On the potential role of source and species of diacylglycerol in phospholipase-dependent regulation of TRPC3 channels

Guillermo Vazquez,1,2,* Jean-Yves Tano1 and Kathryn Smedlund1

1Department of Physiology and Pharmacology; and 2Center for Diabetes and Endocrine Research at the University of Toledo College of Medicine; Health Science Campus; Toledo, OH USA

Key words: TRPC3, phospholipases, diacylglycerol, Ca2+ influx, cation channels, calcium signaling, channel modulation

Introduction

In non-excitable cells calcium (Ca2+) mobilizing agonists acting on G-protein coupled (GPCR) or receptor tyrosine kinase (RTK) membrane receptors typically activates downstream receptor-dependent stimulation of phospholipases, either by store-operated or non-store operated mechanisms. TRPC3, a member of the TRPC3/6/7 subfamily, has been largely studied mostly due to its ability to function in one or the other modes, depending on cell type and expression conditions. The role of TRPC3 as a non-store operated channel has been attributed to its ability to respond to diacylglycerol (DAG) either exogenously applied or endogenously produced following activation of receptor-stimulated phospholipases. Despite the vast amount of information accumulated on this topic, some critical aspects related to phospholipase-dependent DAG-mediated regulation of TRPC3 remain unclear and/or unexplored. Among these, the source and species of native DAG, modulation by different DAG-generating phospholipases and protein kinase C-dependent inhibition of TRPC3 in its native environment are just few examples. The present essay is intended to compile existing knowledge on the nature of phospholipase-derived DAGs, their biophysical properties and current evidence on phospholipase-dependent regulation of TRPC3, to speculate on potential scenarios that may eventually provide answers to some of the above questions.

Members of the Transient Receptor Potential Canonical (TRPC) family of channel forming proteins are among the most important Ca2+-permeable cation channels in non-excitable cells. Physiologically, TRPC channels are activated downstream receptor-dependent stimulation of phospholipases, either by store-operated or non-store operated mechanisms. TRPC3, a member of the TRPC3/6/7 subfamily, has been largely studied mostly due to its ability to function in one or the other modes, depending on cell type and expression conditions. The role of TRPC3 as a non-store operated channel has been attributed to its ability to respond to diacylglycerol (DAG) either exogenously applied or endogenously produced following activation of receptor-stimulated phospholipases. Despite the vast amount of information accumulated on this topic, some critical aspects related to phospholipase-dependent DAG-mediated regulation of TRPC3 remain unclear and/or unexplored. Among these, the source and species of native DAG, modulation by different DAG-generating phospholipases and protein kinase C-dependent inhibition of TRPC3 in its native environment are just few examples. The present essay is intended to compile existing knowledge on the nature of phospholipase-derived DAGs, their biophysical properties and current evidence on phospholipase-dependent regulation of TRPC3, to speculate on potential scenarios that may eventually provide answers to some of the above questions.

Members of the Transient Receptor Potential Canonical (TRPC) family of channel forming proteins are among the most important Ca2+-permeable cation channels in non-excitable cells. Physiologically, TRPC channels are activated downstream receptor-dependent stimulation of phospholipases, either by store-operated or non-store operated mechanisms. TRPC3, a member of the TRPC3/6/7 subfamily, has been largely studied mostly due to its ability to function in one or the other modes, depending on cell type and expression conditions. The role of TRPC3 as a non-store operated channel has been attributed to its ability to respond to diacylglycerol (DAG) either exogenously applied or endogenously produced following activation of receptor-stimulated phospholipases. Despite the vast amount of information accumulated on this topic, some critical aspects related to phospholipase-dependent DAG-mediated regulation of TRPC3 remain unclear and/or unexplored. Among these, the source and species of native DAG, modulation by different DAG-generating phospholipases and protein kinase C-dependent inhibition of TRPC3 in its native environment are just few examples. The present essay is intended to compile existing knowledge on the nature of phospholipase-derived DAGs, their biophysical properties and current evidence on phospholipase-dependent regulation of TRPC3, to speculate on potential scenarios that may eventually provide answers to some of the above questions.

Introduction

In non-excitable cells calcium (Ca2+) mobilizing agonists acting on G-protein coupled (GPCR) or receptor tyrosine kinase (RTK) membrane receptors typically activate a phosphoinositide-specific phospholipase C (PI-PLC, e.g., PLCβ, PLCγ) that catalyzes the hydrolysis of phosphatidylinositol 4,5-bis-phosphate (PIP2) to generate inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers the release of Ca2+ from endoplasmic reticulum Ca2+ stores which results in a transient increase in cytosolic Ca2+ concentration. In the so called biphasic Ca2+ response, such Ca2+ transient is simultaneously accompanied, or followed by Ca2+ influx through plasma membrane Ca2+-permeable channels which provides a more sustained elevation of cytosolic Ca2+.1 When present, the Ca2+ influx component can take place either immediately or after a short delay following receptor activation; therefore, Ca2+ influx can participate in Ca2+-dependent events that occur very early, late or in a sustained manner throughout the signaling process. This Ca2+ entry can occur through a store-operated Ca2+ (SOC) entry route (SOCE, triggered by depletion of Ca2+ stores) and/or through a non-store-operated Ca2+ (non-SOC) entry path (non-SOCE)2,3 and the channels involved may exhibit more or less selectivity towards Ca2+ depending on their molecular structure and/or the cell type being examined.

