



Disruption of palmitate-mediated localization; a shared pathway of force and anesthetic activation of TREK-1 channels[☆]

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ABSTRACT

TWIK related K⁺ channel (TREK-1) is a mechano- and anesthetic sensitive channel that when activated attenuates pain and causes anesthesia. Recently the enzyme phospholipase D2 (PLD2) was shown to bind to the channel and generate a local high concentration of phosphatidic acid (PA), an anionic signaling lipid that gates TREK-1. In a biological membrane, the cell harnesses lipid heterogeneity (lipid compartments) to control gating of TREK-1 using palmitate-mediated localization of PLD2. Here we discuss the ability of mechanical force and anesthetics to disrupt palmitate-mediated localization of PLD2 giving rise to TREK-1's mechano- and anesthetic-sensitive properties. The likely consequences of this indirect lipid-based mechanism of activation are discussed in terms of a putative model for excitatory and inhibitory mechano-effectors and anesthetic sensitive ion channels in a biological context. Lastly, we discuss the ability of locally generated PA to reach mM concentrations near TREK-1 and the biophysics of localized signaling. Palmitate-mediated localization of PLD2 emerges as a central control mechanism of TREK-1 responding to mechanical force and anesthetic action. This article is part of a Special Issue entitled: Molecular biophysics of membranes and membrane proteins.

1. Introduction

TWIK related K⁺ channel (TREK-1) is a mechano- and anesthetic-sensitive member of the two-pore-domain potassium (K2P) family of channels [1]. Inhaled anesthetics (e.g. isoflurane, chloroform, diethyl ether) activate TREK-1 and genetic deletion of TREK-1 decreases anesthesia sensitivity in mice [2–4]. Mechanical force also activates TREK-1 [5–9], making TREK-1 an inhibitory mechanosensitive channel (i.e. attenuates mechanosensation) and a permissive anesthetic or analgesic channel (i.e. facilitates anesthesia).

Initial theories for TREK-1 activation by anesthetics and force assumed distinct direct interactions [10–12]. This assumption was based on the widely accepted theory that anesthetics bind directly to the transmembrane domains of channels [13–15], and it is consistent with computational modeling of anesthetics [3,16] to TREK-1. Additionally, the ‘force from lipid’ [17] mechanism (which requires changes in the membrane to directly lead to channel gating) was proposed to primarily regulate force in cellular membranes based on force induced opening of TREK-1 in purified lipid vesicles [10,11].

Recently indirect (non-canonical) mechanisms for force transduction have emerged based on disruption of protein-protein and protein-

lipid interaction [18–20]. For example cells sense disassembly of caveolae and disruption of G-protein interactions [19,20]. Another mechanism relies on palmitate mediated sequestration of the enzyme phospholipase D2 (PLD2) with monosialotetrahexosylganglioside1 (GM1) lipids (sometimes called lipid rafts or lipid domains) away from its substrate [18,21]. Disruption of lipids by mechanical force disrupts PLD2 localization and leads to activation. This PLD2-based mechanosensation was found to be the tension sensor for endocytosis [22] and appears to function upstream of force transduction through mTOR signaling [23]. PLD2 is also important for TREK-1 since PLD2 activates the channel by local production of the signaling lipid phosphatidic acid (PA) [24]. And similar to endocytosis and mTOR, this has led to a two-step mechanism for TREK-1 mechanosensation in a biological membrane [25].

Anesthetics also disrupt ordered membranes [26–29] and shift their melting temperature [30–33]. Recently we showed inhaled anesthetics activate PLD2 by disruption of palmitate-mediated localization and substrate presentation giving rise to anesthetic sensitivity in TREK-1 [28] (preprint).

This review discusses the evidence for palmitate-based clustering and its similarities and differences between mechanical force and

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anesthetics in TREK-1 activation. We contrast the induced de-clustering of PLD2 and its causative effect on TREK-1 channel activation through lipid binding and competition [34,35]. The molecular underpinnings of the two-step membrane mechanism discussed here for TREK-1 is conceptually very distinct from canonical models of ion channel activation. Much work needs to be done to fully appreciate its extent in other biological systems. The review starts by summarizing recent advancements in lipid domains and their organization of select proteins. Raft disruption and palmitate-mediated localization is then discussed as a regulator of PLD2 and the central pathway for membrane-mediated activation of TREK-1 by anesthetics and mechanical force. Challenges for establishing localization-based mechanisms conclude the review. Details on TREK-1 specific cellular responses to anesthetics and mechanical force are only briefly discussed as they have been reviewed extensively elsewhere; see [2,36,37] for details on canonical TREK-1 anesthesia and [38] for canonical mechanosensation.

2. Lipid domains and cholesterol-induced palmitoyl de-clustering

The membrane is a heterogeneous mixture of fluid and gel-like lipids densely packed with proteins [39,40]. Proteins, including cytoskeletal proteins, compartmentalize the membrane and restrict lateral diffusion [41–44]. Membrane regions enriched in a particular type of lipid are called lipid domains, clusters, or rafts and they are also thought to reduce lateral movement [40,43]. The best characterized domains contain sphingomyelin (GM1) and are functionally modulated by cholesterol [21,45].

