol suggests that VPA may perturb activity of V-ATPase by a mechanism that is independent of inositol, which is beyond the scope of this work.

Loss of function of V-ATPase results in a *vma*⁻ phenotype partially characterized by enlarged, poorly acidified vacuoles, and inability of cells to grow at high pH and in high calcium (Kane, 2006). Cells grown in the presence of VPA show enlarged vacuoles but are able to grow at pH 7.5 and 100 mM calcium, suggesting that VPA compromises the activity of the V-ATPase, but not to the level of complete loss of function. This is consistent with the findings of Oluwatosin and Kane (1998) that yeast cells must lose 70-

75% of V-ATPase function to develop a *vma⁻* growth phenotype (Oluwatosin and Kane, 1998).

It was previously suggested that V-ATPase activity may be modulated by $PI3,5P_2$ (Weisman, 2003; Baars et al., 2007), but no mechanism was proposed to explain this possibility. Recently, Li *et al*. (2014) reported that V-ATPase is stabilized and activated by $PI3,5P_2$ (Li et al., 2014). In support of the role of $PI3,5P_2$, the *fab1Δ* mutant, which cannot synthesize $PI3,5P_2$, has decreased V-ATPase activity (Li et al., 2014) and enlarged vacuoles (Yamamoto et al., 1995), phenotypes similar to those caused by VPA. This suggested that the inositol-containing phosphoinosides in the vacuolar membrane may be altered in response to VPA. I therefore hypothesized that inositol depletion caused by VPA alters the levels of $PI3,5P_2$. One possible consequence of this is perturbation of the binding between $PI3,5P_2$ and V_0 subunits, possibly triggering a conformational change that decreases the efficiency of pumping protons across the vacuolar membrane. Consistent with this possibility, H^+-pumping (carried out by the membrane embedded V_0 sector) was highly dependent on inositol supplementation (Figs. 3.3*B* and3.4*B*).

At least three possibilities may account for this finding. $PI3,5P_2$ may stabilize the structural conformation of V-ATPase by tethering the V_0/V_1 V-ATPase complexes together. Chan et al. (2012) have shown that tethering of the V_0/V_1 complexes is essential for V-ATPase coupling (Chan et al., 2012). Therefore, it is possible that altered levels of $PI3,5P_2$ may partially disturb this tethering and thus cause a slight uncoupling of

the V-ATPase, but not the complete dissociation of the V_0/V_1 complex seen in *fab1Δ* and *vac14Δ* cells (Li et al., 2014). Alternatively, $PI3,5P_2$ may bind or recruit factors required for activation of the V-ATPase. A third possibility is that $PI3,5P_2$ may provide a source of phosphate required for phosphorylation and activation of subunits A and B of the V-ATPase (Makrantoni et al., 2007).

The finding that *vmaΔ* mutants were sensitive to VPA (Fig. 3.6) raised the possibility that VPA may increase the cytosolic pH or cytosolic calcium, conditions that are lethal to the *vma⁻* mutants. However, the pHluorin assay shows that cytosolic pH is not altered by VPA, even when V-ATPase activity is compromised (Fig. 3.7). Additionally, WT cells were able to grow at high pH in the presence of VPA (Fig. 3.5). An increase in cytosolic calcium cannot be ruled out, especially as VPA induces vacuolar fusion within an hour of exposure to the drug (Figs. 3.9*A,B*), a phenomenon that has been associated with increased release of calcium from intracellular stores, including from the vacuole. A recent report by Zhang et al. (2013) showed that VPA induced an increase in cytoplasmic calcium caused by influx from extracellular medium via the Cch1-Yam8 channel complex. However this argues against the increase due to release from endogenous sources.

Increased vacuolar fusion resulting in enlarged vacuoles is a phenotype that is associated with decreased levels of $PI3,5P_2$ (Yamamoto et al., 1995). As mentioned previously, $PI3,5P_2$ comprises less than 0.1% of all phosphoinositides in the cell (Michell et al., 2006). The use of the GFP-tagged FLARES enabled me to monitor changes in the

localization and levels of PI3,5P$_2$ over time and under different conditions. The changes

in membrane dynamics that preceded vacuolar fission were intriguing (Fig. 3.8). The

emergence of PI3,5P$_2$-enriched membranes from the localized dense formations on the

vacuolar membrane suggests that an increase in PI3,5P$_2$ synthesis is a prerequisite for

vacuolar fission, as several small vacuoles could be seen following protrusion of the

PI3,5P$_2$ –packed membranes into the vacuolar space (Fig. 3.8). Zieger and Mayer (2012)

reported similar structures, which they referred to as "membranous structures devoid

of protein." The findings that protrusions did not form in the VPA-treated cells (+VPA)

(Fig. 3.8), and that the vacuoles did not divide, but instead, fused together and enlarged

(Figs. 3.8 and 3.9*A,B*) are consistent with perturbation of PI3,5P$_2$ by VPA, and suggest

that VPA perturbs vacuolar fission. It is tempting to speculate that this observation of

the changes in PI3,5P$_2$ dynamics could identify a novel role for PI3,5P$_2$ whereby a

transient increase in this phosphoinositide initiates vacuole fission, which otherwise

does not occur in mutants that lack PI3,5P$_2$.

The osmotic shock experiments further support perturbation of PI3,5P$_2$

metabolism by VPA. It is well documented that hyperosmotic shock triggers a 20-fold

increase in levels of PI3,5P$_2$ along with an increase in vacuole number (Dove et al., 2002;

Bonangelino et al., 2002). However, the mechanism underlying this response is not

determined (Michell, 2013). Osmotically shocked VPA-treated cells did not show the

same level of increase in PI3,5P$_2$ as the untreated cells (Fig. 3.10*A*). Consistent with this,

osmotic shock did not cause an increase in the number of multivacuoled cells treated

with VPA, in contrast to untreated cells (Fig. 3.10*B,C*). The vacuoles remained enlarged

and did not divide into multiple smaller ones (Fig. 3.10*B,C*). Taken together, these findings suggest that VPA impairs $PI3,5P_2$ production and that this impairment persists even under osmotic stress.

The mechanism whereby VPA causes perturbation of $PI3,5P_2$ synthesis is most likely due to inositol depletion. This is based on the observation that *ino1Δ* cells starved for inositol show the same phenotype as the VPA-treated cells (Fig. 3.8), and that inositol rescues the perturbed V-ATPase pumping. Using the GFP-tagged FLARE for PI3P, I did not observe changes in PI3P levels in VPA treated cells (Fig. 3.11). Fab1p, the kinase that generates $PI3,5P_2$, is activated by Vac7p and Vac14, and inhibited by Atg18p (Dove et al., 2002; Gary et al., 2002; Bonangelino et al., 2002). It is possible that perturbation of $PI3,5P_2$ synthesis by VPA is mediated through upstream regulators of Fab1p.

Potential implications for the therapeutic effect of VPA

The V-ATPase plays an essential role in the acidification and proper functioning of intracellular compartments, including lysosomes, secretory vesicles and synaptic vesicles. The proton gradient generated by the V-ATPase helps in the uploading and storage of neurotransmitters (Schuldiner et al., 1995). Specific inhibition of V-ATPase activity has been shown to block uptake of L-glutamate and γ-amino butyric acid (GABA) into vesicles (Moriyama et al., 1990; Hell et al., 1988). Several neurotransmitter receptor blockers have been shown to dissipate the pH gradient established by the V-ATPase, thereby altering the uptake of dopamine, GABA and glutamate. The V_0 complex of the

V-ATPase has also been shown to be involved in docking and exocytosis (Poea-Guyon et al., 2013). A number of studies indicate that neurotransmission is altered in response to VPA (Cunningham et al., 2003; Martin and Pozo, 2004; Martin and Pozo, 2004; Yoshida et al., 2010). Functional conservation is demonstrated between molecules involved in vesicular trafficking in yeast and synaptic vesicle trafficking in mammalian neurons. This suggests that the mechanistic link between inositol depletion caused by VPA and perturbation of the V-ATPase observed in yeast may be pertinent to altered neurotransmission as depicted in our model (Fig. 3.12).

In conclusion, this study shows that VPA-mediated inositol depletion leads to impairment of V-ATPase function and decreased levels of vacuolar PI3,5P$_2$. I speculate that altered levels of PI3,5P$_2$ in the vacuolar membrane may disrupt the conformational structure of the V$_0$ sector of the V-ATPase, impairing the efficiency V-ATPase function. This is the first demonstration of a link between inositol depletion and the V-ATPase.
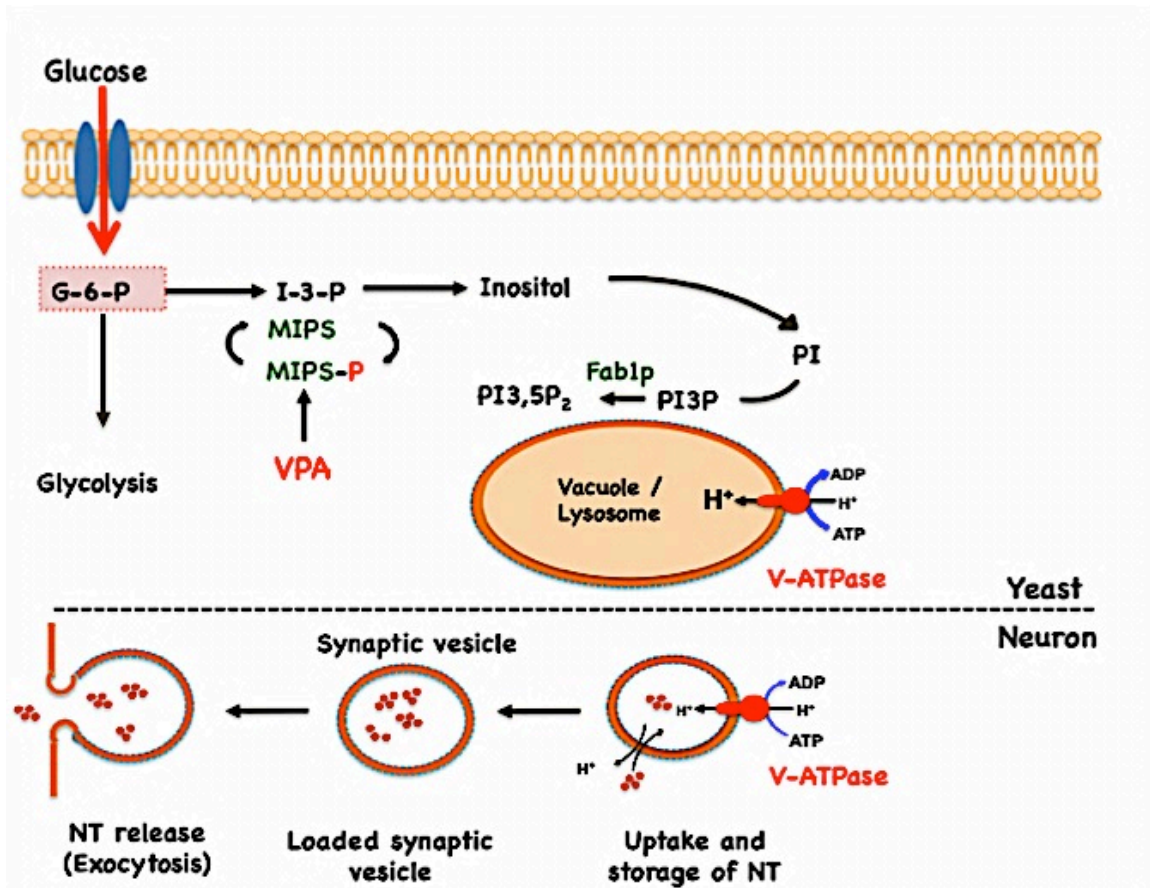
FIGURE 3.12. **Model: Inositol depletion perturbs the vacuolar V-ATPase.** VPA inhibits biosynthesis of inositol, the precursor of PI needed for the synthesis of phosphoinositides, including $PI3,5P_2$ which is required for stabilization of the V-ATPase (Li et al., 2014). The compromised synthesis of $PI3,5P_2$ in response to VPA leads to decreased activity and coupling of the V-ATPase. In neurons, the V-ATPase plays an essential role in the uploading and packaging of neurotransmitters, and in vesicle docking. This suggests that VPA could modulate neurotransmission as a consequence of inhibiting the V-ATPase, suggesting a novel therapeutic mechanism of VPA.

