


Control of protein translation by IP₃R-mediated Ca²⁺ release in *Drosophila* neuroendocrine cells

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ABSTRACT

The inositol 1,4,5-trisphosphate receptor (IP₃R) is one of two Ca²⁺ channels that gates Ca²⁺ release from ER-stores. The ligand IP₃, generated upon specific G-protein coupled receptor activation, binds to IP₃R to release Ca²⁺ into the cytosol. IP₃R also mediates ER-store Ca²⁺ release into the mitochondria, under basal as well as stimulatory conditions; an activity that influences cellular bioenergetics and thus, cellular growth and proliferation. In *Drosophila* neuroendocrine cells expressing a hypomorphic mutant of IP₃R, we observed reduced protein translation levels. Here, we discuss the possible molecular mechanism for this observation. We hypothesize that the cellular energy sensor, AMPK connects IP₃R mediated Ca²⁺ release into the mitochondria, to protein translation, via the TOR pathway.

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Introduction

Ionic Ca²⁺ plays an important role in various cellular reactions and in order to utilize it for specific functions in a spatio-temporal manner, cells carefully manage Ca²⁺ levels in the cytosol as well as various organelles. Under resting conditions, the endoplasmic reticulum (ER) typically is the major store for Ca²⁺ with concentrations in the range 100–500 μM, while cytosolic Ca²⁺ is maintained at ~100 nM.¹ This large concentration gradient is maintained by the Sarco-Endoplasmic Reticulum Calcium ATP-ase (SERCA), which continuously pumps Ca²⁺ from the cytosol into the ER. Release of ER-store Ca²⁺ can be mediated by two known intracellular Ca²⁺ channels, the ryanodine receptor (RyR) and the inositol 1,4,5-trisphosphate receptor (IP₃R). Although the two receptors share structural homology, they are activated by unique ligands and mediate distinct effects inside the cell. The physiological ligand for IP₃R is IP₃, which is generated by the action of phospholipase Cβ (PLCβ) on the lipid phosphatidylinositol 4,5-bisphosphate (PIP₂). PLCβ in turn is activated by G-protein coupled receptors (GPCRs), typically by the Gαq subunit.

Agonist mediated GPCR activation thus results in increased cytosolic Ca²⁺, via IP₃R mediated Ca²⁺ release from the ER-store. This cytosolic Ca²⁺ potentiates a dazzling array of cellular processes, depending on the cell type and context.² Release of ER-Store Ca²⁺ is also known to activate the Store-operated Ca²⁺ entry pathway that brings Ca²⁺ from the extracellular milieu into the cytosol.³ In certain cell types, IP₃R can also be activated by receptor tyrosine kinases (RTKs) via PLC-γ. However in *Drosophila*, no genetic interaction has been observed between *small wings* (*sl*), the gene locus for the sole fly PLC-γ, and IP₃R.⁴

While mammals have three IP₃R isoforms coded by three distinct genes, in *Drosophila melanogaster* there is only one IP₃R gene, which shares 57–60% sequence homology to mammalian IP₃R1 and broad structural domain homology. Importantly, the rat IP₃R1 can functionally complement *Drosophila* IP₃R at the cellular as well as organismal level.⁵ There are three domains recognized on the IP₃R: an N-terminal IP₃/ligand binding domain, a central regulatory domain, followed by a C-terminal channel/pore forming domain. IP₃R functions as a tetrametric channel, and

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a recently published cryo-EM structure predicts a major role for the N-terminal domain in gating channel properties despite a large number of residues between the two.⁶

To elucidate how IP₃R function in distinct cellular contexts can influence systemic animal functioning, our lab generated *Drosophila* single point mutants for IP₃R using an EMS mutagenesis based approach.⁷ Several point mutants were recovered, but none were homozygous viable. We have thus been utilizing IP₃R hypomorphic heteroallelic mutant combinations to uncover systemic phenotypes and then tracking down the cellular basis for the manifestations of such phenotypes. One such study utilized a heteroallelic mutant with a combination that mutates S224F in the ligand binding domain, and G1891S in the regulatory domain. This mutant IP₃R strain is referred to as *itpr^{ku}*. In this strain, IP₃R protein is expressed to the same levels as WT,⁸ whereas functionally, larval neurons exhibit attenuated Store-operated Ca²⁺ entry.⁹