Cells contain a second class of lipid heterogeneity comprised of the anionic lipids phosphatidyl inositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 3,4,5 triphosphate (PIP₃). Early studies in detergent resistant membranes (DRMs) found PIP₂ associated with GM1 lipids [46–48]. However, mass spectrometry showed PIP₂ is mostly unsaturated [49–51] which is inconsistent with GM1 localization [52]. And recent development of super resolution technology [21] (e.g., direct stochastic optical reconstruction microscopy (dSTORM) and stimulated emission depletion (STED) [53–56])) found PIP₂ and PIP₃ cluster separate from GM1 lipids [57–59] and from each other [18,60,61] suggesting PIP₂ may have associated with DRMs through protein interactions (i.e. PIP₂ binding proteins) after disrupting of the membrane with detergent. For all raft types, the imaging limited their putative size to less than 100 nm [62]; however, the exact composition of lipid clusters, in particular their exact size and transient existence, are still heavily debated [40,63].

Palmitoylation is a post translational modification that covalently attaches a saturated lipid (e.g., a 16 carbon palmitate) to a protein [64]. Among other roles, palmitoylation is the primary signal that traffics and targets proteins to lipid rafts [65–67]. Numerous channels and regulatory proteins are palmitoylated [68–70] including PLD2. PLD2 is palmitoylated at cysteines near its pleckstrin homology (PH) domain, and these palmitoylations are confirmed to localize PLD2 to lipid rafts [71–74]. The PH domain also binds PIP₂ which opposes the localization by palmitoylation [21,75]. Absent substrate, sequestration to the GM1-enriched domain renders PLD2 inactive [18]. This process by which a palmitoylated enzyme is sequestered to a GM1 domain and then released to its substrate is termed ‘substrate presentation’ [18].

Super resolution imaging shows that removal of cholesterol with methyl- β -cyclodextrin (M β CD) disrupts clustering of cholera toxin B (CTxB) labeled GM1 lipids in a cellular membrane [18,63]. The role of lipid raft disruption has been widely studied in particular for immune cell regulation [40,76–81]. In C2C12 myoblastoma muscle cells, disruption was identified as a decrease in both cluster size and number [18]. The result is complicated by a technical difficulty to quantify a cluster size and the fact that CTxB is pentadentate (binds five GM1 lipids) resulting in CTxB potentially inducing artificial clustering (see below for a detailed discussion on the caveats of super resolution imaging) [63]. Nonetheless, a change in size is an indicator of some

type of perturbation in the biological membrane.

Cholesterol-dependent de-clustering of GM1 lipids is consistent with de-clustering of PLD2 [76]. In C2C12 cells and in neuroblastoma 2a (N2a) cells, super resolution imaging showed that cholesterol depletion with M β CD led to translocation of PLD2 out of GM1 lipids [18,28]. The PLD2 then translocated to PIP₂ lipids near its substrate phosphatidylcholine (PC) [18]. A live cell PLD2 assay confirmed the translocation causes PC hydrolyzes, further establishing a mechanism of substrate presentation for its enhanced activity [18]. PLD2 activation by substrate presentation is further supported by the observation that PLD2 products are unsaturated [82]. Unsaturated lipids reside in the disordered region with PIP₂, not GM1 domains, so unsaturated PC is hydrolyzed by its localization with unsaturated PIP₂. PLD2 is constitutively active in vivo and invitro [83], suggesting the many other signaling molecules that regulate PLD2 [84] may also work by enhancing translocation and substrate presentation. For example, binding of a prenylated regulatory factor would alter PLD2 localization and increase access to substrate.

Similar to cholesterol depletion, shear force de-localizes PLD2 with GM-1 domains [18,25]. Shear force (3 dynes/cm²) applied to a cell membrane caused PLD2 to translocate from GM1 to PIP₂ domains where the enzyme mixed with its substrate phosphatidylcholine (PC) to produce PA [25], very similar to the process of cholesterol depletion [25]. In addition to binding PIP₂, PLD2 also binds directly to the C-terminus of TREK-1 and activates the channel through local production of phosphatidic acid [24,35], presumably in the disordered region of the cell membrane [18]. This is significant since previous work also implicated TREK-1C-terminus in mechanosensitivity [85].

Similar to mechanical force, super resolution imaging shows inhaled anesthetics disrupt lipid rafts. Inhaled anesthetics are a chemically diverse collection of compounds [86]. In a live cell PLD2 assay, activation was pronounced across all classes of inhaled anesthetics tested (diethyl ether, chloroform, isoflurane, and xenon) [28]. Consistent with this result and substrate presentation, anesthetics caused a pronounced translocation of PLD2 out of GM1 domains almost identical to mechanical force [28,87]. However, the effect of anesthetics to GM1 domain structure is opposite from mechanical force, anesthetics increased the size and number of GM1 domains; force reduces the size and number. Hence, the size of GM1 domain structure is not the key intermediary for anesthesia activation of PLD2. Rather, palmitate-mediated localization of the enzyme is the key intermediary [28].

3. Shared molecular pathway of force and anesthesia signaling to TREK-1

Palmitate-mediated localization is the first step central to both anesthetic and force activation of TREK-1. This was further established using electrophysiology experiments. The addition of a catalytically dead PLD2 mutant completely blocked the effects of force and anesthetic to TREK-1 channels [25,28]. Importantly the anesthetic sensitivity could be transferred to an anesthetic insensitive channel TRAAK by transferring the PLD2 binding site to TRAAK.

