CA^{2+}-SELECTIVE TRPM CHANNELS REGULATE IP_3-DEPENDENT CA^{2+} OSCILLATIONS IN THE *C. ELEGANS* INTESTINE

By

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LIST OF ABBREVIATIONS

AA.....Arachidonic acid

aBoc .....Anterior body wall muscle contraction

ARC .....Arachidonate-regulated Ca^{2+} channels

BAPTA .....1,2-Bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

$[Ca^{2+}]_i$.....Cytoplamic Ca^{2+} concentration

CRAC current, I_{CRAC}.....Ca^{2+} release-activated Ca^{2+} current

DAG.....Diacylglycerol

DIC.....Differential interference contrast

DMSO.....Dimethyl sulfoxide

dsRNA.....double stranded RNA

DVF solution.....Divalent cation-free solution

EGTA.....Ethylene glycol tetracetic acid

Emc .....Enteric muscle contraction

ER .....Endoplasmic reticulum

Fluo-4 AM .....Fluo-4 acetoxymethyl

GFP .....Green fluorescence protein

GPCR.....G-protein coupled receptor

IP_3 .....Inositol 1,4,5-trisphosphate

IP_3R.....IP_3 receptor

LNA .....Linolenic acid

NCX .....Na^+/Ca^{2+} exchangers

OAG 1-oleoyl-2-acetyl-sn-glycerol

ORCa current, I_{ORCa} .....Outwardly rectifying Ca^{2+} current

pBoc .....Posterior body wall muscle contraction

PI .....Phosphoinositide or phosphatidylionitol

PI(4)P .....Phosphotidylinositol 4-monphosphate

PIP_2 .....Phosphotidylinositol 4,5-bisphosphate

$PI(3,4)P_2$.....Phosphotidylinositol 3,4-bisphosphate

$PI(3,5)P_2$.....Phosphotidylinositol 3,5-bisphosphate

$PI(3,4,5)P_3$.....Phosphotidylinositol 3,4,5-trisphosphate

PLC .....Phospholipase C

PMCA .....Plasma membrane Ca^{2+} ATPase

PolyK .....Poly-L-Lysine

RNAi.....RNA interference

RTK.....Receptor tyrosine kinase

ROCCs .....Receptor-operated Ca^{2+} channels

SERCA.....Sarcoplasmic reticulum Ca^{2+} ATPase

SOCCs.....Store-operated Ca^{2+} channels

SOCE .....Store-operated Ca^{2+} entry

SMOCCs.....Second messenger-operated Ca^{2+} channels

TRP .....Transient receptor potential

TRPA .....Ankyrin TRP channels

TRPC.....Canonical TRP channels

TRPM.....Melastatin TRP channels

TRPML .....Mucolipidin TRP channels

TRPP .....Polycystin TRP channels

TRPV .....vanilloid TRP channels

CHAPTER I

INTRODUCTION

Overview of calcium signaling in nonexcitable cells

Calcium is a ubiquitous cellular second messenger that is responsible for controlling numerous cellular processes including fertilization, gene transcription, exocytosis, secretion, cell differentiation and proliferation, and programmed cell death (Berridge et al., 2000). Perturbations in normal intracellular Ca^{2+} concentrations underlie many common pathological conditions (Missiaen et al., 2000).

Cytoplasmic Ca^{2+} concentration is tightly regulated

Intracellular Ca^{2+} concentration is ~100nM in resting cells and rises to 1μM or more upon stimulation. The localization, duration and amplitude of intracellular Ca^{2+} changes are tightly regulated and cells extract specific information from details of cytoplasmic Ca^{2+} changes to carry out downstream tasks (Berridge et al., 2000). At any moment in time, the level of intracellular Ca^{2+} is determined by equilibrium between the 'on' reactions that trigger Ca^{2+} increase in the cytoplasm and the 'off' reactions through which Ca^{2+} concentration is lowered by pumps, exchangers and buffers.

The Ca^{2+} 'on' reactions include pathways that generate Ca^{2+} signals through both internal and external sources. In nonexcitable cells, the major internal Ca^{2+} stores are the endoplasmic reticulum (ER). The major mechanism for mobilizing such stores

involves the classical phosphoinositide (PI) pathway. Essentially, the binding of many hormones to specific receptors on the plasma membrane leads to the activation of phospholipase C (PLC) that hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) to produce the intracellular messenger inositol 1,4,5-trisphosphate (IP_3). IP_3 is water soluble and diffuses into the cell interior where it encounters IP_3 receptors on the ER. The binding of IP_3 changes the conformation of IP_3Rs and opens the channel pore, thus allowing the Ca^{2+} stored at high concentrations in the ER to enter the cytoplasm (Figure 1).

Calcium that enters the cell from the outside is also a principal source of signal during the 'on' reactions (Figure 1). Entry of Ca^{2+} is driven by a large electrochemical force created by the ~20,000 fold concentration gradient for Ca^{2+} across the plasma membrane and the hyperpolarized resting membrane potential. Cells use this external source of signal Ca^{2+} by activating various Ca^{2+} channels with widely different properties.

Ca^{2+} is removed from the cytoplasm through various **'off' mechanisms**. These include the reuptake of Ca^{2+} back to ER through sarcoplasmic reticulum Ca^{2+} ATPases (SERCA) and Ca^{2+} extrusion via plasma membrane Ca^{2+} ATPases (PMCA). Calcium is also extruded from the cell by transporters such as the Na^+/Ca^{2+} exchangers (NCX) that utilizes Na^+ gradient to provide the energy to transport Ca^{2+} up its electrochemical gradient out of the cell. Therefore, the termination of intracellular Ca^{2+} signaling generally depends on the inactivation of intracellular and plasma membrane Ca^{2+} channels, as well as Ca^{2+} removal from the cytoplasm by the reuptake into intracellular organelles and plasma membrane extrusion (Figure 1).

Many cellular proteins can bind to Ca^{2+} over a wide range of affinity from nM to mM. These Ca^{2+}-binding proteins, which become loaded with Ca^{2+} during the on reactions and unload during the off reactions, function to fine-tune the spatial and temporal properties of Ca^{2+} signals.

Intracellular Ca^{2+} signaling has high degree of spatial and temporal diversity. Many Ca^{2+}-signaling components are organized into macromolecular complexes in which Ca^{2+} signaling functions within highly localized environments. The close association between components of the on reactions and their downstream effectors is particularly relevant for rapid responses. Ca^{2+} changes also occur over a diverse range of time scales. At the fast end of the scale, for example, at the synaptic junctions, Ca^{2+} triggers exocytosis within microseconds. Moving up the timescale, the Ca^{2+} transients tend to last longer (over minutes to hours) to drive events such as gene transcription and cell proliferation. During prolonged stimulation, Ca^{2+} transients often occur repetitively generating Ca^{2+} oscillations. Continuous Ca^{2+} oscillations can form intracellular or intercellular calcium waves within cells and tissues, respectively.

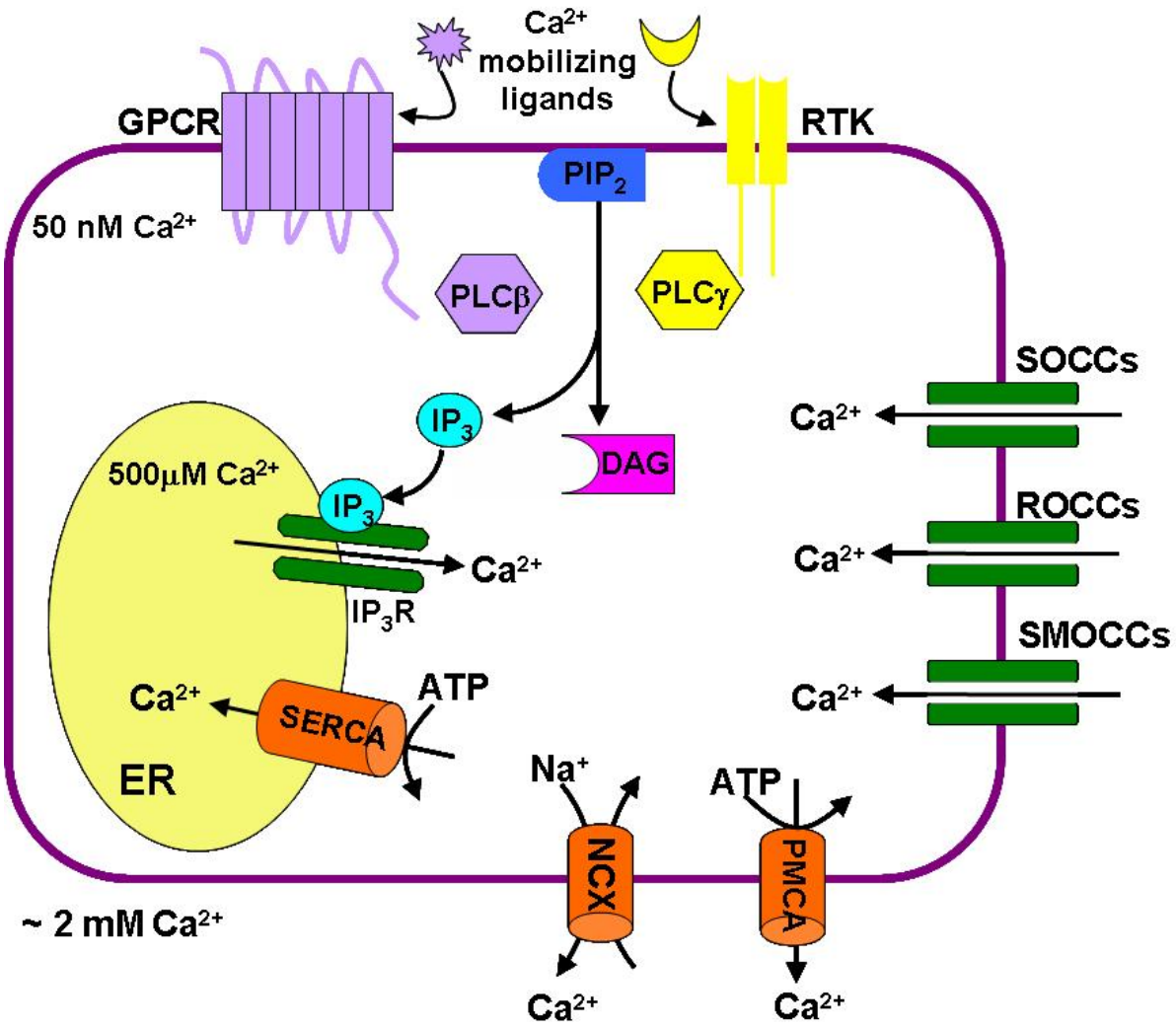


Figure 1. Molecular machinery of intracellular Ca^{2+} signaling pathways in nonexcitable cells. Ligands activate cell surface receptors (GPCR or RTK), which leads to the activation of downstream PLCs (PLCγ and PLCβ). Activated PLCs then catalyze PIP_2 into IP_3 and DAG. IP_3 binds to and opens IP_3R located on ER membrane, which induces Ca^{2+} release from ER. Extracellular Ca^{2+} entry is mediated by plasma membrane Ca^{2+} channels including SOCCs, ROCCs, and SMOCCs. Ca^{2+} influx from external space may function to contribute to cytoplasmic Ca^{2+} changes directly, to refill the ER Ca^{2+} store, and/or regulate Ca^{2+} release from the ER. To terminate the Ca^{2+} signal, cytoplasmic Ca^{2+} is pumped back to the ER through SERCAs and also extruded out of the cell by PMCA and/or NCX. GPCR, G protein coupled receptor; RTK, receptor tyrosine kinase; PLC, phospholipase C; PIP_2, phosphatidyl inositol-3,4-diphosphate; IP_3, inositol trisphosphate; DAG, diacylglycerol; IP_3R, IP_3 receptor; ER, endoplasmic reticulum; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SOCCs, store-operated Ca^{2+} channels; ROCCs, receptor-operated Ca^{2+} channels; SMOCCs, second messenger-operated Ca^{2+} channels; PMCA, plasma membrane Ca^{2+} ATPase; NCX, Na^+/Ca^{2+} exchanger.

Ca^{2+} entry across the plasma membrane is essential for sustained Ca^{2+} signaling

Many key cellular processes require a sustained increase in intracellular Ca^{2+} that can only be accomplished through Ca^{2+} entry into the cell. Ca^{2+} mediated events are terminated within a few seconds to minutes due to the exhaustion of the finite intracellular Ca^{2+} store when extracellular Ca^{2+} is unavailable. Resting cells generally have a low permeability to Ca^{2+}, but a large electrochemical driving force for Ca^{2+} entry. An increase in Ca^{2+} permeability by opening Ca^{2+}-permeable ion channels on the plasma membrane can result in large Ca^{2+} influx into the cytoplasm. Ca^{2+} influx through plasma membrane Ca^{2+} channels plays an important role in generating intracellular Ca^{2+} signals by helping to refill the ER Ca^{2+} stores, by modulating the frequency of Ca^{2+} oscillations, and/or by directly contributing to the elevation of cytoplasmic Ca^{2+} levels.

Plasma membrane Ca^{2+} entry pathways in nonexcitable cells

In excitable cells such as neurons and cardiac myocytes, Ca^{2+} entry across the plasma membrane is mostly through voltage-operated Ca^{2+} channels (VOCCs) activated by membrane depolarization. In electrically nonexcitable cells, Ca^{2+} entry is mediated by other channel types including store-operated Ca^{2+} channels (SOCCs), second messenger-operated Ca^{2+} channels (SMOCCs), and receptor-operated Ca^{2+} channels (ROCCs).

Store-operated Ca^{2+} channels (SOCCs): The study of Ca^{2+} entry in nonexcitable cells has been largely dominated by the so-called store-operated Ca^{2+} channels. Activation of these channels is, by definition, exclusively dependent on the depletion of internal ER Ca^{2+} stores (Putney, Jr., 1986). Although the store-operated

mode of Ca^{2+} entry was first described more than two decades ago, the molecular nature of such channels has remained enigmatic. However, recently the stromal interacting molecule proteins (STIM1) and the pore forming protein-Orai were identified through candidate-based and genome wide RNAi screens in *Drosophila* S2 cells(Zhang et al., 2005a; Prakriya et al., 2006). It is now well established that STIM proteins are the ER Ca^{2+} sensors, which primarily locate on ER membrane, sense the depletion of Ca^{2+} from ER, oligomerize, translocate to junctions adjacent to the plasma membrane, organize Orai channels into clusters and open the channels to bring about Ca^{2+} entry(Putney, Jr., 2007). Store-operated Ca^{2+} entry has been observed in almost every type of cell examined, yet the roles of SOCCs in physiologically relevant responses have largely been limited to studies in lymphocytes (Lewis and Cahalan, 1989)and mast cells (Hoth and Penner, 1992). Previous studies in our laboratory have also demonstrated that STIM and Orai are essential for sheath cell and spermatheca contractile activity required for ovulation and knockdown of *stim-1* or *orai-1* causes complete sterility in *C. elegans* (Yan et al., 2006; Lorin-Nebel et al., 2007). In other cell types, the relevance of SOCCs to actual physiological responses is far from clear. SOC entry has been proposed to function as a failsafe mechanism to prevent Ca^{2+} store depletion under pathophysiological and stress conditions (Yan et al., 2006).

Second messenger-operated Ca^{2+} channels (SMOCCs): One of the most well characterized examples of SMOCCs is arachidonate-regulated Ca^{2+} channels (ARC) discovered by T. Shuttleworth and colleagues about 10 years ago (Mignen and Shuttleworth, 2000). Recent studies have demonstrated that the molecular composition of the ARC channels also involves members of the Orai proteins. Mammalian Orai1 and

Orai3 appear to contribute to form the ARC channel pore (Mignen et al., 2009). ARC channels are shown to provide the predominant route of Ca^{2+} entry, particularly at lower, more physiologically relevant, levels of stimulation (Shuttleworth, 2004). Under these conditions, activation of the cells often results in the generation of oscillatory Ca^{2+} signals, and here the principal role of Ca^{2+} entry is to modulate the frequency of Ca^{2+} oscillations (Girard and Clapham, 1993; Bootman et al., 1996; Shuttleworth and Thompson, 1996).

Receptor-operated Ca^{2+} channels (ROCCs): Activation of hormone-specific receptors on the plasma membrane not only leads to generation of second messenger IP_3, which then mobilizes Ca^{2+} from internal Ca^{2+} store, but also can activate plasma membrane Ca^{2+} channels that mediate Ca^{2+} entry from external space. These channels are referred to as receptor-operated Ca^{2+} channels. The mechanisms through which these channels are activated are highly variable and, in some cases, remain controversial. Many of these channels belong to the large transient receptor potential (TRP) ion channel family.

The TRP channel superfamily

TRP channels: a brief overview

The transient receptor potential (TRP) protein superfamily consists of a diverse group of cation channels that bear structural similarities to *Drosophila* TRP. TRP

channels appear to assemble as homo- or heterotetramers of subunits containing six putative transmembrane domains and cytoplasmic N- and C-terminal tails (Figure 2). Based on amino acid homology, the mammalian TRP superfamily can be divided into six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin). Despite the structural similarities, TRP channels are distinct from the known families of ion channels in that they display a wide array of cation selectivities, activation mechanisms, and physiological functions (Pedersen et al., 2005; Owsianik et al., 2006; Venkatachalam and Montell, 2007).

Whereas all functionally characterized TRP channels are cation channels, the relative cation selectivity varies among isoforms. Only two TRP channels are exclusively permeable to monovalent cations, but not to Ca^{2+} or Mg^{2+} (TRPM4 (Launay et al., 2002) and TRPM5 (Hofmann et al., 2003)), and two others are highly Ca^{2+} permeable (TRPV5 (Nilius et al., 2000) and TRPV6 (Yue et al., 2001)). TRPM6 and TRPM7 are highly permeable to Mg^{2+}. TRP channels are activated by a wide range of stimuli including intra- and extracellular messengers, physical factors such as temperature, voltage, or mechanical stress and chemical factors such as pH or reactive oxygen species (reviewed by (Pedersen et al., 2005),(Venkatachalam and Montell, 2007)). TRP channels participate in a diversity of cellular functions in both excitable and nonexcitable cells. They play critical roles in sensory modalities, such as touch, hearing, taste, olfaction, vision, and thermal sensation, in animals ranging from worms to flies, mice, and humans. In addition, TRP channels function to regulate fluid and hormone secretion, endothelial cell function and vascular tone, neurite outgrowth and growth cone guidance, and epithelial Ca^{2+} and Mg^{2+} transport.

TRP channels are important for human health. At least four channelopathies have been identified in which a defect in a TRP channel-encoding gene is the direct cause of disease (Table 1). Given their various roles as receptors for noxious temperature, chemical compounds, and inflammatory mediators, TRP channels have also been connected to a broad range of systemic diseases. For example, TRPV1 channel is shown to be involved in neuropathic pain, hyperalgesia, allodynia, and spontaneous burning pain. Other indications of the involvement of TRPs in disease come from correlations between the levels of channel expression and disease symptoms. For example, TRPM1 has been suggested to be a tumor suppressor and a decrease in expression of TRPM1 appears to be a prognostic marker for metastasis in patients with localized malignant melanoma (Duncan et al., 1998). Expression of TRPM8 (Zhang and Barritt, 2004) and TRPV6 (Wissenbach et al., 2001)is reported to be up-regulated in prostate cancer.

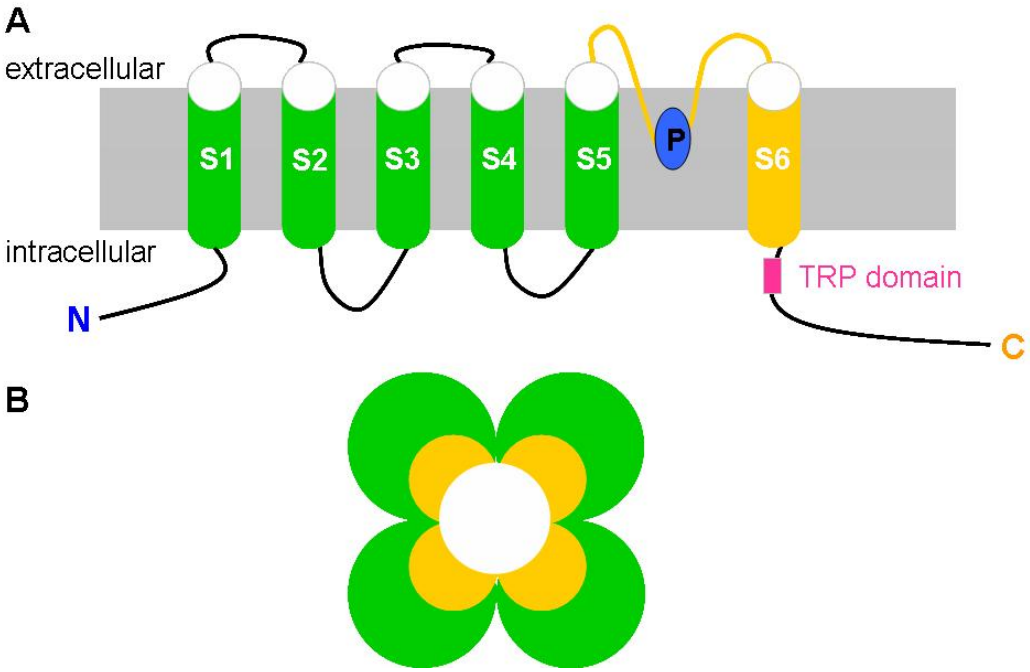


Figure 2. Schematic diagram of a TRP channel subunit (A) which has cytoplasmic N- and C- terminal tails and six transmembrane domains with the pore forming region located between S5 and S6 and tetrameric assembly (B).

Table 1. TRP genes related channelopathies

	Channelopathy	References
TRPC6	Focal and segmental glomerulosclerosis	(Reiser et al., 2005; Winn et al., 2005)
TRPM6	Hypomagnesemia with secondary hypocalcemia (HSH)	(Schlingmann et al., 2002; Walder et al., 2002)
TRPP2	Autosomoal dominant polycystic kidney disease	(Sutter and Germino, 2003)
TRPML1	Mucolipidosis IV	(Raychowdhury et al., 2004)

Roles of TRP channels in calcium signaling

Changes in the cytoplasmic Ca^{2+} concentrations control numerous fundamental cellular processes including muscle contraction, transmitter release, gene transcription, cell proliferation and cell death (Berridge et al., 2000). Many TRP channels play an important role in Ca^{2+} signaling and they contribute to intracellular Ca^{2+} changes by providing plasma membrane Ca^{2+} entry pathways, by modulating the driving force for the Ca^{2+} entry, and also by providing pathways for Ca^{2+} release from intracellular organelles.

The Ca^{2+} influx channels of the TRP family are comprised of all the TRPCs, all TRPVs, TRPM1, 2, 3, 6, 7 and 8, TRPA1, TRPP2, 3, and 5 and TRPML1, 2, and 3 (Pedersen et al., 2005; Owsianik et al., 2006). These TRP channels provide important Ca^{2+} entry pathways across the plasma membrane in various cell types and regulate a plethora of Ca^{2+}-dependent cell functions ranging from gene expression to cell death.

Some TRP channels contribute to cellular Ca^{2+} changes by modulating the driving force for Ca^{2+} influx. The widely expressed TRPM4 channel appears to function as a brake on Ca^{2+} influx in many nonexcitable cells. TRPM4 channels are voltage-modulated, Ca^{2+} activated and selective for mono-valent cations. Activation of TRPM4 following receptor-mediated Ca^{2+} mobilization was shown to depolarize the membrane potential and, with it, decrease the driving force for Ca^{2+} entry through other calcium channels (Launay et al., 2002). Nonselective TRPC3 channels have recently been shown to couple to the Na^+/Ca^{2+} exchanger (NCX1) both physically and functionally, such that Na^+ entry via TRPC3 leads to reverse mode operation of NCX1 resulting in an increase in intracellular Ca^{2+} (Rosker et al., 2004).

A number of recent studies indicate that members of the TRP superfamily including TRPV1 (Turner et al., 2003), TRPM2 (Lange et al., 2009), and TRPM8 (Zhang and Barritt, 2004) may function as intracellular Ca^{2+} release channels in addition to their roles as plasma membrane Ca^{2+} channels. Some of the less studied TRP channels, including TRPML1 (Raychowdhury et al., 2004) and TRPP2 (Koulen et al., 2002), appear to be mainly localized on intracellular membranes and have been proposed to serve as a new type of Ca^{2+} release channel.

Regulation of TRP channels by PI(4,5)P_2

TRP channels are regulated by a broad variety of stimuli, but recent evidence suggests that a common theme is their modulation by lipid messengers, and in particular by phosphatidylinositol 4, 5-bisphosphate (PIP_2) (Nilius et al., 2008). PI(4,5)P_2 is largely confined to the cytoplamic leaflet of the plasma membrane, where it constitutes about 1% of the total cellular phospholipids and forms the precursors of important signaling molecules such as IP_3, DAG and PIP_3 (McLaughlin and Murray, 2005). Importantly, PIP_2 itself is a signaling molecule that modulates the functions of various ion channels and transporters (Gamper and Shapiro, 2007; Suh and Hille, 2008). The first channel shown to be modulated by PIP_2 was the ATP-inhibited inwardly rectifying K^+ (Kir) channel (Hilgemann and Ball, 1996). All members of the Kir channel family and the KCNQ (Kv7.x) voltage gated K^+ channel family have now been shown to require the presence of PIP_2 for activity (Logothetis et al., 2007; Suh and Hille, 2008). In this section, I will

summarize the current knowledge on the modes of modulation of TRPs by PIP_2 and the mechanisms of interaction between TRPs and PIP_2.

A large number of TRP channels that have been reported to be regulated by PIP_2 (reviewed by (Nilius et al., 2008)). However, the effects of PIP_2 are quite variable between members of the TRP superfamily. Below I will discuss the specific roles of PIP_2 in the regulation of TRP channels in detail.

PIP_2 and TRPM channels

The picture of PIP_2 regulation is probably the clearest among TRPM channels, yet there are a number of apparent contradictions. Published reports indicate that PIP_2 activates three members of the mammalian TRPM subfamily: TRPM4, TRPM5, and TRPM8 (Zhang et al., 2005b; Liu and Liman, 2003b; Liu and Qin, 2005; Zhang et al., 2005c; Rohacs et al., 2005; Nilius et al., 2006). PIP_2 is required for channel activity and the breakdown of PIP_2 upon activation of PLC leads to current rundown. Application of exogenous PIP_2 both activates the channels directly and restores current rundown.