CHAPTER 4

VPA DECREASES ENDOCYTOSIS IN YEAST AND MAMMALIAN CELLS

INTRODUCTION

Bipolar disorder (BD) was first categorized as an illness in 1854 (Angst and Sellarno, 2000). It is a major psychiatric disease that is manifested as recurrent episodes of mania and depression, and was previously referred to as "folie circulaire" or circular madness. According to a recent international study, BD affects approximately 2.4% of the worldwide population (Merikangas et al., 2011), and was ranked by the World Health Organization as the sixth leading cause of disability worldwide. The cause of BD is not known, but a genetic predisposition has been reported (Badner and Gershon, 2002; Merikangas et al., 2002; Glahn et al., 2004). Mitochondrial dysfunction has been suggested by Kato and Kato (2000) as the molecular basis of BD based on impaired energy metabolism in the brain, increased mitochondrial gene deletions, and global reduction of mitochondria-related gene expression. Deficiency of omega-3-fatty acids

from the diet has also been suggested as a contributing factor towards the development of BD (Balanza-Martinez et al., 2011). Neuroimaging studies of BD patients showed structural and functional alterations in specific regions in the brain, including the prefrontal and temporal cortices, cerebellum, basal ganglia and limbic system (Soares and Mann, 1997). A significant decrease in glial cells, neuronal density, and plasticity in the prefrontal cortex has also been observed (Rajkowska, 2002). Perturbation of density and post-translational modification of ion channels including Na^+, K^+ and Ca^{+2} channels, were reported (Jan et al., 2014; Hori et al., 2014; Imbrici et al., 2013). Alterations in neurotransmission regulation involving serotonin, dopamine, γ-aminobutyric acid (GABA), and glutamate have also been implicated in the pathogenesis of BD (Ackenheil, 2001; Shiah and Yatham, 2000; Dager et al., 2004). While many mechanisms have been proposed, the mechanism(s) that contribute to the pathophysiology of BD is/are not known.

Treatment of BD patients often involves combinations of mood stabilizers, antipsychotics, and antidepressants to counteract both mania and depression (Bowden, 2003). Valproate (VPA) has been successfully used as a monotherapeutic agent for the treatment of patients with mania and depressive symptoms (Bowden, 1995; Swann et al., 1997). However, less than 50% of patients respond effectively to this drug (Lewis, 2000). Several side effects are associated with VPA, including hepatotoxcicity, teratogenicity, and weight gain. Furthermore, drug dosage must be continuously

monitored and adjusted, and for treatment to be effective, intake of the drug cannot be interrupted. Thus, there is a clear need for better treatment for BD.

The development of improved drugs is hindered by the fact that neither the pathophysiology of BD, nor the therapeutic mechanism of drugs used for its treatment is understood. Although several targets of VPA have been identified, including *myo*-inositol 3-phosphate synthase (MIPS) (Shaltiel et al., 2004; Ju et al., 2204), histone-deacetylase (HDAC) (Phiel et al., 2001), protein kinase C (PKC) (Chen et al., 1994), and glycogen synthase kinase (GSK3-β) (Chen et al., 1999), the contributions of these targets to the therapeutic effect of VPA have not been elucidated. In the studies summarized in Chapter 3, I identified two new targets of VPA action, the vacuolar V-ATPase and the phosphoinositide PI3,5P$_2$. Because PI3,5P$_2$ is required for stabilization of the V-ATPase complex, this suggested that VPA may perturb a cellular process that involves these two components. One likely possibility is that the drug affects membrane trafficking. The V-ATPase is required for endocytosis at the early endosome level (Recchi et al., 2006), while PI3,5P$_2$ is required for regulating flux through the endocytic pathway and for sorting endocytosed cargo at the multivesicular body (Shaw et al., 2003). Perturbations in the synthesis of either V-ATPase or PI3,5P$_2$ lead to trafficking defects (Perzov et al., 2002; Phelan et al., 2006).

Because of the intermittent cycling between mania and depression, BD has often been associated with altered neurotransmission (Higgs et al., 2006; Knable et al., 2004; Sanacora and Saricicek, 2007). Consistent with this possibility, a number of studies

indicate that neurotransmission is altered in response to VPA (Cunningham et al., 2003 ; Martin and Pozo, 2004; Martin and Pozo 2004; Yoshida et al., 2010). Synaptic inhibition in the mammalian brain is mainly elicited by GABA, the chief inhibitory neurotransmitter in the mammalian central nervous system. By binding to its receptors, GABA induces the uptake of chloride ions, resulting in the inhibitory hyperpolarization of the postsynaptic membrane. The anticonvulsant effect of VPA has been largely attributed to potentiation of the inhibitory GABAergic response (Loscher, 2002). However, whether this is due to altered levels of GABA or modulation of the response to GABA remains controversial. Regulation of cell surface stability of $GABA_A$ receptors is essential for synaptic inhibition. The receptors undergo extensive clathrin-dependent endocytosis as a mechanism for internalization and recycling (Jacob et al., 2008). Altered trafficking of $GABA_A$ receptors is associated with epilepsy and anxiety disorders (Rudolph and Mohler, 2004). Therefore, these receptors are clinically relevant as targets for anticonvulsant and sedative drugs (Jacob et al., 2008). For example, benzodiazepines were found to exert their anticonvulsant effect by binding to these receptors (Jacob et al., 2008). Surprisingly, there are no published studies addressing the effect of VPA on trafficking of $GABA_A$ receptors.

Another indicator that membrane trafficking may be relevant to the therapeutic effect of VPA is the finding that VPA increases size of sensory neuron growth cones (Williams et al., 2002) and promotes neurogenesis and neuroprotection (Yuan et a., 2001; Hao et al., 2004). These findings suggest that VPA may induce processes that promote an increase in cell surface area, such as an increase in synthesis of membrane

phospholipids, or an imbalance between endocytic and exocytic processes. A study by Xu et al. (2007) suggested that VPA reduces endocytic trafficking in *Dictyostelium* through the attenuation of phospholipid signaling caused by a decrease in PI3,4,5P$_3$. Miyatake et al. (2007) showed that, in the fission yeast *Schizosaccharomyces pombe*, VPA inhibits the glycosylation and secretion of acid phosphatase, suggesting that VPA affects membrane trafficking.

The screen described in this chapter was originally done to identify regulators of the enzyme MIPS that, when overexpressed, inhibit the enzyme, thereby causing increased sensitivity to the drug as a consequence of decreased inositol levels. No such regulators were identified. However, the finding that overexpression of trafficking genes increased sensitivity to VPA (this chapter), and the earlier finding that VPA decreased V-ATPase and PI3,5P$_2$ (Chapter 3), both of which play a role in endocytosis, suggested that endocytosis may be perturbed by VPA. The studies described here show that VPA decreases endocytosis in yeast and human cells, and suggest that this decrease is due to perturbation of PI4,5P$_2$ in the plasma membrane.

MATERIALS AND METHODS

Strains, media and growth conditions - The *Saccharomyces cerevisiae* BY4741 wild type strain *MATa his3Δ1, leu2Δ0, met15Δ0, ura3Δ0* was obtained from invitrogen. The GSK3 quadruple mutant, a null mutant for the four GSK3 homologs in yeast, herein referred to as *Δgsk-3*, was a generous gift from Dr. Akira Kikuchi-Hiroshima University School of Medicine (Andoh et al., 2000). Cells were maintained on YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose). Synthetic minimal medium (SM) contained all the essential components of Difco® yeast nitrogen base (minus inositol), 2% glucose, 0.2% ammonium sulfate, vitamins, and the four amino acids histidine (20 mg/L), leucine (60 mg/L), methionine (20 mg/L), and lysine (20 mg/L), and the nucleobase uracil (40 mg/L). Adenine (20 mg/L), was added to media used for growth of *Δgsk-3.* Where indicated, inositol was supplemented at a concentration of 75 µM. For induction of protein expression, 2% galactose and 2% raffinose were substituted for 2% glucose. Appropriate amino acids were omitted for selection of cells carrying plasmids. For solid media, 2% agar was added. Growth in liquid cultures was monitored spectrophotometrically by measuring absorbance at 550 nm. All incubations were at 30°C unless otherwise stated.

Screening the cDNA library – A *GAL1*-regulated yeast cDNA expression library (a generous gift from Dr. Anthony Bretscher) was used to screen for genes whose overexpression increases sensitivity to VPA. For this purpose, BY4741 cells were grown

to the mid-log phase in YPD. Competent cells were prepared and transformed with the cDNA library using the lithium acetate one-step transformation method (Ito et al., 1983). Conditions were optimized to get about 300 colonies per plate. Cells transformed with the empty vector pRS316 were used as a negative control, and the *Δgsk-3,* which is sensitive to VPA, was used as a positive control. Cells were plated onto ura- plates containing 2% galactose and 2% raffinose and incubated at 30°C for 3-4 days. Colonies were counted and then replica plated onto 3 different plates: I+, I-, and I- medium containing VPA (I- /VPA). Colonies that did not grow, or grew poorly in the presence of VPA, were picked from the I+ plate for further confirmation. All the colonies that were picked were purified by streaking to obtain single colonies, from which freezer stocks were prepared. All freezer stocks were reconfirmed for sensitivity to VPA by testing for single colony formation: cells were revived on ura- plates supplemented with inositol, grown to the mid-log phase, and then plated on selective media in the presence or absence of inositol and VPA.

Recovery of cDNA from yeast for gene identification - The cDNA plasmids were extracted from clones that were confirmed for increased sensitivity to VPA. *E. coli* was used to amplify the plasmids, which were then purified and digested to determine the length of the cDNA insert. Uniquely sized cDNAs were sequenced using the *GAL1*-cDNA flanking primer 5'- TGCATAACCACTTTAACT-3'. All sequencing was carried out by ACGT, Inc. The sequences obtained were blasted against the *S. cerevisiae* genome and

identified using the Saccharomyces Genome Database (SGD). Genes were assembled into groups based on functional relevance (Table 4.2).

Fluorescence microscopy analysis - To visualize vacuolar membranes *in vivo*, yeast cells were stained with the lipophilic dye FM4-64 as described (Vida and Emr, 1995) with slight modifications. Cells were grown overnight at 30°C with constant shaking. Cells collected from 1 ml cultures were resuspended in fresh medium containing 10 μM FM4-64. Samples were removed at 5, 30, 60, 120, and 240 min, washed, and resuspended in a small volume of fresh medium with 50 mM PIPES. To immobilize cells, slides were coated with a thin layer of 1% agarose dissolved in SM. Cells were examined using a fluorescence microscope. The plasmids pRS416-GFP-2XPH^{Osh2} and pRS426-GFP-2XPH$^{PLC}\delta^1$ used to localize PI4P and PI4,5P$_2$, respectively, were a kind gift from Dr. Stephen Jesch (Cornell University).

To visualize mammalian cells, U251 glioblastoma cells and HEK293 human embryonic kidney cells (a generous gift from Dr. Mustafa Kandouz) were grown on coverslips in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Cells were treated with 1 mM VPA for 30 min. FM4-64 was then added and cells were incubated on ice for 30 min to allow for incorporation of the dye into the membrane. Following incubation at 37°C, samples were removed at 5, 15, 30, 60, and 120 min. At each time point, cells were placed on ice, washed with phosphate buffered saline (1x) pH 7.4, acid-stripped, washed, and immediately fixed with 4% paraformaldehyde.