Fig. 1 shows a cartoon representation of the two PLD2 pathways (anesthetic and mechanical) for TREK-1 activation. First anesthetic or force perturb GM1 domain structure. For mechanosensation the rafts are degraded; for anesthetics the rafts remain intact and expand. In the second step, regardless of its structural state, the perturbations lead to identical disruption of palmitate-mediated localization and PLD2 translocation. The size and number of GM1 domains are either of no consequence to PLD2 mediated anesthesia and force transduction, or their contribution is insignificant compared to palmitate-mediated localization of PLD2.

The molecular basis as to why both disruption (mechanical force) and expansion (anesthetics) lead to PLD2 translocation is unclear. Among the more probable reasons include that the density changes or high-order packing energetics are being perturbed by the addition of

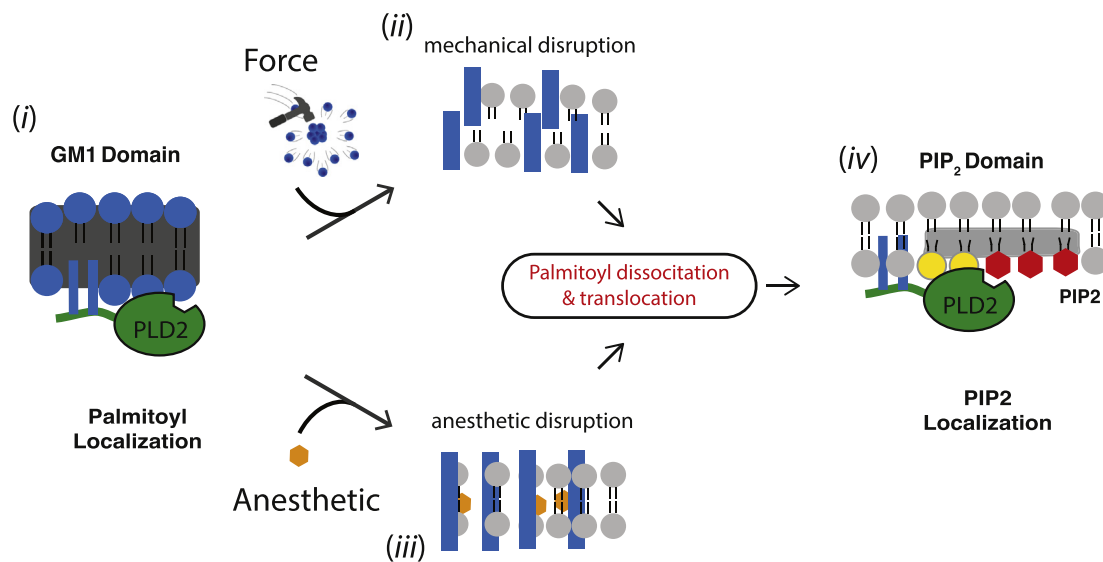


Fig. 1. Palmitate-mediated localization, a central component of TREK-1 mechanosensation and anesthesia. (i) Phospholipase D2 (PLD2, green) is shown with two palmitoylation sites that localize the enzyme to GM1 lipids (blue spheres). (ii) Mechanical force disrupts lipid domains resulting in smaller dispersed domains within the plasma membrane (indicated by disordered blue rectangles). (iii) Anesthetics (orange hexagon) disrupt lipid domains causing them to expand and become larger while remaining relatively ordered (indicated by intact blue rectangles). Both types of raft disruption perturb palmitate mediated localization of PLD2 to GM1 lipids resulting in PLD2 translocation, PLD2 binding to PIP₂ lipids (red hexagon), substrate (phosphatidylcholine, grey spheres) presentation, and phosphatidic acid (PA, yellow spheres) production. PA then activates TREK-1 (not shown). The horizontal grey rectangle indicates clustered lipids on the inner leaflet (PIP₂ domain).

these compounds and force. The expansion of rafts by anesthetics is consistent with studies in artificial membranes—anesthetics expand artificial membranes [29,88]. The requirement for PLD2 to translocate does not rule out anesthetics indirectly affecting PLD2 or TREK-1 through a lipid independent pathway, e.g. phosphorylation, or direct anesthetic binding, but these other pathways appear secondary to PLD2 translocation. Further study is needed to better explain the biophysical changes which lead to PLD translocation.

Local anesthetics also disrupt palmitate-mediated localization of PLD2 to GM1 domains [87], yet they inhibit TREK-1 [89]. Surprisingly the mechanism of inhibition was shown to be primarily through PLD2. Local anesthetics directly bind to and potentially inhibit PLD2 [87]. Hence, PLD2 translocates to its substrate and is bound to the C-terminus of TREK-1, but the direct inhibition of the enzymes blocks PA production leading to TREK-1 inactivation [87]. This showed for the first time how the disordered C-terminus could regulate a channel despite having no structure or a hydrophobic binding site suitable for an anesthetic.