In our recent report, *itpr^{ku}* larvae were observed to have lower rates of protein synthesis.¹⁰ Genetic studies revealed that loss of IP₃R in *dimm⁺* neuroendocrine (NE) cells partially phenocopied the mutant. Interestingly, systemic phenotypes were also recapitulated at the cellular level: *dimm⁺* NE cells from *itpr^{ku}* were observed to have lower levels of protein translation over a 30-minute time period, similar to levels seen in NE cells treated with the protein synthesis inhibitor cyclohexamide.¹⁰ *dimm* is a transcription factor expressed by many, but not all *Drosophila* neuroendocrine cells.¹¹ *Dimm* regulates neuroendocrine cell fate as well as the scaling up of the protein machinery required for the regulated secretory pathway.¹² *dimm⁺* NE cells synthesize and secrete a large variety of neuropeptides (NPs), which directly or indirectly, influence development and physiology profoundly.¹³ Given the important role for NPs in metabolism, and that certain external stimuli can require immediate and robust over-expression as well as secretion of NPs,¹⁴ an effect of IP₃R on protein translation in NE cells might hold regulatory significance. Indeed, loss of IP₃R in *dimm⁺* NE cells reduces pupariation of larvae subject to late-stage nutritional deprivation. As over-expression of WT IP₃R in *itpr^{ku}* NE cells was sufficient to restore basal cellular protein translation to levels as seen in the control, as well as improve systemic protein levels in *itpr^{ku}* larvae,¹⁰ it appears that IP₃R is a positive

regulator of protein synthesis. This commentary speculates on the molecular mechanism that may link IP₃R to protein translation.

In previous studies, depletion of ER-Ca²⁺ store in mammalian cell lines or non-neuronal primary cultures, either by chemicals that cause ER stress, or by activation of IP₃R by hormones, has been reported to reduce protein synthesis, via eIF2 α phosphorylation.¹⁵ This is opposite to what we observe, and may perhaps be explained on the basis of two major differences between these studies and the experiments we performed. First, we observe this effect specifically in NE cells, and not in neurons, a distinct population not previously explored in the context of IP₃R functioning and protein translation. Perhaps this cell-specific effect of IP₃R is restricted to cells with large protein synthetic requirements. Second, we observed protein translation defects in the absence of ER-Store Ca²⁺ depletion i.e., under basal non-stimulatory conditions, while previous studies have been undertaken in the presence of ER stress by addition of inhibitors such as thapsigargin or ionomycin, or stimulation of IP₃R Ca²⁺ release by hormones. This may be significant in the context of a study by Foskett et al., that reported a role for low level constitutive Ca²⁺ leak by IP₃R in cellular bioenergetics.¹⁶

IP₃R and AMPK

IP₃R mediated transfer of Ca²⁺ from the ER to the mitochondria is now a well established cellular process, studied mainly for its role in apoptosis and autophagy.¹⁷ However, it is also well known that there is a basal requirement for Ca²⁺ inside the mitochondria for the production of ATP. For example, Ca²⁺ increases the activity of dehydrogenases of the TCA cycle.¹⁸ Transport of Ca²⁺ relies on local areas of ER-mitochondria contact points called Mitochondrial Associated Membranes, which are enriched with several proteins including IP₃R.¹⁹ In effect, IP₃R mediated Ca²⁺ release affects mitochondrial functioning. Fitting this view is the observation that under normal, nutrient-rich conditions, genetic knockout of the three mammalian IP₃R genes or inhibition of IP₃Rs by Xestospongins (XeB) compromised ATP production in the mitochondria.¹⁶ Increased AMP/ADP:ATP ratio is sensed by AMP-activated protein kinase (AMPK). Consequently, AMPK is phosphorylated (pAMPK) and, in effect reduction of IP₃R, via pAMPK results in

higher levels of basal autophagy.¹⁶ Interestingly, loss of IP₃R in many different cell lines results in increased basal autophagy and pAMPK.^{16,20}

As a central regulator of cellular energy homeostasis pAMPK exerts a strong effect on protein and lipid biosynthesis. Because it is activated under low ATP conditions, pAMPK activates biosynthetic pathways that generate energy like fatty acid β oxidation, and inhibits high-energy consuming activities like protein synthesis and lipogenesis.²¹ Thus, AMPK may link IP₃R Ca²⁺ release activity with protein translation.