Following DAPI-staining, cells were washed, mounted onto slides, and visualized using the fluorescence microscope. For transferrin uptake experiments, U251 and HEK293 cells were serum-starved for 2 h prior to treatment with 1 mM VPA for 30 min. Transferrin-AlexaFluor (20 µg/ml) was added, and cells were incubated at 37°C. Samples (on coverslips) were removed at 5, 15, 30, 60, and 120 min, and immediately placed on ice to arrest endocytosis. Cells were acid-stripped, washed, fixed, stained with DAPI, and visualized using the fluorescence microscope.

Lucifer yellow uptake – Yeast cells were grown to the mid-log phase, harvested, washed with dH$_2$O, and resuspended in medium to which 4 mg/ml of lucifer yellow carbohydrazide (LY-CH) were added. Cells were incubated for 2 h with gentle shaking, harvested, washed 3x in 1x PBS (phosphate buffered saline), and resuspended in 50 µl of the same buffer.

Sensitivity to cell wall perturbing agent - Wild type yeast cells were precultured in SM to the mid-log phase, washed, and spotted in a 10-fold serial dilution onto plates supplemented with the cell wall perturbing agent Calcufluor white (CFW) at a concentration of 50 mM. Plates were incubated at 30°C for 3-4 days.

Immunoblot analysis of the cell wall integrity pathway - Wild type yeast cells were grown in SM at 30°C with or without inositol to the mid-log phase, treated with 2 mM

VPA, and then shifted to 39°C for 1 h. Cells were harvested on ice and immediately stored at -80°C. For protein extraction, cells were resuspended in lysis buffer (0.25M NaOH and 1% BME). Protein was precipitated using 20% TCA, washed twice with ice-cold acetone, and then resuspended in 3M urea, 10% DTT, and 1% SDS. Protein was quantified using the Lowry method (Lowry et al., 1951). A total of 350 µg protein were loaded into each well of a 10% polyacrylamide gel, subjected to electrophoresis and then transferred onto 0.2 µm PVDF membranes. Detection of Phospho-Slt2/Mpk1p was carried out using Rabbit Phospho-p44/42 MAPK (Cell signaling; 1:2000 dilution), and goat anti-rabbit IgG-horseradish peroxidase conjugate (Cell signaling; 1:10000 dilution). Detection was carried out using ECL™ Prime Western Blotting Detection Reagent (Amersham – GE Healthcare).

TABLE 4.1. **Yeast strains and plasmids used in this study.**

Strains	Genotype or Characteristics	Source or Reference
BY4741	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0*	Invitrogen
ino1Δ	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, ino1Δ::KanMX4*	Invitrogen
bck1Δ	*MATa, his 3Δ1, leu 2Δ0, met 15Δ0, ura 3Δ0, bck1Δ::KanMX4*	Invitrogen
Δgsk-3	*MATa his3 leu2 ura3 trp1 mck1::TRP1 mds1::HIS3 mrk1 yol128c::LEU2*	Andoh et al., 2000
pRS316	*CEN6 / ARSH6, URA3*	Liu et al., 1992
pRS316-*GAL1*-cDNA	*Derivative of pRS316*	Liu et al., 1992
pRS416-GFP-2XPH^{Osh2}	*URA3, 2μ, GFP-2XPH^{Osh2}*	Baird et al., 2008
pRS426-GFP 2XPH^{PLC}δ^1	*URA3, 2μ, GFP-2XPH^{PLC}δ^1*	Stefan et al., 2002

RESULTS

Identification of genes that confer sensitivity to VPA when overexpressed:

Wild-type yeast cells were transformed with a *GAL1*-driven yeast cDNA library. A total of about 21,000 transformed yeast colonies were screened for sensitivity to VPA. For this purpose, growth of colonies on selective medium containing 1 mM VPA was compared to that of cells transformed with an empty vector (as a negative control), and to *Δgsk-3* cells that are highly sensitive to VPA (Azab et al., 2007) (as a positive control). In the initial screen, 127 clones showed sensitivity to the drug. All clones were spotted on a master plate for a second round of screening. Clones that showed weaker sensitivity were eliminated, reducing the number to 84 clones that were highly sensitive to VPA. All clones were streaked to obtain single colonies, which were used for subsequent experiments. To make sure that each clone had only one type of cDNA , plasmids were extracted from each yeast clone and amplified in *E. coli*. The amplified plasmids were purified and digested with two restriction enzymes that cut at flanking regions of the cDNA insert. The cDNAs were sequenced, identified, and assembled into groups based on functional similarities (Table 4.2). Three major groups were identified: vesicular trafficking proteins, glycolytic enzymes, and ribosomal proteins. Nine smaller groups were also identified, with genes involved in DNA replication, protein and lipid synthesis, and cell wall proteins. In the current study, I focused on one group, the vesicular trafficking genes. The two other major groups will be discussed in Chapter 5.

TABLE 4.2. **Genes leading to increased sensitivity to VPA when overexpressed**.

Category	Genes	#
Ribosomal proteins	*RPS0B, RPS4A, RPS5, RPS6A, RPS7A, RPS17B, RPS19B, RPS21A, RPS23B, RPS25A, RPP0, RPP2B, RPL2A , RPL2B, RPL3, RPL38, RPL43B, RDN37-2, MRPS35, UTP15, SNR30*	25
Glycolytic enzymes	*PGK1, TDH1, TDH2, **TDH3**, ADH1, PDC1, **ENO2**, TPI1, GPM1, SHB17*	16
Vesicular transport	*VPS21, YAP1801, SEC15, YSC84, VAM3, EGD2, AKL1, SHR3, PIL1*	10
V-ATPase, ATPase related	*VMA2, OLA1, SKP1, PET9, ATP5, SSB2*	7
Transcription and elongation factors	*TAF10, NPL3, EFT1, TEF2, EFB1*	7
Lipid biosynthesis	*NCP1, URA7, PHM8, ADK1*	5
DNA replication, histones	*SIM1, FUN30, HTB1*	3
Amino acid/nucleotide synthesis	*RNR4, CHA1, APT1*	3
Cell wall protein	*CCW12*	2
G-protein regulators	*PLP1, YRB1*	2
Mitochondrial morphology	*PCP1*	1
Heat shock proteins	*HSP104*	1

Underlined genes: identified twice. Underlined and bold: identified tree times.

VPA decreases endocytosis in yeast cells:

Membrane trafficking is a conserved process that is highly regulated. Overexpression of proteins that negatively regulate trafficking is expected to increase the sensitivity of cells, particularly if this process is already compromised by VPA. Nine genes that encode vesicular trafficking proteins were identified in the cDNA screen (Table 4.2). Interestingly, seven of these genes are associated with endocytosis, including *VPS21, AKL1, SHR3, YSC84, VAM3, PIL1,* and *YAP180-1.*

One of these genes, *AKL1,* codes for a Ser-Thr kinase that negatively regulates endocytosis by phosphorylating Pan1, a protein that forms part of an endocytic complex that regulates the internalization step of endocytosis (Takahashi et al., 2006). Overexpression of *AKL1* is expected to perturb endocytosis. I monitored the uptake of lucifer yellow, a fluid phase endocytic marker, in wild-type yeast cells carrying either the empty vector, or the vector with the *AKL1* gene (Fig. 4.1). Although the dye was initially internalized in cells overexpressing *AKL1,* it accumulated in prevacuolar compartments, unlike in cells carrying the empty vector. Interestingly, the same pattern was observed in VPA-treated cells (Fig. 4.1) suggesting that VPA perturbs trafficking in a manner similar to that caused by overexpression of *AKL1*.

Another approach to determine if VPA affects endocytosis is to monitor the uptake of the lipophilic fluorescent dye FM4-64, which is routinely used as a marker for endocytic trafficking (Vida and Emr, 1995; Perzov et al., 2002). Yeast cells were treated with VPA for 1 h before the addition of FM4-64. Uptake of the dye was observed over a period of 4 hours. In untreated cells, the dye reached the vacuoles in the first hour (Fig.
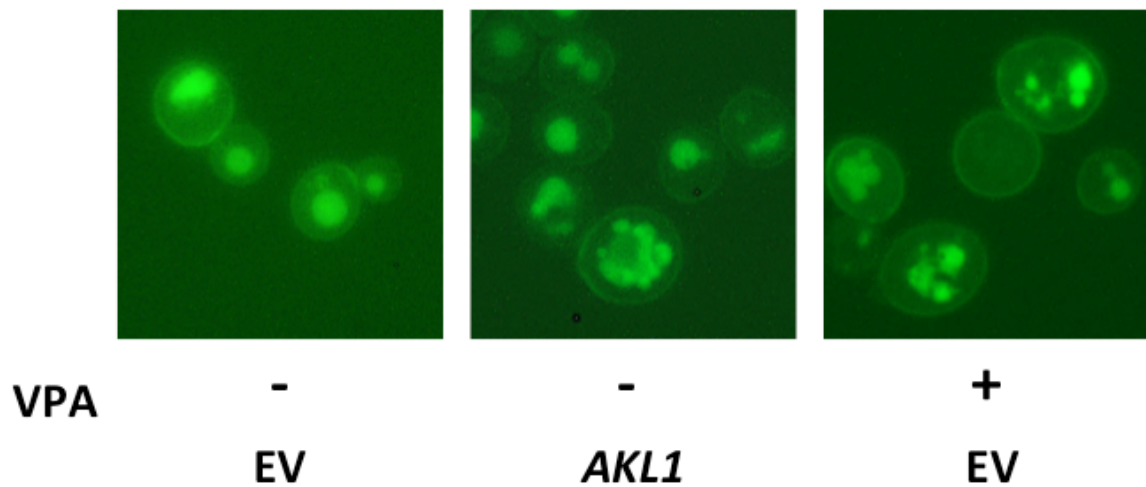
FIGURE 4.1. **VPA treatment phenocopies overexpression of *AKL1*.** Wild-type yeast cells containing an empty vector pRS316 (EV) or the vector expressing *AKL1* were grown to the mid-log phase in SM, treated with 2 mM VPA, and stained with the endocytic marker lucifer yellow, as described in Materials and Methods. All images were taken at the same magnification (1000X).
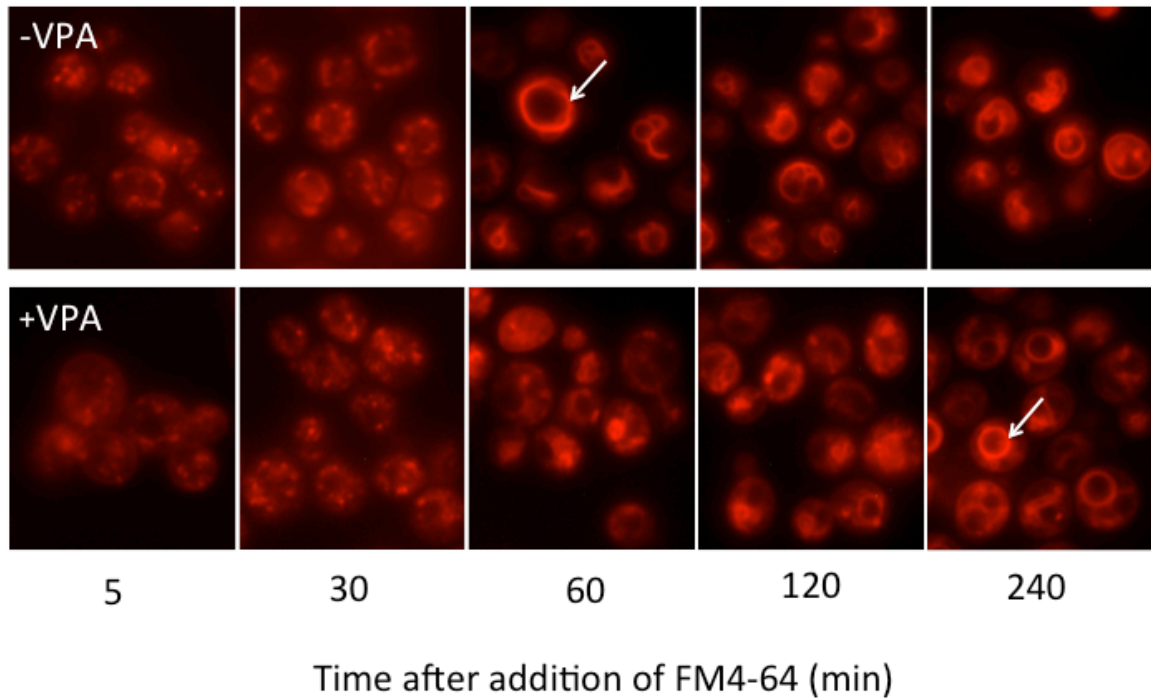
FIGURE 4.2. **Endocytosis is decreased in VPA-treated cells.** Wild-type yeast cells were grown to the mid-log phase in SM, treated with 2 mM VPA, and stained with FM4-64, as described in Materials and Methods. To monitor internalization and uptake of the dye, samples were removed at the indicated times following addition of the dye. All images were taken at the same magnification (1000X).