4. Lipid signaling and inhibitory and excitatory mechano- and anesthetic-effectors

The shared mechano- and anesthetic-pathway for TREK-1 activation yields potentially novel insights into how this process could generate a physiological response (see Fig. 2A–D). TREK-1 is an inhibitory mechanosensor or an inhibitory effector of mechanical force. When activated, TREK-1 inhibits neuronal firing by hyperpolarizing the cell (Fig. 2A, channel type i) [90]. Combining an inhibitory mechanosensor with an excitatory mechanosensor (Fig. 2A, channel type ii) would attenuate the signal depending on the ratio of inhibitory to excitatory mechano-effectors. This principle was shown experimentally by Brohawn et al. by overexpressing TRAAK, a mechanosensitive homolog of TREK-1, with endogenously expressed Piezo in neuroblastoma N2a cells [11]. Mechanical activation of over-expressed TRAAK counteracted almost all of the Piezo specific current (Fig. 2E, F) [11].

Fig. 2C, D shows a model of signaling lipids (e.g., PA and PIP₂) and putative combinations of inhibitory and excitatory mechano-effectors tuning a graded response to force. The model is based on the limited information that mechanical force tends to activate both inhibitory and

excitatory mechanosensitive channels (Fig. 2A, channel types i and ii respectively). In Fig. 2B, the model shows a rapid activation by lipids of both channel types and the ratios of surface expression (inhibitory vs. excitatory) dictating the overall rate and strength of depolarization. Surface expression levels of channels are known to regulate excitability of a synapse [91]. In addition to channel surface expression, the concentration of anionic lipid could dictate the effective number of open channels. If the addition of anionic signaling lipids proves pivotal to mechanosensation, it would add a layer of complexity allowing for fast regulation and dynamic control of channels within the membrane compartment [52].

TREK-1's physiological role in anesthesia [2–4] and the dependence of TREK-1 on the lipid membrane for anesthetic action [28] suggests the plasma membrane is a target for anesthetics and it plays a role in anesthesia. For now, this role is only conclusively established for TREK-1 [28]. However, the ability to transfer anesthetic sensitivity from TREK-1 to anesthetic insensitive TRAAK channels using the PLD2 binding domain suggests that an indirect, lipid-based anesthetic sensitivity can be modular and transferable [28]. Other anesthetic channels with potential to be influenced by anesthetic disruption of lipid regulation include the nicotinic acetylcholine receptor (PA activates), GABA_A (PIP₂ bound in a cryoEM structure), and NMDA (PIP₂ regulates), among others [92–94].

In contrast to the apparent activation of mechanical channels, anesthetics both activate and inhibit anesthetic-sensitive channels. Rather strikingly, almost all classes of anesthetics have the opposite effect on excitatory and inhibitory channels [15]. They activate inhibitory channels and inhibit excitatory channels. Most of the inhibited channels are neurotransmitter-gated [15]. One explanation for this opposite regulation would be if an intermediary, such as a lipid, has the opposite regulatory effect on the channel types (Fig. 2C) and then the anesthetics are affecting the regulatory molecule. An intermediary would better explain the observed action on ion channels compared to direct binding of a ligand to allosteric sites which one would expect a more random distribution of activation vs. inhibition among inhibitory and excitatory channel types respectively.

Due to the added inhibitory contribution to anesthesia compared to mechanosensation, we expect a slightly different model. A speculative