Similar PIP_2 regulation of TRPM7 has been proposed by Runnels et al (Runnels et al., 2002). They have shown that depletion of PIP_2 by Gq coupled receptors inhibits TRPM7, and PIP_2 stimulates single channel activity. However, Takezawa et al. challenged this conclusion by showing that activation of Gq coupled thrombin receptor had no effect on TRPM7 activity, which suggests that PLC mediated PIP_2 hydrolysis is not a major regulator of TRPM7 (Takezawa et al., 2004). This discrepancy may depend on TRPM7 expression level as suggested by Takezawa et al. Furthermore, Langeslag et al. observed that in perforated patch experiments, stimulation of PLC-activating receptors

causes TRPM7 opening rather than closure, which suggests that PIP_2 might play dual roles in regulating TRPM7 function (Langeslag et al., 2007).

PIP_2 and other TRP channels

Additional studies have indicated interactions between PIP_2 and other TRP channels. PIP_2 both activates and inhibits TRPV1 and the mode of action is dependent on the degree of stimulation by channel agonists such as capsaicin (Lukacs et al., 2007b). TRPV5 and TRPV6, on the other hand, were reported to be activated by PIP_2 (Lukacs et al., 2007b; Lee et al., 2005; Thyagarajan et al., 2008). TRPC channels and their non-mammalian homologues are activated by G protein coupled receptors that activate PLC and hydrolyze PIP_2. The exact mechanism of how PLC activates these channels and the role of PIP_2 are not clear, and both could be diverse with-in the subfamily. Drosophila TRPL and mammalian TRPC4 channels were reported to be inhibited by PIP_2 (Estacion et al., 2001; Otsuguro et al., 2008). TRPC3, 6 and 7 have been shown to be activated by PIP_2 (Lemonnier et al., 2008; Kwon et al., 2007). However, PIP_2 has also been reported to have an inhibitory effect on native TRPC6 activity in mesenteric artery myocytes (Albert et al., 2008). The effect of PIP_2 on TRPC5 is complex. Inclusion of PIP_2 in the patch pipette inhibited TRPC5 current. Paradoxically, when single channel activity is examined in excised patches, the channels are robustly activated by PIP_2. The authors proposed that PIP_2 might have two distinct functions in regulating TRPC5 channel activity (Trebak et al., 2009).

Little data are available on PIP_2 regulation of more distantly related TRP channel subfamilies. TRPP2 or PKD2 is inhibited by PIP_2 and EGF activates TRPP2 by PIP2 breakdown and the ensuing relief from this inhibition (Ma et al., 2005). The situation is

controversial for TRPA1. Two studies have shown that PIP_2 activates (Karashima et al., 2008) or inhibits TRPA1 activity (Kim et al., 2008b), respectively, suggesting that PIP_2 might have a dual effect on TRPA1 activity.

How does PIP_2 interact with TRP channels?

The head-group of PIP_2 has a high negative charge density and therefore it is likely to electrostatically interact with proteins that have clustered positive residues. At this point, there are experimental data suggesting the existence of multiple distinct types of PIP_2 interaction sites in the TRP family, all of which are characterized by an abundance of positively charged residues (summarized in Figure 3).

The TRP domain in the proximal C terminus is the most conserved region of TRP channels among the TRPC, TRPV and TRPM families. Mutations of positively charged residues in this domain reduce in the apparent affinity of PIP_2 activation of TRPM8, TRPV5 and TRPM5 channels, suggesting that these residues are critical in TRP-PIP interactions(Rohacs et al., 2005).

Neutralization of the equivalent residues in TRPM4 does not have significant effects on PIP_2 sensitivity of the channel. A more distal C-terminal region, which also contains clusters of positively charged residues, was shown to play critical roles in channel activation by PIP_2 (Zhang et al., 2005b; Zhang et al., 2005c). Similarly, a region containing eight positive charges in the C terminus of TRPV1 was identified as a possible PIP_2 interaction site responsible for PIP_2-mediated channel inhibition (Prescott and Julius, 2003).

Kwon et al. found that neutralization of basic residues in the calmodulin binding site of TRPC6 affected PIPs (including PIP_2 and PIP_3) binding and channel function, and

that PIPs disrupts the interaction between calmodulin and the C-terminus of the channel, suggesting that PIPs interact directly with the calmodulin-binding site (Kwon et al., 2007). Otsuguro et al studied the effects of PIP_2 on two different splice isoforms of TRPC4 and found that TRPC4α is inhibited by PIP_2, whereas TRPC4β, which lacks 84 amino acids (Δ84AA) in the C terminus, is PIP_2 insensitive. This suggests that this stretch of 84 AA contains all or part of a PIP_2 interaction site (Otsuguro et al., 2008).

Clearly, there is a considerable variability in the putative PIP_2 interacting sites among the TRP channel superfamily, and yet another layer of complexity could be added to the mechanisms of TRP-PIP_2 interaction. Recent evidence indicates that PIP_2-dependent regulation of TRPV1 occurs through an accessory protein, Pirt. PIP_2-binding is dependent on a cluster of basic residues in the C terminus of Pirt, and this binding enhances TRPV1 channel activity (Kim et al., 2008a). These results indicate that mutations that alter the interaction between the channel and a PIP_2-binding accessory protein would also alter the PIP_2 sensitivity of the channel and therefore putative PIP_2-interacting sites defined purely by mutagenesis studies should be carefully interpreted.

In summary, regulation by PIP_2 represents a general mechanism for modulation of the majority of TRP channels. There is strong evidence that PIP_2 can activate a variety of TRP channels (TRPC3, TRPM4, 5, 8 and TRPV5, 6), and possibly also inhibit others (TRPC4, TRPP2, dTRPL/TRP). PIP_2 may interact with TRP channels through disparate PIP_2-binding sites or through accessory PIP_2-binding proteins. However, we are only starting to understand the variety and importance of PIP_2-TRP interactions. Little is currently known about the physiological implications of PIP_2-TRP interactions and how binding of PIP_2 influences TRP channel gating. Individual TRP channel-PIP_2 interactions

have to be studied in detail to reveal their physiological roles. We would anticipate answers to the latter question arising through the study of reconstituted channel activity in pure systems and ultimately by high-resolution structures of TRP channels.

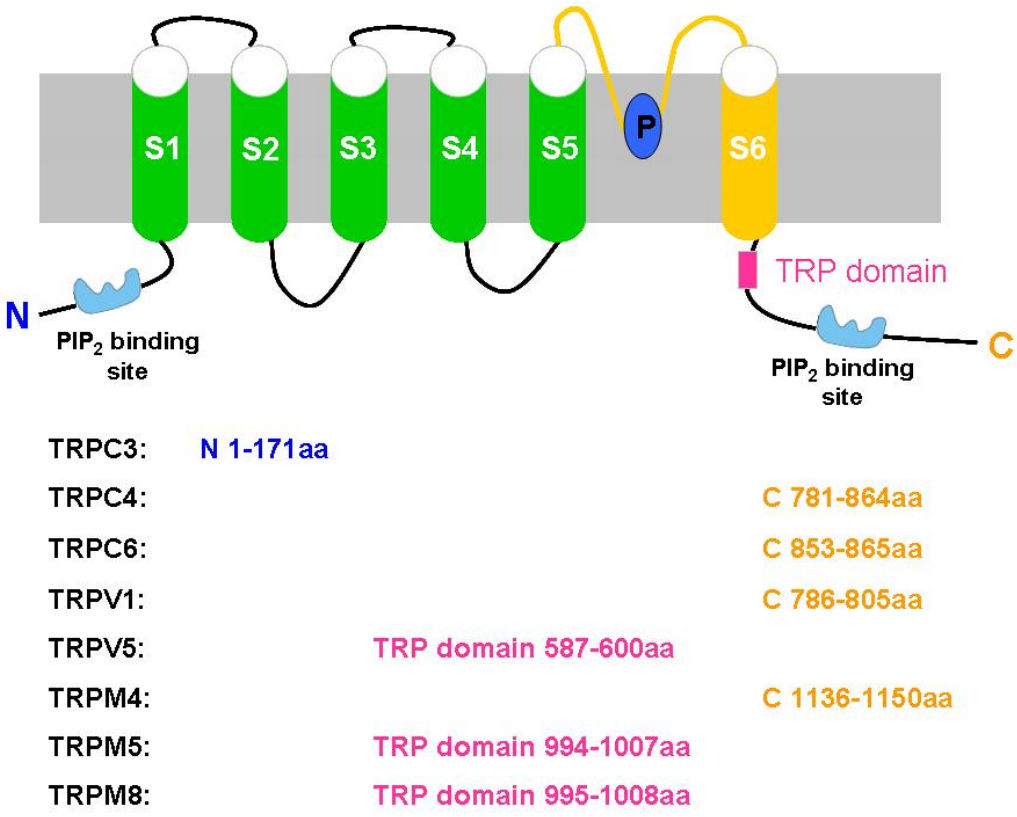


Figure 3. Schematic diagram of PIP_2 interacting sites on TRP channels. Known binding sites of PIP_2 on TRP channels are shown and their localizations are indicated.

IP_3-dependent Ca^{2+} signaling in the nematode *C. elegans*

Functions of the IP_3 receptor in *C. elegans*

Inositol 1,4,5-trisphosphate (IP_3) activates receptors (IP_3Rs) that mediate intracellular Ca^{2+} release, thereby modulating intracellular calcium signals and regulating important aspects of cellular physiology and gene expression (Berridge, 1993). A single gene, *itr-1*, encodes the IP_3R in *C. elegans* (Dal Santo et al., 1999). The IP_3R protein (ITR-1) is approximately 42 % identical with known IP_3Rs and possesses conserved structural features. When the putative IP_3 binding domain was expressed in *E. coli*, specific binding of IP_3 was detected (Baylis et al., 1999). ITR-1 is strongly expressed in the *C. elegans* intestine, pharynx, nerve ring, excretory cell and gonad (Baylis et al., 1999) and it functions to regulate several physiological processes including the defecation cycle (Dal Santo et al., 1999), pharyngeal pumping (Walker et al., 2002), ovulation and fertility(Yin et al., 2004), epidermal cell migration during embryogenesis (Thomas-Virnig et al., 2004), and male mating behavior (Gower et al., 2005). The high degree of structural and functional conservation of IP_3Rs from nematodes to mammals demonstrates that *C. elegans* can be utilized as a model system for studies on IP_3R mediated signaling.

IP_3-dependent Ca^{2+} oscillations in the intestine control rhythmic *C. elegans* defecation

The digestive tract of *C. elegans* consists of a pharynx, intestine and rectum (Figure 4A). *C. elegans* is a filter feeder and the pharynx is a muscular organ that pumps food into the pharyngeal lumen, grinds it up and then moves it into the intestine. Twenty epithelial cells with extensive apical microvilli form the main body of the intestine, which is approximately 750μm long in a full-grown adult worm. Intestinal cells are filled with numerous granules that likely contain lipids, proteins, and carbohydrates.

C. elegans exhibits a number of relatively simple stereotyped behaviors that have formed the bases for powerful forward genetic screens. The defecation cycle is one such behavior. While they are feeding, nematodes defecate rhythmically once every 45-50 seconds with little variation (Figure 4B). Defecation is initiated by contraction of the posterior body wall muscles (pBoc). After relaxation of these muscles, the anterior body wall muscles contract (aBoc) and then expulsion occurs by enteric muscle contraction (Emc)(Thomas, 1990). Laser ablations experiments identified two motor neurons that are required for the contraction of the anterior body wall and the enteric muscles(McIntire et al., 1993). In contrast, extensive neuronal laser ablation and mutations that disrupt neurotransmission have no effect on the posterior body wall contraction, suggesting that neuronal mechanisms do not regulate this part of the cycle.

It is now well established that pBoc rhythm is largely controlled by rhythmic Ca^{2+} oscillations in the *C. elegans* intestine. A proposed model is illustrated in Figure 5. IP_3-depedent Ca^{2+} oscillations may control the secretion of protons from the intestinal epithelium that act on H^+ gated ion channels in neighboring muscle cells and triggers

contraction of the surrounding posterior body wall muscles (pBoc) that drives defecation (Dal Santo et al., 1999; Espelt et al., 2005a; Pfeiffer et al., 2008; Beg et al., 2008).

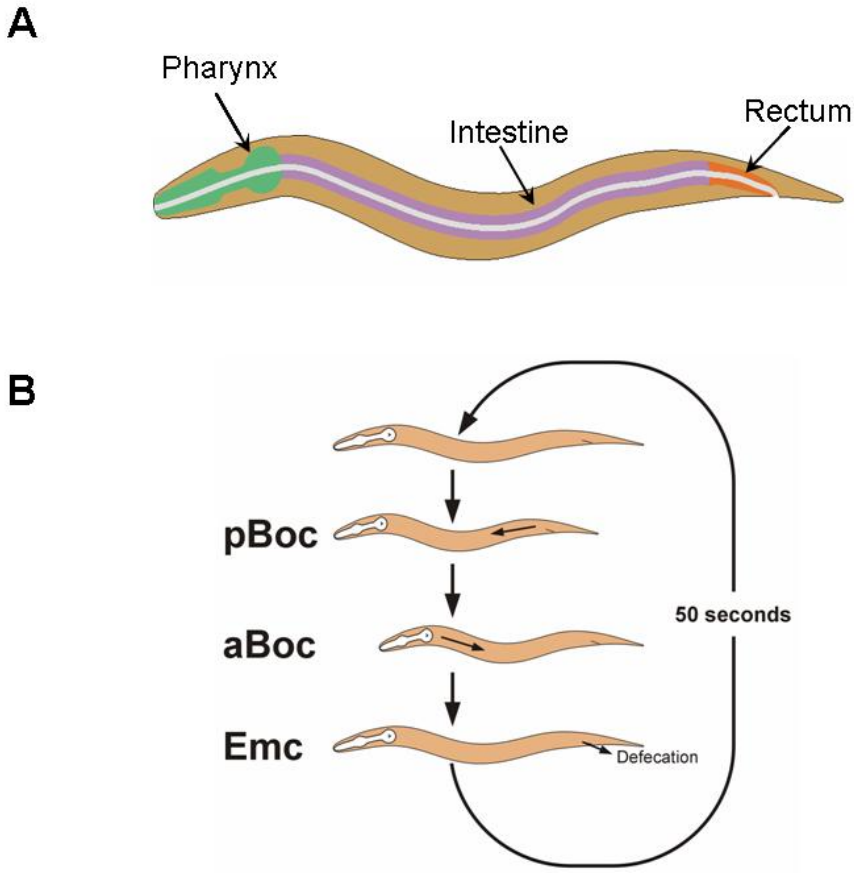


Figure 4. Cartoon illustrating muscle contractions that mediates *C. elegans* defecation. (A) Schematic diagram of the *C. elegans* digestive tract. Twenty epithelial cells with extensive apical microvilli form the main body of the intestine. (B) Diagram illustrating muscle contractions that mediates defecation. Cycle is mediated by sequential contraction of the posterior body wall muscles (pBoc), the anterior body wall muscles contract (aBoc) and the enteric muscles (Emc). The cycle repeats itself every 45-50 sec.

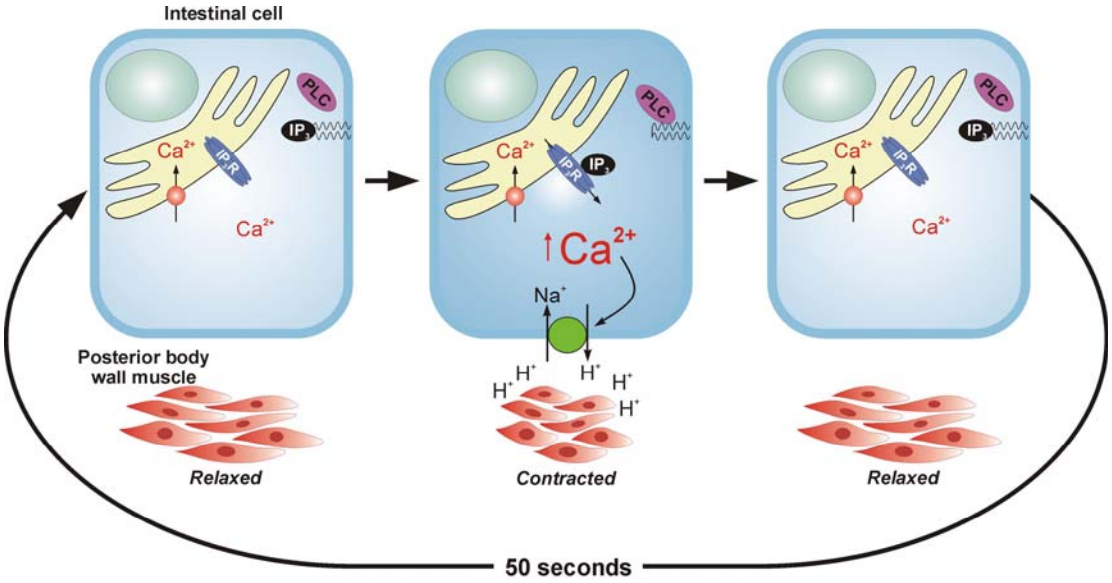


Figure 5. Role of intestinal Ca^{2+} oscillations in regulating *C. elegans* pBoc rhythm. A model has been proposed to illustrate possible role of intracellular Ca^{2+} in regulating defecation cycle. Cyclical elevation of cytoplasmic Ca^{2+} levels is driven by IP_3-dependent intracellular Ca^{2+} release. Increased Ca^{2+} concentration triggers proton secretion through the Na^+/H^+ exchanger. H^+ then acts at proton gated ion channels in the muscle cells and induces muscle contraction. The cycle repeated itself once every 50 sec.

Develop *C. elegans* intestine as a model system to study the molecular mechanisms of Ca^{2+} oscillations in nonexcitable cells

Genetic model organisms provide a number of powerful experimental advantages for defining the genes and genetic pathways involved in biological processes such as Ca^{2+} signaling. The nematode *C. elegans* is a particularly attractive model system. *C. elegans* is well suited for mutagenesis and forward genetic analysis and has a fully sequenced and well annotated genome. Gene expression in nematodes is relatively easy and economical to manipulate using RNA interference (RNAi), knockout, and transgenesis (Strange, 2003). To study oscillatory Ca^{2+} signaling events directly, we developed an isolated intestine preparation that allows physiological access to the intestinal epithelium. We have previously shown that isolated intestines exhibit spontaneous, rhythmic Ca^{2+} oscillations that occur with the same frequency as pBoc.

Physiological and genetic analyses demonstrate that rhythmicity of the intestinal Ca^{2+} oscillations require the combined function of PLCγ and PLCβ homologues and the IP_3 receptor. PLCγ functions primarily to generate IP_3 that regulates IP_3R activity while PLCβ function in a separate, yet to be defined pathway. The molecular and genetic tractability of *C. elegans* combined with the physiological accessibility of the isolated intestine preparation provides a powerful new model system in which to develop an integrated genetic and molecular understanding of oscillatory Ca^{2+} signaling. Mutations in intestine-expressed genes that disrupt the pBoc and Ca^{2+} oscillation rhythm are likely to play a role in IP_3-dependent oscillatory Ca^{2+} signaling in the intestinal cells.

C. elegans intestinal cells express store-independent and store-operated Ca^{2+} conductances

As noted above, the *C. elegans* intestine provides a unique model system in which to characterize the molecular details of IP_3-dependent oscillatory Ca^{2+} signaling. Our laboratory developed methods to culture *C. elegans* embryonic cells. Isolated embryonic cells differentiate within 24 h into the various cell types that form the newly hatched L1 larva and this allows direct electrophysiological characterization of *C. elegans* somatic cells (Christensen et al., 2002). Culturing embryo cells from worms expressing cell-specific GFP reporters allows identification of differentiated cell types. The *C. elegans* *elt-2* gene encodes a gut-specific GATA transcription factor. When ectopically expressing *elt-2*::GFP in intact animals, the transgene is only expressed in nuclei of cells from the gut lineage (Fukushige et al., 1998). Primary cultures were prepared from worms strains expressing *elt-2*::GFP transgene. Figure 6 shows combined DIC and fluorescence micrographs of a transgenic worm expressing *elt-2*::GFP (left) and an intestinal epithelial cell (right) cultured from *elt-2*::GFP expressing worms (Estevez et al., 2003). The cytoplasm of the cultured intestinal cells contains numerous granules (Figures 6A and 6B, arrowheads), which is a prominent characteristic that also allows identification of these cells in culture.

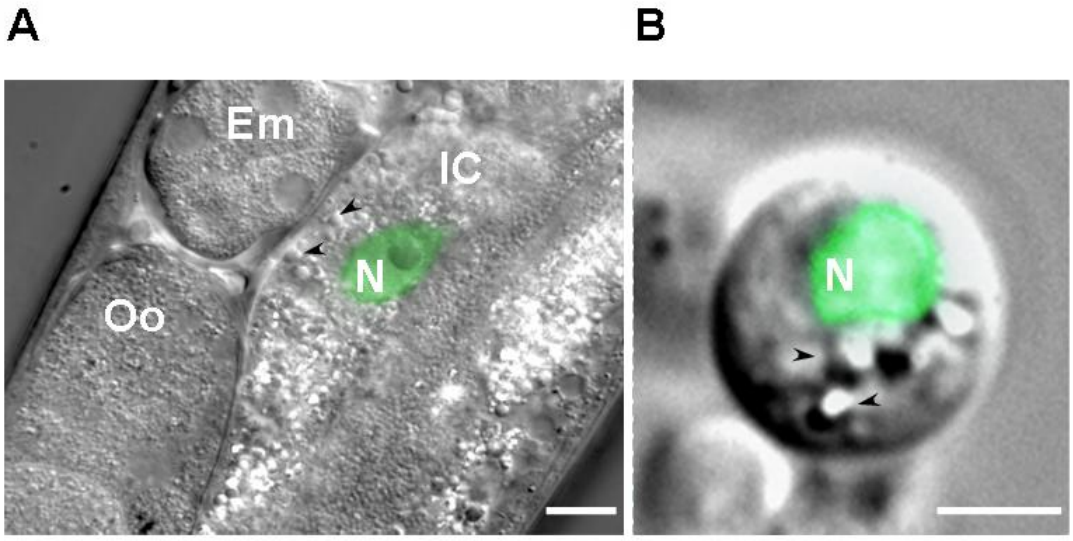


Figure 6. Primary culture of *C. elegans* intestinal cells. Images are overlays of differential interference contrast (DIC) and fluorescence micrographs of a transgenic worm (A) and a cultured intestinal cell (B) expressing *elt-2*::GFP in the cell nucleus. GFP fluorescence is shown in green. Scale bar are 10μm and 2.5 μm for the whole animal and the cultured intestinal cell, respectively. Em, developing embryo in uterus; Oo, oocyte in proximal gonad; IC, intestinal cell; N, intestinal cell nucleus. Arrow heads denote refractile granules.

To begin defining the functional roles and regulation of calcium channel involved in Ca^{2+} signaling events, Estevez et al. performed patch clamp analysis of intestinal cells cultured in vitro and identified two highly Ca^{2+}-selective conductances in the cultured intestinal cells (Estevez et al., 2003). One conductance, I_{ORCa}, is constitutively active, exhibits strong outward rectification, is 60~70-fold more selective for Ca^{2+} than Na^{+}, is inhibited by intracellular Mg^{2+} with a $K_{1/2}$ of 692 μM, and is insensitive to Ca^{2+} store depletion. Detailed characterization of the ORCa channel reveals that it has similar biophysical properties as TRPM7 channels observed in mammalian cells (Figure 7). The biophysical similarities between TRPM7 currents and ORCa suggest that the channels may have a common molecular origin.

Inhibition of I_{ORCa} with high intracellular Mg^{2+} concentrations revealed the presence of a small amplitude conductance that is activated by depletion of intracellular Ca^{2+} stores. The store-operated conductance resembles the Ca^{2+} release activated channel (I_{CRAC}) current (Estevez et al., 2003). Recent studies in our laboratory have demonstrated that *C. elegans* CRAC channel activity is mediated by ORAI-1 and STIM-1 proteins. RNAi mediated-suppression of either ORAI-1 or STIM-1 fully inhibits I_{CRAC} without affecting the pBoc cycle and Ca^{2+} oscillations in isolated intestine (Yan et al., 2006; Lorin-Nebel et al., 2007). This suggests that store-operated calcium entry (SOCE) is not essential for intestinal IP_3-dependent oscillatory Ca^{2+} signaling. The focus of this thesis work is to determine the molecular identity of the ORCa channel and to define its role in oscillatory Ca^{2+} signaling.

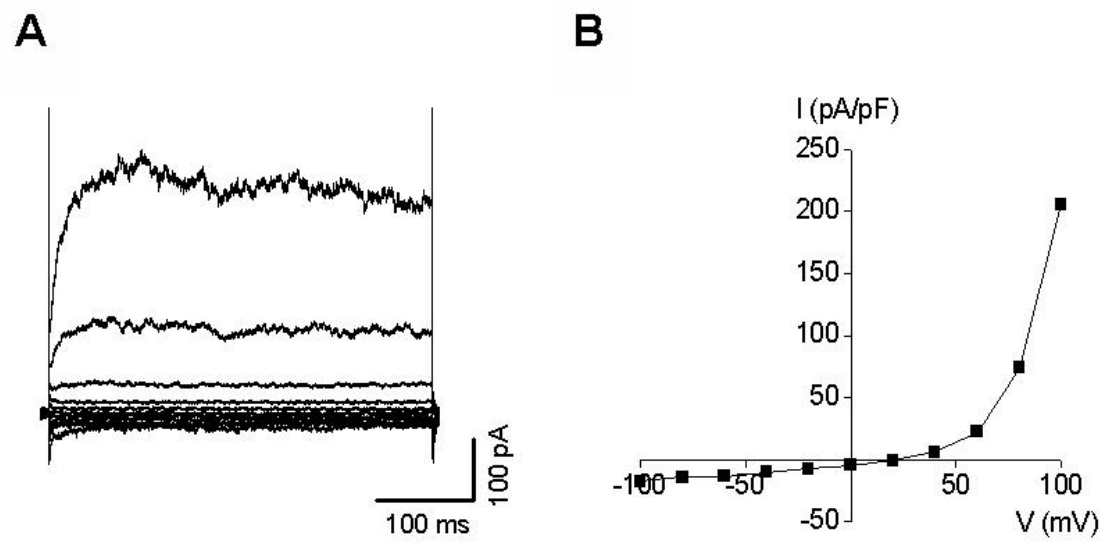


Figure 7. Whole cell ORCa current recorded in cultured *C. elegans* intestinal cells. (A) Whole-cell outwardly rectifying Ca^{2+} (ORCa) currents recorded from a cultured intestinal cell. Currents were elicited by stepping membrane voltage from -100 mV to +100 mV in 20 mV steps from a holding potential of 0 mV. Voltage steps were 400 msec long. (B) Steady-state I-V relationship for the whole-cell currents shown in A.