4.2). However, in VPA-treated cells, the FM4-64 dye was not apparent in vacuolar membranes until the 4th hour, suggesting that VPA treatment markedly slows the endocytic process in yeast cells.

VPA compromises PI4,5P$_2$ levels:

VPA causes a decrease in intracellular inositol (Vaden et al., 2001), which is required for the synthesis of phosphoinositides. The phosphoinositide PI4,5P$_2$, which is abundant in the plasma membrane (Thorner and Strahl, 2007), binds and controls more than 30 membrane proteins that regulate endocytosis, either by binding to the actin cytoskeleton, or by regulating other proteins of the endocytosis machinery (Smythe and Ayscough, 2006; deHart et al., 2003). Thus perturbation of PI4,5P$_2$ levels may destabilize these proteins. This led me to hypothesize that VPA causes a decrease in PI4,5P$_2$ synthesis thereby causing a decrease in endocytosis. The presence of a large pool of PI4,5P$_2$ in the plasma membrane makes it difficult to detect changes in newly synthesized PI4,5P$_2$ (King et al., 2009). Therefore, I used the indirect approach of monitoring levels of PI4P, the sole precursor of PI4,5P$_2$ in yeast (Thorner and Strahl, 2007), as a marker for changes in PI4,5P$_2$ synthesis in response to VPA. PI4P is delivered by Golgi vesicles to the plasma membrane, where it is phosphorylated by the PI4P 5-kinase Mss4 to generate PI4,5P$_2$ (Homma et al., 1998). As Fig. 4.3 shows, the fluorescent punctae representing the GFP-tagged PI4P-specific FLAREs carried by Golgi vesicles are absent from VPA treated-cells grown in the absence of inositol, suggesting that in the

I+   **I-**   **I-**   **I+**

Control   **+ 2mM VPA**
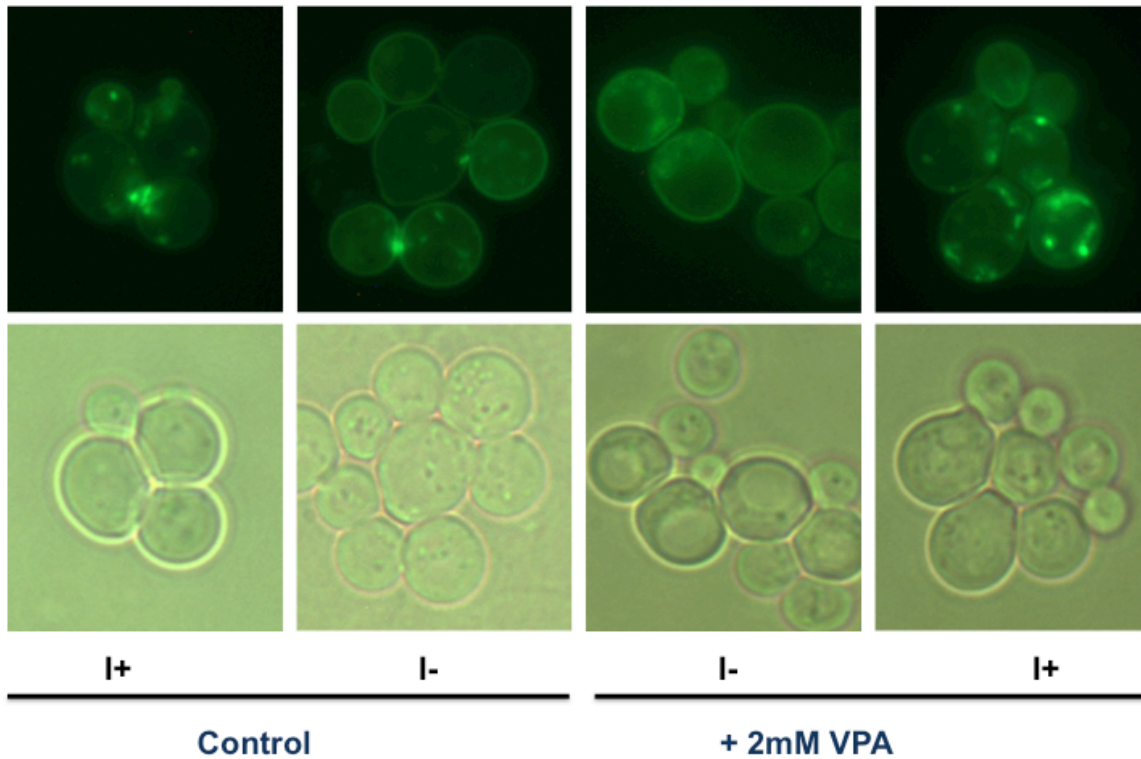
FIGURE 4.3. **VPA alters the trafficking of PI4P.** Wild type cells constitutively expressing a GFP-tagged PI4P-binding probe were grown in SM at 30°C to the mid-log phase. VPA was added to a final concentration of 2 mM. Cells were visualized by fluorescence microscopy.

absence of sufficient precursor, levels of $PI4,5P_2$ are compromised and may, thus, cause destabilization of membrane proteins that regulate endocytosis.

VPA inhibits processes regulated by Rho1, a protein that is dependent on $PI4,5P_2$:

One of the proteins that are activated and stabilized by $PI4,5P_2$ in the plasma membrane is the small G protein, Rho1 (Levin, 2011). Rho1 activates a wide array of targets that regulate several processes, including organization of the actin cytoskeleton, β-glucan synthesis, gene expression, exocytosis, and PKC-cell wall integrity (CWI) signaling (Levin 2011). The actin cytoskeleton is a major regulator of endocytosis and membrane trafficking in yeast (Smythe and Ayscough, 2006). Thus, decreased $PI4,5P_2$ levels are expected to destabilize Rho1 and thereby disturb the organization of the cytoskeleton required for the endocytic process. In the same manner, other processes that are regulated by Rho1 are expected to be perturbed.

β-1,3-glucan, the main structural component of the yeast cell wall, is synthesized by a glucan synthase complex, the regulatory subunit of which is Rho1 (Drgonova et al., 1996; Qadota et al., 1996; Mazur and Baginsky, 1996). Cells grown in the presence of the cell wall perturbing agent CFW exhibited marked sensitivity to VPA (Fig. 4.4*A*), suggesting that VPA causes defects in cell wall biosynthesis. This is consistent with perturbation of β-1,3-glucan synthesis, which is regulated by Rho1. The observation that inositol rescues this defect supports the hypothesis that decreased $PI4,5P_2$ may destabilize Rho1.
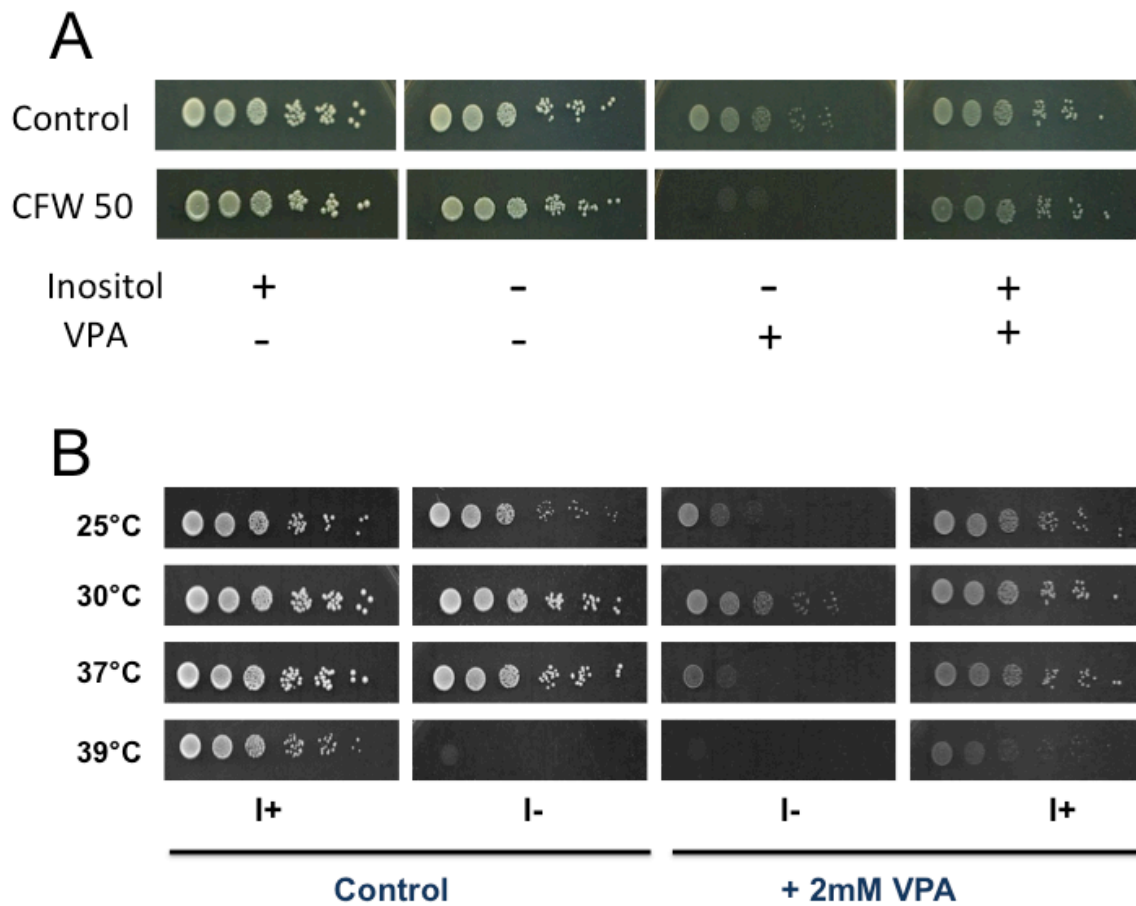
FIGURE 4.4. **VPA causes cell wall defects and increases thermosensitivity.** Wild type yeast cells were grown to the mid-log phase and spotted in ten-fold serial dilutions on *A*, plates supplemented with 50 mM Calcufluor white and incubated at 30°C, or *B*, SM plates incubated at the indicated temperatures for 3-4 days.

The PKC-CWI pathway is also regulated by the $PI4,5P_2$-dependent Rho1 (Fig. 4.5*A*). It is employed by yeast cells to protect cells in response to environmental stresses, including heat stress (Levin, 2011), hypo-osmotic stress (Davenport et al., 1995) and cell wall biogenesis mutations (De Noble et al., 2000). VPA-treated cells showed thermosensitivity at 37°C, and growth was completely abolished at 39°C (Fig.4.4*B*), suggesting that in the presence of the drug, cells are unable to respond to heat stress.