Transient Receptor Potential Canonical (TRPC) channels are now recognized amongst the most important Ca2+-permeable cation channels in nearly all non-excitable cells.4-6 Regardless of expression conditions—i.e., heterologous vs. native-TRPC proteins form non-voltage gated, Ca2+-permeable non-selective cation channels that are activated downstream stimulation of PI-PLCs and therefore, now for more than a decade, they have been considered good candidates for mediating SOCE and/or non-SOCE under physiological conditions of receptor stimulation.5 In this context TRPC3, a member of the TRPC3/6/7 subfamily, has maintained a leading role, but its ability to be activated by mere depletion of Ca2+ stores—i.e., to operate as a SOC channel—has been, and still is, a controversial issue (reviewed in refs. 5, 7 and 8). The demonstration that the activity of TRPC3—as well as that of its close relatives TRPC6 and 7—can be enhanced by direct application of synthetic DAG analogues or by increasing endogenous DAG levels with the combined use of DAG lipase and DAG kinase inhibitors,9-11 provided a reasonable explanation as to how those channels could be activated physiologically in a PI-PLC-dependent but store-independent manner. Nevertheless, whereas the role of TRPC3 as a non-SOC channel has been somewhat less questioned than its role as a genuine SOC (reviewed in refs. 5 and 7), TRPC3 activation by DAG is not exempt of mysteries. Indeed, some critical aspects related to phospholipase-dependent, DAG-mediated regulation of TRPC3, have received little attention and several questions remain to be
answered. Do the source and species of endogenous DAG have an impact on TRPC3 function? Can TRPC3, in its physiological environment, be subject to simultaneous modulation by different DAG-generating phospholipases? How does protein kinase C (PKC)-dependent inhibition of TRPC3 fit within a physiological model whereby DAG is the endogenous activator and/or modulator of the channel? In the following sections we take in consideration existing knowledge on the nature of phospholipase-derived DAGs, their biophysical behavior in biological membranes and current evidence on phospholipase-dependent regulation of TRPC3 to speculate on potential scenarios that may eventually provide answers—or at least provoke new thoughts and/or experimental efforts—to some of the above questions. The focus will be on TRPC3 because the vast majority of experimental data on DAG-sensitive TRPCs comes from studies on this member of the TRPC family. However, many of the concepts or speculations made here can eventually apply to its close DAG-sensitive relatives TRPC6 and 7.

**DAG Sources, DAG Species and Regulation of TRPC3**

Whereas there is compelling evidence supporting a role for DAG, particularly that derived from PI-PLC activity, as an endogenous activating signal for TRPC3 channels (reviewed in refs. 7 and 8), it is still debatable whether the action of DAG results from direct interaction with the channel or if ancillary channel proteins endowed with DAG binding properties are responsible for mediating DAG’s activating effect. The latter scenario is supported by the following: (a) DAG-dependent regulation of TRPC7, a close TRPC relative, is lost in excised patches;—this however is not the case for TRPC6; an explanation to this apparent discrepancy was recently discussed in—or by preventing resynthesis of polyphosphoinositides; (b) TRPC3 activity induced by 1-oleyl-2-acetyl-sn-glycerol (OAG) exhibits an obligatory requirement for the tyrosine kinase Src; (c) TRPC3 channels newly inserted into the plasma membrane show constitutive activity but are DAG-insensitive; (d) so far, no canonical DAG binding sites have been identified on the TRPC3 protein-candidate DAG-binding auxiliary proteins have not been found either.

**Synthetic vs. native DAG species in regulation of TRPC3.**

Experimental evidence showing DAG-induced activation of TRPC3 comes mostly from studies involving exogenous application of synthetic membrane permeable analogues of DAG, such as OAG or 1,2-dioctanoyl-sn-glycerol (DOG). Importantly, both the fatty acid composition of this class of DAG analogues and probably their mode of accumulation and/or distribution in the plasma membrane poorly resemble that of endogenous PI-PLC-derived DAG species (vide infra). Moreover, in those instances where activation by endogenous DAG has been presumed—e.g., using inhibitors of DAG metabolism—the ultimate source of DAG responsible for channel activation is either uncertain, or it is assumed that DAG derives from PI-PLC-mediated hydrolysis of PIP$_2$. However, activation of either GPCRs or RTKs results in most instances in a biphasic production of DAG, characterized by a rapid and transient phase, mostly due to PI-PLC activity, and a second sustained phase that is associated to activation of PLD- and/or PLC-mediated hydrolysis of phosphatidylcholine (PC). Activation of PC-PLC represents an alternative direct source of receptor-regulated production of DAG from PC, as PC-PLC catalyzes the hydrolysis of PC—and other phosphatides, e.g., sphingomyelin, phosphatidylethanolamine—to generate phosphocholine and DAG. PLD-dependent generation of DAG from PC however, is a two step process, with PLD-mediated hydrolysis of PC producing phosphatidic acid (PA) which then is converted to DAG by the action of a phosphatidate phosphohydrolase (PAP). Unlike PI-PLC, the role of DAGs derived from the activity of PC-PLD in regulation of TRPC3 has been scarcely explored. It was not until recently that some basic evidence became available suggesting a link between PC-PLD and TRPC3 function in both native and expression systems (vide infra). As for PC-PLC, whereas its activity and regulation by GPCRs and RTKs is well described in mammalian cells, there is no evidence yet on a potential role of this phospholipase in TRPC3 function.

OAG, a synthetic membrane permeable analogue of DAG, is by far the most popular pharmacological tool to induce TRPC3 activity, particularly when TRPC3 is ectopically expressed in heterologous cell systems. This is so despite the poor resemblance of OAG’s fatty acid composition to that of endogenous DAG species generated upon receptor-dependent stimulation of phospholipases. As mentioned above, endogenous DAG species can derive from different sources—e.g., PI-PLC, PC-PLD and PC-PLC—and they differ in their fatty acid profiles (Fig. 1A). DAGs accumulated upon receptor stimulation generally contain: (a) a saturated fatty acid at position sn-1 of glycerol—e.g., stearoyl—and an unsaturated fatty acid in position sn-2—e.g., arachidonoyl or oleyl; or (b) a saturated acyl chain in position sn-1 and a mono- or unsaturated fatty acid in position sn-2. The relative abundance of one or the other fatty acids is dictated by the original phospholipase substrate, i.e., PIP$_2$, or PC, whether PI-PLC or PC-PLD/PC-PLC are involved, respectively. It has been demonstrated that DAGs derived from PI-PLC activation are, for the most part, polyunsaturated, while DAGs generated by PC-PLD are saturated or monounsaturated. Of relevance within the context of TRPC3 regulation, this differential lipid profile of DAGs is known to strongly affect their ability to activate PKC (vide infra). A native-like fatty acid profile resembling PI-PLC-derived DAGs is well preserved in the DAG analogue SAG (1-stearoyl-2-arachidonoyl-sn-glycerol). Nevertheless, the poor membrane permeability of SAG compared to OAG has limited its use in intact cells, where bath application of SAG has shown, if anything at all, much less potency than OAG to activate cation influx and/or currents (reviewed in ref. 22). Of note, the original study by Hoffmann et al. on the activating effect of DAG on TRPC3 and 6 clearly showed a more efficient activation of TRPC6 with SAG than with OAG or 1-stearoyl-2-linoleoyl-sn-glycerol (SLG)—this was not tested on TRPC3—already suggesting the importance of preserving native fatty acid composition. However, the vast majority of studies that came thereafter opted for OAG as the DAG analogue of preference (reviewed in refs. 7 and 8), even if the possibility existed for the investigator...
to add membrane impermeant DAGs by either accessing the cell’s interior or the cytosolic leaflet of the membrane in the whole-cell or excised patch configurations, respectively, of the patch-clamp technique.