Summary of chapter I

Fluctuating intracellular Ca^{2+} concentrations control numerous cellular processes. Keeping this in mind, it is not surprising that abnormal intracellular Ca^{2+} homeostasis leads to a plethora of diseases. Over the last two decades, physiologists have gained impressive understanding of Ca^{2+} signaling events, although many fundamental questions remain unanswered. The nematode *C. elegans* offers substantial experimental advantages to study Ca^{2+} signaling events. *C. elegans* pBoc is an easily observable and quantifiable behavior that is controlled by intestinal Ca^{2+} oscillations. The central focus of my thesis work is to determine the molecular identity of the plasma membrane ORCa channel in *C. elegans* intestinal cells, define its roles in regulating intestinal Ca^{2+} oscillations, and study how the channel is regulated. Given that ORCa current shares similar biophysical properties as TRPM currents in mammalian cells, we hypothesized that ORCa may have a common molecular origin as mammalian TRPM channels and focused our study on characterizing the roles of *C. elegans* TRPM homologues in regulating IP_3-dependent oscillatory signaling pathway. Our long-term goal is to utilize the powerful forward and reverse genetic screens of pBoc to identify the genes, gene networks, and molecular mechanisms that underlie intestinal Ca^{2+} oscillations, which are common to eukaryotic nonexcitable cells. Thorough molecular understanding of Ca^{2+} signaling will lead to a better understanding of numerous diseases related to disruption of Ca^{2+} homseostasis.

CHAPTER II

HIGHLY CA^{2+}-SELECTIVE TRPM CHANNELS REGULATE IP_3-DEPENDENT OSCILLATORY CA^{2+} SIGNALING IN THE *C. ELEGANS* INTESTINE.

This paper has been published under the same title in *J Gen Physiol.* 131(3):245-55.

Summary

Posterior body wall muscle contraction (pBoc) in the nematode *Caenorhabditis elegans* occurs rhythmically every 45–50 s and mediates defecation. pBoc is controlled by inositol-1,4,5-trisphosphate (IP_3)–dependent Ca^{2+} oscillations in the intestine. The intestinal epithelium can be studied by patch clamp electrophysiology, Ca^{2+} imaging, genome-wide reverse genetic analysis, forward genetics, and molecular biology and thus provides a powerful model to develop an integrated systems level understanding of a nonexcitable cell oscillatory Ca^{2+} signaling pathway. Intestinal cells express an outwardly rectifying Ca^{2+} (ORCa) current with biophysical properties resembling those of TRPM channels. Two TRPM homologues, GON-2 and GTL-1, are expressed in the intestine. Using deletion and severe loss-of-function alleles of the *gtl-1* and *gon-2* genes, we demonstrate here that GON-2 and GTL-1 are both required for maintaining rhythmic pBoc and intestinal Ca^{2+} oscillations. Loss of GTL-l and GON-2 function inhibits I_{ORCa} ~ 70% and ~90%, respectively. I_{ORCa} is undetectable in *gon-2;gtl-1* double mutant cells.

These results demonstrate that (a) both *gon-2* and *gtl-1* are required for ORCa channel function, and (b) GON-2 and GTL-1 can function independently as ion channels, but that their functions in mediating I_{ORCa} are interdependent. I_{ORCa}, I_{GON-2}, and I_{GTL-1} have nearly identical biophysical properties. Importantly, all three channels are at least 60-fold more permeable to Ca^{2+} than Na^{+}. Epistasis analysis suggests that GON-2 and GTL-1 function in the IP_3 signaling pathway to regulate intestinal Ca^{2+} oscillations. We postulate that GON-2 and GTL-1 form heteromeric ORCa channels that mediate selective Ca^{2+} influx and function to regulate IP_3 receptor activity and possibly to refill ER Ca^{2+} stores.

Introduction

The genetic model organism *Caenorhabditis elegans* provides numerous experimental advantages for developing an integrative genetic and molecular understanding of fundamental physiological processes (Barr, 2003; Strange, 2003). These advantages include a short life cycle, forward genetic tractability, a fully sequenced and well-annotated genome and relative ease and economy of characterizing gene function using transgenic and RNA interference methods.

C. elegans intestinal epithelial cells generate rhythmic inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} oscillations that control posterior body wall muscle contraction (pBoc) (Dal Santo et al., 1999; Teramoto and Iwasaki, 2006; Espelt et al., 2005b; Peters et al., 2007). pBoc is part of a motor program that mediates defecation and can be observed readily through a dissecting microscope making it amenable to forward and reverse genetic screening (Thomas, 1990; Liu and Thomas, 1994; Iwasaki et al., 1995).

Intestinal Ca^{2+} signaling can be quantified by imaging methods in isolated intestines (Espelt et al., 2005b; Teramoto and Iwasaki, 2006; Peters et al., 2007) or in vivo using genetically encoded Ca^{2+} indicators (Teramoto and Iwasaki, 2006; Yan et al., 2006; Peters et al., 2007). Recent development of primary cell culture methods (Christensen et al., 2002; Strange et al., 2007) has made it possible to characterize intestinal ion channels using patch clamp methods. The ability to combine direct physiological measurements of IP_3–dependent oscillatory Ca^{2+} signals and associated ion channel activity with forward and reverse genetic screening is unique to *C. elegans*. The worm intestinal epithelium thus provides a powerful model system in which to define the genetic and molecular details and integrative physiology of oscillatory Ca^{2+} signaling in nonexcitable cells.

Intestinal Ca^{2+} oscillations are strictly dependent on Ca^{2+} release from the endoplasmic reticulum (ER) via ITR-1, the single IP_3 receptor encoded by the *C. elegans* genome (Dal Santo et al., 1999; Teramoto and Iwasaki, 2006; Espelt et al., 2005b). Extensive studies in vertebrate (reviewed by (Hogan and Rao, 2007; Venkatachalam et al., 2002; Parekh and Putney, 2005) and *Drosophila* cells (Yeromin et al., 2004) have demonstrated that depletion of ER Ca^{2+} stores activates store-operated Ca^{2+} channels (SOCCs). SOCCs are widely believed to be an essential and ubiquitous component of Ca^{2+} signaling pathways, functioning to refill ER Ca^{2+} stores and modulate intracellular Ca^{2+} signals (e.g., (Hogan and Rao, 2007; Venkatachalam et al., 2002; Parekh and Putney, 2005). The most egxtensively studied and characterized SOCC is the Ca^{2+} release activated Ca^{2+} (CRAC) channel (Parekh and Putney, 2005). The CRAC channel pore is comprised of Orai1/CRACM and channel activation is mediated by STIM1, which

functions as an ER Ca^{2+} sensor (reviewed by (Hogan and Rao, 2007; Lewis, 2007; Putney, Jr., 2007).

C. elegans intestinal cells express robust CRAC channel activity (Estevez et al., 2003). RNAi silencing of *orai-1* or *stim-1*, which encode worm Orai1/CRACM and STIM1 homologues, dramatically reduces CRAC channel expression and function, but surprisingly has no effect on intestinal Ca^{2+} signaling (Yan et al., 2006; Lorin-Nebel et al., 2007). These findings suggest that CRAC channels are not essential components of IP_3-dependent Ca^{2+} signaling in the intestine and indicate that other Ca^{2+} entry mechanisms must function to maintain intestinal Ca^{2+} oscillations.

In addition to CRAC channels, intestinal cells express a store-independent outwardly rectifying Ca^{2+} (ORCa) channel that has biophysical properties resembling those of mammalian TRPM channels (Estevez et al., 2003). Three TRPM homologues are encoded by the *C. elegans* genome, GON-2 (abnormal gonad development), GTL-1 (gon-2 like 1) and GTL-2 (Baylis and Goyal, 2007; Kahn-Kirby and Bargmann, 2006). They share approximately 23% identity with TRPM1, TRPM3, TRPM6 and TRPM7 (Baylis and Goyal, 2007). The conserved structural motifs in these channels are the transmembrane domains, the TRP domain and portions of the cytoplasmic N-terminus. GFP reporter studies have demonstrated that intestinal cells express *gon-2* and *gtl-1* (Teramoto et al., 2005); cited as unpublished observations in (Baylis and Goyal, 2007)WormBase; http://www.wormbase.org/). The goal of the present study was to define the roles these genes play in intestinal Ca^{2+} signaling. Our results demonstrate that GON-2 and GTL-1 are both required for ORCa channel activity and for maintaining rhythmic Ca^{2+} oscillations. We propose that *gon-2* and *gtl-1* encode the ORCa channel.

We also suggest that ORCa channels comprise a major Ca^{2+} entry pathway in intestinal epithelial cells and that they function to regulate IP_3 receptor activity and refill ER Ca^{2+} stores.

Material and methods

C. elegans strains

Nematodes were cultured using standard methods on Nematode Growth Medium (NGM) (Brenner, 1974). Wild type worms were the Bristol N2 strain or *elt-2::gfp* worms that express a transcriptional GFP reporter in intestinal cell nuclei. Worms homozygous for the *gon-2* loss-of-function allele *gon-2(q388)* or the *gtl-1* deletion allele *gtl-1(ok375)* were used for studies of GON-2 and GTL-1 function. *gon-2;gtl-1* double mutant worms were generated by crossing the *gtl-1(ok375)* and *gon-2(q388)* strains (Teramoto et al., 2005). The *gon-2;gtl-1* double mutant worm strain exhibits greatly slowed larval development on NGM. To improve development and fertility sufficiently for experiments to be performed, double mutants were grown on NGM supplemented with 20 mM Mg^{2+} (see (Teramoto et al., 2005). All worm strains were maintained at 16-20 °C. Growth temperatures used in specific experiments are described below.

Construction of transgenic worms

Full-length *gtl-1* cDNA was a genenous gift from Dr. Howard Baylis. Translational GFP reporter was generated using a PCR-fusion based method (Hobert, 2002) and expression of the GFP reporter was driven by 2kb of promoter sequence upstream of the *gtl-1* start codon. This sequence was amplified by PCR from *C. elegans* N2 genomic DNA. Transgenic worms were generated by DNA microinjection as described by Mello et al. using *rol-6* as a transformation marker (Mello et al., 1991).

Characterization of pBoc cycle

gon-2(q388) is a temperature sensitive allele and the mutant phenotype is observed at growth temperatures of 25 °C (Sun and Lambie, 1997). For posterior body wall muscle contraction (pBoc) measurements, eggs from wild type and mutant worm strains were cultured in a 25 °C incubator until adulthood. The times required for wild type, *gon-2* mutant, *gtl-1* mutant and double mutant worms to reach adulthood at 25 °C were 2-3 days, 3-4 days, 3-4 days and 5 days, respectively.

pBoc was monitored by imaging worms on growth agar plates using a Zeiss Stemi SV11 M^2BIO stereo dissecting microscope (Kramer Scientific Corp., Valley Cottage, NY) equipped with a DAGE-MTI (Michigan City, IN) DC2000 CCD camera. A minimum of ten pBoc cycles was measured in each animal. Measurements were performed at a room air temperature of 22-23 °C. Agar temperature was monitored during the course of pBoc measurements using a thermistor (Model 4600, Yellow Springs Instruments, Yellow Springs, OH) and was 24-25 °C.

Dissection and fluorescence imaging of intestines

Worms were cultured as described above for pBoc measurements. Calcium oscillations were measured in isolated intestines as described previously (Figure 8) (Espelt et al., 2005b). The *C. elegans* digestive tract consists of a pharynx, intestine, and rectum (Figure 8A). Worms were placed in control saline (137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 10 mM HEPES, 5 mM Glucose, 2 mM L-asparagine, 0.5 mM L-cysteine, 2 mM L-glutamine, 0.5 mM L-methionine, 1.6 mM L-tyrosine, 28 mM sucrose, pH 7.3, 340 mOsm) and cut behind the pharynx using a 26-gauge needle. The hydrostatic pressure in the worm spontaneously extruded the intestine, which remained attached to the rectum and the posterior end of the animal. Isolated intestines were incubated for 15 min in bath saline containing 5 μM fluo-4 AM and 1% bovine serum albumin (BSA). Imaging was performed using a Nikon TE2000 inverted microscope, a Superfluor 40X/1.3 N.A. oil objective lens, a Photometrics Cascade 512B cooled CCD camera (Roper Industries, Duluth, GA) and MetaFluor software (Molecular Devices Corporation, Sunnyvale, CA). Room temperature was maintained at 25-26 °C. Fluo-4 was excited using a 490-500BP filter and a 523-547BP filter was used to detect fluorescence emission. Fluorescence images were acquired at 0.2 or 1 Hz. Changes in fluo-4 intensity were quantified in posterior-to-anterior moving Ca^{2+} waves using region-of-interest selection and MetaFluor software (Molecular Devices Corporation). Figure 8B shows the differential interference contrast (DIC) micrograph (left, bright field) and fluorescence micrograph (right, dark field) of an isolated intestine loaded with fluo-4 AM. Under control conditions, isolated intestines from wild type animals exhibit spontaneous rhythmic intracellular Ca^{2+} oscillations with a period of ~ 50s (Figure 8C), which is not

significantly ($P>0.2$) different from the mean pBoc period of 56 ± 1 sec observed in intact animals.

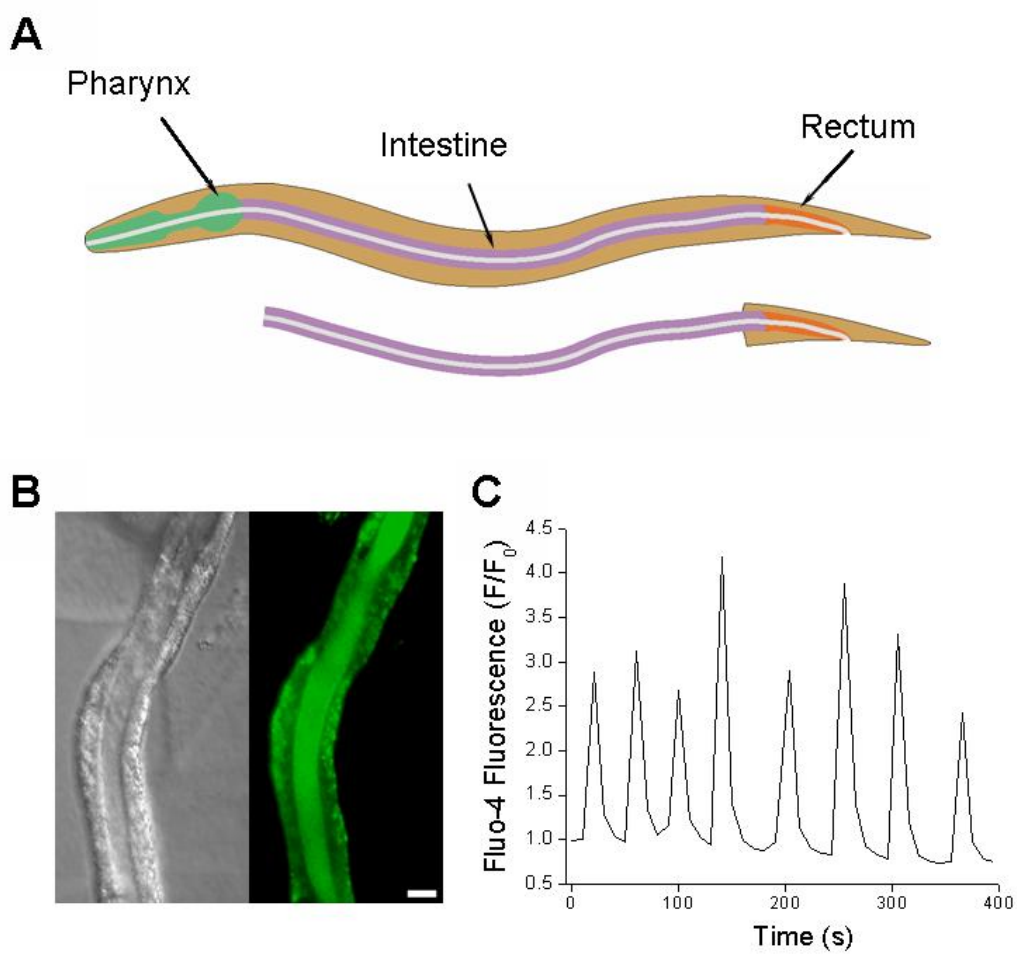


Figure 8. Isolated *C. elegans* intestines exhibited spontaneous Ca^{2+} oscillations. (A) Schematic diagrams of worm digestive tract and isolated intestine. (B) Differential interference contrast (DIC) and fluorescence micrographs of an isolated intestine loaded with fluo-4 AM. Bar, 20μm. (C) Intracellular Ca^{2+} oscillations in an intestine isolated from a wild type worm. Images were acquired at 5s intervals. The Ca^{2+} oscillation period for this intestine was 49 s.

C. elegans embryonic cell culture and patch clamp electrophysiology

Newly hatched wild type and mutant worm L1 larvae were cultured at 25 °C until adulthood. Embryonic cells were cultured for 2-3 days at 25 °C on 12 mm diameter acid-washed glass cover slips using established methods (Strange et al., 2007; Christensen et al., 2002). To maximize suppression of GON-2 activity, cells isolated from *gon-2* and *gon-2;gtl-1* double mutant worms were cultured in the presence *gon-2* double strand RNA (dsRNA) using methods described previously (Yan et al., 2006; Lorin-Nebel et al., 2007). *gon-2* dsRNA was from synthesized from a 640 bp (4041-4681 bp) *gon-2* cDNA that was amplified from a *C. elegans* cDNA library.

Cover slips with cultured embryo cells were placed in the bottom of a bath chamber (model R-26G; Warner Instrument Corp., Hamden, CT) that was mounted onto the stage of a Nikon TE2000 inverted microscope. Bath temperature was maintained at 25 °C using a Warner Instruments model SC-20 dual in-line heater/cooler, a model CL-100 bipolar temperature controller, and a PHC series heater/cooler jacket for the bath chamber. Cells were visualized by fluorescence and video-enhanced DIC microscopy. Intestinal cells were identified in culture by expression of the intestine specific reporter *elt-2*::GFP or by morphological characteristics (Fukushige et al., 1998; Estevez et al., 2003).

Patch electrodes were pulled from soft glass capillary tubes (PG10165-4, World Precision Instruments, Sarasota, FL) that had been silanized with dimethyl-dichloro silane. Pipette resistance was 4-7 MΩ. Bath and pipette solutions contained 145 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM HEPES, 20 mM glucose, pH 7.2 (adjusted with NaOH), and 147 mM sodium gluconate (NaGluconate), 0.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM

EGTA, 10 mM HEPES, 2 mM Na_2ATP, 0.5 mM Na_2GTP, pH 7.2 (adjusted with CsOH), respectively. The osmolality of bath and pipette solutions were adjusted to 345-350 mOsm and 325-330 mOsm using sucrose.

Whole cell currents were recorded using an Axopatch 200B (Axon Instruments, Foster City, CA) patch clamp amplifier. Command voltage generation, data digitization, and data analysis were carried out on a 2.79 GHz Pentium computer (Dimension 9150; Dell Computer Corp.) using a Digidata 1322A AD/DA interface with pClamp 10 software (Axon Instruments). Electrical connections to the amplifier were made using Ag/AgCl wires and 3 M KCl/agar bridges.

Currents were elicited using a ramp or step voltage clamp protocol. For the ramp protocol, membrane potential was held at 0 mV and ramped from -80 mV to +80 mV at 215 mV/sec every 5 sec. Step changes in whole cell current were elicited by stepping membrane voltage from -80 to +80 mV in 20 mV steps from a holding potential of 0 mV. Voltage steps were maintained for 400 msec. Cell capacitances for all cells studied ranged from 1-4 pF.

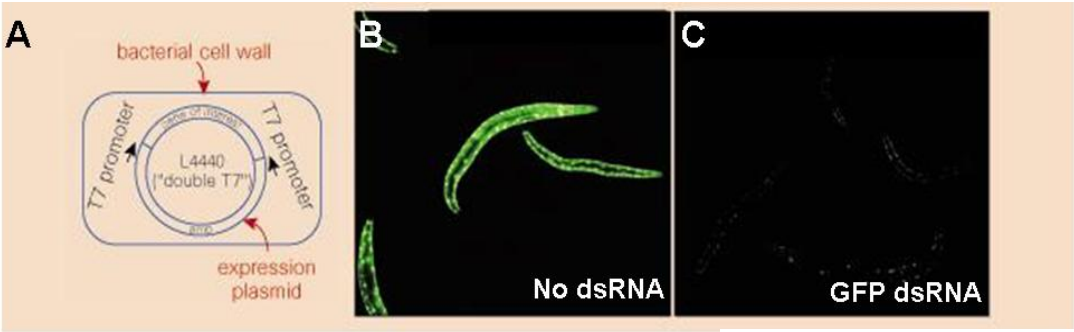
As we described previously, I_{ORCa} is outwardly rectifying with a strongly positive reversal potential (Estevez et al., 2003). In the present study, we also observed that currents in *gon-2* and *gtl-1* mutant cells reversed at strongly positive membrane potentials and exhibited outward rectification. Outwardly rectifying currents with reversal potentials <10 mV were deemed to be excessively contaminated with non-specific leak current and were rejected from final datasets.

Ion substitution studies were performed by replacement of bath Na^+ with various test cations. Cells were patch clamped initially in control bath solution until whole cell

current had stabilized and then switched to a Ca^{2+}- and Mg^{2+}-free medium containing 1 mM EGTA. Changes in reversal potential (E_{rev}) were measured after replacement of 150 mM bath NaCl with 150 mM NMDG-Cl, 130 mM NMDG-Cl and 10 mM $CaCl_2$ or 130 mM NMDG-Cl and 10 mM $MgCl_2$. Liquid junction potential changes were calculated using pClamp 10. Reversal potentials during ion substitution experiments were corrected for liquid junction potentials. Relative permeabilities were calculated from E_{rev} changes as described previously (Estevez et al., 2003).

Induction of RNA interference by double strand RNA feeding

RNA interference was induced by feeding *gon-2;gtl-1* double mutant worms bacteria producing double stranded RNA (dsRNA) (Figure 9) (e.g., (Kamath et al., 2000; Rual et al., 2004) homologous to PLCγ or PLCβ. RNAi bacterial strains were engineered as described previously (Figure 9) (Yin et al., 2004). Bacterial strains were streaked to single colonies on agar plates containing 50 µg/ml ampicillin and 12.5 µg/ml tetracycline. Single colonies were used to inoculate LB media containing 50 µg/ml ampicillin and cultures were grown at 37 °C for 16-18 h with shaking. Three hundred microliters of each bacterial culture were seeded onto 60 mm NGM agar plates containing 20 mM Mg^{2+}, 50 µg/ml ampicillin and 1 mM IPTG to induce dsRNA synthesis. After seeding, plates were left at room temperature overnight. Eggs were transferred to the RNAi feeding plates and grown at 25 °C.



Timmons and Fire, 1998

Figure 9. Induction of RNA interference following ingestion of dsRNA-expressing bacteria by *C. elegans.* (A) General scheme for dsRNA production. Segments of the target genes were cloned between flanking copies of the T7 promoter into a bacterial plasmid. A bacterial strain expressing the T7 polymerase gene was used as a host and dsRNA was produced by these bacteria. (B) A GFP-expressing *C. elegans* strain (PD4251) fed on a naive bacterial host. Animals show high GFP fluorescence in body muscles. (C) GFP fluorescence was significantly reduced in PD4251 animals that were reared on bacteria expressing dsRNA corresponding to the gfp coding region

Statistical analysis

Data are presented as means ± S.E. Statistical significance was determined using Student's two-tailed *t* test for unpaired means. When comparing three or more groups, statistical significance was determined by one-way analysis of variance with a Bonferroni post-hoc test. P values of ≤ 0.05 were taken to indicate statistical significance. The rhythmicity of the pBoc cycle and intestinal Ca^{2+} oscillations is quantified as coefficient of variance (CV), which is the standard deviation expressed as a percentage of the sample mean.

Results

Removal of extracellular Ca^{2+} causes rapid cessation of intestinal Ca^{2+} oscillations

Calcium is taken up into the ER via the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) while plasma membrane pumps and exchangers continuously extrude Ca^{2+} from the cell (Hogan and Rao, 2007; Berridge et al., 2003). Because of the presence of plasma membrane Ca^{2+} extrusion mechanisms, some Ca^{2+} will be lost from the cell during ER Ca^{2+} release. Repeated and/or prolonged ER Ca^{2+} release will eventually deplete ER Ca^{2+} stores and prevent further IP_3–dependent Ca^{2+} signals unless plasma membrane Ca^{2+} entry mechanisms are also active. To determine whether such Ca^{2+} entry mechanisms are required for IP_3-dependent Ca^{2+} signaling in the intestine, we monitored Ca^{2+} oscillations during removal of bath Ca^{2+}. As shown in Figure 10, total intracellular

fluo-4 fluorescence dropped and Ca^{2+} oscillations ceased rapidly when extracellular Ca^{2+} was removed. Calcium oscillations recovered when Ca^{2+} was added back to the bath. These results demonstrate that Ca^{2+} entry mechanisms are active in the intestine and that Ca^{2+} oscillations are strictly dependent on extracellular Ca^{2+} influx. Calcium entry almost certainly functions to refill ER stores. In addition, Ca^{2+} influx may modulate IP_3 receptor activity and/or contribute to the total increase in cytoplasmic Ca^{2+} concentration during Ca^{2+} oscillations.