AMPK is a highly conserved Ser/Thr kinase heterotrimer consisting of a catalytic α subunit and, regulatory β and γ subunits. While there are multiple genes for each subunit in mammals, *Drosophila* contains a single gene for α , β and γ respectively. There is ~60% homology between mammalian and *Drosophila* AMPK, but importantly there is functional conservation.²² Firstly, at the structural level, AMPK functions as a heterotrimer in both systems; Second, the key site for AMPK phosphorylation on the so called “activation loop” of the α subunit (T172 in humans, T184 in flies) is conserved and necessary for AMPK activity; Third, both are activated by AMP, although the degree of stimulation of the *Drosophila* kinase is to a lower level (4.5-fold compared with 22-fold)²³; Fourth, downstream targets of AMPK activity such as acetyl-CoA carboxylase (ACC) are conserved. One major difference between the fly and mammalian AMPK is lipidation: Gly2 of the β subunit in mammalian systems is myristoylated and this plays a key role in tethering AMPK to the plasma membrane, which further allows it to be phosphorylated by LKB1. However, lack of the myristoylation does not prevent the activation of AMPK by LKB1 as in an LKB *Drosophila* mutant, low but detectable levels of pAMPK was observed.²⁴ Further, even in mammalian systems, basal pAMPK was detectable in the absence of LKB1.²⁵ Another well known activator of AMPK is Ca²⁺ calmodulin-dependent protein kinase kinase 2 (CaMKK2), a cytosolic kinase that is activated upon increased Ca²⁺ concentrations. A *Drosophila* homologue (CG17698) for CaMKK2 has been annotated, but as yet there is no biochemical evidence regarding its role and therefore, it is not discussed further.

In a preliminary experiment (Fig. 1), whole larval lysates were probed for pAMPK levels, as well two markers to indicate activity of the insulin/TOR signaling pathway, pAkt and pS6K. While levels of pAKT

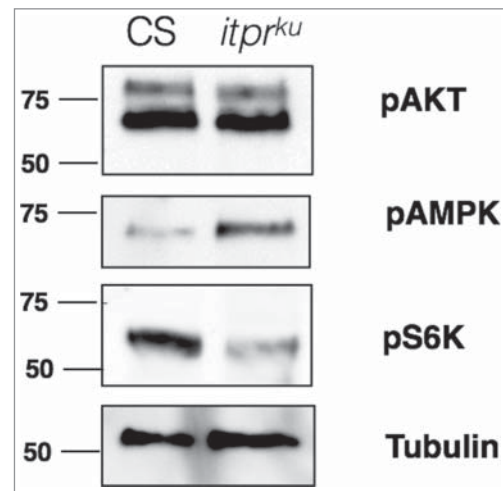


Figure 1. Western blot of whole larval lysates. 30 μ g of lysates prepared from 3rd instar larvae were probed with 1:1000 pAKT (Cell Signalling), 1:1000 pAMPK (Cell Signalling), 1:1000 pS6K (Cell Signalling) and 1:5000 tubulin (DHSB).

were unchanged between control and *itpr^{ku}*, pS6K was reduced. Interestingly, pAMPK was increased in *itpr^{ku}*. Thus, at the systemic level AMPK appears overactive in the IP₃R mutant, and in agreement with reports that pAMPK inhibits the TOR signaling pathway (discussed in more detail below), pS6K levels were lowered. Another link that suggests that AMPK function is affected in *itpr^{ku}* stems from the observation that *itpr^{ku}* has higher levels of TAGs (Triacylglycerides) both in larval as well as adult stages.^{10,26} While at a cellular level activated AMPK promotes lipolysis, at a systemic level it can have an opposite effect. For example, activation of AMPK in mice POMC (Pro-opiomelanocortin) neurons causes an increase in NPY, Neuropeptide Y and Agouti Related Peptide, which leads to more feeding, and a concomitant increase in body weight.²⁷ This is similar to *itpr^{ku}* which as adults display hyperphagia and increased body weight, both of which are rescued by overexpression of IP₃R in NE cells,²⁸ some of which are functionally analogous to human POMC neurons. Also, in a *Drosophila* AMPK hypomorphic mutant, decreased levels of TAGs were observed²⁴ which suggests that up-regulation of AMPK may promote a systemic increase in TAGs.