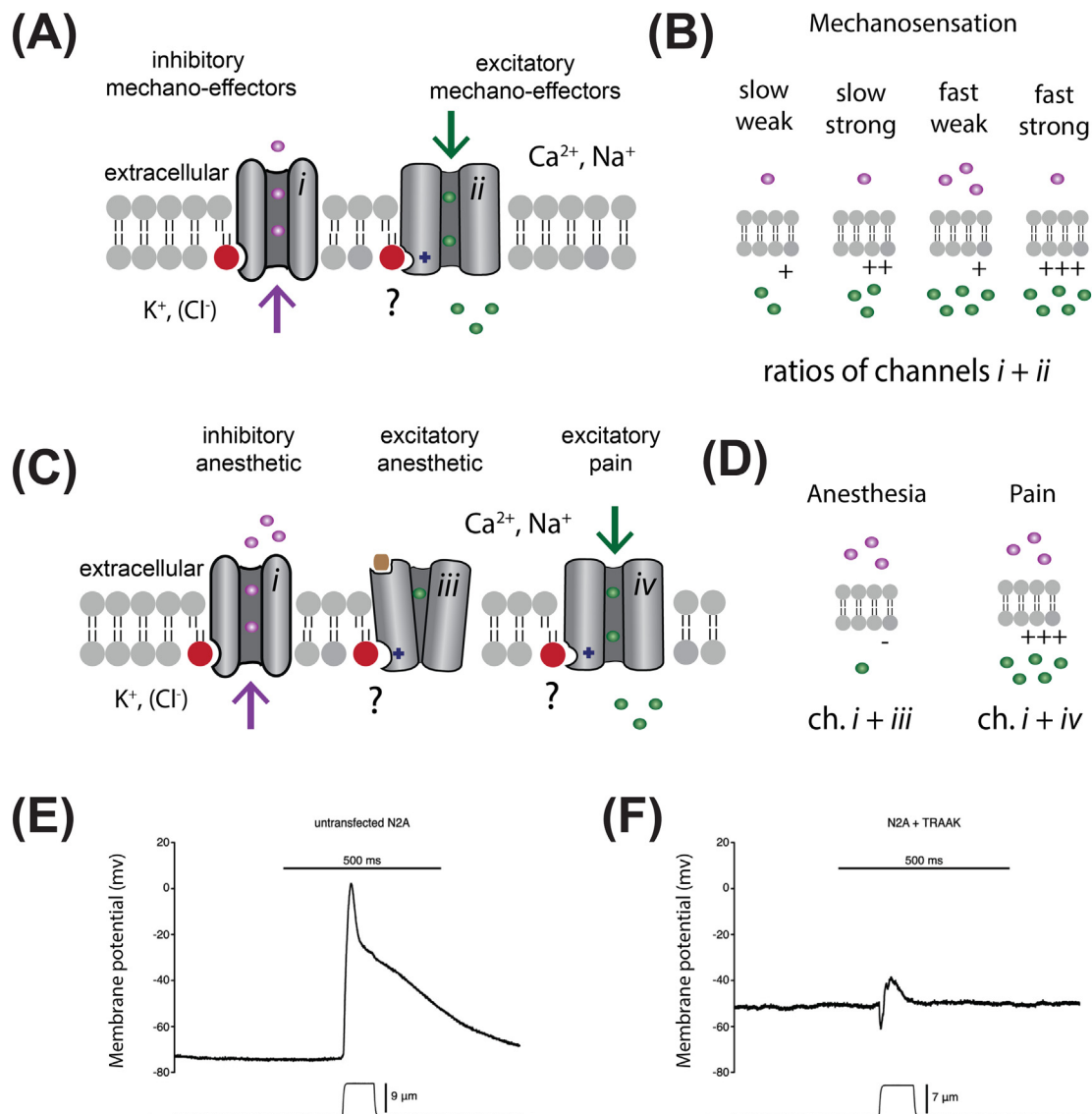


Fig. 2. A putative role for anionic lipid regulation in mechanosensation and anesthesia. **(A–B)** A model of graded mechanical response based on lipid activation. **(A)** A lipid (red circle) is shown activating inhibitory K⁺ channel (grey cylinders) (*i*) and excitatory Ca²⁺ and Na⁺ channels (*ii*). Inhibitory K⁺ ions (purple spheres) are shown flowing out of the cell. Inhibitory Cl⁻ ions flow into the cell (not shown). Excitatory Ca²⁺ and Na⁺ (green spheres) are shown flowing into and depolarizing the cell. A '?' indicates ion channels that are known to be regulated by an anionic signaling lipid but the role of the lipid has not been directly linked to the mechano- or anesthetic- sensitivity of the channel. **(B)** Four putative ratios of inhibitory and excitatory mechano-effector channels. The amount of conductance for each ion is indicated by the number spheres (1–5). Mechano-sensory currents are elicited when more excitatory effectors are active than inhibitory. Having many channels present and open leads to faster and stronger firing. **(C–D)** A model of an anesthetic response based on lipid activation. An anionic signaling lipid, produced in response to anesthetic, is shown activating a potassium channel (e.g. TREK-1, *i*) and inhibiting neurotransmitter-gated (brown square) Ca²⁺ and Na⁺ channels (*iii*) (all channels are shown as grey cylinders). A second class of anesthetic sensitive excitatory channels are shown activated by anesthetics (e.g. TRPV1, *iv*). **(D)** A lipid driven binary response to anesthetics is shown where lipid dependent external K⁺ is high and internal Ca²⁺ and Na⁺ remains low resulting in anesthesia. **(E–F)** Traces of membrane potentials from neuroblastoma 2a (N2a) cells measured under current clamp with mechanical poke by a glass rod. The application and distance of the mechanical poke is shown underneath the trace. Data are taken from Brohawn et al., PNAS, 111 (2014) pg. 3614–9. **(F)** The same conditions in **(E)** with over expressed TRAAK channel, from the same source.

model for lipid regulation in anesthesia is shown in Fig. 2D. Lipids and/or direct binding of anesthetic are shown potentiating potassium and chloride channels (e.g. TREK-1 and GABA_A respectively, Fig. 2D channel type *i*) and inhibiting calcium and sodium channels (Fig. 2D channel type *iii*). Only TREK-1 has been shown to work almost entirely through indirect lipid activation [28]. For other anesthetic channels (e.g. GABA_A, nAChR, and NMDA) that bind lipids [92–94] it is not known if any of the anesthetic sensitivity is transmitted to the channel through the lipid or how a lipid would inactivate these channels.

The combined activation and inhibition of excitatory and inhibitory

channels respectively would, in theory, lead to a much more binary response compared to the gradation expected from the ratio of mechanical effectors (Fig. 2C, D). The finding that the levels of anionic lipid are modulated by anesthetics strongly suggests there will be some influence from indirect lipid binding. But, data for these models is extremely limited and much work is needed to elucidate the roles of anionic signaling lipids in anesthesia through ion channels.

Paradoxically, anesthetics can cause pain when applied to a pain fiber [95,96]. Anesthetics are known to activate excitatory Ca²⁺ conducting transient receptor potential (TRP) channels (Fig. 2C channel

type iv) contributing to pain [97,98]. The channels are highly regulated by signaling lipids, but it is not known to what degree the lipid transmits the anesthetic sensitivity. Assuming a major contribution of the lipid similar to TREK-1, then in nociceptive membranes with both types of channels, the ratio of lipid-activated channel likely contributes to the outcome of excitable pain (Fig. 2D). In tissues with lipid activating TRP channels, the anesthetic induced potassium/chloride current would be insufficient to counteract TRP and the pain nerve would fire. Supporting a role for anionic lipids in pain, PA increases the threshold for pain sensitivity in flies [25], the signaling lipid sphingosine 1-phosphate decreases the threshold in mice [99], and PIP₂ lowered the mechanical sensitivity threshold in B-cells [100].