To investigate if temperature sensitivity is due to perturbation of the CWI pathway, cells were grown at 30°C, treated with VPA, and then transferred to 39°C for 1 h, a condition that induces CWI signaling. Activation of the CWI pathway is typically monitored by phosphorylation of the Slt2/Mpk1 MAP kinase (de Noble et al., 2000). As shown in Fig. 4.5*B*, at 39°C, in the absence of inositol, untreated cells showed a high level of phosphorylation of Slt2 indicating activation of the CWI pathway. However, phosphorylated Slt2 was not observed in VPA-treated cells. Supplementation with inositol moderately rescued this effect (Fig. 4.5*B*). These results indicate that VPA inhibits activation of the CWI pathway at elevated temperatures as a consequence of inositol depletion. Taken together, these data demonstrate that three processes regulated by the $PI4,5P_2$-dependent Rho1, are disrupted by VPA, including endocytosis, cell wall biosynthesis, and CWI signaling. This suggests that by decreasing $PI4,5P_2$ levels, VPA may destabilize membrane proteins, including Rho1. In addition to the observed decrease in PI4P (Fig. 4.3), this provides further supportive evidence that
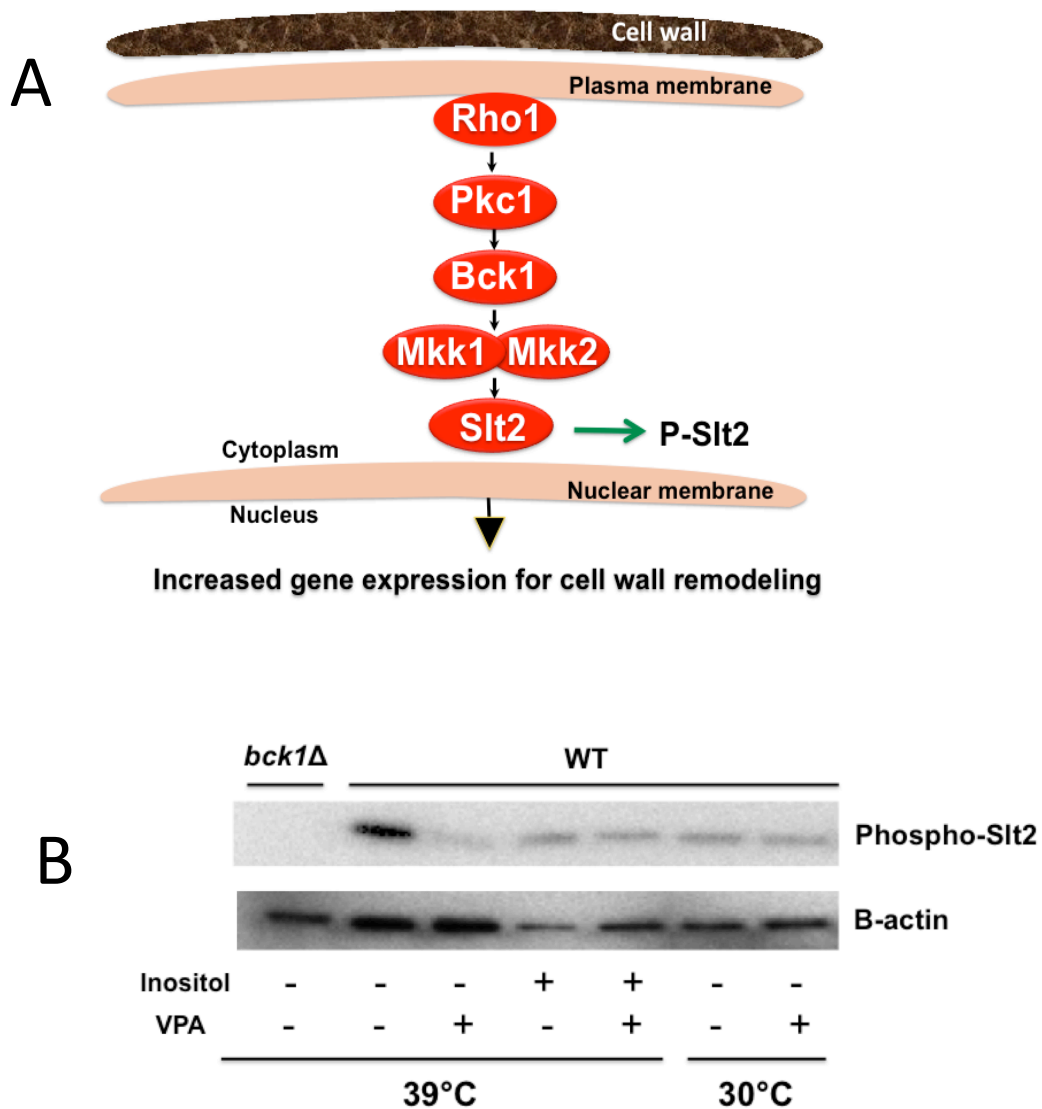
FIGURE 4.5   **VPA perturbs the cell wall integrity pathway.** *A,* Signaling cascade of the

CWI pathway. *B,* Wild type yeast cells were precultured in SM at 30°C in the presence or

absence of inositol and treated with VPA for 1 h. Cells were then transferred to 39°C for

1 h. Total protein extracts were fractionated by SDS-PAGE and transferred to PVDF

membranes. Phosphorylated Slt2 was detected using rabbit Phospho-p44/42 MAPK, and

B-actin was used as a loading control.

decreased endocytosis in VPA-treated cells may be due to decreased $PI4,5P_2$ in the plasma membrane.

VPA decreases constitutive endocytosis in mammalian cells:

To determine if perturbation of endocytosis by VPA is conserved in mammalian cells, incorporation of FM4-64 was observed in glioblastoma cells treated with VPA. In mammalian cells, this dye is taken up by endocytic vesicles and accumulates in endosomes (Henkel et al., 1996). As shown in Fig. 4.6, the untreated cells show clear punctae indicative of accumulation of the dye in vesicles. However, the rate of incorporation was significantly less in VPA-treated cells, as labeling was diffuse even after 120 min.

Internalization of transferrin is decreased in VPA-treated cells:

Clathrin-mediated endocytosis (CME) is the mechanism responsible for internalization of many cell surface receptors, including the $GABA_A$ receptors (Kittler et al., 2000). These receptors have been implicated in the therapeutic mechanism of anticonvulsants (Jacob et al., 2008). To explore if VPA perturbs CME, I examined the effect of VPA on internalization of Alexa-fluor-conjugated transferrin, a marker that is routinely used for detection of CME (Le Roy and Wrana, 2005). As shown in Fig. 4.7, at 15 min, decreased accumulation of endocytic vesicles was observed in VPA-treated cells, suggesting that VPA may decrease the internalization of the transferrin receptors. Following internalization, transferrin remains bound to its receptor and is then recycled back to

the surface, where it is released into the medium. The disappearance of the dye from the perinuclear region in untreated cells (after 90 min) was not detected in VPA-treated cells. This suggested that cells treated with VPA delay recycling of transferrin.
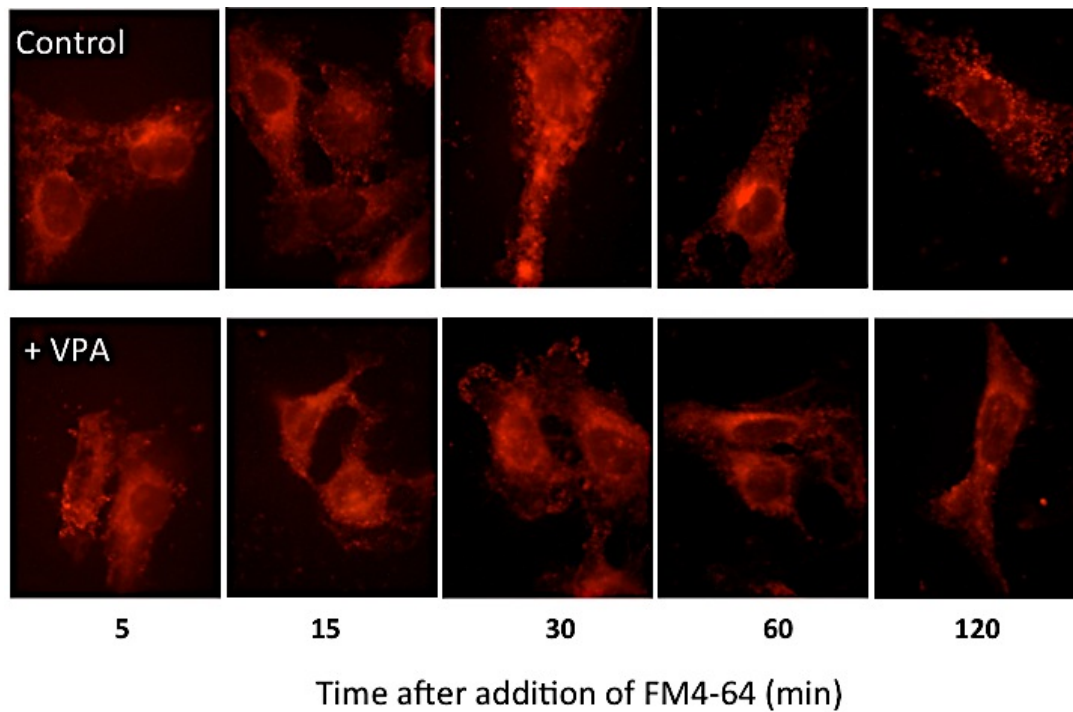
FIGURE 4.6. **Endocytosis is decreased in VPA-treated glioblastoma cells.** Glioblastoma

U251 cells were grown in DMEM with 10% FBS. Serum-starved cells were treated with 1

mM VPA and the endocytic marker FM4-64 was added, as described in Materials and

Methods. To monitor internalization and uptake of the dye, samples were removed at

the indicated times following addition of the dye. All images were taken at the same

magnification (1000X).

Control

VPA

| 5 | 15 | 30 | 45 | 90 |

Time after addition of Transferrin (min)
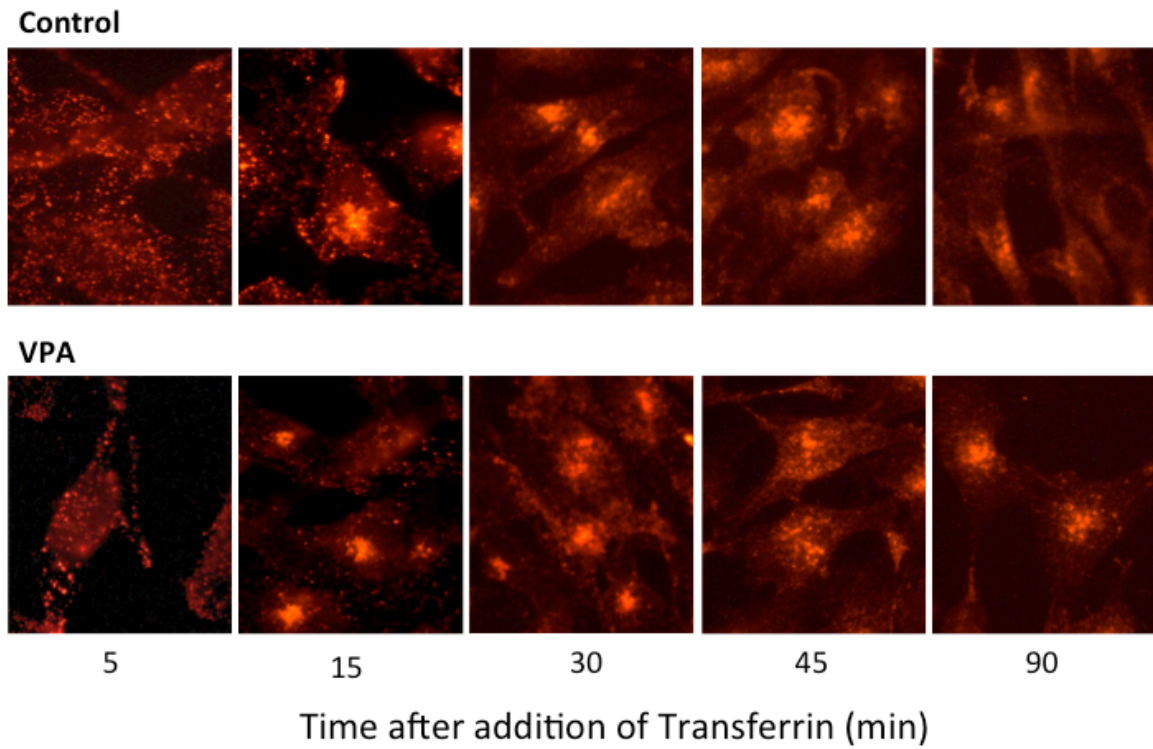
FIGURE 4.7. **Internalization of transferrin is decreased in VPA-treated glioblastoma cells.** Glioblastoma U251 cells were grown in DMEM with 10% FBS. Serum-starved cells were treated with 1 mM VPA. Uptake of transferrin was monitored at the indicated times. All images were taken at the same magnification (1000X).