pAMPK and protein translation via Insulin/TOR signaling

itpr^{ku} is deficient in its ability to pupariate under nutritional stress.^{10,29} Over-expression of wild type

IP₃R or genes of the Insulin/ TOR signaling pathways, in NE cells, was sufficient to rescue the pupariation deficiency of *itpr^{ku}*.¹⁰ Further, in NE cells, loss of InR (Insulin Receptor), a key regulator of the insulin pathway, resulted in larvae which were deficient in pupariation under nutritional stress; a deficiency that could be over-come when wild type IP₃R was over-expressed in NE cells. Together, these genetic experiments suggested that IP₃R and the Insulin/TOR pathway can functionally compensate for each other, and are sufficient to ultimately affect a systemic, organism-level adaptation to nutrient stress. The Insulin and TOR pathways are well known growth signaling pathways that link nutritional status to protein synthesis either through growth factors such as insulin, or nutrients themselves, such as amino acids. Notably, many components of the two pathways are conserved between *Drosophila* and mammals.³⁰ There is also considerable cross-talk between the two pathways – for example, pAKT can inhibit TSC2, alleviating the repression of Rheb and thus promoting TOR activation.³⁰

In mammalian systems, pAMPK is known to suppress protein synthesis by its action at two points in the TOR signaling pathway: 1) It can phosphorylate TSC2, which inactivates TOR, leading to a reduction in pS6K and an increase in p4eBP³¹, and 2) it can phosphorylate Raptor, a key scaffold of the mTORC1 complex, thereby preventing the complex from recruiting S6K and 4eBP.³² Information regarding regulation of TOR signaling by AMPK in *Drosophila* is scarce. In the adult fly gut, loss of AMPK did not change pS6K levels and over-expression of TSC1/2, or TOR^{DN} did not rescue muscle phenotypes of an AMPK hypomorphic mutant.²⁴ However, specific over-expression of AMPK in adult fly neurons led to increased pAMPK and decreased pS6K.³³ This is similar to mouse cortical neurons, where over-activation of AMPK reduces axonal growth and specification, via its inhibitory effect on the mTOR pathway.³⁴ Thus AMPK functionality in the context of TOR signaling appears to be cell-specific.

Future directions

At present, the link between IP₃R and AMPK has been established only in mammalian cells, where the physiological significance remains to be understood. However, tools are available to investigate their connection in *Drosophila*. In mammals, the Hsp70

family chaperone GRP75 (Glucose-regulated protein 75) binds IP₃R and places it in close juxtaposition to VDAC1 (Voltage Dependent Anion Channel), located on the mitochondrial outer membrane. Ca²⁺ uptake into the inner mitochondrial membrane is then facilitated by the Mitochondrial Calcium Uniporter, MCU.³⁵ There are *Drosophila* homologues for Hsp70 and VDAC family of proteins, but no conserved genetic homologue of GRP75 or VDAC1. MCU is significantly conserved; a recent paper describes *Drosophila* MCU (CG18769) having evolutionarily conserved Ca²⁺ transporter function as well as genetic interaction with IP₃R.³⁶ Notably, oxidative stress by application of tert-butyl hydroperoxide on larval muscles resulted in increased [Ca²⁺]_{mito}, that was abrogated upon IP₃R knock-down.³⁶ Moreover, in *Drosophila* larval neuroblasts, ER-mitochondria contact sites were proposed to be stabilized by the rhoGTPase Miro, which promotes mitochondrial Ca²⁺ uptake, and positively regulates mitochondrial metabolism.³⁷ Loss of IP₃R or porin (a *Drosophila* homologue of the VDAC family) decreased basal mitochondrial Ca²⁺ levels in Miro over-expressing NBs, suggesting a functional connection between IP₃R, Porin and Miro.³⁷