Based on the above considerations, it becomes intuitive to think on the potential effect of DAG’s fatty acid composition on TRPC3 function and its modulatory consequences, particularly under physiological situations. How and at which stage of channel activation/regulation do DAG species play a role? Regardless of the action of DAGs on TRPC3 being direct or indirect (vide supra) it is plausible that the fatty acid composition of a particular DAG species may affect: (a) its affinity for the channel protein, should DAG/TRPC3 interactions occur; (b) its affinity for the ancillary DAG-binding protein, should the action of DAG be indirect; (c) the orientation of DAG within the inner membrane leaflet and consequently, its interaction with surrounding lipids, TRPC3 or TRPC3-auxiliary proteins. In fact, the nature of the fatty acids attached to the glycerol backbone of DAG—i.e., saturated vs. unsaturated—is determinant for DAG’s actual membrane miscibility and will determine the resultant spatial structure and accommodation of DAG within the membrane (reviewed in ref. 23). Another aspect to be considered is that exogenously applied DAG analogues are unlikely to accumulate in and/or distribute within the plasma membrane as endogenous DAGs do. Of importance, studies on membrane localization of PI-PLC-derived DAG indicate that this lipid accumulates in the membrane in the form of discrete droplets that localize within the microdomains of the site of its generation,24 a situation that dramatically contrasts with the massive and extensive membrane insertion that may follow exogenous addition of OAG.

### Biophysical properties of DAGs in biological membranes and regulation of TRPC3

An interesting property of DAGs, at least in synthetic lipid bilayers is that as their concentration increases, they induce lateral phase separation and, subsequently, an alteration in membrane curvature. This is believed to be due to the immiscibility of DAG with phospholipids, which favors the occurrence of hydrophobic interactions between DAG and intrinsic membrane proteins. This property of DAGs explains the fact that when they are incorporated in artificial phospholipid bilayers their presence promotes transitions from lamellar to non-lamellar phases, such as inverted hexagonal and inverted cubic phases.23 In any case, the practical consequence of an increase in DAG concentration in a biological membrane is that the cytosolic membrane leaflet will “tend” to transition from a lamellar to a non-lamellar state; whereas

---

**Figure 1.** (A) DAG can be generated by receptor dependent stimulation of PI-PLCs (e.g., PLCβ, PLCδ), PC-PLC or PC-PLD. PIP₂-derived DAG (DAG₁) mostly contains a saturated fatty acid at position sn-1 of glycerol and an unsaturated fatty acid in position sn-2. Instead, PC-derived DAG (DAG₂) in general presents with a saturated fatty acid at sn-1 and a mono- or unsaturated fatty acid at sn-2. Designation as DAG₁ or DAG₂ is arbitrary to emphasize the fact that they represent different DAG species. For comparison, the acyl chain composition of most commonly used DAG analogues (SAG, DOG, OAG; see text for details) is shown. PC: phosphatidylcholine; PI: phosphoinositide; PIP₂: phosphatidylinositol 4,5-bis-phosphate; PA: phosphatidic acid; PAP: phosphatidate phosphohydrolase; p-choline: phosphocholine. (B) Receptors (R) coupling to both PI-PLC and PC-PLD (or PC-PLC) can generate two different DAG species in terms of their fatty acid profiles (DAG₁ and DAG₂). DAGs can act directly on TRPC3 (T₃) or indirectly through a DAG binding ancillary protein (DBAP). The cartoon at the bottom provides an idealized model of the potential temporal interplay between DAG species, PKC activity and TRPC3 function (see text for details): T₃ function (stage 1 in model) is increased by receptor-generated DAGs (stages 2 and 3 in model) with DAG₁ and DAG₂ accounting, respectively, for rapid and sustained channel activation. DAG₁ species are good activators of PKC, but their cellular levels decrease rapidly due to the action of DAG metabolizing systems and desensitization of the PI-PLC pathway; while DAG₂ species are not potent activators of PKC, as DAG₂ levels increase this may compensate for their lower affinity towards PKC and thus support kinase activity, which downregulates channel function (stage 4 in model). PKC activity and the decrease of DAG levels eventually lead to the return of TRPC3 function to basal levels (stage 5 in model).
such change does not actually take place in a cell’s membrane, the direct consequence of the “transitional propensity” is a change in what is referred to as “membrane curvature packing stress”. DAGs derived from either PI-PLC, PC-PLC or PC-PLD activity have indeed been shown to affect curvature packing stress of native membranes, altering the activity of glycosyltransferases, vesicle fusion at synapses and the binding of ArfGAP1 to membranes in HeLa cells. In the case of PC-PLD, the immediate products of PC hydrolysis are PA and phosphocholine. PA exhibits a conical shape that fits well in membranes exhibiting negative curvature, such as hexagonal H2 phase. Further conversion of PA to DAG by the action of PAP increases both the hydrophobicity and stability of the membrane surface particularly in regions where membrane curvature is already negative. Instead, DAG derived from PIP2 fits better in planar phospholipid bilayers, due to higher degree of unsaturation in the fatty acids. On a theoretical basis, TRPC3 protein, or the ancillary channel proteins that bind DAG, may exhibit more or less preference for one or the other DAG species, or the efficiency of the DAG recognizing site to interact with DAG—whether on TRPC3 or on the DAG binding protein—may vary depending upon the extent of hydrophobicity imposed to the membrane by the newly formed DAG.