DISCUSSION

This study was carried out to identify novel pathways or processes that are altered in response to VPA-treatment and may, thus, underlie the therapeutic effect of the drug. The majority of the genes identified in the cDNA screen fell into three major categories: ribosomal proteins, glycolytic enzymes, and proteins involved in vesicular trafficking (Table 4.2). I focused on trafficking as my previous studies showed that VPA perturbs the vacuole by decreasing V-ATPase activity and perturbing $PI3,5P_2$ levels, both of which are involved in endocytic trafficking. This led me to hypothesize that VPA perturbs endocytosis. The following are my findings: 1) VPA decreases endocytosis in yeast; evidence suggests that decreased endocytosis is due to perturbed $PI4,5P_2$ levels in the plasma membrane. 2) Perturbation of endocytosis by VPA is conserved in mammalian cells.

The observations that VPA causes a phenotype similar to that resulting from overexpression of *AKL1,* which perturbs endocytic trafficking (Fig. 4.1), and that FM4-64 trafficking to the vacuolar membrane is delayed in VPA-treated cells (Fig. 4.2), suggest that VPA delays but does not completely inhibit endocytosis. A possible mechanism whereby VPA affects endocytic trafficking is by decreasing levels of $PI4,5P_2$, which activates and stabilizes membrane proteins that regulate endocytosis (DeHart et al., 2007). $PI4,5P_2$-dependent membrane proteins anchor the actin cytoskeleton, which is a major regulator of membrane trafficking in yeast, and also, recruit other proteins required for the endocytic process (Smythe and Ayscough, 2006; DeHart et al., 2007).

Temperature-sensitive *mss4* mutants were shown to be defective in receptor internalization (Derevieres et al., 2002), indicating that PI4,5P$_2$ is required for endocytic trafficking.

King et al. (2009) have shown that the presence of a large PI4,5P$_2$ pool in the plasma membranes, and the dynamic changes associated with its role as a precursor for the signaling molecules IP$_3$ and DAG, obscure detection of changes in levels of this phosphoinositide. This is in agreement with a study I have done using a GFP-tagged FLARE that specifically binds PI4,5P$_2$, where the high intensity of fluorescence observed did not reveal differences in PI4,5P$_2$ levels in plasma membranes of VPA-treated and untreated cells (data not shown). Therefore, I used two indirect approaches to determine if VPA alters PI4,5P$_2$ levels; (i) visualization of PI4P, the precursor of PI4,5P$_2$, and (ii) assays of PI4,5P$_2$-dependent functions.

Studies by Pawolleck and Williams (2009) and Xu et al. (2007) have shown that VPA causes a decrease in PI4P and PI4,5P$_2$, an effect that was phenocopied by attenuation of phosphoinositide signaling in *Dictyostelium*. Furthermore, VPA was shown to decrease PI4P and PI4,5P$_2$ levels in a dose- and time-dependent manner (Chang et al., 2011). These reports are consistent with my finding that VPA causes a decrease in PI4P (Fig. 4.3) and support my conclusion that PI4,5P$_2$ is decreased in response to VPA treatment of yeast cells.

The rationale for the second approach, assay of PI4,5P$_2$-dependent functions, was that if decreased endocytosis caused by VPA is due to decreased PI4,5P$_2$, then other processes that are dependent on PI4,5P$_2$, would be disrupted in the presence of VPA.

Rho1, the functional ortholog of the mammalian RhoA (Qadota et al., 1994), is a small GTPase that regulates several processes, including actin organization, CWI, and β-glucan synthesis through independent effectors. It resides in the plasma membrane where it is activated and stabilized by binding to $PI4,5P_2$ via a polybasic domain (Yoshida et al., 2009). *mss4* mutants, which are defective in $PI4,5P_2$ production, have been shown to display defects in actin organization and CWI at elevated temperatures (Desrevieres et al., 1998), thus, confirming the importance of $PI4,5P_2$ for Rho1 activity.

Three processes regulated by Rho1 were perturbed in response to VPA. This suggests that VPA perturbs Rho1 activity, either by inhibiting Rho1 directly, or by perturbing $PI4,5P_2$ which is required for activation and stabilization of Rho1. The observation that inositol supplementation rescued the cell wall defect (Fig, 4.4*A*) and the temperature sensitivity of VPA-treated cells (Fig. 4.4*B*) suggests that observed perturbations caused by VPA are inositol dependent, and likely due to a decrease in $PI4,5P_2$.

The PKC-CWI pathway protects the cell from environmental stress that could damage the protective cell wall, such as heat stress (Kamada et al., 2005) and hypo-osmotic shock (Davenport et al., 1995). Yeast cells grown in I- medium at 30°C and shifted to 39°C showed increased levels of phosphorylated Slt2 (Fig. 4.5*B*), which indicates activation of CWI. However, under the same conditions, VPA-treated cells did not show an equivalent increase in P-Slt2, suggesting that VPA inhibits activation of the CWI pathway. Interestingly, heat shock activates the PI4P 5-kinase Mss4, causing an

increase in $PI4,5P_2$ (Desrivieres et al., 1998; Aduhya and Emr, 2002). It has been suggested that this increase is required for activation of the CWI pathway at high temperatures (Levin, 2011). The finding that VPA inhibits activation of CWI at 39°C, and that inositol supplementation rescues this effect (Fig. 4.5), suggests that attenuation of CWI by VPA is due to perturbed synthesis of $PI4,5P_2$. Another significance of this result is that attenuation of CWI signaling by VPA signifies that VPA inhibits PKC. Although VPA has been shown to inhibit mammalian PKC in cell extracts (Chen et al., 2004), the effect of VPA on PKC in yeast has not been reported before, and may be of importance to regulation of inositol biosynthesis, as discussed in Chapter 3.

Taken together, the results presented here suggest that VPA-mediated perturbation of cell wall biosynthesis, the CWI pathway, and endocytosis are due to decreased $PI4,5P_2$ synthesis. Although these findings are indirect, two different approaches support the conclusion that the decrease in $PI4,5P_2$ may underlie the mechanism whereby VPA causes decreased endocytosis. Nevertheless, the possibility remains that other mechanisms may be involved as well (Fig. 4.8).

Perturbation of V-ATPase and $PI3,5P_2$ by VPA (Chapter 3) is consistent with decreased endocytosis shown here. V-ATPase null mutants have a markedly decreased rate of endocytosis (Perzov et al., 2002). It is possible that reduced V-ATPase activity in response to VPA results in decreased lumenal acidification of vesicles. This may affect membrane recruitment of components required for the endocytic machinery. Furthermore, $PI3,5P_2$ is required for regulating flux through the endocytic pathway and

for sorting endocytosed cargo at the multivesicular body (Shaw et al., 2003). It is possible that more than one mechanism may underlie perturbation of endocytosis caused by VPA.

How might decreased endocytosis relate to the therapeutic effect of VPA? The mechanism that underlies the GABAergic effect caused by VPA is a subject of controversy. An increase in GABA levels following VPA treatment has been observed in cultured mammalian cells, human plasma, and rat brains (MacDonald et al., 1979; Loscher and Schmidt, 1980; Patsalos et al., 1981). The mechanism that underlies the increase in GABA levels in response to VPA is not known. Several mechanisms have been proposed. For example, VPA was shown to elevate GABA levels by increasing the availability of the GABA precursor, α-ketoglutarate, through the inactivation of α-ketoglutarate dehydrogenase (Luder et al, 1990). Decreased breakdown of GABA by the two catabolic enzymes, GABA transaminase and succinate semialdehyde dehydrogenase (SSD), was also reported (Loscher, 1993; Whittle and Turner, 1978). Modulation of the post-synaptic response has also been suggested as a possible mechanism for the VPA-induced GABAergic effect. Cunningham et al. (2003) showed that VPA prolongs the post-synaptic inhibitory response by binding to the regulatory site of the GABA receptors on the postsynaptic membrane. Thus, there is no consensus as to how VPA elicits its GABAergic inhibitory effect.

In glioblastoma cells, VPA decreased the incorporation of FM4-64 into vesicles (Fig. 4. 6). It also decreased the internalization and recycling of transferrin (Fig. 4. 7).

This suggests that VPA decreases clathrin-dependent endocytosis, the same mechanism responsible for internalization of $GABA_A$ receptors. Therefore, it is tempting to speculate that VPA may decrease internalization of $GABA_A$ receptors, prolonging their exposure to the inhibitory effect of GABA present in the synapse. In support of this speculation, phosphorylation of the $GABA_A$ receptors by PKC and GSK-β is required for internalization and trafficking of these receptors (Tyagarajan et al., 2011; Brandon et al., 2000; Clayton et al., 2010). Interestingly, both kinases are inhibited by VPA (Chen et al., 1994; Chen et al., 1999; Kim et al., 2005), suggesting that inhibition of PKC and GSK-β by VPA may be another mechanism whereby VPA inhibits endocytosis.

In conclusion, the aim of this study was to identify cellular functions that are affected by VPA. Here I show that VPA perturbs endocytosis, and that this perturbation is conserved in yeast and human cells. Decreased endocytosis by VPA may be due to: 1) decreased V-ATPase activity and $PI3,5P_2$ levels, 2) decreased $PI4,5P_2$ levels, or 3) inhibition of PKC and GSK-β. While several mechanisms have been suggested to explain the GABAergic effect caused by VPA, none have addressed the trafficking of $GABA_A$ receptors. I propose decreased endocytosis as a novel effect of VPA that may underlie its anticonvulsant effect.
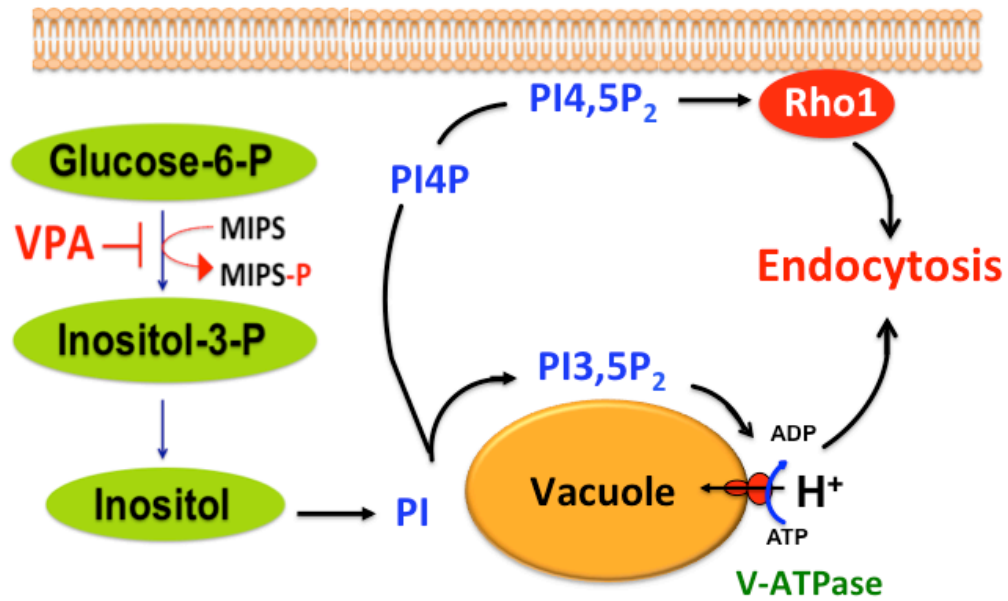
FIGURE 4.8. **Model: VPA causes a decrease in endocytosis**. VPA inhibits biosynthesis of inositol, the precursor of phosphatidylinositol (PI) required for the synthesis of the two phosphoinositides PI3,5P$_2$ and PI4,5P$_2$. Two mechanisms that may underlie the decrease in endocytosis observed in VPA-treated yeast cells are perturbation of PI3,5P$_2$ which decreases V-ATPase coupling, and perturbation of PI4,5P$_2$ required for stabilization of membrane proteins that regulate endocytosis.