Taken together, a molecular mechanism linking IP₃R to protein translation via AMPK is proposed (Fig. 2). This mechanism is likely to be relevant in cells/ tissues where the IP₃R affects protein translation. Increased levels of pAMPK in whole larval lysates suggest that the IP₃R may affect protein translation in other cell types, in addition to NE cells. This aspect needs to be addressed in future studies. Validation of the proposed mechanism will require loss-of-function and gain-of-function studies with Porin, Miro and AMPK in *Drosophila* NE cells, in the context of protein translation, at the cellular as well as systemic level. The proposed hypothesis predicts that loss of IP₃R Ca²⁺ channel activity would activate AMPK, which in turn would inhibit TOR signaling, thereby reducing protein translation. Under nutrient-rich conditions, nutrients and insulin signaling may over-ride this inhibitory effect, by providing positive regulation of the insulin/TOR signaling pathway, to produce a small net decrease in protein translation, resulting in a small systemic effect. Hence, protein synthesis in *itpr^{ku}* is lower, but not low enough to be detrimental to organismal survival in nutrient-rich conditions. Under conditions of nutrient deprivation, insulin/TOR signaling is turned off, and therefore, these pathways are

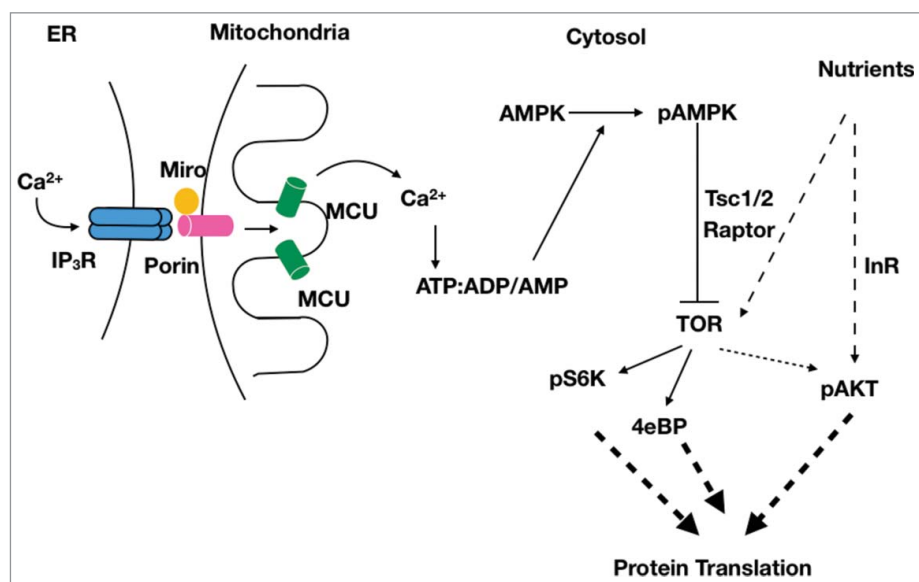


Figure 2. Hypothetical schematic connecting basal IP₃R mediated Ca²⁺ release to protein translation, via AMPK and the TOR signalling pathway. Basal transfer of Ca²⁺ from the ER to the inner mitochondria via Porin and Mitochondrial Calcium Uniporter (MCU), promotes ATP synthesis and inhibits phosphorylation of AMP-activated kinase (AMPK). Reduced IP₃R mediated Ca²⁺ release decreases ATP production, leading to increased levels of AMP and ADP, that activates AMPK, which inhibits protein synthesis via its negative regulation of the TOR pathway. Thus, even under nutrient-rich conditions, overall protein synthesis levels would be lower in cells with reduced IP₃R function. However, cell survival is not affected as nutrients can keep the insulin/TOR signalling pathway active, thereby over-riding the inhibitory effects of pAMPK on protein synthesis. In conditions where both nutrients are withdrawn as well as IP₃R function is compromised, the net reduction in protein synthesis assumes physiological significance.

unable to compensate for reduced protein translation in cells where IP₃R mediated Ca²⁺ release is also compromised. Particularly in cells like NE cells, that need to scale their secretory process and NP synthesis in response to starvation, i.e., increase their protein synthesis under starvation, the loss of IP₃R mediated Ca²⁺ release may have stronger systemic consequences, such as the inability of larvae to pupariate under nutrient stress. We are currently in the process of identifying NPs that operate in the context of IP₃R signaling and are required for adaptation to nutrient stress.

The regulation of protein translation by IP₃R has not been previously reported in NE cells. It would be interesting to understand if similar mechanisms are at play in mammalian NE cells. Given the far-reaching consequences that products of some neuroendocrine cells, such as POMC neurons, have on integrating nutritional status to feeding and metabolism, our studies in *Drosophila* provide the basis to investigate regulation of protein synthesis by IP₃R mediated Ca²⁺ release in a novel context.

Disclosure of potential conflicts of interest

The authors report no conflict of interest.

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