A model intended to explain how changes in membrane curvature may affect ion channel function through membrane stretching has been recently discussed (reviewed in ref. 30). Briefly, this model postulates that if the thickness of the transmembrane portion of the channel were to increase when the channel opens, then the phospholipid bilayer would have to stretch to prevent exposure of hydrophobic areas of the channel protein to water or to the polar head groups of the membrane phospholipids. Because such a stretching requires curving of the membrane immediately surrounding the channel, lipids endowed with a conical shape, such as PA or DAGs, would favor membrane curvature. In a study aimed at determining the effects of mechanical stretching on the function of TRPC6 channels, Spassova et al. proposed a model in which PI-PLC-derived DAG, generated in the inner leaflet of the membrane, would increase membrane curvature as a major determinant of the physical stretch leading to channel activation. The conclusion was also made that OAG, because of its membrane permeability, would reach the inner bilayer leaflet and affect membrane curvature in a DAG-like manner. Whereas is possible that endogenously generated DAG impacts membrane curvature in the channel’s vicinity, it is highly unlikely that OAG, which massively enters the cell and partitions throughout all cellular membranes (vide supra), will end up selectively localized in a delimited area of the inner membrane leaflet. Notably, the idea that DAGs may exert a physiological role through their ability to modify membrane curvature was proposed by Michell more than thirty years ago. Whereas the findings by Spassova et al. are in line with operation, at least for TRPC6, of a membrane stretching model, further studies are required to determine if this is a general phenomenon or a feature of certain TRPCs. Nevertheless, operation of a membrane stretching mechanism could be of particular relevance for channels endowed with a lipid annulus. The lipid annulus—annular or boundary lipids—is present in many ion channels and transporters such as the nicotinic acetylcholine receptor, the potassium channel KcsA from Streptomyces lividans, the Ca2+/Mg2+-ATPase or the mechanosensitive channel of large conductance (MscL) from Myobacterium tuberculosis, among others, and is critical for proper function. It can be defined as a lipid shell surrounding, to different extents, the transmembrane segment of the channel protein, and is immobilized relative to the bulk membrane phospholipids. The lipid annulus is present in a liquid-ordered (Lo) phase with a higher degree of order than the bulk lipid in the membrane, and plays a critical role in modulating channel gating as well as the interaction of the channel protein with bulk lipids, ligands and pharmacological agents (reviewed in refs. 30, 37 and 38). It remains to be explored if a lipid annulus exists for TRPC3 channels when embedded in biological membranes, and if so, how DAGs, either from outside or inside the annular lipid, may influence channel function.

Interestingly, OAG-induced lateral phase separation of phospholipids is facilitated by the presence of Ca2+ in the surrounding medium. This could explain some disparate effects of OAG on TRPC3 in the presence or absence of extracellular Ca2+. Lintschinger et al. observed that when Ca2+ was present in the bath at physiological concentrations (e.g., 2 mM), OAG-induced currents in HEK293 cells stably overexpressing human TRPC3 were smaller than when nominally Ca2+-free medium was used, and this behavior was different whether TRPC3 was alone or forming heteromultimers with TRPC1. It is possible that when Ca2+ is present OAG-dependent disturbance of phospholipids surrounding the channel occurs, which in turn interferes with channel gating. The interpretation provided by Lintschinger et al. still applies, in that TRPC3 homomultimers vs. TRPC3/TRPC1 heteromultimers likely exhibit differential sensitivity towards OAG-induced disturbances of the phospholipid microenvironment. Functional TRPC3 channels are thought to be formed by either homo- or hetero-tetrameric arrangements of four TRPC proteins by means of interactions between specific domains in the N-terminus, the pore region and C-terminal domain. If we consider an idealized combinatorial arrangement of all possible tetramers made of DAG sensitive TRPCs—i.e., TRPC3, 6 and 7.twenty one different oligomers are theoretically possible—assuming that all combinations are feasible and that all three members co-exist within the same cell at a particular time and in stoichiometrically sufficient proportions. Based on this, it is reasonable to consider that different tetrameric arrangements may alter the DAG affinity of individual TRPC subunits, or alternatively, their ability to interact more or less efficiently with ancillary DAG-binding proteins.

Role of DAG in the negative regulation of TRPC3 by PKC.
As mentioned above, endogenously generated DAGs are believed to accumulate within short distances of the site of production, within the boundaries of the inner leaflet of the plasma membrane. This is a situation that contrasts dramatically with the massive and extensive membrane insertion that likely follows exogenous addition of membrane permeable DAG analogues. And it could in part explain a puzzling observation related to DAG-dependent regulation of TRPC3, which is the fact that in most cells known to endogenously express TRPC3—the same applies for most cells expressing TRPC6 and/or 7 proteins-, native
Channels are unresponsive to DAG analogues in that no cation influx and/or currents are detected upon application of OAG—some exceptions exist, see for instance. This has been logically interpreted as a manifestation of the well known sensitivity of TRPC3/6/7 proteins to PKC-dependent inhibition. Whereas TRPC3 activation by DAG analogues is independent of PKC (reviewed in refs. 7 and 8), massive PKC activation by OAG—or other DAG analogues—will mask the ability of OAG to activate native channels, as channel inhibition will prevail over stimulation. That native TRPC3—and TRPC7—channels are however able to respond to exogenously applied OAG has been shown by measuring OAG-activated cation influx and currents under conditions of PKC inhibition. However, if phospholipase-derived DAG is responsible for activation of native channels, those findings and the above interpretation do not explain how concomitant channel inhibition by PKC is prevented or defeated under physiological conditions. One possibility is that the fatty acid composition of native DAG species may allow for a fine spatiotemporal control of channel vs. kinase activation (see model in Fig. 1B). It is reasonable to consider that different DAG species might exhibit differential affinity towards the TRPC3 protein, the hypothetical ancillary DAG binding protein or the DAG-sensitive PKCs that mediate channel inhibition. It is also possible that a significant degree of compartmentalization of the signaling route exists, so that DAGs act on the immediate channel’s vicinity but remain inaccessible to PKC. For example, if a lipid annulus exists for TRPC3 and DAG is generated within the boundaries of the annulus, the intra-annular DAG would be unlikely to be available for PKC activation (Fig. 2). Moreover, as DAGs accumulate in the channel’s lipid microenvironment, an activating threshold is reached and further DAG accumulation may result in PKC activation (C; see text for details). DAGs can act directly on TRPC3 or indirectly through a DAG binding ancillary protein (DBAP).