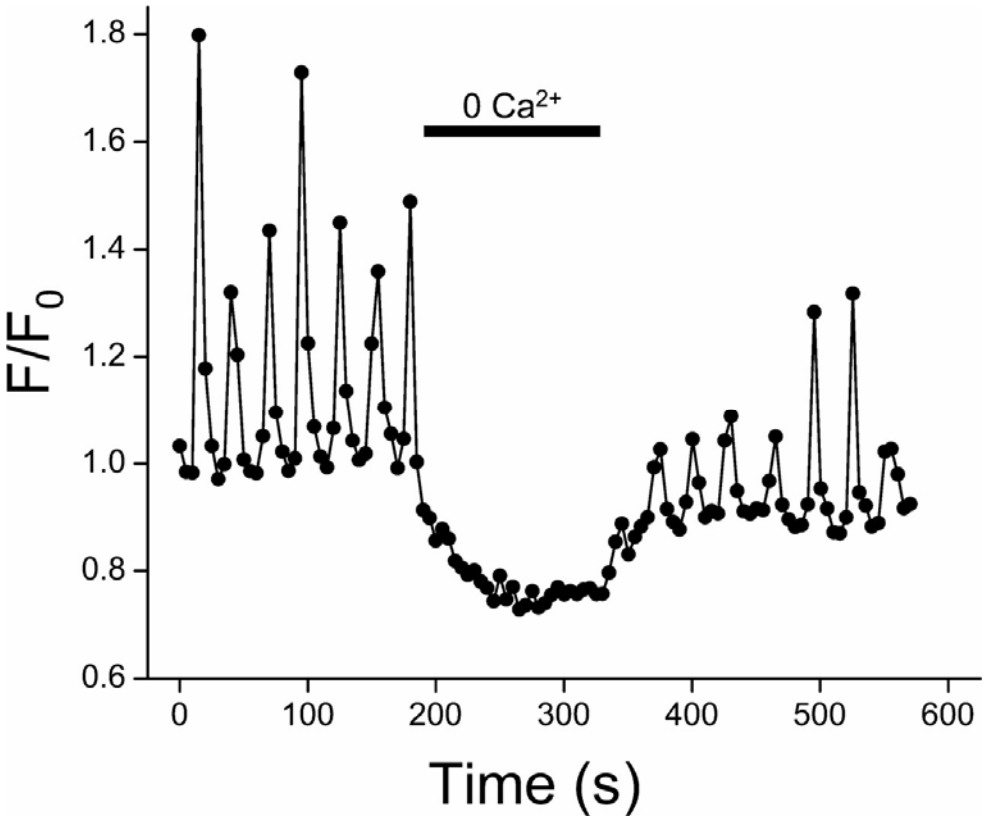


Figure 10. Requirement of intestinal Ca^{2+} oscillations on extracellular Ca^{2+}. Representative experiment showing the effect of extracellular Ca^{2+} removal on Ca^{2+} oscillations. Calcium-free extracellular solution was buffered with 1 mM EGTA. Removal of bath Ca^{2+} causes rapid inhibition of Ca^{2+} oscillations and drop in total fluo-4 fluorescence (similar results were observed in 5 out 5 intestines). Addition of Ca^{2+} back to the bath causes a rapid increase in fluo-4 fluorescence and recovery of Ca^{2+} oscillations (similar results were observed in 4 out 4 intestines).

The TRPM channels GON-2 and GTL-1 are required for normal *C. elegan* pBocs

As discussed in the Introduction, loss of function of CRAC channels and the ER Ca^{2+} sensor STIM-1 has no effect on oscillatory Ca^{2+} signaling in the *C. elegans* intestines. Other channels must therefore mediate Ca^{2+} entry. *gon-2* and *gtl-1* are reported to be expressed in the worm intestine (Teramoto et al., 2005)cited as unpublished observations in (Baylis and Goyal, 2007)WormBase; http://www.wormbase.org/). We have generated transgenic worms expressing full-length GTL-1 fused to GFP. Expression was driven by ~ 2 kb of the *gtl-1* promoter located upstream of the start codon. Prominent expression of GTL-1::GFP was detected in the apical membrane of *C. elegans* intestine (Figure 11). We then quantified pBoc and intestinal Ca^{2+} oscillations in animals harboring loss-of-function mutations in these genes. *gtl-1(ok375)* is a 2,714 bp deletion allele that deletes all of the predicted transmembrane domains of GTL-1 and is almost certainly null. *gon-2(q388)* is a point mutation in which glutamate 955 is mutated to lysine (West et al., 2001). Glutamate 955 is highly conserved in human, mouse, *Drosophila* and *C. elegans* TRP channels and mutation to lysine most likely causes temperature-sensitive disruption of a step in GON-2 synthesis (West et al., 2001). The E955K mutation induces a severe loss-of-function phenotype when worms are grown at 25 °C (Sun and Lambie, 1997; Church and Lambie, 2003). As noted earlier, the *gon-2;gtl-1* double mutant was derived from a cross of *gtl-1(ok375)* and *gon-2(q388)* worms (Teramoto et al., 2005).

Figure 12A shows pBoc cycles in individual wild type and channel mutant worms. Coefficients of variance were calculated as a measure of cycle rhythmicity. Wild type worms exhibited a highly rhythmic pBoc cycle with coefficients of variance for

individual animals ranging from 2-5%. In striking contrast, loss of activity of either channel disrupted pBoc rhythmicity. Coefficients of variance ranged from 3-33% and 7-28% for GTL-1 and GON-2 mutant worms, respectively. Disruption of pBoc was more severe in the double mutant worms where coefficients of variance ranged from 10-67%.

pBoc cycle data are summarized in Figure 12B. Mean cycle periods and coefficients of variance were increased significantly ($P<0.05$) in *gtl-1* mutant, *gon-2* mutant and double mutant worms. In addition, the mean coefficient of variance was significantly ($P<0.01$) greater in the double mutant worms compared to either GTL-1 or GON-2 mutant animals.

As discussed in the Material and Methods section, double mutant worms develop and reproduce poorly unless the Mg^{2+} concentration in the growth agar is increased to 20 mM. To determine whether high Mg^{2+} has any effect on the pBoc cycle, we grew wild type worms for one generation on high Mg^{2+} plates. Mean ± S.E. pBoc period and coefficient of variance were 43 ± 1 sec and 3.5 ± 0.7% (n=6), respectively, and were not significantly ($P>0.3$) different from those of worms grown on standard NGM agar (see Figure 12B).

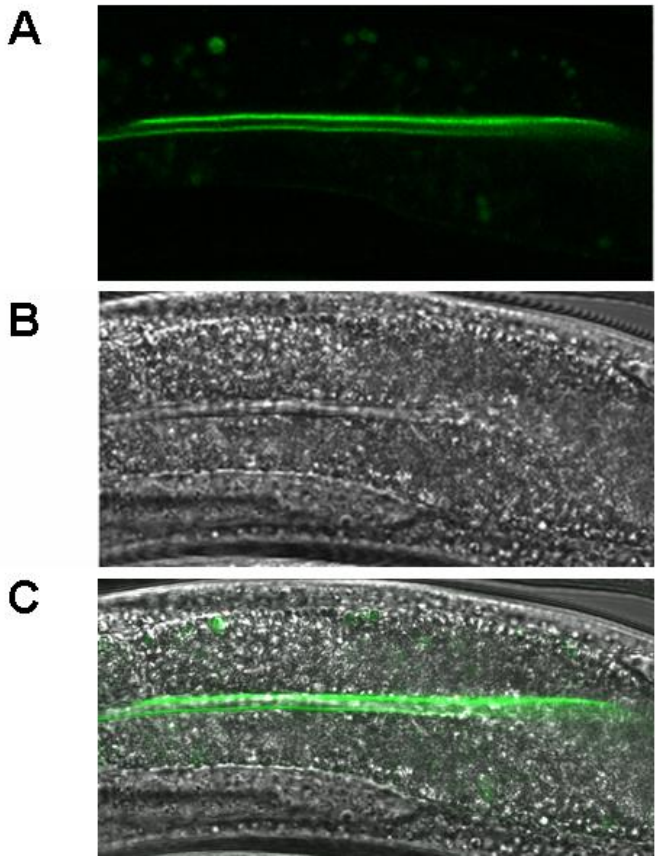


Figure 11. GTL-1::GFP expression in the *C. elegans* intestine. High magnification confocal images of a worm intestine. GTL-1::GFP specifically localized in the apical membrane of the intestine. GFP micrograph (A), differential interference contrast (DIC) micrograph (B), and overlay image of both micrographs (C) of a worm intestine expressing GTL-1::GFP translational reporter are shown.

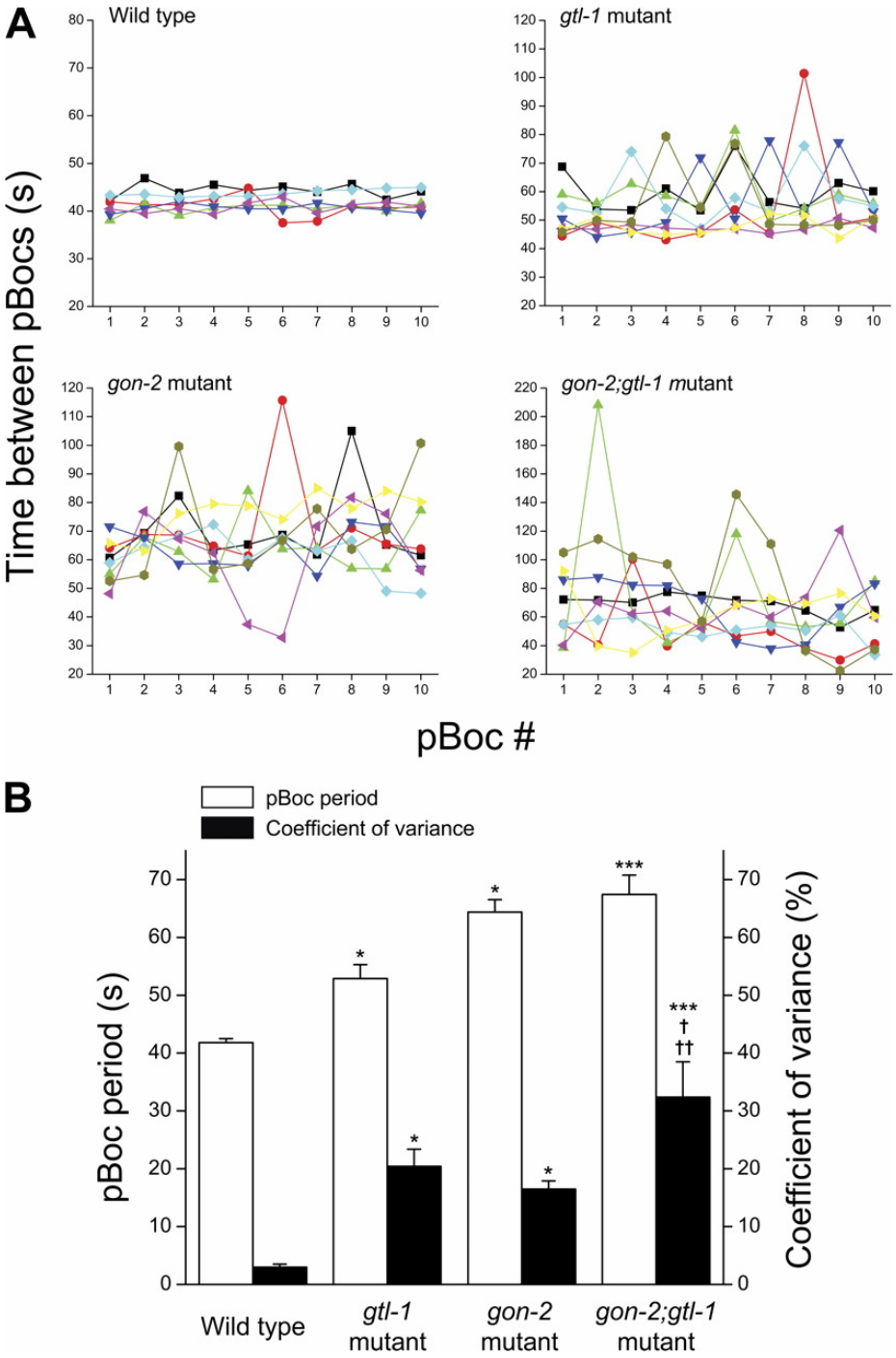


Figure 12. Effect of *gtl-1* and *gon-2* loss-of-function mutations on pBoc period and rhythmicity. (A) pBoc cycles in individual wild type, GTL-1 mutant, GON-2 mutant and double mutant worms. Different colors and symbols represent different animals. (B) pBoc periods and rhythmicity for wild type and GTL-1 and GON-2 mutant worm strains. Cycle rhythmicity is quantified as coefficient of variance. Values are means ± S.E. (N=6-23). *P<0.05 and ***P<0.001 compared to wild type worms. †P<0.01 compared to GTL-1 mutant worms. ††P<0.001 compared to GON-2 mutant worms. All worm strains were grown at 25 °C.

GTL-2 is not required for rhythmic *C. elegans* pBocs

As discussed in the introduction, *C. elegans* have three TRPM channels. GON-2 and GTL-1 were shown to play essential role in regulating *C. elegans* pBoc rhythm (Figure 12). We then analyzed the pBocs in *gtl-2* RNAi worms. Figures 13A and 13B show pBoc cycles in individual wild type worms fed control RNAi or *gtl-2* RNAi, respectively. Coefficients of variance were calculated as a measure of cycle rhythmicity. Wild type worms fed with both control and *gtl-2* RNAi exhibited a highly rhythmic pBoc cycle with coefficients of variance for individual animals ranging from 4-10%. pBoc cycle data are summarized in Figure 13C. Mean cycle periods were 47.7 ± 1.8 sec and 53.1 ± 0.6 sec (means ± S.E., N= 5~6) for *control* and *gtl-2* RNAi worms, respectively. Coefficients of variance were 6 ± 1 % and 8 ± 1 % (means ± S.E., N= 5~6) for *contro*l and *gtl-2* RNAi worms, respectively. Both mean cycle periods and coefficient of variance were not significantly different between *control* and *gtl-2* RNAi worms. These data suggested that GTL-2 activity is not required for rhythmic pBocs. Hereafter, we focused our studies on characterizing the functions of GON-2 and GTL-1 in oscillatory Ca^{2+} signaling pathways in the *C. elegans* intestine.

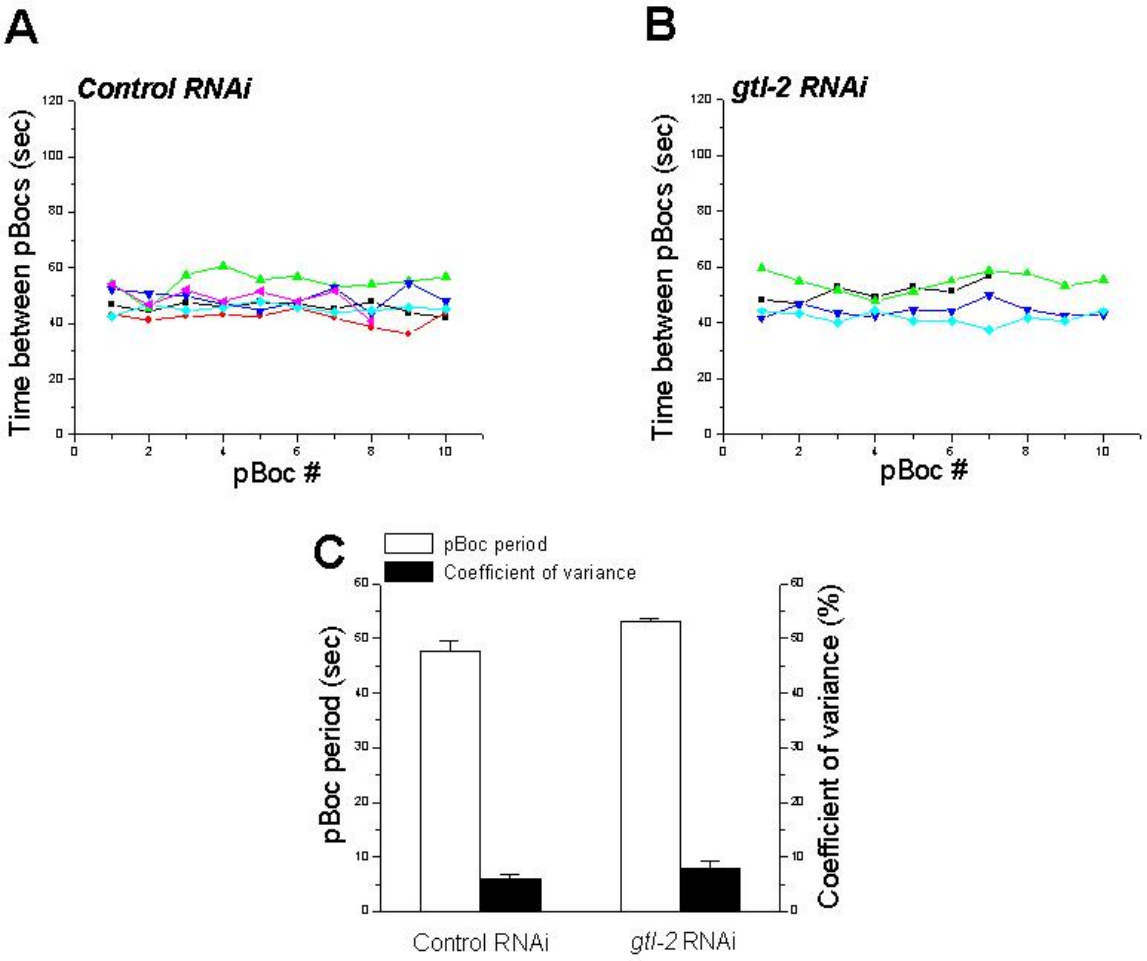


Figure 13. Effect of *gtl-2* RNA interference on pBoc period and rhythmicity. (A, B) pBoc cycles in individual wild type worms fed with *control* (A) or *gtl-2* RNAi (B). Different colors and symbols represent different animals. (C) pBoc periods and rhythmicity for wild type worms feeding *control* or *gtl-2* RNAi. Cycle rhythmicity is quantified as coefficient of variance. Values are means ± S.E. (N= 5~6).

GON-2 and GTL-1 are required for generating and maintaining rhythmic intestinal Ca^{2+} oscillations in *C. elegans*

As discussed in chapter I, *C. elegans* intestinal IP_3-dependent Ca^{2+} oscillations drive pBoc (Figure 5) (Beg et al., 2008; Dal Santo et al., 1999; Teramoto and Iwasaki, 2006; Espelt et al., 2005b; Peters et al., 2007). To determine whether GTL-1 and GON-2 function in Ca^{2+} signaling, we quantified Ca^{2+} oscillations in intestines dissected from wild type and mutant animals. Calcium oscillations were arrhythmic in intestines isolated from GTL-1, GON-2 and double mutant worms (Figure 14A). Mean coefficients of variance were increased significantly ($P<0.05$) by 2.3-3.2 fold in the single and double mutants (Figure 14B). Due to intracycle and animal-to-animal variability, the mean oscillation periods were not significantly ($P>0.05$) different for the three groups of mutant worms and wild type animals (data not shown). Oscillation kinetics as measured by oscillation rise and fall times were unaffected ($P>0.05$) by channel mutations (data not shown). We conclude from data shown in Figures 12 and 14 that GTL-1 and GON-2 are both required for maintaining the rhythmicity of Ca^{2+} oscillations in the *C. elegans* intestinal epithelium.

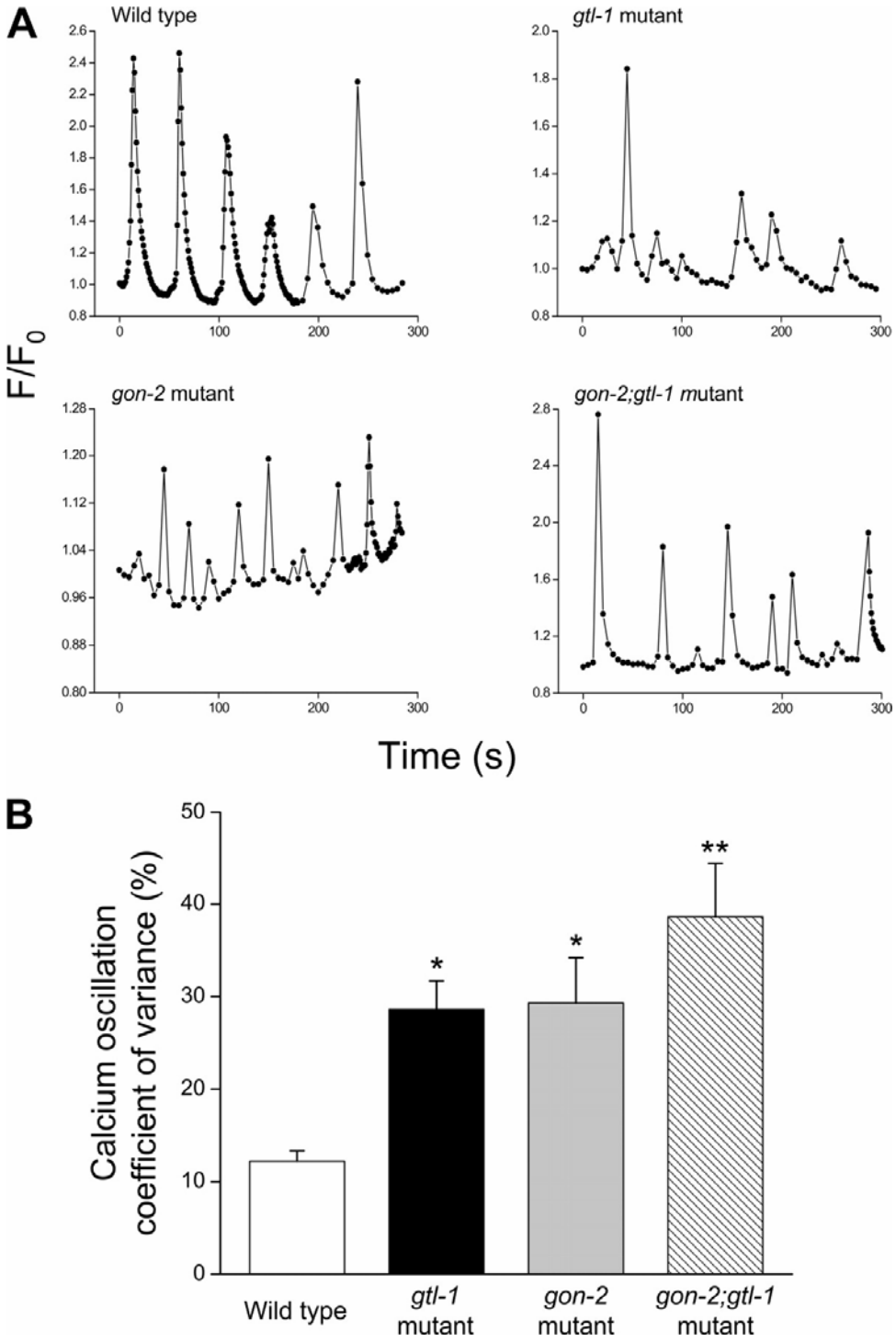


Figure 14. Effect of *gtl-1* and *gon-2* loss-of-function mutations on intestinal Ca^{2+} oscillation rhythmicity. (A) Calcium oscillations in single intestines isolated from wild type, GTL-1 mutant, GON-2 mutant and double mutant worms. (B) Calcium oscillation rhythmicity in wild type and mutant worm intestines. Rhythmicity is quantified as coefficient of variance. Values are means ± S.E. (N=6-10). *P<0.05 and **P<0.001 compared to wild type worms. All worm strains were grown at 25 °C.

GON-2 and GTL-1 mediate whole cell outwardly rectifying Ca^{2+} currents

We suggested previously that I_{ORCa} may play an important role in generating intestinal Ca^{2+} oscillations (Estevez and Strange, 2005; Estevez et al., 2003). To determine whether the ORCa channel is encoded by *gon-2* and/or *gtl-1*, we characterized whole cell cation currents in intestinal cells cultured from wild type, *gon-2* mutant, *gtl-1* mutant and *gon-2;gtl-1* double mutant worms. I_{ORCa} in wild type intestinal cells is constitutively active and undergoes additional slow activation for 1-2 min after whole cell recording is initiated (Estevez et al., 2003). Mean ORCa current density at +80 mV measured 4-5 min after membrane rupture in wild type cells was 266 pA/pF (Figure 15A). The mean ± S.E. reversal potential (E_{rev}) of I_{ORCa} was 18 ± 1 mV (n=22). The positive reversal potential is expected for a Ca^{2+}-selective channel (Estevez et al., 2003).

Whole cell current density was strikingly and significantly ($P<0.01$) suppressed in intestinal cells cultured from both *gon-2* and *gtl-1* mutant worms. In both groups of cells, the majority of currents we observed were outwardly rectifying with a strongly positive E_{rev} similar to that of I_{ORCa}. In 2 out 11 *gon-2* mutant cells, whole cell current exhibited an E_{rev} close to zero and a near-linear current-to-voltage relationship. We interpreted these observations as indicating that loss of function of *gon-2* in these cells completely suppressed I_{ORCa} and that whole cell conductance was due largely to a non-selective leak current. Mean current density was 26.5 pA/pF in *gon-2* cells and 83.5 pA/pF in *gtl-1* cells (Figure 15A). Currents recorded from all *gtl-1* cells showed outward rectification and had a mean ± S.E. E_{rev} of 19 ± 1 mV (n=21). The mean ± S.E. E_{rev} value for the outwardly rectifying currents observed in *gon-2* mutant cells was 18 ± 2 mV (n=9). Mean

reversal potentials of outwardly rectifying currents in *gon-2* and *gtl-1* mutant cells were not significantly ($P<0.05$) different from that observed in wild type cells.

In 5 out of 5 *gon-2;gtl-1* double mutant cells, a small current with a near-linear current-to-voltage relationship was detected. The mean ± S.E. E_{rev} for this current was 1.1 ± 2.7 mV (n=5), which is not significantly ($P>0.7$) different from 0 (Figure 15B). To determine whole cell current properties in the absence of I_{ORCa}, we patch clamped wild type intestinal cells and bathed them with 100 μM La^{3+}, which completely inhibits ORCa channel activity (see Figure 17A). A small near-linear current with an E_{rev} (mean ± S.E. = -1.6 ± 1.5 mV; n=5) not significantly ($P>0.3$) different from 0 was recorded in these cells (Figure 15B). We define this current as non-selective leak current. Mean ± S.E. whole cell currents measured at -80 mV and +80 mV in *gon-2;gtl-1* double mutant cells and wild type cells treated with 100 μM La^{3+} were -3.5 ± 1.8 pA/pF and 4.0 ± 1.8 pA/pF (n=5) and -1.9 ± 3.2 pA/pF and 3.1 ± 0.4 pA/pF (n=5), respectively, and were not significantly ($P>0.6$) different (Figure 15B). Treatment of *gon-2;gtl-1* mutant cells with 100 μM La^{3+} had no significant ($P>0.2$) on whole cell current amplitude (mean ± S.E. whole cell currents measured at -80 mV and +80 mV were -4.9 ± 1.0 pA/pF and 8.3 ± 3.8 pA/pF, respectively; n=3). These results demonstrate that combined loss of GON-2 and GTL-1 activity completely suppresses I_{ORCa}. We therefore conclude that I_{ORCa} is mediated by the function of both channels.