CHAPTER 5

FUTURE DIRECTIONS

The studies described in this dissertation show novel findings pertaining to regulation of inositol biosynthesis and cellular consequences of inositol depletion. These findings will, hopefully, contribute to a better understanding of the therapeutic mechanism of action of the inositol-depleting drug, VPA. Although my studies have answered some questions raised by those who embarked on this project before me, I now raise new questions based on the work I have done, and hope that they enthuse future students to continue with this exciting project. Highlighted in this chapter are some unanswered questions that require continued investigation.

Kinases that regulate *myo* inositol 3-phosphate synthase (MIPS):

The inositol biosynthesis pathway is highly conserved, and is regulated at the level of the first and rate-limiting enzyme, MIPS. In Chapter 2, I showed that MIPS is postranslationally regulated, and that at least three phosphosites that are conserved in yeast and human MIPS play a role in its regulation. Phosphosite prediction analysis showed that the two inhibitory phosphosites, S184 and S374, lie within recognition sequences for PKA and PKC, respectively, while S296, is within a recognition sequence for GSK3. Preliminary experiments showed that both PKC and PKA decreased MIPS activity *in vitro*, while GSK3 increased activity (data not shown). While these results

seem to correlate with the predictions, further experiments are necessary to elucidate the role of these kinases in regulating MIPS. *In vitro* experiments using purified and dephosphorylated MIPS should clearly show the effect of each kinase on phosphorylation and activity of MIPS. The use of the site mutants as controls would greatly facilitate these studies.

Another question of fundamental importance to the mechanism of VPA is, which of the identified residues (if any) are phosphorylated in response to VPA? MIPS purified from VPA-treated cells shows a higher level of phosphorylation than enzyme from untreated cells. Answering this question, would be crucial to demonstrate the mechanism by which VPA inhibits MIPS and which is targeted by VPA. Identification of the kinase may help identify upstream regulators of MIPS that may be directly affected by VPA.

In a previous study, Ju and Greenberg (2003) showed that VPA causes an increase in the transcriptional level of *INO1*, the gene coding for MIPS, concomitant with a decrease in inositol biosynthesis. This indicates that inositol depletion does not result from perturbation of *INO1* transcription. The current study shows that VPA increases MIPS phosphorylation. It would be interesting to determine if there is a direct correlation between increased phosphorylation and decreased inositol levels in response to VPA. What is the time frame during which the protein is modified by phosphorylation? These studies were previously hindered by the lack of available antibodies that detect MIPS. Now that the phosphosites have been identified, antibodies could be designed that specifically recognize phosphorylated MIPS.

Regulation of human MIPS – Surprisingly, not much is known about regulation of human MIPS. In Chapter 2, I showed that the three regulatory phosphosites initially identified in yeast MIPS, are conserved in the human homolog. Shaltiel et al. (2004 and 2007) showed that VPA decreased inositol biosynthesis in human prefrontal cortex by inhibiting MIPS. Thus, it is likely that, similar to what we observed in yeast, VPA may decrease human MIPS activity by increasing its phosphorylation. Although we have shown that VPA increases the phosphorylation of yeast MIPS, similar experiments were not done with the human enzyme. It is particularly important to determine if the kinases PKC and GSK3 regulate human MIPS, because although earlier studies have shown that VPA inhibits mammalian PKC and GSK3 (Chen at al. 1994; Chen et al., 1999), no connection has been made between inositol depletion and the inhibition of these two enzymes. Elucidating the effect of PKC and GSK3 on human MIPS would be of direct relevance to the mechanism by which VPA causes inositol depletion in human.

VPA as a tool to uncover regulation of Fab1:

In Chapter 3, I showed that VPA inhibits the $PI3,5P_2$-dependent osmotic stress response. Cells respond to hyperosmotic stress by activating two pathways - the high osmolarity glycerol (Hog1) pathway, which initiates a long term response, and the $PI3,5P_2$-dependent immediate, short term response (Bonangelino et al., 2002). The latter leads to a 20-fold increase in $PI3,5P_2$, which triggers an increase in the number of small vacuoles, thereby increasing total vacuole membrane surface area.   The finding that VPA blocks the rapid increase in $PI3,5P_2$ suggests that VPA may perturb activation of

Fab1, the kinase that generates $PI3,5P_2$ from PI3P. Fab1 is regulated by two activators, Vac7 and Vac14, which form part of a multimeric complex on the vacuolar membrane. There are no published studies describing how these two activators respond to stress. Are they activated by phosphorylation, for example by the Hog1 kinase? Is it a coincidence that both pathways are activated at the same time in response to osmotic stress? Does VPA perturb activation of Vac7 and Vac14, thus negatively affecting Fab1 and, hence, preventing an increase in $PI3,5P_2$? One approach to address these questions is to determine if the Fab1 pathway is regulated by the Hog1 kinase, in which case the pathway would be altered by deleting or overexpressing *HOG1*. A complementary approach would be to ascertain if Vac7 and Vac14 are phosphoproteins, and if VPA affects their activity by altering phosphorylation of these proteins.

While the HOG1 stress response pathway has been extensively studied, not much is known about the osmotic stress response that depends on $PI3,5P_2$. This is a very promising area because it is becoming increasingly evident that $PI3,5P_2$ has many more functions than previously thought (Ho, 2012). Answering the questions addressed here would not only identify the mechanism by which VPA inhibits the $PI3,5P_2$-dependent response to osmotic stress, but may also uncover a novel regulatory mechanism of $PI3,5P_2$ synthesis.

Does VPA cause temperature sensitivity as a consequence of altered plasma membrane lipid composition?

In Chapter 4, I showed that VPA inhibits growth of cells at elevated temperatures. The response of *Scaccharomyces cerevisiae* to heat stress involves two phases. Initially, growth is arrested for about an hour (Shin et al., 1987), during which cells gain thermotolerance. This involves an increase in trehalose accumulation and activation of heat shock proteins (Weimken, 1990; Lindquist and Craig, 1988). In the second phase, degradation of trehalose takes place, and cells resume growth (Hottiger et al., 1992). The heat stress response is also characterized by a dramatic, transient increase in sphingolipids and ceramides within the first 15 min and 1 hr, respectively (Jenkins et al., 1997; Chen et al., 2013). In two separate studies, Desrevieres et al. (1998) and Audhya and Emr (2002) showed that heat shock causes an increase in $PI4,5P_2$, which is predominantly found in the plasma membrane. Interestingly, the synthesis of the three lipids, sphingolipids, ceramides, and $PI4,5P_2$, is perturbed in inositol limiting-conditions (Henry et al., 2014). Thus, it is likely that thermosensitivity caused by VPA may be due to the inositol-depleting effect of the drug. It would be interesting to find out if VPA alters the phospholipid content of cells grown at elevated temperature. For this purpose, it would be essential to measure the lipids at different times following the transfer of cells to a high growth temperature.

Another interesting finding in Chapter 4 was that VPA perturbs activation of the CWI pathway when cells are shifted from 30°C to 39°C for 1 h. This signaling pathway is activated in response to growth at elevated temperatures (Kamada et al., 1995; Zarzov et al., 1996). However, its activation is detectable after about 20 min (Kamada et al.,

1995), suggesting that CWI signaling is detecting a secondary effect of exposure to high temperature (Levin, 2011). It is tempting to speculate that one or more of the phospholipids $PI4,5P_2$, sphingolipids, or ceramides may constitute the secondary effect in question, and that perturbation of these lipids as a result of VPA-mediated inositol depletion leads to decreased activation of CWI signaling. Deletion mutants for synthesis of each of the phospholipids, or commercially available inhibitors (Jesch et al., 2010) could be used to determine if any of these phospholipids are required for CWI activation.

In the same context, I would like to draw attention to glycosylphosphatidyl inositol (GPI) lipids, which are synthesized from PI and used to anchor several cell wall proteins (Pittet and Conzelmann, 2007). Surprisingly, the effect of VPA on this group of inositol-lipids has never been addressed in our lab. It would be interesting to find out if levels of GPI are altered in response to VPA, as this may uncover new cellular processes affected by the drug.

What is the mechanism underlying decreased endocytosis in response to VPA?

One of the main aims of this study was to elucidate the mechanism of action of VPA by identifying cellular processes that are affected by this drug. The studies in Chapter 4 show that VPA perturbs endocytosis in both yeast and mammalian cells. This is by no means the end of the story. I hypothesize that VPA potentiates the inhibitory GABA-mediated effect (Johannessen, 2000; Loscher, 2002) by decreasing the internalization of the $GABA_A$ receptors, thus prolonging exposure of the receptors to GABA. The

hypothesis is supported by the finding that VPA perturbs clathrin-mediated internalization depicted by Fig. 4.7 shown in Chapter 4. A further test of the hypothesis would be to determine if VPA perturbs the internalization of $GABA_A$ receptors in mammalian cells. This experiment is challenging but doable. A decrease in internalization of the receptors would suggest a new mechanism that may explain how VPA may potentiate the inhibitory effect.

What is the mechanism responsible for the VPA-mediated decrease in endocytosis? Several studies have shown that phosphoinositides, ceramides, and sphingolipids play a role in regulating endocytosis by recruiting proteins, acting as signaling molecules, or by playing a structural role (Munn et al., 1999; Chen et al., 1995; Zanolari et al., 2000). While it is possible that the VPA-mediated defect may be due to altered phospholipid composition of the plasma membrane, an intriguing possibility is that the defect may be due to inhibition of the kinases PKC and GSK3ß, both of which are required for phosphorylation of proteins essential for the internalization step of the GABA receptors (Brandon et al., 2000; Tyagarajan et al., 2011). Interestingly, both kinases are inhibited by VPA (Chen et al., 1994; Chen et al., 1999). If inhibition of these two enzymes is responsible for the observed VPA-mediated decrease in endocytosis, this would uncover a novel effect of VPA that has not been identified before.

Is there cross talk between the glycolytic and inositol biosynthetic pathways?

The cDNA screen discussed in Chapter 4 showed that overexpression of any of ten different glycolytic enzymes caused increased sensitivity to VPA (Table 4.2 and Fig. 5.1).

This finding raises the possibility that accumulation of glycolytic enzymes or products causes sensitivity to the drug. Overexpression was not deleterious to cells in the absence of VPA. At least three possibilities may explain these findings:

1) Glycolytic intermediates negatively affect a process already compromised by VPA. For example, some intermediates may inhibit enzymes of the inositol biosynthetic pathway, causing a further decrease in inositol levels. In support of this, the study by Shi et al. (2005) showed that dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G-3-P), two glycolytic intermediates, inhibit the activity of MIPS. Thus, it is possible that accumulation of other intermediates may inhibit MIPS and cause a further decrease in inositol biosynthesis. Inositol levels could be quantified in each of the clones expressing the glycolytic genes and compared to levels in wild-type cells. Clones that show decreased inositol levels may identify new MIPS inhibitors.