**Figure 2.** (A) Signaling compartmentalization (black bordered box, expanded in B and C) and/or the existence of a lipid annulus (symbolized by darker phospholipids) may explain the paradoxical regulation of TRPC3 by DAGs. DAGs can be generated by receptor dependent stimulation of PI-PLC or PC-PLD (see text and Fig. 1). PI-PLC-derived DAGs (DAG1), of a more transient nature than PC-derived DAGs (DAG2), may account for initial channel activation, while DAG2 can support more sustained channel activity (B). When generated within the immediate channel’s vicinity DAGs remain inaccessible to PKC. As DAGs accumulate in the channel’s lipid microenvironment, an activating threshold is reached and further DAG accumulation may result in PKC activation (C; see text for details). DAGs can act directly on TRPC3 or indirectly through a DAG binding ancillary protein (DBAP).
above of signaling compartmentalization and TRPC3 forming part of signaling microdomains, it is possible that acute channel activation occurs subsequently to DAGs generated by phospholipases in close proximity to the channel microenvironment—with DAG metabolizing systems accounting for rapid DAG conversion and thus minimal PKC activation—while distal DAG-generating phospholipases account for the more sustained phase of channel function and eventually, PKC activation.

DAG species, PKC function and negative regulation of TRPC3. Notably, whereas in vitro data shows that essentially all DAG species are able to activate DAG-sensitive PKCs, it is not clear if that is the case in vivo. In fact, it seems that in intact cells PI-PLC-generated DAG species are more efficient in terms of PKC activation than PC-PLD-derived DAGs, a phenomenon likely related to the nature of the acyl chains in the glycerol backbone. For instance, DAGs containing polyunsaturated fatty acids in sn-2 position, like those derived from PI-PLC-mediated hydrolysis of PIP_2, are more efficient in activating PKCs than DAGs containing saturated or monounsaturated fatty acids—derived from PC hydrolysis. This is well exemplified by the observation that 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) and 1-stearoyl-2-docosahexaenoyl-sn-glycerol (SDG) both exhibit similar potencies towards PKC activation, while the effects of 1-stearoyl-2-eicosapentaenoyl-sn-glycerol (SEG) are significantly lower. Therefore, whenever PC-PLD-derived DAGs were to activate TRPC3, it is plausible to consider that the inefficiency of those DAG species to support PKC activity would allow for a more sustained activation of the channel, as PKC-mediated inhibition will be minimal or inexistent. However, whenever robust and/or sustained PC-PLD activation occurs, this may give rise to concentrations of DAG in the channel’s microdomain high enough to activate PKC, as the higher DAG level would compensate for the lower affinity of PKC for that particular DAG species (see model in Fig. 1B).

There is no question on the inhibitory action of PKC on TRPC3 activity, and a PKC phosphorylation site responsible for such effect has been unequivocally identified. However, the identity of the PKC isoform/s involved remains unknown. So far, available functional evidence linking PKC with TRPC3 activity is for the most part pharmacological. Despite the limited isoform selectivity of the PKC inhibitors traditionally used in those studies, their spectrum of action suggests that the isoform/s involved are likely to belong to the Ca^{2+}- and DAG-dependent category. If this is so, it is then possible that rapid DAG-dependent activation of the channel, a membrane delimiting step, will drive the Ca^{2+} influx that, in concert with rising DAG levels, will provide the appropriate environment for PKC recruitment and activation. This scenario would be compatible with a temporal sequence of events where DAG-dependent activation precedes DAG-mediated stimulation of PKC and subsequent negative feedback regulation of the channel.

Pi-PLC and PC-PLD in DAG-Dependent Regulation of TRPC3: Mutually Exclusive, Complementary or Alternating?

Activation of PI-PLC by membrane receptors is subject to rapid desensitization, and DAG generated through PIP_2 hydrolysis accumulates in a rapid but transient manner, declining to pre-stimulation levels in a matter of seconds to few minutes (reviewed in refs. 21 and 53). As mentioned above, receptor-dependent generation of DAG is biphasic, with the second, more delayed phase of DAG production occurring right about when the concentration of PI-PLC-dependent DAG begins to subside. This second phase, which accounts for sustained DAG levels in the cell, is for the most part associated to activation of PLD-mediated hydrolysis of PC. The scenario for phospholipase-dependent regulation of TRPC3 gets puzzling when we consider receptors exhibiting dual coupling in terms of those two DAG-generating phospholipases—i.e., PI-PLC and PC-PLD coupled receptors. Considering the above mentioned biphasic nature of phospholipase-mediated generation of DAG in biological membranes, one would intuitively think that PIP_2-derived DAG would be responsible for early channel activation and as stimulation persists, PC-derived DAG may take over to compensate for the desensitization of the PI-PLC pathway (Fig. 2B). Based on the above considerations on fatty acid composition of PI-PLC vs. PC-PLD derived DAGs, such a model implies two main assumptions: (a) the channel—or the ancillary DAG binding protein—should be able to interact equally well with DAGs regardless of their origin; or (b) channel activation and regulation are differentially managed by different DAG species—interestingly, TRPC3-mediated Ca^{2+} influx and whole-cell currents in general exhibit a somewhat biphasic time course if remains to be determined whether this is related to intrinsic channel gating properties or the resultant of bimodal regulation by different DAG species.