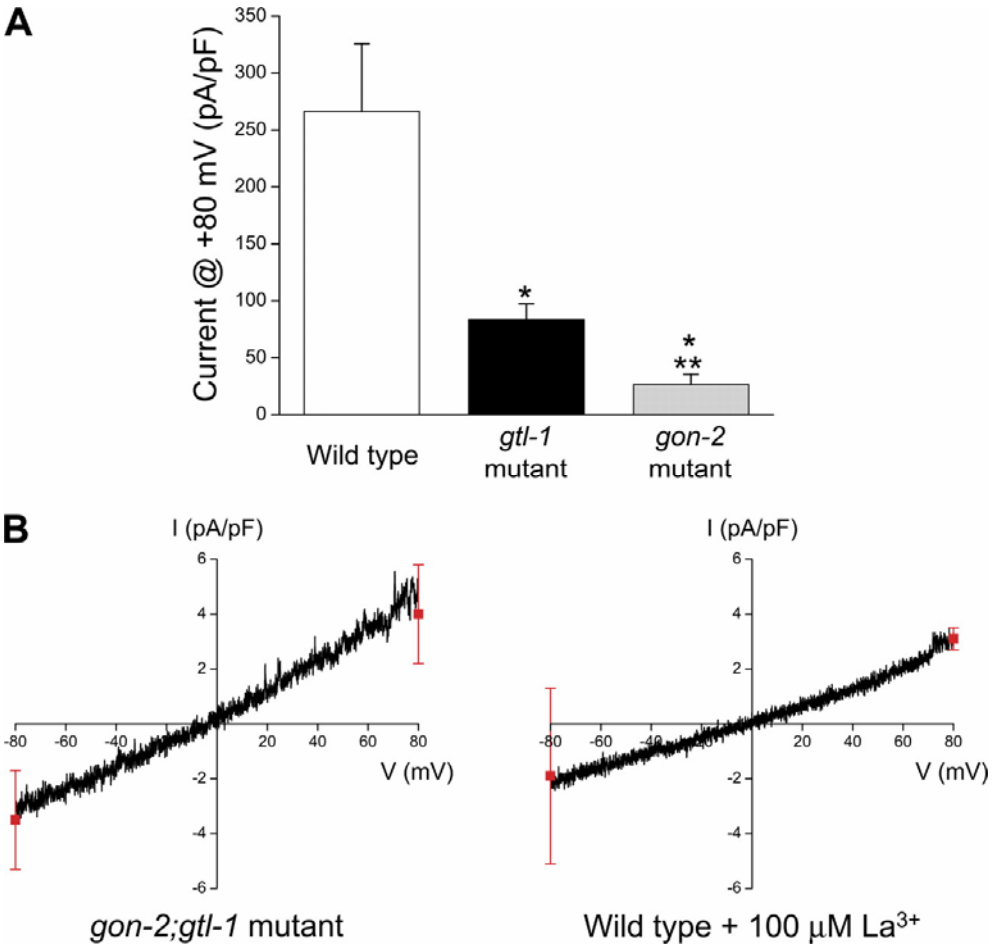


Figure 15. Effect of *gtl-1* and *gon-2* loss-of-function mutations on whole cell ORCa current. (A) Mean whole cell current amplitude in intestinal cells cultured from wild type, *gtl-1* mutant and *gon-2* mutant worms. Values are means ± S.E. (N=11-22). *P<0.01 compared to wild type worms. **P<0.002 compared to *gtl-1* mutant worms. All worm strains were grown at 25 °C. (B) Current-to-voltage relationships of whole cell currents measured in intestinal cells cultured from wild type and *gon-2;gtl-1* double mutant worms. Wild type intestinal cells were patch clamped in a bath solution containing 100 mM La^{3+}, which completely inhibits I_{ORCa} (see Figure 17A). Remaining current shows a near linear current-to-voltage relationship and E_{rev} near 0 mV and is defined as leak current. Current-to-voltage relationship of *gon-2;gtl-1* double mutant whole cell currents is also near linear with a near 0 E_{rev}. Data shown are the means of currents recorded from 4 wild type cells and 5 *gon-2;gtl-1* double mutant cells. Currents were elicited by ramping membrane potential from -80 mV to +80 mV at 215 mV/sec. Red symbols and error bars are mean ± S.E. error currents measured at holding potentials of -80 mV and +80 mV. Mean currents measured at -80 mV and +80 mV and E_{rev} values were not significantly (P>0.3) different for wild type cells treated with La^{3+} and *gon-2;gtl-1* double mutant cells.

Functional properties of GON-2 and GTL-1 mediated whole cell currents

The inhibitory effects of loss of GON-2 or GTL-1 alone on I_{ORCa} are not additive; whole cell current density was reduced ~90% and ~70% in *gon-2* and *gtl-1* mutant cells, respectively (Figure 15A). These results indicate that 1) GON-2 and GTl-1 can function independently as ion channels, but 2) their functions in mediating I_{ORCa} are somehow interdependent (see Discussion). We define the currents observed in *gon-2* and *gtl-1* mutant cells as I_{GTL-1} and I_{GON-2}, respectively.

To further define the roles of GON-2 and GTL-1 in mediating I_{ORCa}, we characterized the biophysical properties of I_{GTL-1} and I_{GON-2}. Figure 16 shows representative ORCa (i.e, wild type), GON-2 and GTL-1 currents and relative current-to-voltage relationships. All three currents show similar outward rectification. However, relative inward GTL-1 currents at -20 mV to -80 mV were slightly but significantly ($P<0.001$) greater than that of I_{ORCa} (Figure 16B).

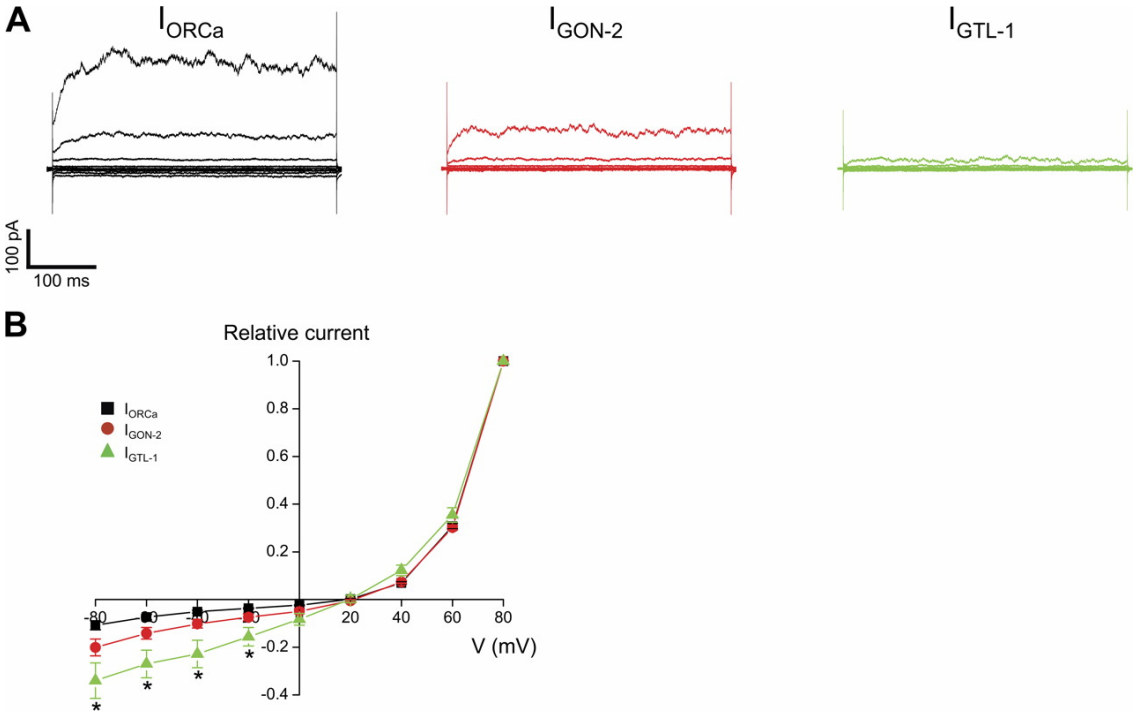


Figure 16. Current-to-voltage characteristics of I_{ORCa}, I_{GTL-1} and I_{GON-2}. (A) Representative whole cell currents recorded from wild type, *gtl-1* mutant and *gon-2* mutant worm intestinal cells. Currents were elicited by stepping membrane voltage from -80 mV to +80 mV in 20 mV steps from a holding potential of 0 mV. (B) Relative current-to-voltage relationships of I_{ORCa}, I_{GTL-1} and I_{GON-2}. Values are means ± S.E. (N=9-22). *P<0.001 compared to I_{ORCa}. I_{GTL-1} and I_{GON-2} are currents measured in intestinal cells isolated from *gon-2* and *gtl-1* mutant worms, respectively.

I_{ORCa} was inhibited by extracellular La^{3+} with a mean ± S.E. IC_{50} of 3.7 ± 0.6 μM (n=6). The La^{3+} dose-response relationships for I_{GON-2} and I_{GTL-1} were superimposable with that of I_{ORCa} (Figure 17A). Mean ± S.E. La^{3+} IC_{50} values were 5.7 ± 1.8 μM (n=6) and 5.3 ± 1.5 μM (n=4) for I_{GON-2} and I_{GTL-1}, respectively, and were not significantly ($P>0.05$) different from that of I_{ORCa}.

Figure 17B shows cation permeabilities measured under bi-ionic conditions of the ORCa, GON-2 and GTL-1 channels relative to Na^+ (i.e, P_{cation}/P_{Na}). The P_{NMDG}/P_{Na}, P_{Ca}/P_{Na} and P_{Mg}/P_{Na} for the channels were not significantly ($P>0.05$) different and ranged between 0.07-0.1, 57-66 and 3-6, respectively.

Increasing intracellular Mg^{2+} concentration inhibits I_{ORCa} (Figure 17C) (Estevez et al., 2003). The Mg^{2+} dose-response relationships for I_{ORCa}, I_{GON-2} and I_{GTL-1} were similar (Figure 17C). IC_{50} values derived from fits to mean values in the datasets were 420 μM for I_{ORCa}, 440 μM for I_{GON-2} and 260 μM for I_{GTL-1}. Comparison of the fits indicated that the three datasets were not significantly ($P>0.05$) different from one another.

It has been suggested that GON-2 and GTL-1 play a central role in intestinal Mg^{2+} uptake (Teramoto et al., 2005). The ORCa, GON-2 and GTL-1 channels clearly have measurable Mg^{2+} permeabilities under bi-ionic conditions. However, given that the relative Ca^{2+} permeabilities of the channels are at least an order of magnitude greater than that of Mg^{2+} (Figure 17B and (Teramoto et al., 2005)), a more physiologically relevant question is whether significant Mg^{2+} permeation occurs when Ca^{2+} is present in the extracellular medium. To address this question, we patch clamped wild type intestinal cells in a modified standard bath solution containing 130 mM NaCl and 30 mM NMDG-Cl and the normal Ca^{2+} and Mg^{2+} concentrations of 1 mM and 5 mM, respectively. When

current amplitude had stabilized, the NMDG-Cl was replaced with 15 mM $MgCl_2$. In the presence of 1 mM bath Ca^{2+}, the mean ± S.E. shifts in E_{rev} and current density at -80 mV observed when bath Mg^{2+} levels were raised 4-fold were 0.7 ± 0.5 mV (n=4) and -1.6 ± 1.7 pA/pF (n=4), respectively (Figure 18). These values were not significantly ($P>0.3$) different from zero suggesting that Mg^{2+} permeation through the ORCa channel is very low in the presence of Ca^{2+}. Studies designed to directly quantify net Mg^{2+} influx through the ORCa channel under physiologically relevant conditions are needed to fully define its role in intestinal Mg^{2+} uptake and whole animal Mg^{2+} homeostasis.

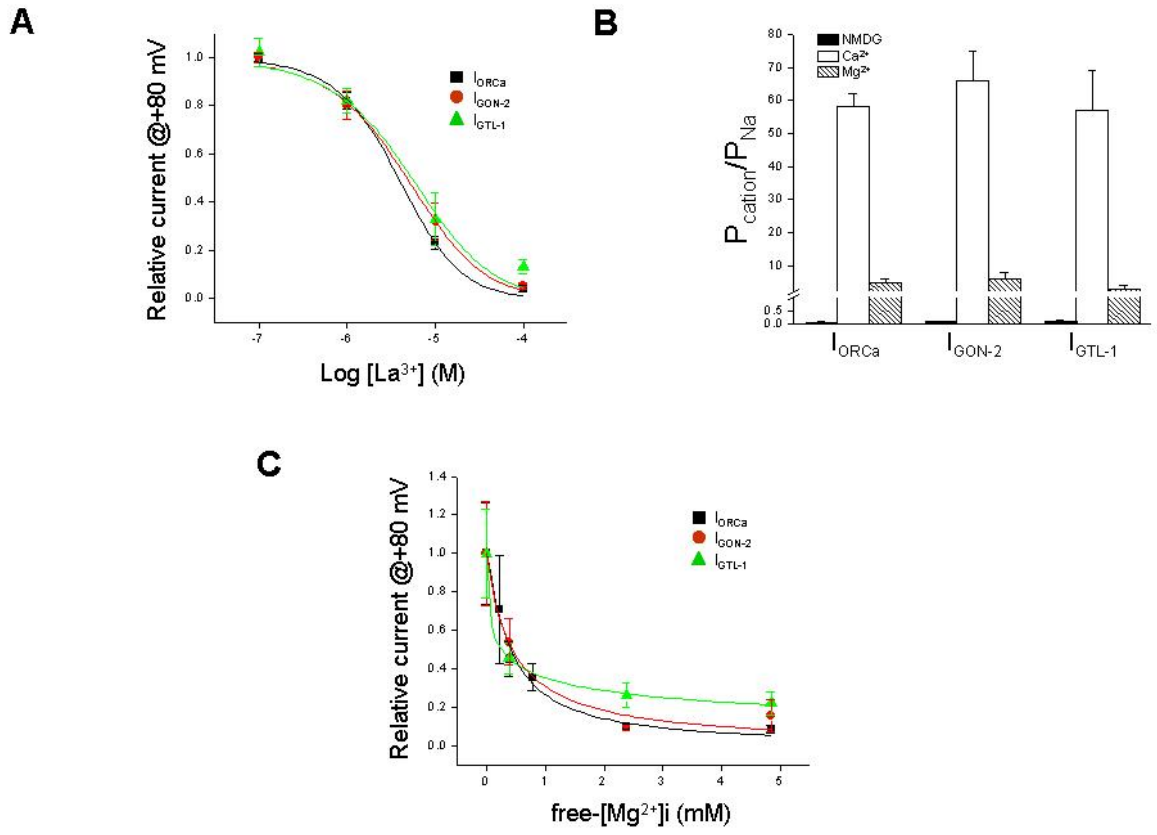


Figure 17. Lanthanum and Mg^{2+} sensitivity and relative cation permeabilities of the ORCa, GTL-1 and GON-2 channels. (A) Dose-response relationship for the inhibitory effect of extracellular La^{3+} on I_{ORCa}, I_{GTL-1} and I_{GON-2}. Data were fit using the equation $I = 1/1 + ([La^{3+}]/IC_{50})^n$. Values are means ± S.E. (n=4-6). (B) Relative cation permeabilities of the ORCa, GTL-1 and GON-2 channels. Values are means ± S.E. (n=4-9). (C) Dose-response relationship for the inhibitory effect of intracellular Mg^{2+} on I_{ORCa}, I_{GTL-1} and I_{GON-2}. Data were fit using the equation $I = 1/1 + ([Mg^{2+}]/IC_{50})^n$. Values are means ± S.E. (n=4-8). I_{GTL-1} and I_{GON-2} are currents measured in intestinal cells isolated from *gon-2* and *gtl-1* mutant worms, respectively.

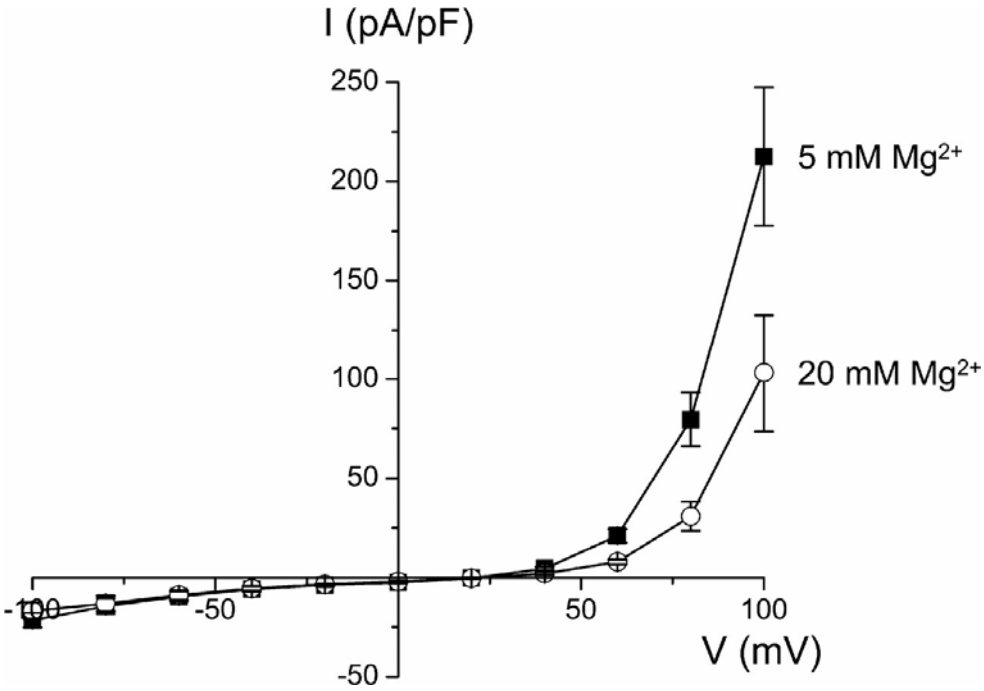


Figure 18. Effect of increasing bath Mg^{2+} concentration on whole cell current amplitude and E_{rev} in the presence of 1 mM Ca^{2+}. No significant (P>0.3) shift in E_{rev} or inward current was detected when Mg^{2+} concentration was raised 4-fold to 20 mM. Values are means ± S.E. (n=4).

Physiological roles of GON-2 and GTL-1

As shown in Figures 12 and 14, loss of *gon-2* and/or *gtl-1* activity dramatically disrupts pBoc rhythmicity and intestinal Ca^{2+} signaling. Teramoto et al. (Teramoto et al., 2005) observed that the pBoc cycle was prolonged and apparently arrhythmic in *gon-2;gtl-1* double mutant worms and that the defect was fully rescued by increasing the Mg^{2+} concentration of the growth agar to 40 mM. They suggested that the altered defecation cycle was due to an alteration in the physiological state of the intestine resulting from Mg^{2+} deficiency. In our hands, the pBoc defect in double mutant worms was unaffected by external Mg^{2+} levels of either 20 mM (Figure 12) or 40 mM (unpublished observations).

Given the lack of effect we observed of high external Mg^{2+} concentration on pBoc and the high relative Ca^{2+} permeabilities of the ORCa, GON-2 and GTL-1 channels (Figure 17B), it is reasonable to postulate that they play a direct role in regulating and/or maintaining IP_3-dependent intestinal Ca^{2+} oscillations. To address this possibility, we performed genetic epistasis experiments. Epistatic analysis can be utilized to determine interaction among genes that control a common phenotype (Figure 19). PLCγ and PLCβ homologues function together to regulate pBoc and generate Ca^{2+} oscillations in the *C. elegans* intestine. Loss of function of either enzyme causes striking arrhythmia of both pBoc and oscillatory Ca^{2+} signaling. Combined loss of function of both enzymes is additive giving rise to severe Ca^{2+} signaling and pBoc defects (Espelt et al., 2005b). These results suggest that PLCγ and PLCβ function in different signaling pathways. Epistasis analysis using mutant alleles predicted to elevate intracellular IP_3 levels indicates that PLCγ functions primarily to generate IP_3 and regulate IP_3 receptor activity

whereas PLCβ functions in a distinct and yet to be defined signaling pathway required for normal Ca^{2+} signaling (Espelt et al., 2005b). The localization of PLCβ to sites of cell-cell contact (Miller et al., 1999) suggests that the enzyme may play a role in regulating intestinal Ca^{2+} waves that coordinate muscle contractions required for defecation (Peters et al., 2007).

To determine whether GON-2 and GTL-1 may play a role in the IP_3 receptor signaling pathway, we fed *gon-2;gtl-1* double mutant worms bacteria expressing dsRNA homologous to either PLCγ or PLCβ. As shown in Figure 20A, PLCγ RNAi had no additive effect on the pBoc arrhythmia induced by loss of function of both channels. Mean ± S.E. pBoc period and coefficient of variance for *gon-2;gtl-1;PLCγ(RNAi)* worms were 73 ± 7 sec and 49 ± 7% (n=14). These values were not significantly (P>0.09; see Figures 12A-B) different from that observed in *gon-2;gtl-1* double mutant worms fed normal bacteria. In contrast, knockdown of PLCβ in *gon-2;gtl-1* mutant worms induced a pBoc defect that was much more severe than that observed with the channel mutations alone (Figures 20B-C). Over a 20 min measurement period, no more than 7 pBocs were observed in any of the *gon-2;gtl-1;plcβ(RNAi)* worms. The mean number of pBocs observed in 20 min in these animals was 3 (n=12). One of the 12 animals examined exhibited no pBocs in this time period. This phenotype is remarkably similar to that induced by combined loss of function of PLCγ and PLCβ (Espelt et al., 2005b) and suggests that GON-2 and GTL-1 function together with PLCγ to regulate IP_3 receptor activity and ER Ca^{2+} release.

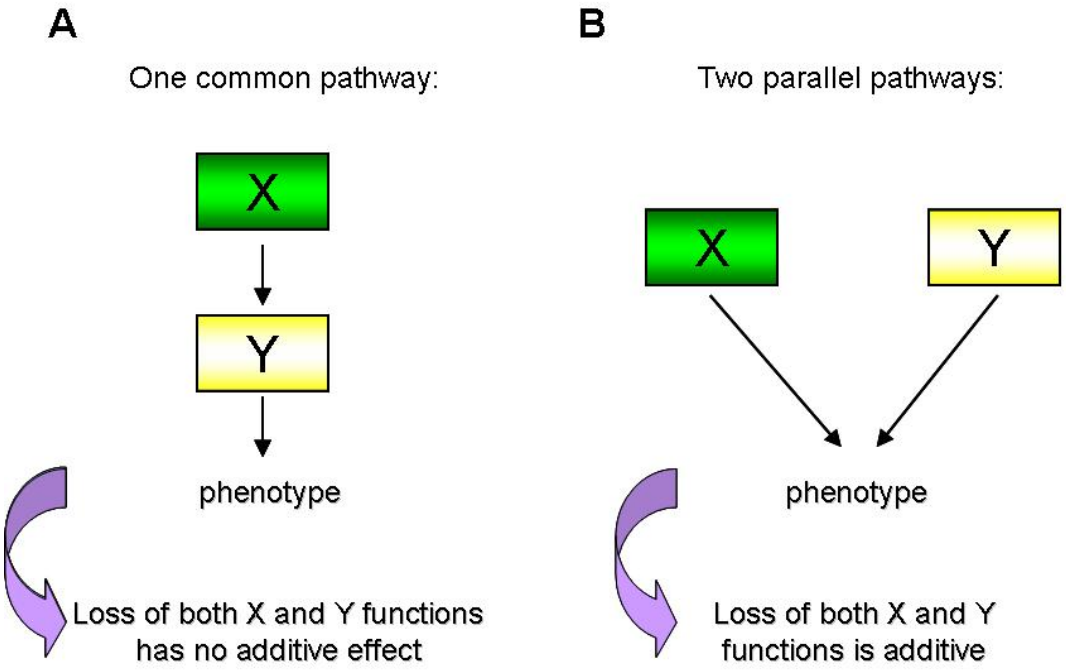


Figure 19. Epistatic analysis can be utilized to determine interaction among genes that control a common phenotype. If X and Y function in the same pathway to regulate the same phenotype, loss of both X and Y functions has no additive effect (A). If X and Y function in two separate pathways to control the same phenotype, loss of both X and Y functions is additive (B).

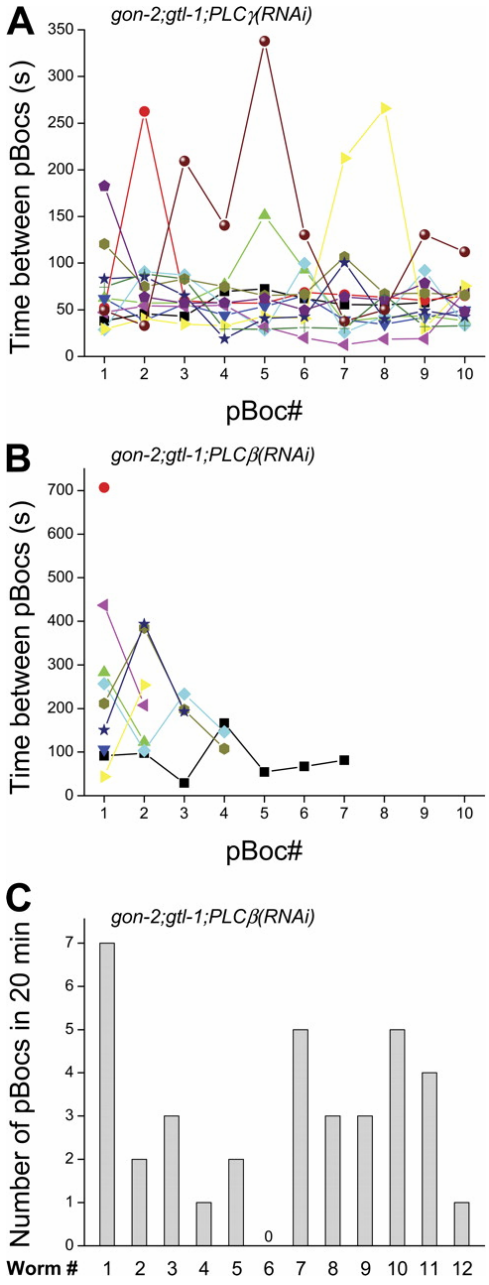


Figure 20. Effects of loss of PLCγ and PLCβ function on pBoc rhythmicity in *gon-2;gtl-1* double mutant worms. (A, B) pBoc cycles in individual *gon-2;gtl-1* mutant worms fed bacteria producing dsRNA to either PLCγ (A) or PLCβ (B) Different colors and symbols represent different animals. (C) Number of pBocs measured in 12 *gon-2;gtl-1;PLCβ(RNAi)* worms over 20 min observation period.