5. Localized signaling

The demonstration that a local production of PA could activate TREK-1 [24] convincingly established the existence of lipid gradients within the membrane [21]. These gradients depend on restricted movement of lipids. Live-imaging of the GM1 domains, which regulates TREK-1 through PLD2 sequestration, showed the domains to be dynamic but stable over long timescales (minutes) [18]. The partitioning of lipids is thought to involve both lipid and protein-mediated partitioning (e.g. phase separation and cytoskeletal 'picket fence' respectively) [39]. This level of control at nanoscale distances allows for an estimation of the time and concentration it would take to initiate a signaling event through TREK-1 in response to the mechanical disruption of lipid domains.

We find that the local production of PA through PLD2 allows for theoretical gating of TREK-1 on sub millisecond time scales, which is sufficiently fast to allow typical mechanosensitive signaling. We estimate a single PA molecule within 16 nm results in an effective concentration of ~5.5 mM, a concentration 275× the measured K_d (Fig. 3) [35]. For PLD2 the rate of production is thought to be 1 PA molecule every ~45 ms [101] which is halved when considering that each TREK-1 protein contains two PLD2 binding sites. This means that it would only require an effective time of ~300 μs to reach a K_d on par with PA for TREK-1. Importantly, this rate applies to an unstimulated PLD2, the actual rate of PA production can be much higher if PLD2 is bound to PIP₂ or some other activator. When added to the latency previously calculated for the activation of PLD2 from domain disruption [18] a

conservative estimate of ~0.9 ms is sufficient to activate TREK-1 in a PLD2-dependent raft-disruption model of mechanosensation. This places mechanically-induced second-messenger signaling to TREK-1 well within the temporal limit previously thought to be only achieved by direct-to-channel mechanosensitive mechanisms.

6. Considerations of dSTORM imaging

For dSTORM, a molecule of interest must be fluorescently labeled, and this introduces perturbations to the system. Fluorescently labeled CTxB is the most common label for GM1 lipids. Fig. 4A shows differences in CTxB domain size from 3 different cells types, (primary mouse neurons, N2a, and C2C12). CTxB is pentadentate and clusters GM1 lipids in live cells [63,102,103]. In fixed cells the result is less clear. GM1 is a lipid and if the lipid is mobile after fixation then the CTxB could still induce artificial clustering.

Fixing with either paraformaldehyde (PFA) and glutaraldehyde (GA) or osmium tetroxide reduces the movement of protein and lipids and the potential for artificial clusters [104]. Fluorescent recovery after photobleaching (FRAP) showed very little movement of sphingomyelin labeled lipid after osmium tetroxide treatment. This is consistent with our findings that 3% PFA and 0.1% GA also shows little movement of CTxB labeled lipid using FRAP (Fig. 4B, data not previously published). However, single particle tracking for 100 ms showed substantial movement [105]. The movement was over a relatively small area, on the order of a 100 nm cluster (~0.01 μm²). Combining these data, lipids in fixed membranes appear to have some mobility in very confined spaces but not over long distances. Hence determining an absolute cluster or domain size is unlikely to be accurate below 100 nm in fixed cells, but determining the distance between fixed domains and/or measuring the translocation of a molecule from one domain to the another is likely accurate and useful.

When quantifying the diameter of these small clusters, the length of the labeling molecules adds to the inherent error. For example, CTxB can add up to ~5 nm of distance to the measurement and antibodies (primary + secondary) up to ~25 nm (Fig. 4A). Antibodies are bidentate meaning they can also cause increased clustering and artificial generation or enlargement of a domain or cluster (Fig. 4A) to unfixed protein or lipids. For this reason, along with the technical inhibitions of accurately measuring domain size with many techniques, conclusions