2) Glycolysis and the inositol biosynthetic pathway depend on glucose-6-phosphate (G-6-P) as the primary precursor. Two enzymes, MIPS and phosphoglucose isomerase (PGI), use G-6-P as a substrate. MIPS converts G-6-P to inositol 3-P, while PGI converts G-6-P to fructose-6-P (Fig. 5. 1). It is possible that overexpressing glycolytic enzymes shifts the reaction in favor of glycolysis, and away from inositol biosynthesis, leading to increased sensitivity to VPA. What determines the direction of the reaction? It is tempting to speculate that the flux of the reaction may be regulated by a metabolic signal, for example, an allosteric regulator that responds to altered ATP levels. In the event of ATP shortage, the regulator may direct the flux towards glycolysis.
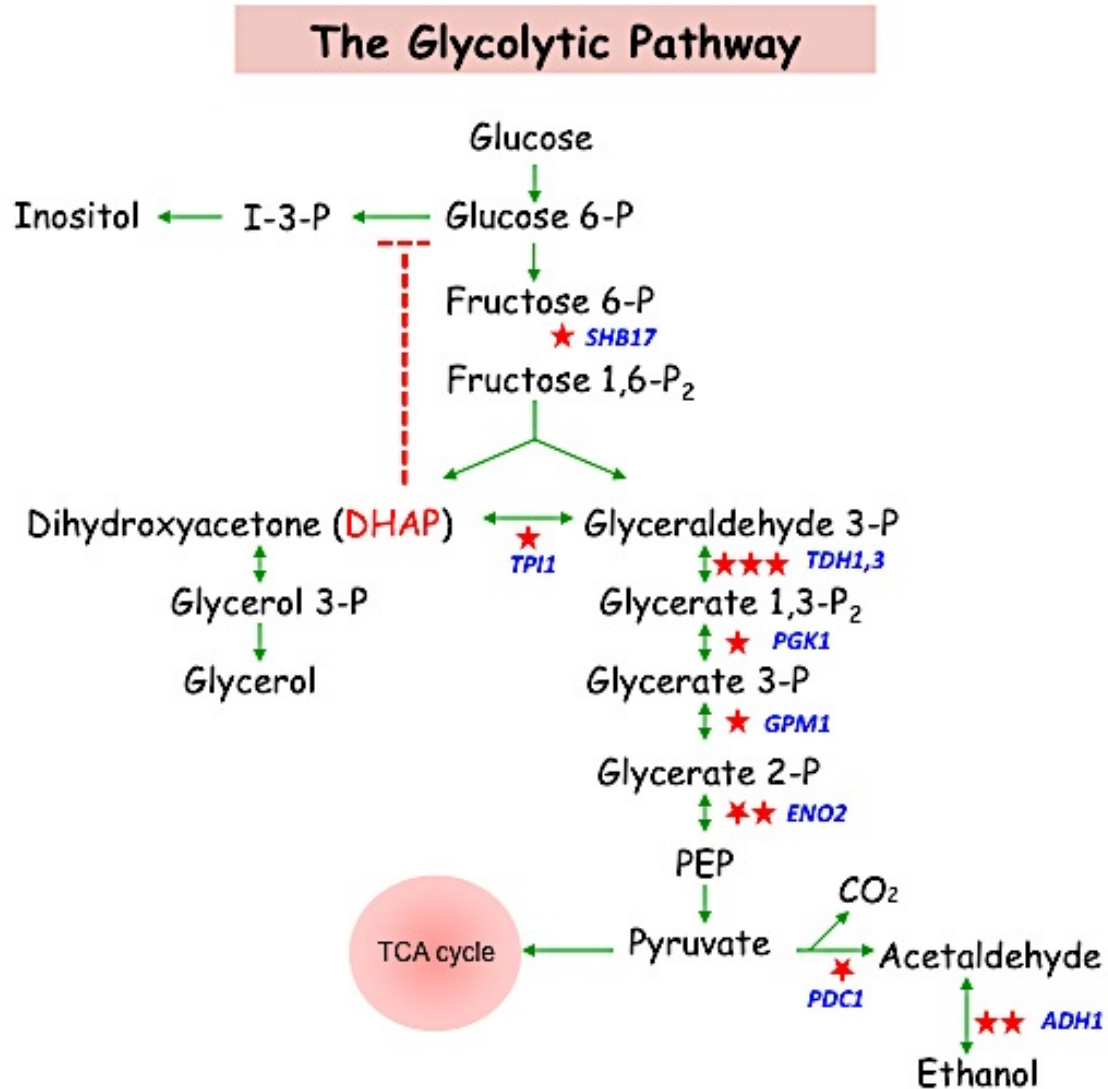
FIGURE 5.1 **Overexpression of glycolytic enzymes increases sensitivity to VPA.** Shown

in blue are the genes encoding the glycolytic enzymes that were identified in the cDNA

screen described in Chapter 4.

3) Some glycolytic enzymes have additional roles. For example, glyceraldehyde phosphate dehydrogenase (GAPDH), which converts glyceraldeyde-3-phosphate to glycerate 1,3-bisphosphate is involved in several non-glycolytic processes, including apoptosis (Tarze et al., 2007), transcription activation, ER to Golgi shuttling (Tisdale et al., 2004), and fast axoplasmic transport (FAT) (Zala et al., 2013). GAPDH provides glycolytic energy for FAT, which is responsible for vesicular transport and movement of organelles, including synaptic vesicles, to and from the neuron cell body along the axoplasm. Interruption of this process is associated with neurodegenerative diseases (Chevalier-Larsen and Holzbaur, 2006). The cDNA screen (Chapter 4) showed that overexpression of triose phosphate dehydrogenase (TDH), the yeast homolog of GAPDH, confers sensitivity to VPA. This raises the possibility that by increasing glycolytic flux (and decreasing inositol biosynthesis), VPA may increase activation of GAPDH/TDH. In neurons, this would increase the efficiency of FAT, which would be of great significance if FAT is defective in neurons of BD patients. In yeast, however, the only two known functions of TDH are in gluconeogenesis and glycolysis (Boucherie et al., 1995). Thus, a study to determine if TDH plays a role in vesicular trafficking in yeast may uncover a novel mechanism that has not been reported previously for this enzyme!

Does VPA perturb the unfolded protein response (UPR) pathway?

The UPR pathway is generally activated in response to ER stress caused by accumulation of misfolded protein in the ER lumen. Three mechanisms help alleviate the ER stress, including attenuation of protein synthesis, targeting the misfolded proteins for

degradation, and properly folding existing proteins (Kim et al., 2005). Results of several screens for VPA sensitivity indicate that VPA may perturb the UPR pathway. A study by Shulin Ju (2005 PhD dissertation) showed that cells exhibit increased resistance to VPA in response to overexpression of Ubi4, a ubiquitin that marks proteins and targets them for selective degradation. Thus, VPA may perturb selective degradation of misfolded proteins. In Chapter 4, I showed that overexpression of ribosomal proteins increases sensitivity to VPA. It is possible that an increase in ribosome biogenesis ensues, leading to increased protein synthesis and accumulation of misfolded proteins. It is also possible that overexpression of proteins stresses cells and causes accumulation of misfolded proteins. Thus, perturbation of the UPR by VPA may explain the increased sensitivity of cells when proteins are overexpressed.

Furthermore, ribosomal proteins have extra-ribosomal functions, including regulation of gene expression, DNA repair, DNA replication, and repression of translation (Warner and McIntosh, 2009; Weisberg, 2008). The *INO1* gene is particularly sensitive to defects in general transcription factors (Henry et al., 2014). It is possible that some of the ribosomal proteins identified in the screen may perturb the transcription of *INO1* and, thus, account for the increased sensitivity to VPA.

In conclusion, VPA is a drug that, for years, has been successfully used for the treatment of two devastating illnesses, BD and epilepsy, without knowing how it elicits its therapeutic effect. It is very likely that the effect involves more than one mechanism and numerous targets. The identification of novel targets and cell processes that are perturbed by VPA, undoubtedly uncovers new leads towards potential mechanisms of

action of this drug. These mechanisms may be responsible for the therapeutic effect, side effects, or may even shed light on the pathophysiology of a disorder that remains poorly understood.

And a final thought...

During my work on this project, I had the chance to train a student who is afflicted with bipolar disorder. Needless to say, she is one of the smartest, most thoughtful, creative, and cheerful persons I have ever met. I have often thought of her during my work.

It is heart-wrenching to see the increase in number of people affected by mental disorders and the devastating consequences that ensue. In spite of all the advancements that are generated by science, there is no proper cure for these disorders.

I hope that my work contributes to the advancement of research in this area.

For all future students working on the bipolar project, I pass the torch to you....

Good luck,

Rania

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ABSTRACT

REGULATION OF INOSITOL BIOSYNTHESIS AND CELLULAR CONSEQUENCES OF INOSITOL DEPLETION: IMPLICATIONS FOR THE MECHANISM OF ACTION OF VALPROATE

by

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Inositol is a six-carbon cyclitol that is ubiquitous in biological systems. It is a precursor for the synthesis of numerous biologically important compounds, including inositol phosphates and phosphoinositides that are essential for cell function and viability. Inositol compounds play a role in membrane formation, gene regulation, signaling, regulation of ion channels, and membrane trafficking. Furthermore, inositol regulates hundreds of genes, including those involved in the biosynthesis of inositol and phospholipids. While transcriptional regulation of inositol biosynthesis has been extensively studied and well characterized, regulation of inositol biosynthesis at the enzymatic level has not been addressed. The current study shows that *myo*-inositol 3-phosphate synthase (MIPS), the enzyme that catalyzes the rate-limiting step in inositol biosynthesis, is a phosphoprotein. Mass spectrometry analysis identified five phosphosites, three of which are conserved in yeast and human MIPS. Analysis of phosphorylation-deficient and phosphomimetic site-mutants of both

yeast and human MIPS indicated that the three conserved sites affect MIPS activity. Two of the phosphosites are inhibitory, and one is critical for activity.

Previous studies have shown that valproate (VPA), a branched chain fatty acid that has been successfully used for the treatment of bipolar disorder, epilepsy, and migraine, causes inositol depletion by inhibiting MIPS *in vivo* but not *in vitro*, which suggests that inhibition is indirect. Elimination of the two inhibitory phosphosites caused an increase in MIPS activity, conferred a growth advantage, and partially rescued sensitivity to VPA, suggesting that VPA-mediated inositol depletion may result from phosphorylation of MIPS.

Decreased IP_3 signaling caused by Inositol depletion has been proposed as a mechanism that underlies the therapeutic effect of VPA. However, no direct correlation between altered IP_3 signaling and the therapeutic effect has been established. Because of the versatility of inositol compounds, inositol depletion may have more far-reaching consequences that may account for the effect of VPA. This study showed that inositol depletion caused by VPA or by starvation of *ino1Δ* cells, which cannot synthesize inositol, perturbs vacuolar structure, decreases vacuolar ATPase (V-ATPase) proton pumping, and causes partial un-coupling of the V-ATPase. These perturbations were rescued by inositol supplementation. Furthermore, VPA compromised the synthesis of $PI3,5P_2$, which is necessary for stabilization of the V-ATPase complex. Osmotic stress, known to increase $PI3,5P_2$ levels, did not rescue the compromised $PI3,5P_2$ levels, nor did it induce vacuolar fragmentation in VPA-treated

cells, suggesting that perturbation of the V-ATPase is a consequence of altered PI3,5P$_2$ homeostasis under inositol-limiting conditions. These findings identify novel consequences of inositol depletion and provide evidence for a previously unidentified link between inositol levels and the V-ATPase.

To identify novel pathways and processes that are altered in response to VPA, a yeast cDNA library was screened for genes that increase sensitivity to VPA when overexpressed. One of the major categories identified was endocytic trafficking genes, which led to the hypothesis that VPA perturbs endocytosis. The study showed that VPA perturbs endocytosis in yeast and human cells. Evidence showed that the likely mechanism underlying decreased endocytosis by VPA is decreased PI4,5P$_2$ levels.

Taken together, my studies led to three major novel findings. First, I identified a regulatory mechanism of inositol biosynthesis characterized by phosphorylation of the rate-limiting enzyme *myo*-inositol 3-phosphate synthase (MIPS). Second, I demonstrated that the highly conserved vacuolar ATPase (V-ATPase) is a target of VPA. Third, my studies indicated that VPA-mediated inositol depletion perturbs endocytosis in both yeast and mammalian cells. These findings suggest new mechanisms that may underlie the therapeutic action of VPA, and identify potential targets that may be used for the development of more effective and safer drugs.

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