Discussion

The ORCa channel is encoded by the TRPM homologues *gon-2* and *gtl-1*

The TRP cation channel superfamily is subdivided into TRPC, TRPV, TRPM, TRPML, TRPP, TRPN and TRPA subfamilies. All TRP channels are comprised of six predicted transmembrane domains and intracellular N- and C-termini. Functional TRP channels are formed from homomeric or heteromeric association of four TRP subunits. TRP channels function in diverse physiological processes including sensory transduction, epithelial transport of Ca^{2+} and Mg^{2+}, Ca^{2+} signaling and modulation of membrane potential (Owsianik et al., 2006; Nilius et al., 2007).

The mammalian TRPM subfamily consists of TRPM1-8 (Kraft and Harteneck, 2005). GON-2 and GTL-1 share approximately 23% identity with TRPM1, TRPM3, TRPM6 and TRPM7 (Baylis and Goyal, 2007). The conserved structural motifs in these channels are the transmembrane domains, the TRP domain and portions of the cytoplasmic N-terminus.

Amino acids that comprise the pores of TRPM6 and TRPM7 have been identified by mutagenesis and patch clamp analysis (Li et al., 2007; Topala et al., 2007; Chubanov et al., 2007). The homologous pore domains are nearly identical in GON-2 and GTL-1. This is consistent with our findings that the two channels have similar biophysical properties (Figures 16-17).

As shown in Figures 15A and 16A, I_{ORCa} is dramatically inhibited by loss-of-function mutations in either *gon-2* or *gtl-1*. Loss of function of both genes completely

eliminates the current (Figure 15B). There are two possible explanations for these results. Either the ORCa whole cell current is comprised of independent GON-2 and GTL-1 currents, or the ORCa channel is a GON-2/GTL-1 heteromer. Our results suggest that the function of GON-2 and GTL-1 are interdependent. The combined inhibition of I_{ORCa} observed in *gon-2* and *gtl-1* mutant cells is ~160% (Figure 15A). This finding indicates that GON-2 and GTL-1 can function independently as ion channels, but that maximal I_{ORCa} activity requires a functional interaction between them. One possibility is that the ORCa channel is a GON-2/GTL-1 heteromer. Alternatively, loss of either GON-2 or GTL-1 alone may disrupt the trafficking, expression and/or regulation of the other channel.

Numerous studies have provided evidence that closely related TRP channels heteromultimerize (Owsianik et al., 2006; Nilius et al., 2007). Heteromultimers of TRPM6 and TRPM7, homologues of GON-2 and GTL-1, have been described (Li et al., 2006; Chubanov et al., 2004). At present, we favor the hypothesis that the ORCa channel is formed by association of GON-2 and GTL-1 subunits. However, extensive additional work including heterologous expression, mutagenesis and subcellular localization of the two channels in vivo is required to test this idea.

Our electrophysiological findings differ from those of Teramoto et al. (Teramoto et al., 2005). These investigators saw no effect of the *gtl-1* deletion allele on whole cell current whereas the *gon-2* mutation reduced La^{3+}-inhibitable outward current at +100 mV ~75%. Current reduction was similar in intestinal cells cultured from *gon-2* and the *gon-2;gtl-1* double mutant worms. They also observed that the IC_{50} value for inhibition of the wild type current by intracellular Mg^{2+} was 4.7-fold higher than that of the current

observed in *gtl-1* mutant cells. In contrast, we found that I_{ORCa}, I_{GON-2} and I_{GTL-1} exhibit similar sensitivities to intracellular Mg^{2+} (Figure 17C). Teramoto et al. (Teramoto et al., 2005) concluded that GON-2 mainly mediates the outwardly rectifying current and that GTL-1 functions mainly to regulate current Mg^{2+} responsiveness. The reasons for the differences in our findings are unclear.

Role of GON-2 and GTL-1 in oscillatory Ca^{2+} signaling

Most TRP channels described to date have no or relatively low selectivity for Ca^{2+} over Na^{+} (Owsianik et al., 2006). The exceptions to this generalization are TRPV5 and TRPV6, which have P_{Ca}/P_{Na} values >100 and play important roles in epithelial Ca^{2+} absorption (Vennekens et al., 2000; Yue et al., 2001; Owsianik et al., 2006). GON-2, GTL-1 and the ORCa channels exhibit a >60-fold selectivity for Ca^{2+} over Na^{+} (Figure 6B; (Estevez et al., 2003). Mammalian TRPM channels are either impermeable to Ca^{2+} (TRPM4 and TRPM5) or have P_{Ca}/P_{Na} values of 0.1-10 (Owsianik et al., 2006). Heterologously expressed *Drosophila* TRP and TRPL have relative Ca^{2+} permeabilities of 10-12 (Xu et al., 1997). Studies of the native TRP current in wild type *Drosophila* photoreceptor cells indicate that the channel(s) responsible for the current are ~40-fold more permeable to Ca^{2+} than monovalent cations (Hardie and Minke, 1992; Reuss et al., 1997). The endogenous Ca^{2+} conductances in *trp* and *trpl* mutant photoreceptor cells have P_{Ca}/P_{Na} values of ~4 and ~86, respectively (Reuss et al., 1997; Hardie and Minke, 1992). Thus, together with mammalian TRPV5/6 and possibly *Drosophila* TRP, GON-2,

GTL-1 and the ORCa channels have the highest Ca^{2+} selectivity of all characterized TRPs.

Given their exceptionally high Ca^{2+} selectivity and essential roles in maintaining pBoc and Ca^{2+} signaling rhythmicity (Figures 12 and 14), what possible functions could GON-2 and GTL-1 be performing? Data in Figure 20 suggests that the channels function in a signaling pathway together with PLCγ to regulate IP_3 receptor activity. Our previous studies failed to identify a significant role for the canonical store-operated CRAC channel in maintaining intestinal Ca^{2+} oscillations. Thus other Ca^{2+} channels must provide a Ca^{2+} entry pathway that allows for store refilling. It is conceivable that GON-2 and GTL-1 function in part to refill ER Ca^{2+} stores. However, even in the absence of these channels Ca^{2+} oscillations continue albeit arrhythmically (Figures 12 and 14). This indicates that other Ca^{2+} entry pathways must function in the intestine to refill stores under these experimental conditions.

An attractive possibility is that the GON-2 and GTL-1 channels play a direct role in modulating IP_3 receptor activity and controlling oscillation frequency. It is well established that IP_3 receptors are regulated in a biphasic manner by intracellular Ca^{2+}; low levels of Ca^{2+} activate the channels whereas high Ca^{2+} levels feedback and inhibit channel activity (Foskett et al., 2007). Foskett and co-workers (Mak et al., 1998; Foskett et al., 2007) have argued that Ca^{2+} is a true IP_3 receptor agonist and that IP_3 functions only to relieve Ca^{2+} inhibition. In excitable cells, plasma membrane Ca^{2+} influx through voltage- and ligand-gated Ca^{2+} channels can trigger intracellular Ca^{2+} release through ryanodine receptors via a process termed Ca^{2+}-induced Ca^{2+} release (CICR) (Berridge et

al., 2003). Plasma membrane Ca^{2+} influx can also trigger CICR via IP_3 receptors (e.g., (Gordienko et al., 2007; Kapur et al., 2001; Kukuljan et al., 1997).

The disruption of Ca^{2+} oscillation rhythmicity in *gon-2* and *gtl-1* mutants (Figures 12 and 14) suggests that the channels function as part of the timekeeping apparatus that regulates cycle periodicity. We have shown previously that under conditions of low intracellular Ca^{2+} buffering, ORCa channel activity oscillates. Oscillating channel activity is due to a Ca^{2+} feedback mechanism similar to that observed with the IP_3 receptor (Estevez and Strange, 2005). Such oscillating channel activity could provide a source of extracellular Ca^{2+} that functions to modulate IP_3 receptor function. Specifically, Ca^{2+} influx through ORCa channels could trigger IP_3 receptor mediated Ca^{2+} release via CICR. Rising cytoplasmic Ca^{2+} levels would feedback on both the IP_3 receptor and ORCa channels functioning initially to increase and than eventually inhibit their activity. Calcium influx through ORCa channels would raise Ca^{2+} levels in channel microdomains and may also contribute to the overall increase in cytoplasmic Ca^{2+}. Microdomain Ca^{2+} increases as well as the amplitude of the cytoplasmic Ca^{2+} increase would likely play a role in triggering downstream cellular functions.

Several TRP channels are known to be regulated by intracellular Ca^{2+} and play important roles in Ca^{2+} signaling. For example, the nonselective cation channel TRPM4 is activated by increases in intracellular Ca^{2+} (Launay et al., 2002). In T cells, TRPM4-mediated membrane depolarization modulates Ca^{2+} influx via CRAC channels and controls oscillatory Ca^{2+} signaling (Launay et al., 2004). TRPM5 is activated by Ca^{2+} intracellular concentrations of 0.3-1 μM and inhibited by higher Ca^{2+} levels and may function to couple intracellular Ca^{2+} release to membrane electrical activity (Prawitt et al.,

2003). TRPC3 shows modest Ca^{2+} selectivity and initiates Ca^{2+} oscillations when activated by OAG. Increasing intracellular Ca^{2+} levels inhibit the channel (Grimaldi et al., 2003). Extensive additional studies utilizing Ca^{2+} imaging, patch clamp electrophysiology, molecular biology and forward and reverse genetics are needed to define the precise roles played by GON-2 and GTL-1 in intestinal Ca^{2+} signaling.

In conclusion, we have demonstrated that I_{ORCa} requires the combined function the TRPM genes *gon-2* and *gtl-1*. GON-2 and GTL-1 are highly Ca^{2+} selective channels and are essential for maintaining rhythmic Ca^{2+} oscillations in the *C. elegans* intestine. We postulate that GON-2 and GTL-1 form a heteromeric channel that selectively mediates Ca^{2+} influx and functions primarily to regulate IP_3 receptor activity and possibly to refill ER Ca^{2+} stores.

CHAPTER III

PI(4,5)P_2 AND LOSS OF PLCγ ACTIVITY INHIBIT TRPM CHANNELS REQUIRED FOR OSCILLATORY CA^{2+} SIGNALING

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Summary

The *C. elegans* intestinal epithelium generates rhythmic inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} oscillations that control muscle contractions required for defecation. Two highly Ca^{2+} selective TRPM channels, GON-2 and GTL-1, function with PLCγ in a common signaling pathway that regulates IP_3-dependent intracellular Ca^{2+} release. A second PLC, PLCβ, is also required for IP_3-dependent Ca^{2+} oscillations, but functions in an independent signaling mechanism. PLCγ generates IP_3 that regulates IP_3 receptor activity. We demonstrate here that PLCγ via hydrolysis of PI(4,5)P_2 (PIP_2) also regulates GON-2/GTL-1 function. Knockdown of PLCγ but not PLCβ activity by RNA interference (RNAi) inhibits channel activity ~80%. Inhibition is fully reversed by agents that deplete PIP_2 levels. PIP_2 added to the patch pipette has no effect on channel activity in PLCγ RNAi cells. However, in control cells, 10 μM PIP_2 inhibits whole cell current ~80%. Channel inhibition by phospholipids is selective for PIP_2 with an IC_{50} value of 2.6 μM. Elevated PIP_2 levels have no effect on channel voltage and Ca^{2+} sensitivity and

likely inhibit by reducing channel open probability, single channel conductance and/or trafficking. We conclude that hydrolysis of PIP_2 by PLCγ functions in the activation of both the IP_3 receptor and GON-2/GTL-1 channels. GON-2/GTL-1 functions as the major intestinal cell Ca^{2+} influx pathway. Calcium influx through the channel feedback regulates its activity and likely functions to modulate IP_3 receptor function. PIP_2–dependent regulation of GON-2/GTL-1 may provide a mechanism to coordinate plasma membrane Ca^{2+} influx with PLCγ and IP_3 receptor activity as well as intracellular Ca^{2+} store depletion.

Introduction

The genetically tractable model organism *Caenorhabditis elegans* provides numerous experimental advantages for developing integrative genetic and molecular understanding of fundamental physiological processes (Barr, 2003; Strange, 2003). We have exploited *C. elegans* as a model for defining the integrative physiology and molecular details of oscillatory Ca^{2+} signaling in non-excitable cells. *C. elegans* intestinal epithelial cells generate rhythmic inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} oscillations that control body wall muscle contractions required for defecation (Dal Santo et al., 1999; Teramoto and Iwasaki, 2006; Espelt et al., 2005b; Peters et al., 2007; Pfeiffer et al., 2008). Intestinal Ca^{2+} signaling can be readily studied using forward and reverse genetic methods (Thomas, 1990; Liu and Thomas, 1994; Iwasaki et al., 1995), in vitro (Espelt et al., 2005b; Teramoto and Iwasaki, 2006; Peters et al., 2007; Xing et al., 2008) and in vivo (Teramoto and Iwasaki, 2006; Yan et al., 2006; Peters et al., 2007) Ca^{2+}

imaging, and patch clamp electrophysiology (Lorin-Nebel et al., 2007; Estevez and Strange, 2005; Estevez et al., 2003; Yan et al., 2006; Xing et al., 2008). The ability to combine direct physiological measurements of IP_3–dependent oscillatory Ca^{2+} signals and associated ion channel activity with forward and reverse genetic analyses is unique to *C. elegans*.

Intestinal Ca^{2+} oscillations are strictly dependent on Ca^{2+} release from the endoplasmic reticulum (ER) via ITR-1, the single IP_3 receptor encoded by the *C. elegans* genome (Dal Santo et al., 1999; Teramoto and Iwasaki, 2006; Espelt et al., 2005b). Calcium oscillations also require Ca^{2+} influx from the extracellular medium and are rapidly and completely inhibited by external Ca^{2+} removal (Xing et al., 2008; Espelt et al., 2005b). *C. elegans* intestinal cells express two highly selective Ca^{2+} entry pathways, a canonical Ca^{2+} release activated Ca^{2+} (CRAC) channel (Parekh and Putney, 2005; Hogan and Rao, 2007) that is activated by intracellular Ca^{2+} store depletion and a store-independent outwardly rectifying Ca^{2+} (ORCa) channel (Estevez et al., 2003).

The *C. elegans* CRAC channel is encoded by *orai-1* and regulated by STIM-1 (Lorin-Nebel et al., 2007; Yan et al., 2006). ORAI-1 and STIM-1 are homologues of mammalian Orai/CRACM and STIM (Hogan and Rao, 2007). RNAi silencing of either *orai-1* or *stim-1* dramatically reduces CRAC channel and STIM-1 expression and function, but surprisingly has no effect on intestinal Ca^{2+} signaling (Lorin-Nebel et al., 2007; Yan et al., 2006). These findings suggest that CRAC channels are not essential components of IP_3-dependent Ca^{2+} signaling in the intestine and indicate that other Ca^{2+} entry mechanisms must function to maintain intestinal Ca^{2+} oscillations.

The TRP cation channel superfamily is subdivided into TRPC, TRPV, TRPM, TRPML, TRPP, TRPN and TRPA subfamilies. TRP channels function in diverse physiological processes including sensory transduction, epithelial transport of Ca^{2+} and Mg^{2+}, Ca^{2+} signaling and modulation of membrane potential (Owsianik et al., 2006; Nilius et al., 2007). We recently demonstrated that the *C. elegans* TRPM homologues GON-2 and GTL-1 are both required for generating intestinal Ca^{2+} oscillations. The two channels also give rise to the ORCa current and may function together as a heteromultimer (Xing et al., 2008).

GON-2/GTL-1 channels function together with a PLCγ homolog in a common signaling pathway to regulate IP_3-dependent intracellular Ca^{2+} release (Xing et al., 2008). PLCγ generates IP_3 that regulates ITR-1 activity (Espelt et al., 2005b). In the present study, we demonstrate that PLCγ via hydrolysis of $PI(4,5)P_2$ (PIP_2) also regulates GON-2/GTL-1 function. Elevated PIP_2 levels inhibit GON-2/GTL-1 channel activity in a voltage- and Ca^{2+}-independent manner. Hydrolysis of PIP_2 by PLCγ thus functions in the activation of both the IP_3 receptor and GON-2/GTL-1 channels, which serve as the major cellular Ca^{2+} influx pathway. Calcium influx through the channel feedback regulates its activity (Estevez and Strange, 2005) and likely functions to modulate IP_3 receptor function and possibly to refill intracellular stores (Xing et al., 2008). Our studies provide unique insights into mechanisms of oscillatory Ca^{2+} signaling and the regulation of TRPM channels.

Material and methods

C. elegans strains

Nematodes were cultured using standard methods on Nematode Growth Medium (NGM) (Brenner, 1974). Wild type worms were JR1838 (wIs84), which express an *elt-2* transcriptional GFP reporter (*elt-2*::GFP) in intestinal cell nuclei. The *egl-8(n488)* allele was used to assess the role of *egl-8*, which encodes a PLCβ homolog, in channel regulation. Worm strains were maintained at 16-25 °C.

C. elegans embryonic cell culture and patch clamp electrophysiology

Newly hatched wild type and EGL-8 (hereafter referred to as PLCβ) deletion mutant L1 larvae were cultured at 25 °C until adulthood. Embryo cells from these animals were cultured for 2-3 days at 25 °C on 12 mm diameter acid-washed glass cover slips using established methods (Strange et al., 2007; Christensen et al., 2002).

plc-3 encodes a PLCγ homolog. PLC-3 (hereafter referred to as PLCγ) and PLCβ expression were knocked down by culturing wild type embryo cells in the presence of *plc-3* or *egl-8* double strand RNA (dsRNA) using methods described previously (Lorin-Nebel et al., 2007; Yan et al., 2006). dsRNA was synthesized from an 868 bp (2203-3071 bp) *plc-3* cDNA that was amplified from a *C. elegans* cDNA library. PLCβ dsRNA was synthesized from a ~1 kb DNA template that was amplified from an *egl-8* RNAi feeding vector carrying 15219-16355 bp of the *egl-8* genomic DNA.

Cover slips with cultured embryo cells were placed in the bottom of a bath chamber (model R-26G; Warner Instrument Corp., Hamden, CT) that was mounted onto the stage of a Nikon TE2000 inverted microscope. Cells were visualized by fluorescence and video-enhanced differential interference contrast (DIC) microscopy. Intestinal cells were identified in culture by expression of the intestine specific reporter *elt-2*::GFP or by morphological characteristics (Fukushige et al., 1998; Estevez et al., 2003).

Patch electrodes were pulled from soft glass capillary tubes (PG10165-4, World Precision Instruments, Sarasota, FL) that had been silanized with dimethyl-dichloro silane. Pipette resistance was 4-7 MΩ. Bath and pipette solutions contained 145 mM NaCl, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 10 mM HEPES, 20 mM glucose, pH 7.2 (adjusted with NaOH), and 147 mM sodium gluconate (NaGluconate), 0.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES, 2 mM Na_2ATP, 0.5 mM Na_2GTP, pH 7.2 (adjusted with CsOH), respectively. The osmolality of bath and pipette solutions were adjusted to 345-350 mOsm and 325-330 mOsm using sucrose.

For studies on the effects of intracellular Ca^{2+} concentration on whole cell current activity, cells were patch clamped with pipette solutions buffered using 1 or 10 mM BAPTA instead of EGTA. Calcium concentration was adjusted using $CaCl_2$. Free Ca^{2+} levels were calculated using MaxChelator software (http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm).

Whole cell currents were recorded using an Axopatch 200B (Axon Instruments, Foster City, CA) patch clamp amplifier. Command voltage generation, data digitization, and data analysis were carried out on a Pentium computer (Dimension 9150; Dell Computer Corp.) using a Digidata 1322A AD/DA interface with pClamp 10 software

(Axon Instruments). Electrical connections to the amplifier were made using Ag/AgCl wires and 3 M KCl/agar bridges.

Whole cell currents were elicited using a ramp or step voltage clamp protocol. For the ramp protocol, membrane potential was held at 0 mV and ramped from -80 mV to +80 mV at 215 mV/sec every 5 sec. Step changes in whole cell current were elicited by stepping membrane voltage from -100 to +100 mV in 20 mV steps from a holding potential of 0 mV. Voltage steps were maintained for 400 msec. Cell capacitances for all cells studied ranged from 1-4 pF. Measurement of the effects of various experimental maneuvers on current amplitude was performed 5 min after obtaining whole cell access when current run-up was complete.

Drugs, phospholipids and fatty acids

18:0-20:4 PI(4,5)P_2 and inositol-1,4,5-trisphosphate (IP_3) were obtained from Calbiochem (Gibbstown, NJ). DiC16 PI(3,4,5)P_3, arachidonic acid, linolenic acid and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were purchased from Biomol International (Plymouth Meeting, PA). 17:0-20:4 PI(4)P, 17:0-20:4 PI(3,4)P_2 and 17:0-20:4 PI(3,5)P_2 were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Poly-L-lysine (molecular weight 1-5 kD), wortmannin, U-73122 and U-73343 were purchased from Sigma-Aldrich Co. (St. Louis, MO).

PI(4,5)P_2 and PI(3,4,5)P_3 were dissolved in water and IP_3, PI(4)P, PI(3,4)P_2 and PI(3,5)P_2 were dissolved in DMSO to stock concentrations of 10 mM. Poly-L-lysine was dissolved in water as stock solution of 10 mg/ml. Arachidonic and linolenic acids, OAG,

wortmannin, U-73122 and U-73343 were dissolved in DMSO to stock concentrations of 2-100 mM. Stocks were divided into aliquots and frozen at -80°C. Working solutions were prepared daily by dilution of stock aliquots. Patch pipet solution containing phospholipids and fatty acids were bath sonicated for 15 min before use. Final DMSO concentrations in all solutions were 0.1%. Exposure of cells to 0.1% DMSO alone had no effect on current amplitude (data not shown).

Statistical analysis

Data are presented as means ± S.E. Statistical significance was determined using Student's two-tailed *t* test for unpaired means. When comparing three or more groups, statistical significance was determined by one-way analysis of variance with a Bonferroni post-hoc test. P values of ≤ 0.05 were taken to indicate statistical significance.

Results

Regulation of the *gon-2* and *gtl-1* encoded ORCa channel by PLCγ and PIP_2

Two PLCs, a PLCγ and PLCβ homolog, function in separate signaling pathways to maintain rhythmic Ca^{2+} oscillations in the *C. elegans* intestine. PLCγ functions to generate IP_3 that regulates IP_3 receptor activity. The function of PLCβ remains to be defined, but it may play a role in G-protein signaling events that regulate intestinal Ca^{2+}

oscillations (Espelt et al., 2005b). In recent studies, we demonstrated by epistasis analysis that GON-2/GTL-1 function in the same signaling pathway as PLCγ to regulate IP_3 receptor activity and ER Ca^{2+} release (Xing et al., 2008). To further characterize this relationship, we examined the effect of loss of PLCγ and PLCβ activity on whole cell currents.

Figure 21A shows the current-to-voltage relationship for the intestinal cell outwardly rectifying Ca^{2+} (ORCa) channel current we described in detail previously (Estevez et al., 2003) and that is carried by the GON-2 and GTL-1 TRPM channels (Xing et al., 2008). Whole cell current amplitude was not significantly ($P>0.05$) different in intestinal cells cultured from wild type and PLCβ loss-of-function mutant worms (Figure 21B). In contrast, knockdown of PLCγ activity by RNAi inhibited whole cell current approximately 80% ($P<0.01$; Figure 21B).

The *egl-8(n488)* allele is an 1819 bp deletion mutation. It has been suggested by Bastiani et al. (Bastiani et al., 2003) that this allele may encode a neomorphic protein. To further assess the possible role of PLCβ in regulating GON-2/GTL-1 then, we knocked down its expression by RNAi. As shown in Figure 21B, PLCβ RNAi had no effect on whole cell current amplitude. Data in Figure 21B thus demonstrate that PLCγ but not PLCβ activity modulates GON-2/GTL-1 function.

We also examined the effects of acute inhibition of PLCγ on channel activity using the pan-PLC inhibitor U-73122. Incubation of intestinal cells for 10-60 min with 2 μM U-73122 in the bath reduced mean ± S.E. whole cell current to 17 ± 3 pA/pF (n=4). This current value was not significantly different ($P>0.05$) from that observed with PLCγ RNAi (Figure 21B). In contrast, exposure of intestinal cells to 2 μM U-73343, which is

an inactive analog of U-73122, did not significantly ($P>0.05$) alter whole cell current (mean ± S.E. whole cell current = 78 ± 19 pA/pF; n=4). These results demonstrate that both acute and chronic inhibition of PLCγ activity inhibits GON-2/GTL-1.

PLCβ and PLCγ hydrolyze PIP_2 to IP_3 and diacylglycerol (DAG). DAG in turn can be metabolized into arachidonic and other polyunsaturated fatty acids (PUFAs). PIP_2, DAG and PUFAs are known to modulate the activity of numerous TRP channels (Raghu and Hardie, 2009; Beech et al., 2009; Nilius et al., 2008). Loss of PLC activity is expected to increase PIP_2 levels as well as decrease IP_3, DAG and PUFA concentrations. Inhibition of GON-2/GTL-1 activity in PLCγ RNAi cells suggests 1) that IP_3, DAG and/or PUFAs may function normally to activate the channels or 2) that PIP_2 is inhibitory.

We carried out a series of studies to test these possibilities. PLCγ RNAi cells were patched clamped with pipette solutions containing 100 μM OAG, a DAG analog, 10 μM arachidonic acid, 10 μM linolenic acid or 10 μM IP_3. As shown in Figure 22A, these signaling molecules failed to activate ($P>0.05$) GON-2/GTL-1 channels inhibited by PLCγ RNAi. In contrast, inclusion of 10 μM PIP_2 in the patch pipette solution inhibited whole cell current in control cells to the same extent as knockdown of PLCγ (Figures 21B and 22B). However, PIP_2 had no significant ($P>0.05$) additional inhibitory effect in PLCγ RNAi cells (Figure 22B).

To further examine the role of PIP_2 in regulating channel activity, we treated PLCγ RNAi cells with 20 μM wortmannin or 20 μg/ml poly-L-lysine in the patch pipette solution. Wortmannin depletes cellular PIP_2 levels by inhibiting phosphoinositide 4-kinase and PIP_2 synthesis (Nakanishi et al., 1995). Poly-L-lysine is a polyvalent cation that binds to PIP_2 and has been widely used to deplete cellular PIP_2 levels (e.g., (Kozak et

al., 2005). In control cells, wortmannin and poly-L-lysine had no significant ($P>0.05$) effect on whole cell current. Mean ± S.E. control cell current densities at +80 mV observed in the presence of wortmannin and poly-L-lysine were 112 ± 12 pA/pF (n=3) and 88 ± 6 pA/pF (n=4), respectively.