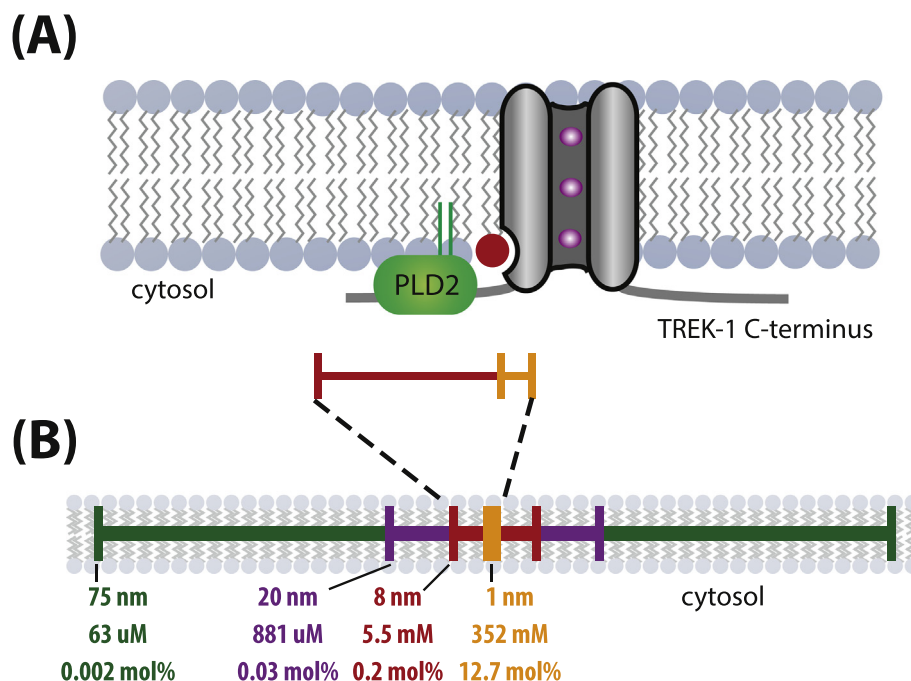


Fig. 3. Generation of mM lipid concentrations by local production. (A) Cartoon illustrating the proximity of phospholipase D2 (PLD2) and phosphatidic acid (PA) production (red sphere) to a TREK-1 channel (grey cylinder) in the plasma membrane. PLD2 bound to the C-terminus of TREK-1 will produce PA within 8 nm of the TREK-1 lipid binding site (red scale bar). Since TREK-1 is a homodimer, an identical setup would exist on the opposite side as well (not shown). (B) The effective molar concentration and mole percent of a single signaling lipid (PA) produced within indicated radii from the channel. The former is calculated by the formula $1/(\pi r^2 h N_A)$, where N_A is Avogadro's number, the height, h , of a lipid leaflet is estimated to be 15 Å, and the distance, r , from TREK-1 is indicated. Mole percent were calculated using the assumption that each μm² of lipid contain 1×10^5 lipids**. Only at radii ~130 nm from the channel are concentrations estimated below the measured K_d of TREK-1 for PA (~20 μM).