Some available evidence shows TRPC3’s ability to concomitantly couple to signaling from different phospholipases—at least from within the same class. For instance, in HEK293 cells stably expressing TRPC3, stimulation of the epidermal growth factor receptor (EGFR) results in robust TRPC3-mediated Ca^{2+} influx, clearly indicating that DAG derived from PLC—i.e., the PLC recruited and activated upon EGFR activation—can activate TRPC3. In the same expression system, TRPC3 is also efficiently activated by stimulation of GPCRs such as the muscarinic receptor type-5, which couples to PI-PLC. Similarly, TRPC3 ectopically expressed in avian B lymphocytes is robustly activated by antibody-induced crosslinking of surface IgM, which triggers a series of tyrosine phosphorylation events that drive recruitment and activation of PLCγ. This is not surprising, as PLCβ and PLCγ both use PIP_2 as a substrate and thus, DAG species derived from their activities are expected to be similar, if not identical, in regards to fatty acid composition and biological properties. Interestingly, IgM-dependent activation of TRPC3 is lost in a PLCγ-deficient variant of DT40 cells whereas stimulation of the M5 muscarinic receptor, which in those cells also couples to PI-PLCβ, is preserved and results in efficient channel activation. In HEK293 cells EGFR also couples to PC-PLD and therefore it is plausible that DAG generated from EGF stimulation is contributed by both PI-PLCγ and PC-PLD—this may actually be the case for several of the receptors known to activate TRPC3. In that situation, it remains to be determined if EGFR-dependent activation of TRPC3 is the result of the action of PI-PLCγ or PC-PLD derived DAG, a concerted effect of both
DAGs, or if, as discussed above, they act sequentially during early and sustained activation of the channel.

The majority of studies examining regulation of TRPC3 by store-dependent or store-independent mechanisms give little consideration to the possibility that those may actually be alternative and/or concomitant routes of channel regulation. In fact, TRPC3 can function either as SOC or as non-SOC channel, with existing variations and discrepancies mostly due to the characteristics of the expression system and/or channel expression level, among others (reviewed in refs. 5 and 60–63). But is likely that the scenario under physiological conditions implies a combination of both influx routes, with a particular receptor triggering the store-operated, store-independent or both mechanisms of channel regulation depending on the coupling phospholipase and/or the sequential stimulation of different phospholipases, whenever dual coupling exists—which in turn, may be cell type dependent or expression level dependent, among other variables. In the case of receptors coupling to PC-PLD rather than PI-PLC, activation of the phospholipase will result in an increase in the local DAG concentration but no IP₃ will be produced; consequently, the system is not expected to promote store depletion and DAG will be the dominating signal. However, in mammalian cells many receptors that promote PC-PLD activity also couple to PI-PLC, giving rise to two potential scenarios: either both PI-PLC and PC-PLD participate in regulation of TRPC3, or only one of them contributes to the process. Recent experimental findings suggest that both scenarios are possible.

Regulation of TRPC3 by PI-PLC and PC-PLD. This situation is illustrated by the recent findings by Kwan et al. Working with HEK293 cells transiently overexpressing TRPC3, these authors showed that histamine promoted Ca²⁺ influx through a non-capacitative pathway subsequent to activation of H₂ type histamine receptors. H₂ histamine receptors couple to both Gᵣ and Gᵣ proteins, and therefore downstream signaling events can be associated to the adenyl cyclase/cyclicAMP/PKA route, the PI-PLC/ DAG/IP₃/ Ca²⁺ path, or a combination of both. The results showed that histamine-dependent activation of TRPC3 did not involve the cyclic AMP/PKA pathway, but rather was subsequent to activation of both PI-PLC and PC-PLD. The PI-PLC inhibitor U73122 or the PC-PLD inhibitor 1-butanol, when used separately, caused a partial reduction of the non-capacitative TRPC3-mediated Ca²⁺ influx, whereas simultaneous inhibition of both phospholipases resulted in complete suppression of channel function. Although the conclusions from this work were based solely on pharmacological inhibition of phospholipase activity, the findings were indicative of a potential involvement of PC-PLD in receptor-activated TRPC3 in HEK293 cells. All experiments in the study by Kwan et al. were conducted in cells in which Ca²⁺ stores were depleted by thapsigargin-mediated inhibition of sarcoplasmic reticulum Ca²⁺ pumps (SERCa), a maneuver that may mask potential effects of SERCA inhibition and/or store depletion on the activity and regulation of membrane phospholipases and/or the channel itself—see for instance. Therefore, it remains to be determined the extent of contribution of PI-PLC and PC-PLD to receptor- and OAG-stimulated TRPC3 function under normal intracellular buffering conditions. Of note, TRPC6 was not affected by inhibition of PC-PLD, suggesting that the role of PC-derived DAG might be specific for TRPC3. If the latter is true, it implies either that highly selective structural recognition site/s exist on the TRPC3 protein—should DAG’s effect on TRPC3 be direct—or the hypothetical DAG-binding ancillary proteins are not only specific for PC-derived DAG but also selectively interact with TRPC3 and not TRPC6.

As mentioned above, DAG-dependent regulation of TRPC7 is lost in excised patches or in cells in which resynthesis of polyphosphoinositides is blocked. Interestingly, all members of the TRPC3/6/7 group have been shown to be sensitive, to different extents, to activation by PIP₂ in excised patches. It is well established that PIP₂ is a critical cofactor for PLD, to the extent that it can profoundly affect the activity, trafficking and receptor-dependent activation of the enzyme (reviewed in ref. 66). Therefore, a decrease in cellular PIP₃ levels—via PI-PLC dependent hydrolysis of PIP₃, PIP₃ scavenging or phosphatase mediated degradation of PIP₃, as it may occur in excised patches—can inhibit PLD activity. It remains to be explored whether PIP₃-dependent activation of TRPC3/6/7 channels is the result of direct channel activation by the lipid, or if it reflects restoration and/or activation of PLD activity that may potentially be required for channel function. The latter is somewhat supported by the observation that addition of PIP₃ to patch pipettes in the whole-cell configuration did not affect pre-existing TRPC7 currents. This was indeed interpreted by Lémonnier et al. as indicative of PIP₃ acting as a co-factor, as sufficient PIP₃ will be present in intact cells to preclude an additional effect from exogenously added lipid to be detected.