As shown in Figure 22B, both wortmannin and poly-L-lysine reversed the inhibitory effect of PLCγ RNAi on whole cell current amplitude. Whole cell current amplitude in PLCγ RNAi cells treated with these agents was not significantly ($P>0.05$) different than that observed in control cells. Taken together, the results shown in Figures 21 and 22 indicate 1) that GON-2/GTL-1 channels are inhibited by loss of PLCγ activity and by PIP_2 added to the patch pipette solution, and 2) that the PLCγ RNAi induced inhibition of the channels is mediated by elevation of cellular PIP_2 levels.

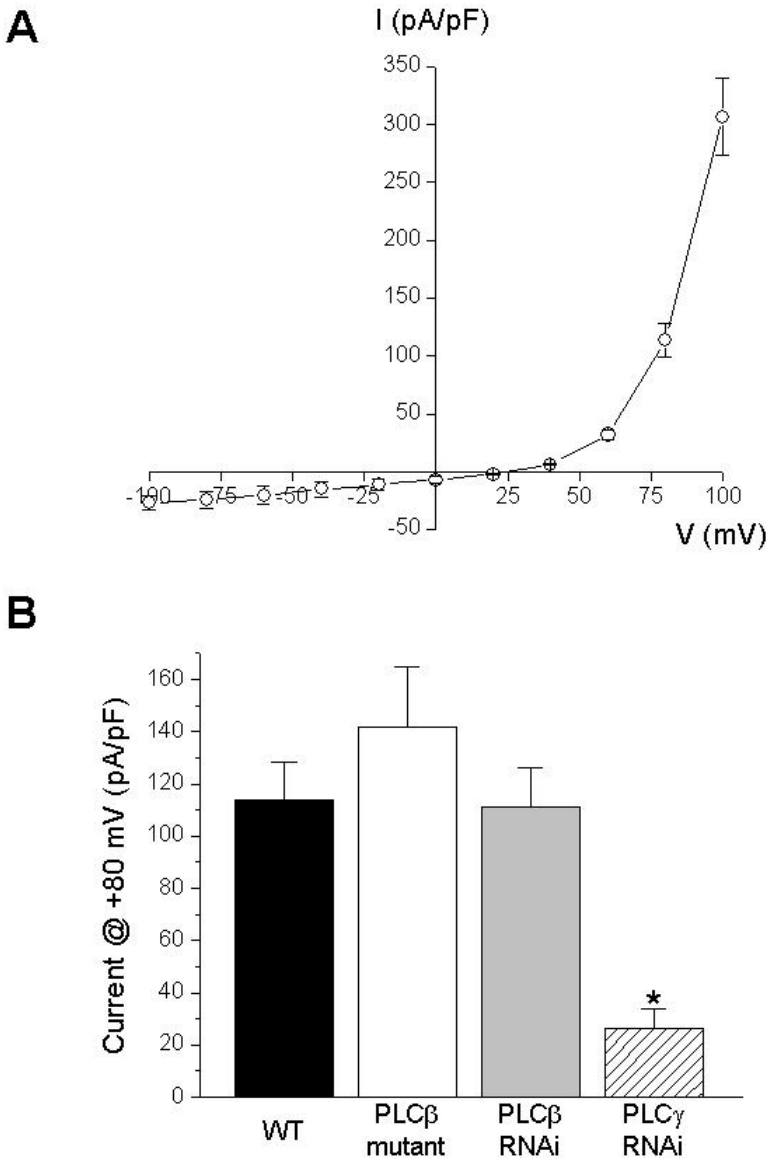


Figure 21. Loss of PLCγ activity inhibits GON-2/GTL-1 mediated whole cell current. (A) Current-to-voltage relationship of whole cell current measured in intestinal cells cultured from wild type worms. Values are means ± S.E. (n=11). (B) Effect of loss of PLCβ and PLCγ activity on whole cell current amplitude. Values are means ± S.E. (n=4-11). *P<0.01 compared to wild type (WT) worms.

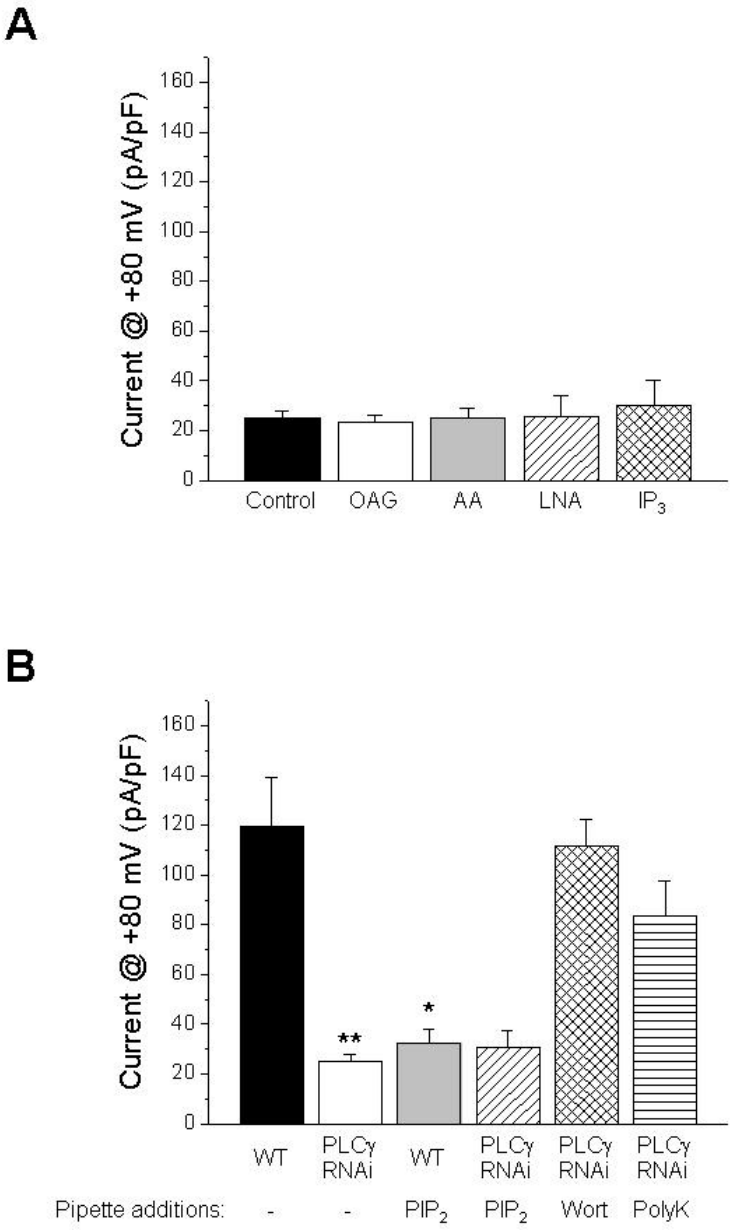


Figure 22. Inhibition of GON-2/GTL-1 mediated whole cell current by PLCγ RNAi is reversed by agents that lower cellular PIP_2 levels. (A) Whole cell current amplitude in PLCγ RNAi cells patch clamped with a control pipette solution or a solution containing 100 μM 1-oleoyl-2-acetyl-sn-glycerol (OAG), 10 μM arachidonic acid (AA), 10 μM linolenic acid (LNA) or 10 μM IP_3. Values are means ± S.E. (n=4-5). (B) Effects of 10 μM PIP_2, 20 μM wortmannin (Wort) or 20 μg/ml poly-L-lysine (PolyK) on whole cell current in wild type and PLCγ RNAi cells. Values are means ± S.E. (n=5-15). *P<0.01 and **P<0.001 compared to untreated wild type cells. Whole cell currents recorded from wortmannin and poly-L-lysine treated cells were not significantly (P>0.05) different from those observed in untreated wild type cells. Data in A and B are plotted on the same scale as Figure 21B to facilitate comparisons.

Sensitivity and specificity of GON-2/GTL-1 to PIP_2

To assess the sensitivity of GON-2/GTL-1 channels to PIP_2, we defined the dose-response relationship for PIP_2 inhibition. As shown in Figure 23A, maximal inhibition is observed at ~10 μM PIP_2. The IC_{50} value for inhibition was 2.6 μM with a Hill coefficient of 0.6. PIP_2 is largely confined to the plasma membranes and represents ~1% of the total anionic phospholipid pool. If dissolved in the cytoplasm, PIP_2 concentration has been estimated to be 4-10 μM (Hilgemann, 2007; Suh and Hille, 2008). Data in Figure 23A thus suggest that PIP_2 likely plays a physiologically relevant role in regulating GON-2/GTL-1 channel activity.

To assess the specificity of PIP_2 inhibition, we quantified the effects of singly phosphorylated PI(4)P or triply phosphorylated $PI(3,4,5)P_3$. Cellular PI(4)P levels are comparable to those of PIP_2 while PIP_3 is much less abundant (Vanhaesebroeck et al., 2001). We also examined the effects of the PIP_2 isomers $PI(3,4)P_2$ and $PI(3,5)P_2$. As shown in Figure 23B, all four phospholipids had small (~30-50%) inhibitory effects on whole cell current amplitude. However, none of these effects achieved statistical significance ($P>0.05$).

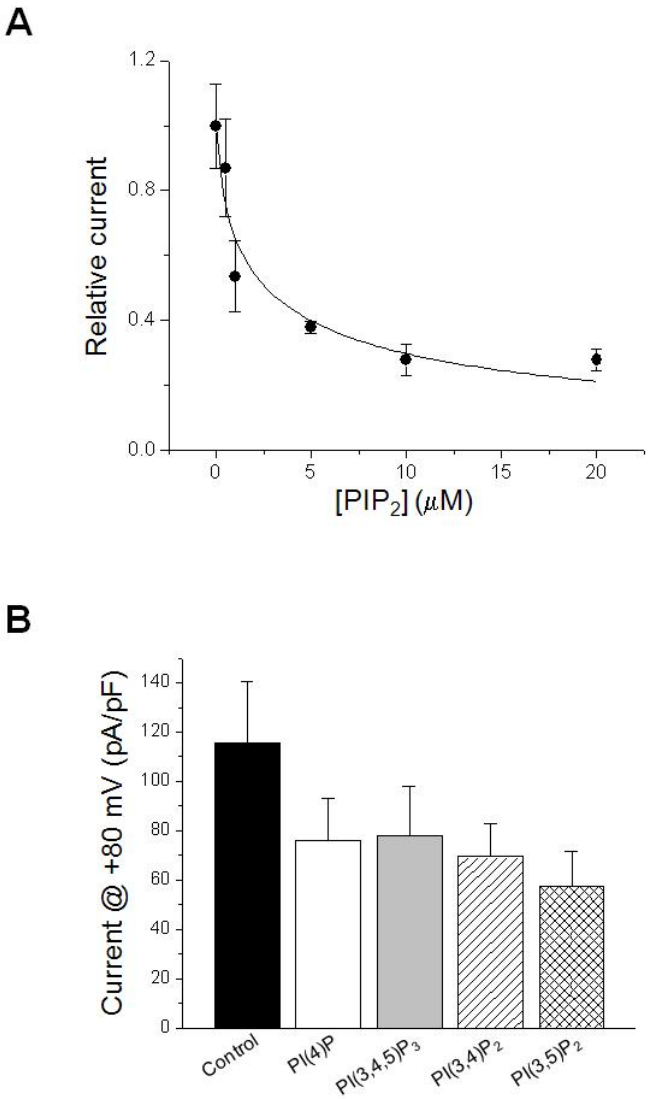


Figure 23. Sensitivity and specificity of GON-2/GTL-1 to PIP_2. (A) Dose-response relationship for the inhibitory effect of PIP_2 on GON-2/GTL-1 currents. Data were fit using the equation $I = 1/1+([PIP_2]/IC_{50})^n$. Values are means ± S.E. (n=4-5). (B) Sensitivity of GON-2/GTL-1 currents to 10 μM PI(4)P, $PI(3,4,5)P_3$, $PI(3,4)P_2$ or $PI(3,5)P_2$. Values are mean ± S.E. (n=5-6). None of the phospholipids had a significant (P>0.05) effect on whole cell current amplitude.

Functional properties of PIP_2 inhibited GON-2/GTL-1 currents

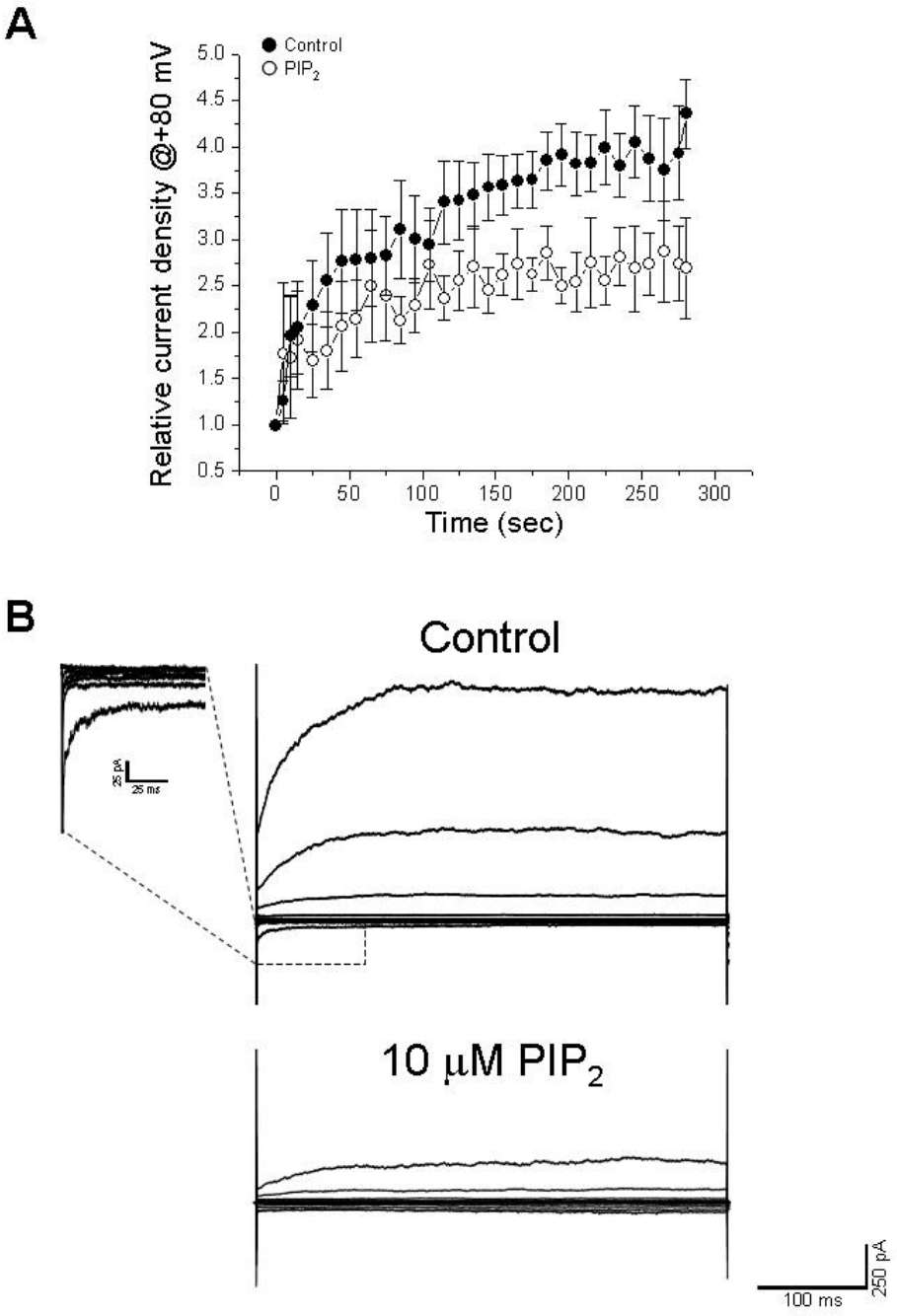
PIP_2 alters the functional properties of several different channel types (e.g., (Zhang et al., 2005b; Nilius et al., 2006; Wu et al., 2002). To determine whether PIP_2 modulates GON-2/GTL-1 functional characteristics, we quantified current properties in control cells and cells treated with 10 μM PIP_2. Upon obtaining whole cell access, GON-2/GTL-1 current shows rapid run-up and then stabilizes within 1-2 min (Figure 24A). Cells exposed to 10 μM PIP_2 showed a similar pattern of run-up (Figure 24A). Mean ± S.E. rates of current activation were 36 ± 6 pA/pF/min (n=10) in control cells and 35 ± 3 pA/pF/min (n=4) in cells dialyzed with 10 μM PIP_2. These rates were not significantly ($P>0.9$) different.

GON-2/GTL-1 currents are voltage and time dependent (Estevez et al., 2003). Strong depolarization and hyperpolarization activated and inactivated, respectively, currents in both control and PIP_2 treated cells (Figure 24B). Current activation and inactivation were both well fit by double exponentials describing fast (τ_f) and slow (τ_s) time constants. Mean ± S.E. τ_f and τ_s at +100 mV were 34 ± 2 ms and 218 ± 34 ms (n=15) for control cells and 30 ± 3 ms and 168 ± 84 ms (n=4) for cells patch clamped in the presence of 10 μM PIP_2. At -100 mV, τ_f and τ_s in control cells were 11 ± 4 ms and 191 ± 25 ms (n=15) and 8 ± 3 ms and 177 ± 46 ms (n=6) in PIP_2 treated cells. Neither activation nor inactivation time constants were significantly ($P>0.4$) altered by PIP_2.

To further assess the effects of PIP_2 on channel gating, we quantified the voltage dependence of steady-state and tail currents. Whole cell currents were normalized to either the maximum steady-state ($I_{max,\ steady\text{-}state}$) or maximum tail current ($I_{max,\ tail}$). Normalized current-to-voltage relationships determined in the presence and absence of 10

μM PIP_2 were superimposable (Figure 24C). These data and the results discussed above demonstrate that PIP_2 does not alter the voltage-dependent gating of GON-2/GTL-1.

The major physiologically relevant ions that permeate GON-2/GTL-1 channels are Ca^{2+} and Na^{+} (Estevez et al., 2003; Xing et al., 2008). Mean ± SE current reversal potentials (E_{rev}) were 25 ± 1 mV (n=15) and 25 ± 3 mV (n=9) in control and PIP_2 cells, respectively. These values were not significantly (P>0.9) different suggesting that channel selectivity was unaffected by PIP_2. To test this directly, we measured relative Ca^{2+} permeability by replacing bath Na^{+} with 130 mM $NMDG^{+}$ and 10 mM Ca^{2+}. Elevation of bath Ca^{2+} increased E_{rev} by 29 ± 1 mV (n=6) in PIP_2 cells. The calculated relative Ca^{2+} to Na^{+} permeability was 72 ± 9:1 (n=6) and was not significantly (P>0.4) different from that we have reported previously (Estevez et al., 2003; Xing et al., 2008).


A
Control
PIP2
Relative current density @+80 mV
Time (sec)
B
Control
25 pA
25 ms
10 μM PIP2
250 pA
100 ms

C

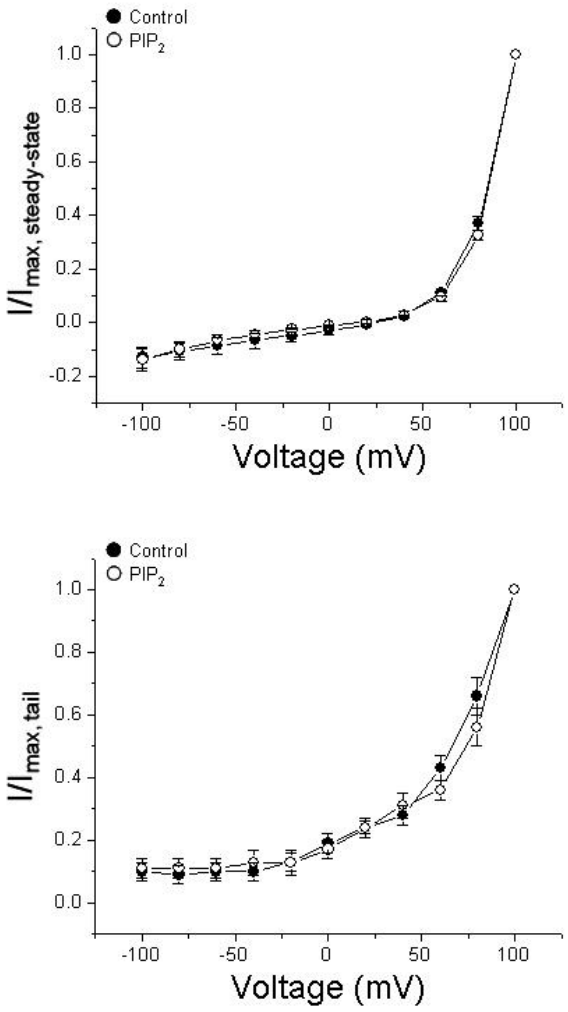


Figure 24. Effects of PIP_2 on current run-up and voltage dependent channel activity. (A) Current run-up after obtaining whole cell access in control cells and cells patch clamped with 10 μM PIP_2. Data are plotted relative to the first measurement recorded after whole cell access was obtained. Values are means ± SE (n=5-12). (B) Whole cell GON-2/GTL-1 currents recorded from a control intestinal cell and a cell dialyzed with 10 μM PIP_2. Currents were elicited by stepping membrane voltage from -100 to +100 mV in 20 mV steps from a holding potential of 0 mV. Voltage steps were held for 400 ms long. Inset shows inactivation behavior observed at hyperpolarized voltages. (C) Voltage dependence of whole cell steady-state and tail currents measured in the presence and absence of 10 μM PIP_2. Currents were elicited by stepping membrane voltage in 20 mV steps from a holding potential of 0 mV to a test potential between -100 and +100 mV. Voltage steps were held for 400 ms. After the test potential, membrane voltage was stepped to -100 mV for 100 ms to inactivate currents. Cells were then stepped to 0 mV and allowed to recover for 20 ms before initiating the next test pulse. Steady-state current was defined as the mean current measured during the last 50 ms of the 400 ms test pulse. Tail current was the peak current measured during the 100 ms step to -100 mV. Values are mean ± S.E. (n=5-6).

Combined role of intracellular Ca^{2+} and PIP_2 in regulating GON-2/GTL-1 channel activity

A number of studies have demonstrated that intracellular Ca^{2+} and PIP_2 co-regulate TRP channels. For example, PIP_2 activates TRPM5 and increases the sensitivity of the channel to intracellular Ca^{2+} (Liu and Liman, 2003a). The GON-2/GTL-1 current is also regulated by intracellular Ca^{2+} (Estevez and Strange, 2005), suggesting that Ca^{2+} and PIP_2 may function synergistically.

To begin testing this idea, we characterized the effect of PLCγ knockdown on oscillating channel activity. As we have described previously (Estevez and Strange, 2005), GON-2/GTL-1 is regulated by Ca^{2+} influx through the channel and Ca^{2+} accumulation in a space very close to the intracellular pore opening. Low concentrations of Ca^{2+} activate the channel whereas higher concentrations are inhibitory. These dual effects of Ca^{2+} are manifested as oscillations in whole cell current amplitude when intestinal cells are patch clamped with pipette solutions containing low concentrations of Ca^{2+} buffers (Figure 25A). We reasoned that if PIP_2 and Ca^{2+} function synergistically, then elevated PIP_2 levels may modify Ca^{2+} dependent channel oscillations. Figure 25B shows whole cells current oscillations in PLCγ RNAi cells patch clamped with a pipette solution containing 1 mM BAPTA. The mean ± S.E. number of oscillations detected was 0.7 ± 0.2 oscillations/min (n=4 cells) in control cells and 0.75 ± 0.1 oscillations/min (n=5 cells) in PLCγ RNAi cells, and were not significantly ($P>0.9$) different.

The overall characteristics of the current oscillations were qualitatively similar. However, peak current amplitude was reduced ~85% ($P<0.0001$) in cells treated with PLCγ dsRNA. The mean ± S.E. peak current was 691 ± 49 pA/pF (n=16 oscillations) and 98 ± 15 pA/pF (n=15 oscillations) in control and PLCγ RNAi cells, respectively.

Reductions in peak oscillatory and steady-state current amplitudes (see Figure 21B) induced by PLCγ knockdown were similar. The lack of an obvious effect of PLCγ RNAi on oscillatory channel behavior indicates that the Ca^{2+} feedback mechanisms regulating channel activity remain unchanged even in presence of maximal PIP_2 induced inhibition and PLCγ knockdown.

To test further for possible regulatory interactions between Ca^{2+} and PIP_2, we quantified the effect of intracellular Ca^{2+} on the rate of current run-up observed after attaining whole cell access. As described previously (Estevez and Strange, 2005), the initial rate of current activation increases with increasing intracellular Ca^{2+} concentration in wild type intestinal cells (Figure 25C). Inclusion of 2.5 μM PIP_2, which is the approximate IC_{50} value determined from data shown in Figure 23A, in the patch pipette solution had no effect on the rate of Ca^{2+} dependent current activation (Figure 25C).

The maximal current that is activated after obtaining whole cell access is also modulated by cellular Ca^{2+} levels (Estevez and Strange, 2005). Figure 25D shows the relationship between Ca^{2+} concentration and peak current amplitude. Inclusion of 2.5 μM PIP_2 in the patch pipette solution inhibited peak current amplitude similarly at all Ca^{2+} concentrations.

At high intracellular Ca^{2+} concentrations (>250 nM), whole cell current activation is transient (Estevez and Strange, 2005). We quantified the initial rate of current inactivation and the final steady-state current levels in cells patch clamped with 500 nM intracellular Ca^{2+} in the presence or absence of 2.5 μM PIP_2. Mean ± S.E. relative rates of inactivation and steady-state current amplitudes relative to peak current were -2.2 ± 0.4 %/min (n=6) and 0.27 ± 0.1 (n=7) in the absence of PIP_2, and -1.6 ± 0.4 %/min (n=4)

and 0.21 ± 0.1 (n=5) with PIP_2. Neither the rate nor extent of current inactivation were significantly (P>0.3) altered by inclusion of PIP_2 in the patch pipette solution. Taken together, our results indicate that Ca^{2+} and PIP_2 act independently to regulate GON-2/GTL-1 channel activity.

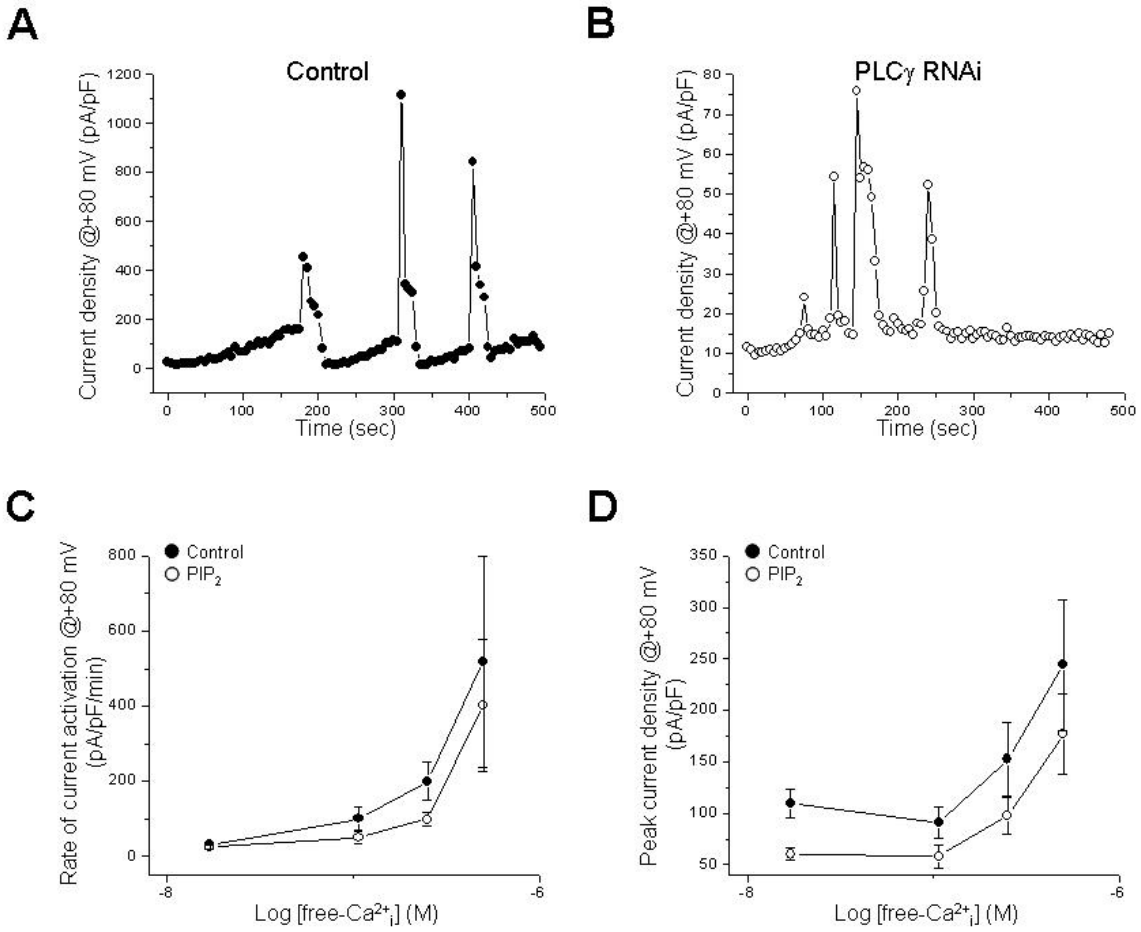


Figure 25. Effects of PIP_2 on Ca^{2+} dependent GON-2/GTL-1 channel activity. (A, B) Examples of whole cell current oscillations in a control and PLCγ RNAi cell. Current oscillations were induced by patch clamping cells with a pipette solution containing 1 mM BAPTA. (C) Relationship between initial rate of current activation after obtaining whole cell access and intracellular free-Ca^{2+} levels in control cells and cell patch clamped in the presence of 2.5 μM PIP_2. (D) Relationship between peak current amplitude and intracellular free-Ca^{2+} levels in control cells and cell patch clamped in the presence of 2.5 μM PIP_2. Values in C and D are mean ± S.E. (n=4-7).

Discussion

The TRPM channels GON-2 and GTL-1 are the major pathway for Ca^{2+} entry into *C. elegans* intestinal cells (Xing et al., 2008) and may also play a role in Mg^{2+} transport (Teramoto et al., 2005). GON-2/GTL-1 function in a common signaling pathway with PLCγ to maintain the rhythmicity of *C. elegans* intestinal Ca^{2+} oscillations (Xing et al., 2008). Loss of either GON-2/GTL-1 or PLCγ activity causes arrhythmic Ca^{2+} oscillations and associated contractions of posterior body wall muscles that mediate defecation (Xing et al., 2008; Espelt et al., 2005b).

The current studies demonstrate that PIP_2 levels regulated by PLCγ modulate GON-2/GTL-1 channel function. Elevated PIP_2 levels inhibit channel activity (Figure 22B). The mechanism by which PIP_2 inhibits GON-2/GTL-1 is unclear. GON-2/GTL-1 exhibits voltage dependence and is regulated by intracellular Ca^{2+} levels (Figures 24 and 25)(Estevez and Strange, 2005). However, unlike other TRPM channels (e.g., (Nilius et al., 2006; Liu and Liman, 2003a; Zhang et al., 2005b), PIP_2 has no effect on the voltage sensitivity or Ca^{2+} responsiveness of GON-2/GTL-1 (Results and Figures 24 and 25). PIP_2 most likely modulates channel open probability, single channel conductance and/or channel trafficking. Both GON-2 and GTL-1 have multiple positively charged domains on their cytoplasmic N- and C-termini that could function as PIP_2 binding sites (reviewed in (Suh and Hille, 2008)). Single channel studies and molecular and biochemical analyses will be needed to define the mode of action of PIP_2.

In addition to its catalytic activity, PLCγ can also play non-catalytic regulatory roles. For example, PLCγ binds to TRPC3 and regulates membrane expression of the channel (van Rossum et al., 2005). The Na^+/H^+ exchanger NHE3 also interacts with

PLCγ. This interaction is dynamic and regulated by changes in Ca^{2+} levels (Zachos et al., 2009). Elevated Ca^{2+} reduces NHE3 activity in part by decreasing membrane expression (Li et al., 2004) suggesting that changes in PLCγ/NHE3 interaction control transporter trafficking. Our data indicate that the regulatory role of PLCγ on GON-2/GTL-1 is mediated through its catalytic activity. Normal channel activity is restored in PLCγ RNAi cells by wortmannin or poly-L-lysine, agents that function to lower PIP_2 levels (Figure 22B).

Numerous ion channels including members of the TRP superfamily (reviewed by (Nilius et al., 2008) have been shown to be regulated by PIP_2. For example, PIP_2 activates TRPM4 channels by increasing voltage and Ca^{2+} sensitivity such that channels open at physiologically relevant membrane voltages and intracellular Ca^{2+} levels (Nilius et al., 2006). TRPP2 is inhibited by PIP_2 (Ma et al., 2005). PIP_2 both activates and inhibits TRPV1 and the mode of action is dependent on the degree of stimulation by channel agonists such as capsaicin (Lukacs et al., 2007a).

In most cases, the physiological relevance of PIP_2 regulation of ion channel activity is uncertain or inferred from knowledge of channel function. Similarly, the physiological role of PIP_2 regulation of GON-2/GTL-1 is unclear at present. However, our current understanding of Ca^{2+} signaling in the *C. elegans* intestine allows us to propose a working model (Figure 26). As we have shown previously (Espelt et al., 2005b), PLCγ generates IP_3 that regulates intracellular IP_3 receptor activity and Ca^{2+} release. It is well established that IP_3 receptors are also regulated by Ca^{2+}. Low intracellular Ca^{2+} concentrations activate IP_3 receptor Ca^{2+} channels while high Ca^{2+} levels inhibit intracellular Ca^{2+} release (Foskett et al., 2007). In excitable cells, Ca^{2+}

influx through voltage- and ligand-gated Ca^{2+} channels regulates intracellular Ca^{2+} release via both IP_3 and ryanodine receptors (e.g., (Gordienko et al., 2007; Kapur et al., 2001; Kukuljan et al., 1997). Similarly, Ca^{2+} influx through GON-2/GTL-1 may control IP_3 receptor activity in *C. elegans* intestinal cells.

Regulation of GON-2/GTL-1 by PIP_2 may function to coordinate PLCγ activity, IP_3 levels and IP_3 receptor activity with plasma membrane Ca^{2+} influx. Foskett and co-workers have shown that the Ca^{2+} sensitivity of IP_3 receptors varies with IP_3 concentration. As IP_3 levels rise, the concentration of Ca^{2+} required to feedback inhibit IP_3 channels increases (Mak et al., 1998; Foskett et al., 2007). Thus, IP_3 channels activate and remain active in the presence of higher local Ca^{2+} concentrations when IP_3 levels are elevated. Under conditions where PIP_2 levels are high and IP_3 concentration is presumably low, the reduced Ca^{2+} influx through PIP_2–inhibited GON-2/GTL-1 may be insufficient to activate IP_3 receptors. As PIP_2 is hydrolyzed to IP_3, increased Ca^{2+} influx through GON-2/GTL-1 could now serve to stimulate IP_3 receptor activity and trigger a rise in intracellular Ca^{2+} concentration (Figure 26).

In addition to its role in regulating IP_3 receptor function, Ca^{2+} also modulates GON-2/GTL-1 activity. As with the IP_3 receptor, low Ca^{2+} concentrations activate and high Ca^{2+} levels inhibit the channel (Estevez and Strange, 2005). Feedback regulation of GON-2/GTL-1 activity by local Ca^{2+} levels may also serve to coordinate intracellular Ca^{2+} release and plasma membrane Ca^{2+} influx (Figure 26).

The nature of the signal that triggers an intestinal Ca^{2+} spike is unknown. No intestinal Ca^{2+} signaling agonist has been identified. Since Ca^{2+} oscillations continue for long periods of time in vitro after isolation of the intestine (Espelt et al., 2005b), it is

likely that Ca^{2+} oscillations are independent of extracellular signaling events. It is conceivable that extracellular Ca^{2+} is the agonist that triggers intracellular Ca^{2+} oscillations. Low levels of Ca^{2+} influx through PIP_2–inhibited GON-2/GTL-1 channels could increase the activity of PLCγ. Increasing PLCγ activity would lower PIP_2 levels and relieve channel inhibition. Increasing Ca^{2+} influx through GON-2/GTL-1 would further stimulate channel activity (Estevez and Strange, 2005) and modulate IP_3 receptor function (Figure 26). Such a mechanism is analogous to that proposed for the PIP_2–dependent regulation of TRPM8 (Rohacs et al., 2005) and TRPV6 channels (Thyagarajan et al., 2008). However, for these channels PIP_2 is required for normal activity. Calcium influx through the channels is postulated to activate PLC thereby depleting PIP_2, which leads to channel inactivation (Rohacs et al., 2005; Thyagarajan et al., 2008).

It is generally accepted that changes in intracellular Ca^{2+} levels regulate the activity of the δ-isoforms of PLCs. However, all PLC isoforms require Ca^{2+} for normal catalytic function (Rebecchi and Pentyala, 2000) and at least one report has shown that PLCγ1 is also activated, albeit less than PLCδ1, by increasing Ca^{2+} levels (Allen et al., 1997). Extensive additional studies are needed to determine whether *C. elegans* PLCγ is regulated by Ca^{2+} changes and whether such regulation contributes to oscillatory Ca^{2+} signaling in the intestine.

We have shown previously that canonical store-operated Ca^{2+} channels do not appear to be required for generating intestinal Ca^{2+} oscillations (Lorin-Nebel et al., 2007; Yan et al., 2006). GON-2/GTL-1 channels may thus play a role in refilling intracellular Ca^{2+} stores. PIP_2 regulation of the channel would provide a means of coupling store Ca^{2+} levels to plasma membrane Ca^{2+} influx. Under conditions of low PLCγ activity and IP_3

levels, store Ca^{2+} release would presumably be low and hence there would be no need for high rates of plasma membrane Ca^{2+} influx. Intracellular Ca^{2+} release triggered by increased PLCγ activity and IP_3 levels would occur concomitantly with falling PIP_2 levels and increased Ca^{2+} influx through GON-2/GTL-1.

In conclusion, PLCγ and GON-2/GTL-1 function in a common signaling pathway to maintain the rhythmicity of IP_3–dependent Ca^{2+} oscillations (Xing et al., 2008). The current studies demonstrate that GON-2/GTL-1 is regulated by PLCγ in a PIP_2 dependent manner. Hydrolysis of PIP_2 functions to both activate plasma membrane Ca^{2+} entry and intracellular Ca^{2+} release. PIP_2–dependent regulation of GON-2/GTL-1 may provide a mechanism to coordinate plasma membrane Ca^{2+} influx with PLCγ and IP_3 receptor activity and intracellular Ca^{2+} store depletion.

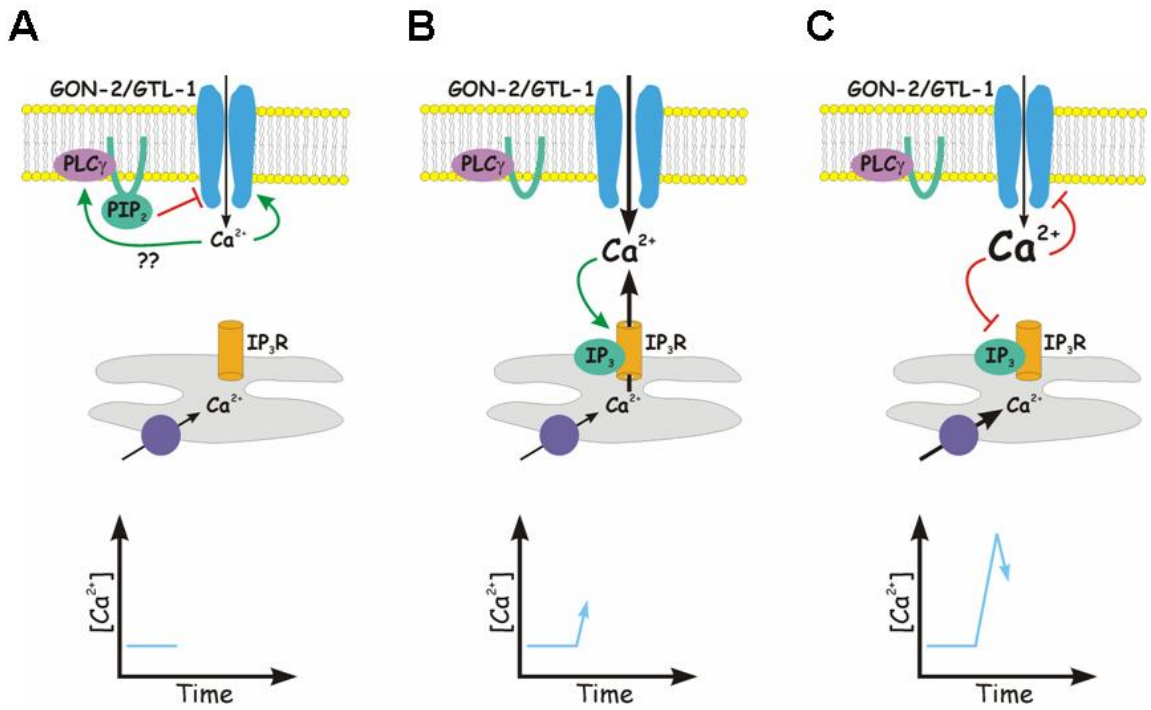


Figure 26. Working model illustrating the established and putative roles of PLCγ, GON-2/GTL-1 and Ca^{2+} in oscillatory Ca^{2+} signaling in the *C. elegans* intestine. Graphs at bottom show expected intracellular Ca^{2+} changes. (A) PIP_2 partially inhibits GON-2/GTL-1 channels under resting conditions. Calcium entering through GON-2/GTL-1 accumulates in a microdomain near the channel mouth and stimulates channel activity. Local Ca^{2+} accumulation may also activate PLCγ. (B) PLCγ hydrolyzes PIP_2 relieving GON-2/GTL-1 inhibition. IP_3 and enhanced Ca^{2+} influx through GON-2/GTL-1 activate IP_3 receptor mediated Ca^{2+} release from intracellular stores generating the rising phase of a Ca^{2+} spike. (C) Elevated intracellular Ca^{2+} feedback inhibits both IP_3 receptor and GON-2/GTL-1 channels. Cytoplasmic Ca^{2+} levels are lowered by reuptake of Ca^{2+} into intracellular stores and extrusion across the plasma membrane. Green arrows and red lines indicate activation and inhibition, respectively.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Fluctuating intracellular Ca^{2+} concentration is a ubiquitous signaling mechanism that controls numerous cellular processes including fertilization, gene transcription, exocytosis, secretion, cell differentiation, and apoptosis (Berridge et al., 2000). Keeping this in mind, it is not surprising that abnormal intracellular Ca^{2+} homeostasis underlies many common pathological conditions and human diseases, such as cardiac hypertrophy and heart failure, ataxia and certain types of epilepsy (Missiaen et al., 2000). The nematode *C. elegans* offers substantial experimental advantages for defining the molecular mechanisms of Ca^{2+} signaling. These advantages include a fully sequenced and well-annotated genome; a short life cycle and a number of stereotyped behaviors that allow forward genetic screening; relative ease of generating transgenic animals and manipulating gene expression by RNA interference; and numerous freely available reagents including worm strains and cosmid and YAC clones spanning the genome (Barr, 2003; Strange, 2003). Direct physiological measurements can be combined with genetic and molecular analyses in *C. elegans*, which make it a unique system for studying oscillatory Ca^{2+} signaling (Espelt et al., 2005b; Estevez et al., 2003; Yan et al., 2006; Lorin-Nebel et al., 2007; Xing et al., 2008).

Posterior body wall muscle contraction (pBoc) in *C. elegans* drives worm defecation and occurs in a rhythmic fashion once ever 45~50 sec(Thomas, 1990). Genetic and physiological analyses have demonstrated that the pBoc cycle is regulated by inositol-1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} oscillations in the intestinal epithelium (Dal Santo et al., 1999; Espelt et al., 2005b). Mutations in two intestinal TRPM channel encoding genes *gon-2* and *gtl-1* disrupt the pBoc rhythm, suggesting that they are likely to play a role in regulating Ca^{2+} oscillations in the intestinal cells. The central goal of studies carried out in this dissertation is to characterize the roles of GON-2 and GTL-1 in oscillatory Ca^{2+} signaling pathways in the *C. elegans* intestine, and to determine how these channels are regulated. Results from this study provide new insights into our understanding of the molecular identity of plasma membrane Ca^{2+} channels and their roles in controlling rhythmic Ca^{2+} oscillations in *C. elegans* intestinal cells. Moreover, this study is an important step toward our long-term goal to utilize the *C. elegans* intestine as a model system to develop an integrated molecular understanding of oscillatory Ca^{2+} signaling pathways in nonexcitable cells.

This final chapter summarizes my conclusions. Studies carried out in this dissertation demonstrated that: (1) Two *C. elegans* TRPM channels GON-2 and GTL-1 are required for rhythmic pBocs and rhythmic Ca^{2+} oscillations in the *C. elegans* intestine; (2) GON-2 and GTL-1 function together to generate the outwardly rectifying Ca^{2+} (ORCa) current and mediate selective Ca^{2+} influx in intestinal cells; (3) Epistasis analyses indicate that GON-2/GTL-1 function in the common signaling pathway with PLCγ and IP_3 receptors to regulate *C. elegans* pBoc rhythm and intestinal Ca^{2+} oscillations; (4) Loss of PLCγ activity inhibits GON-2/GTL-1 current in *C. elegans* intestinal cells by increasing

PIP_2 concentration; (5) Application of exogenous PIP_2 also inhibits GON-2/GTL-1 current in *C. elegans* intestinal cells and PIP_2 regulates GON-2/GTL-1 channel activity in a voltage and calcium independent manner.

These key findings allow us to propose a working modeling illustrating how GON-2/GTL-1 may function together with PLCγ and IP_3 receptors to generate rhythmic Ca^{2+} oscillations in the *C. elegans* intestine that drive worm defecation. Under resting conditions, PIP_2 partially inhibits GON-2/GTL-1 channels and calcium entering through GON-2/GTL-1 accumulates in a microdomain near the channel mouth and stimulates channel activity. Local Ca^{2+} accumulation may also activate PLCγ. Activated PLCγ hydrolyzes PIP_2 relieving GON-2/GTL-1 inhibition. IP_3 and enhanced Ca^{2+} influx through GON-2/GTL-1 activate IP_3 receptor mediated Ca^{2+} release from intracellular stores increasing cytoplasmic Ca^{2+} levels. Elevated intracellular Ca^{2+} feedback inhibits both IP_3 receptors and GON-2/GTL-1 channels. Cytoplasmic Ca^{2+} levels are lowered by reuptake of Ca^{2+} into intracellular stores and extrusion across the plasma membrane. Ca^{2+} influx through GON-2/GTL-1 may also play a role in refilling the ER Ca^{2+} stores and PIP_2 regulation of the channel may provide a means of coupling store Ca^{2+} levels to plasma membrane Ca^{2+} entry (Xing et al., 2009).

Future directions

GON-2 and GTL-1 function together to mediate selective Ca^{2+} current (ORCa) in cultured *C. elegans* intestinal cells. Do they form heterotetrameric channels?

We have identified two *C. elegans* TRPM homologues that mediate the outwardly rectifying Ca^{2+} (ORCa) current in intestinal cells. Our results demonstrate that the function of GON-2 and GTL-1 are interdependent. One possibility is that the ORCa channel is a GON-2/GTL-1 heteromer. Alternatively, loss of either GON-2 or GTL-1 alone may disrupt the trafficking, expression and/or regulation of the other channel.

The ability of different TRP channels to interact physically and functionally is well known. Numerous studies have provided evidence that a number of TRP channel family members form homo- and/or hetero- tetramers including TRPC1 (Barrera et al., 2007), TRPP2 and TRPC1 (Kobori et al., 2009), and TRPV1 (Moiseenkova-Bell et al., 2008). Heteromultimers of TRPM6 and TRPM7, homologues of GON-2 and GTL-1, have also been described (Li et al., 2006; Chubanov et al., 2004). Interactions among different TRP channels have been detected by co-immunoprecipitation and fluorescence resonance energy transfer (FRET), and through the demonstration that co-expression of two different subunits produces channels with properties distinct from those formed after expression of either subunit alone (Li et al., 2006; Chubanov et al., 2007). In most cases, these studies have been performed in heterologous expression systems. Future studies including generating transgenic worms co-expressing GON-2/GTL-1 tagged with different fluorophores and FRET analyses in vivo may provide meaningful insights into our understanding of possible TRPM heterotetramers in their native environments.

Identify novel endogenous regulators of *C. elegans* TRPM channels

My studies have discovered that GON-2 and GTL-1 mediate ORCa current in *C. elegans* intestinal cells and the activity of GON-2/GTL-1 is required for rhythmic pBocs (Xing et al., 2008). Mutations in genes that are required for normal GON-2/GTL-1 channel activity are likely to disrupt pBoc rhythm in *C. elegans*. Further characterization of whole cell ORCa current in intestinal cells carrying these mutations would allow us to determine whether GON-2/GTL-1 activity is affected in these mutant cells. This may provide basis for genetic screens to identify novel endogenous regulators of GON-2/GTL-1 channels.

Novel regulators of GON-2/GTL-1 channels may directly modulate channel activity or may disrupt the trafficking, and/or expression of the two channels. GTL-1 has been shown to specifically localize to the apical membrane of the intestine using transgenic worms expressing GTL-1::GFP translational reporter (Xing, unpublished data), whereas the subcellular localization of GON-2 is still uncharacterized. Future studies using transgenic worms expressing GON-2 translational reporters would provide the basis for RNAi screens to identify novel signaling molecules that regulate the trafficking of GON-2 and/or GTL-1.

Identify PIP_2 interacting domains on GON-2 and GTL-1 and characterize the mechanism underlying PIP_2 inhibition of the two channels

We have demonstrated that application of exogenous PIP_2 significantly inhibits the activity of the ORCa current, which is mediated by *gon-2* and *gtl-1* in *C. elegans* intestinal cells. What is the specific role of PIP_2 in regulating GON-2 and GTL-1? Are

there PIP_2 binding sites on the channels? Both GON-2 and GTL-1 have multiple positively charged domains on their cytoplasmic N- and C-termini that could function as PIP_2 binding sites. Biochemical analyses using purified proteins will be needed to identify possible PIP_2 interacting domains on GON-2 and/or GTL-1.

PIP_2 has no effect on the voltage sensitivity or Ca^{2+} responsiveness of GON-2/GTL-1 and it most likely modulates channel open probability, single channel conductance, and/or channel trafficking. Single channel studies will be needed to define the mode of action of PIP_2 inhibition. However, single channel analysis is not yet technically feasible in primary cultured *C. elegans* cells. Alternatively, characterization of PIP_2 regulation of heterologously expressed GON-2 and/or GTL-1 may be useful in addressing this question. To determine whether PIP_2 affects channel trafficking, cell surface expression of fluorescence protein tagged GON-2 and GTL-1 can be monitored by total internal reflection fluorescence (TIRF) microscopy while cellular PIP_2 levels are manipulated through different maneuvers.

Final remarks

The work described in this dissertation greatly expands our knowledge of the oscillatory Ca^{2+} signaling pathways in the *C. elegans* intestine. My study identified that the plasma membrane ORCa current in *C. elegans* intestinal cells is mediated by two TRPM channel genes, *gon-2* and *gtl-1*, and discovered that these channels are regulated by PLCγ activity and PIP_2 in vivo. Our results indicate that Ca^{2+} influx through GON-2 and GTL-1 is essential for maintaining the rhythmicity of intestinal Ca^{2+} oscillations.

The *C. elegans* intestine provides unique tools that allow us to combine genetic analysis with direct electrophysiological measurements and Ca^{2+} imaging methods to develop an integrated understanding of oscillatory Ca^{2+} signaling pathways. The genetic power of *C. elegans* makes it well suited for identification of the molecular components of signaling pathways. Forward genetic analysis can be used to screen for abnormal phenotypes and/or suppression of mutant phenotypes. Reverse genetic methods can be used to knockdown the expression of specific genes. My work has identified phenotypes that can be useful for forward and reverse genetic screens in *C. elegans* to search for signaling proteins that function together with GON-2/GTL-1. For example, GTL-1 is specifically localized in the apical membrane of the *C. elegans* intestine, which could be used as the basis for RNAi screens to identify novel endogenous regulators of TRPM channel trafficking.

In summary, the work described in this dissertation has expanded our understanding of the physiological roles and regulation of TRPM channels in vivo and the molecular mechanisms underlying the oscillatory Ca^{2+} signaling pathways in nonexcitable cells. Given the highly conserved nature of Ca^{2+} signaling, insights gained from *C. elegans* will likely provide new and important understanding of the Ca^{2+} signaling mechanisms in mammals.

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