Regulation of TRPC3 by PC-PLD. In a recent work aimed at examining the effects of phospholipase inhibitors on excitatory postsynaptic currents (EPSC) induced by metabotropic glutamate receptor type 1 (mGluR1) in rat Purkinje cells, Glitsch showed that glutamate-dependent activation of these currents involved PC-PLD-derived DAG without participation of PI-PLC. When cells were pre-treated with the PI-PLC inhibitor U73122, but not the less active analogue U73343, EPSC currents were not affected. In contrast, incubation with the PC-PLD inhibitor 1-butanol, but not the inactive isomer 2-butanol, completely abrogated EPSC currents. Because in HEK293 cells stably overexpressing human TRPC3 1-butanol did not affect OAG-stimulated Ca²⁺ influx, the conclusion was made that the effects of the alcohol were not the result of direct channel inhibition. But this was not directly tested in Purkinje cells—i.e., the native channel environment—and thus the possibility remains that channel expression level and/or the stoichiometric arrangement of the native channel may exhibit a somewhat different sensitivity to pharmacological manipulations as compared to overexpression conditions (reviewed in ref. 62). Despite this, perhaps the most intriguing observation was that simultaneous inhibition of PI-PLC and PC-PLD did not result in any further reduction in mGluR1-dependent EPSC compared to PC-PLD inhibition alone, suggesting that in Purkinje cells mGluR1-induced activation of TRPC3—i.e., regulated channel function—was entirely dependent upon PC-PLD activity, despite the fact that mGluR1 also couples to PI-PLC in those cells. These findings in Purkinje...
cells somehow exemplify the notion discussed above that TRPC3 either exhibits a high degree of selectivity towards PC-PLD-derived DAG species compared to those from PI-PLC, or a tight association and/or compartmentalization exists between the channel and PC-PLD, so that only locally produced PC-derived DAG within the immediate channel’s vicinity is able to reach the activating threshold for DAG. 

TRPC3 Constitutive, Non-Regulated Function and Phospholipase Activity

The first, and as of this writing the only attempt to address the potential role of phospholipase-associated signaling on TRPC3 constitutive function derives from studies by Albert and Large. These authors described the existence of constitutive TRPC3-like non-selective cation currents (I_{cat}) in rabbit ear artery myocytes and showed that constitutive activity of these currents were likely to be supported by PC-PLD-derived DAG, but not DAG from PI-PLC. In the initial description of I_{cat} a dual effect of OAG on these currents was noticed, i.e., stimulation followed by inhibition. OAG-dependent stimulation of I_{cat} was PKC-independent, whereas OAG-induced inhibition of the current required PKC activity. Under native conditions PKC activation was mediated by PI-PLC-derived DAG. Importantly, PKC-mediated inhibition was found to be tonic, i.e., already operating under constitutive conditions. The fact that I_{cat} was endowed with high constitutive activity that was spontaneously detectable—without the need of PKC inhibition—implied the existence of an additional activating signal to overcome the tonic inhibition by PKC. Moreover, because PKC activity was dependent upon PI-PLC-derived DAG, the channel activating signal should be generated through a pathway other than G_{i}/PI-PLC. Indeed, pharmacological inhibition of PI-PLC enhanced the already constitutively active I_{cat}, in line with the notion of a non-PI-PLC derived signal responsible for supporting I_{cat} constitutive activity. Albert and Large tested the hypothesis that DAG derived from an alternative source was responsible for I_{cat} constitutive activity by a combination of pharmacological and biochemical approaches. First, the PC-PLD inhibitors 1-butanol—but not 2-butanol—and C_{12}-ceramide significantly reduced I_{cat}, whereas D-609 and AACOF3—PC-PLC and PLA_{2} inhibitors, respectively—were devoid of effect on the current. At the single channel level the activity of I_{cat} was strongly augmented by addition of purified PC-PLD to excised membrane patches. Finally, that constitutive currents were supported by PC-PLD-derived DAG and not PA, was demonstrated by the inhibition of I_{cat} by DL-propranolol, an inhibitor of PAP, confirming that I_{cat} activity required conversion of PA to DAG. It remains to be explored if tonic production of DAG through PC-PLD activity also accounts for TRPC3 constitutive activity in other cell types and expression systems.

Concluding Remarks

As the field of TRPC research grows at a fast pace towards understanding their role in physiology and disease, particularly in relation of Ca^{2+}/Na^{+}-influx dependent events located downstream channel activation, our knowledge on the paths leading to activation/regulation in native systems remains poor and still subject to conservative experimental perspectives. Biophysical approaches combined to existing molecular/structural information on the TRPC3 protein are likely to provide a more refined information on the influence of DAG species—as well as other potential modulatory lipids—on TRPC3 activation and regulation in its native environment. Such information is critical not only to improve our understanding of TRPC3 regulation in vivo but also to better focus existing efforts aimed at designing specific pharmacological modulators of TRPC3 function with potential therapeutical use in human disease.

Acknowledgements

Work at Dr. Guillermo Vazquez’s lab is supported by University of Toledo College of Medicine and American Heart Association (SDG0635250N to Guillermo Vazquez).

References

1. Bird GSAO, Lievremont JP, Wedel BJ, Trebak M, Vazquez G, Putney JW Jr. Mechanisms of phospholipase C-regulated calcium entry. Curr Mol Med 2004; 4:291-301.
2. Tran QK, Watanabe H. Calcium signalling in the endothelium. Handbook of Experimental Pharmacology 2006; 176:145-87.
3. Nilius B, Droogmans G. Ion channels and their functional role in vascular endothelium. Physiol Rev 2001; 81:1415-59.
4. Birnbaumer L. The TRPC Class of ion channels: A critical review of their roles in slow, sustained increases in intracellular Ca^{2+} concentrations*. Ann Rev Pharmacol Toxicol 2005; 49:395-426.
5. Vazquez G, Wedel BJ, Aziz O, Trebak M, Putney J, James W. The mammalian TRPC cation channel: Biochemical and Biophysical Acta (BBA)—Molecular Cell Research 2004; 1742:21-36.
6. Ramsey IS, Delling M, Clapham DE. An introduction to TRP channels. Annual Review of Physiology 2006; 68:619-47.
7. Trebak M, Vazquez G, Bird GS, Putney JW Jr. The TRPC3/6/7 subfamily of cation channels. Cell Calcium 2003; 33:451-61.
8. Dietrich A, Kalwa H, Rost BR, Guder mann T. The diacylglycerol-sensitive TRPC3/6/7 subfamily of cation channels: functional characterization and physiological relevance. Pillagors Archiv European Journal of Physiology 2005; 451:72-80.
9. Okada T, Inoue R, Yamazaki K, Maeda A, Kurosaki T, Yamakuni T, et al. Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. Ca^{2+}-permeable cation channel that is constitutively activated and enhanced by stimulation of g protein-coupled receptor. J Biol Chem 1999; 274:7359-70.
10. Trebak M, St. JBG, McKay RR, Birnbaumer L, Putney JW Jr. Signaling mechanism for receptor-activated canonical transient receptor potential 3 (TRPC3) channels. J Biol Chem 2003; 278:16244-52.
11. Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, Schultz G. Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature 1999; 397:259-63.
12. Lemmonier L, Trebak M, Purney JW Jr. Complex regulation of the TRPC3, 6 and 7 channel subfamily by diacylglycerol and phosphatidylinositol-4,5-bisphosphate. Cell Calcium 2008; 43:506-14.

13. Hirsutun C, Kudota Y, Nakamura K, Inoue T, Nakamura T, Maizama T, et al. Regulation of TRPC6 channel activity by tyrosine phosphorylation. J Biol Chem 2004; 279:18887-94.

14. Vazquez G, Wedel BJ, Kawasaki BT, Bird GS, Purney JW Jr. Obligatory role of Src kinase in the signaling mechanism of TRPC3 cation channels. J Biol Chem 2004; 279:40521-31.

15. Smyth JT, Lemmonier L, Vazquez G, Bird GS, Purney JW Jr. Dissociation of regulated trafficking of TRPC6 channels to the plasma membrane from their activation by phospholipase C. J Biol Chem 2006; 281:427-37.

16. Vorland M, Thoresen VAT, Holmsen H. Phospholipase D in platelets and other cells. Platelets 2008; 19:582-94.

17. Hodgkin MN, Pettit TR, Martin A, Michell RH, Pemberton AJ, Wakelam MJ. Diacylglycerols and phosphatidate: which molecular species are intracellular messengers? Trends Biochem Sci 1998; 23:200-4.

18. Eton JH. Phosphatidylincholine breakdown and signal transduction. Biochimica et Biophysica Acta (BBA)—Lipids and Lipid Metabolism 1994; 1212:26-42.

19. Hofmann T. Regulation of phospholipase D. FEBS Letts 2002; 531:58-61.

20. Szun AM, Ralhden SA. Biological role of phosphatidylincholine-specific phospholipase C in mammalian cells. Postepy Hig Med Dosw (Online) 2008; 62:593.

21. Pettit TR, Martin A, Horton T, Liassi C, Lord JM, Wakelam MJ. Diacylglycerol and Phosphatidate Generated by Phospholipase G3 and D, Respectively. Have Distinct Fatty Acid Compositions and Functions. J Biol Chem 1997; 272:17354-9.

22. Gamberucci A, Giurisato E, Pizzo P, Tassi M, Giunti F, et al. Diacylglycerol activates the influx of extracellular cations in T-lymphocytes independently of intracellular calcium-store depletion and possibly involving endogenous TRP6 gene products. Biochem J 2002; 364:245-4.

23. Goni FM, Alonso A. Structure and functional properties of diacylglycerol in membranes. Progress in Lipid Research 1999; 38:1-48.

24. Coleman R, Finean JB, Knutton S, Limbrick AR. A role for phospholipase D in platelets and other cells. Platelets 2008; 19:582-94.

25. Edman M, Berg S, Storm B, Wikstrom M, Vikstrom S, Ohman A, et al. Structural features of glycosyltransferases synthesizing major bilayer and nonbilayer-er membrane lipids in Archeobacteria paludissum and Streptococcus pneumoniae. J Biol Chem 2003; 278:8420-8.

26. Villar AV, Gonzalez E, Eucelitza M, et al. Diacylglycerol effects on phosphatidylinositol-specific phospholipase C activity and vesicle fusion. FEBS Letters 2001; 494:81-87.

27. Pariss A, Rawler M, Reges L, Barkan B, Romman M, Gainer M, et al. Golgi localization determinants in ArfGAP1 and in new tissue-specific ArfGAP1 isoforms. J Biol Chem 2006; 281:3785-92.

28. Rosanna C, Anne NS, Michelle QF, William EH. Phospholipid signalling through phospholipase D and phosphatidic acid. JUBMB Life 2006; 58:457-61.

29. Shulga YY, Myers DS, Ivanova PT, Milne SB, Brown HA, Topham MK, et al. Molecular species of phosphatidylinositol-cycloadd-mediates in the endoplasmic reticulum and plasma membrane. Biochem Soc 2009; 33:127-20.

30. Lee A. The effects of lipids on channel function. Journal of Biology 2009; 8:86.

31. Spassova MA, Hewawitharana T, Xu W, Soboleff J, Gill DL. A common mechanism underlies stretch activation and receptor activation of TRPC6 channels. Proc Natl Acad Sci USA 2006; 103:1686-91.