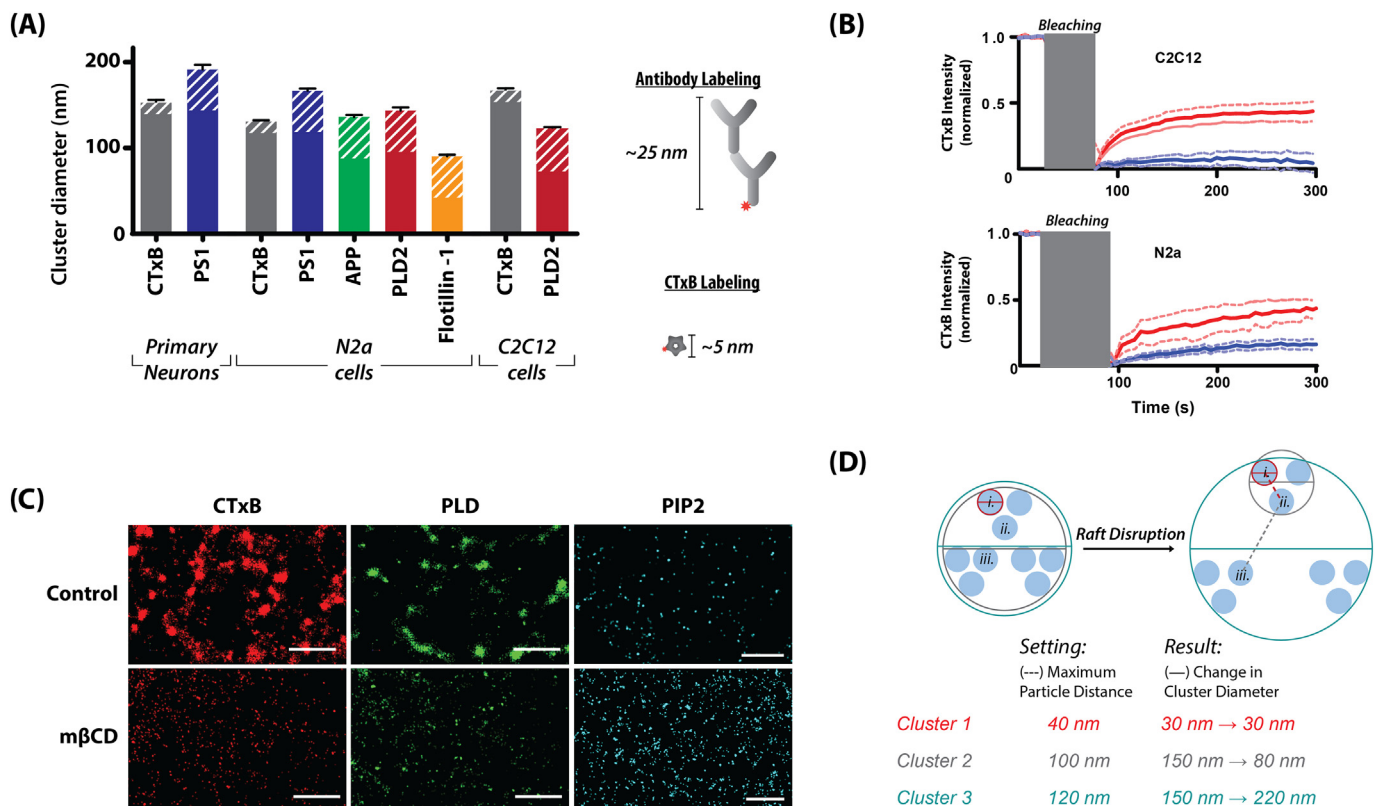


Fig. 4. Acquisition and analysis considerations of super-resolution images. (A) Comparison of the size of CTxB labeled GM1 domains from three cell types with antibody labeled proteins known to localized within the GM1 domains. The potential added diameter from antibody or CTxB labeling is indicated by white stripes. Fixed proteins within the GM1 lipids form clusters approximately the same size as CTxB labeled GM1 clusters, suggesting CTxB does not cluster unfixed lipids after treatment. (Antibodies used: 13891, ab15272, ab41927, MAB5232; secondary Cy3b antibodies and CTxB, along with the protocols used can be found in [18,28]). (B) Fluorescence recovery after photobleaching (FRAP) quantification showing movement of CTxB labeled lipids after fixation. C2C12 cells show negligible recovery in fixed cells vs. live cells while N2a showed a slight increase indicated some diffusion of lipids in N2a cells. (C) Images from C2C12 cells showing changes in lipid and protein clustering upon application of the cholesterol-sequestering molecule methyl- β -cyclodextrin (m β CD). Removal of cholesterol leads to a reduction in diameter in CTxB (80 to 58 nm, red) and PIP₂ clusters (73 to 57 nm, blue). Data taken from Petersen et al. Nature Comm. 7 (2016) 13873 with permission. (D) A hypothetical schematic showing how the selection of the maximum particle distance (MPD) parameter using DBSCAN can change the apparent diameter of a cluster in identical images or across treatments. In the two 'samples' shown, comparison of cluster sizes can lead to either shrinking (Cluster 2), enlargement (Cluster 3), or no apparent change (Cluster 1) in overall size in identical images. Care should be taken in both the selection of MPD used and the resulting interpretation of such data.

about changes in raft sizes should be limited to relative quantification between treated and control samples rather than absolute sizes measured, although with the proper instrumentation and controls these types of measurements may be possible [62].

PLD2 is a membrane associated protein which are not mobile after fixing [105]. In two of three cell types, fixed PLD2 clusters were on average larger than CTxB clusters suggesting CTxB does not increase cluster size in those cell types, at least not in a way that is significant compared to inherent error of the measurement. Of course, the possibility of artificial clustering can never be completely ruled out for multivalent probes.

Monovalent lipid binding domains are available and could be utilized for super resolution imaging to avoid induced clustering. For example, a 15 kDa toxin ostreolysin A (OlyA) labels sphingomyelin in both fixed and live cells [106]. And recently modified OlyA discriminated a cholesterol bound conformation of sphingomyelin from the unbound conformation [107,108]. These will likely be useful tools for imaging. Mutations creating mono and divalent CTxB also retain binding and partition with detergent resistant membranes, but with reduced affinity [109].

Lastly, some user-selected variables can affect the apparent size of a lipid domain. For example, density-based spatial clustering of applications with noise (DBSCAN), a commonly used clustering analysis algorithm, allows for user input on the maximum distance to consider a particle when defining a cluster. This distance can affect the apparent

size of the resulting domain (see Fig. 4D). Even when using the correct parameters, a measurement of an absolute size is uncertain. Rather one must compare two conditions and limit the comparison to a change in domain structure. For example, a relative shift in domain size with and without m β CD, force, anesthetic, etc. (Fig. 4C) can indicate a perturbation to the system if similar preparations and internal controls (such as comparing the total number of localizations for dSTORM) are used. User-selected variables are unlikely a major factor for the observed decrease in CTxB and PLD2 domain size in Fig. 4C due to the clear structural differences in the lipid domains. For PIP₂ a reanalysis of the data with the more automated DBSCAN showed a decrease in size Fig. 4C compared to the manual analysis which showed a slight increase [18].

For determining localization changes between two molecules, pair correlation offers a robust method to quantify these changes [110,111]. Pair correlation is largely unaffected by domain size and the overcounting common in dSTORM making it a preferred method of quantification for localization-based techniques. Pair correlation can identify and quantify relationship changes between two molecules before and after a stimuli. For example, the translocation of PLD2 out of GM1 domains to PIP₂ domains is independent of domain size or number. Hence, observation of PLD2 translocation through changes in pair correlation is definitive evidence for anesthetic, mechanical, and cholesterol induced disruption of palmitate-mediated localization and domain disruption.

7. Perspective and conclusions

Almost all mechanisms of TREK-1 regulation are proposed to depend on the carboxy C-terminus including mechanical force, temperature, pH, arachidonic acid (AA), and anesthetics [90]. This loop is disordered and has no obvious binding sites for hydrophobic molecules like AA or anesthetics nor an obvious structured mechanism for transmitting mechanical or thermal information to a putative gate in the transmembrane domain. Early TREK-1 studies [6,8,90] were unaware of PLD2 mechano and anesthetic sensitivity [18,28] and the direct interaction of PLD2 with TREK-1 [24]. The role of PLD2 appears key for understanding regulation of TREK-1. Recently, the two step PLD2 mechanism nicely explained the role of the C-terminus for local anesthetic inhibition of TREK-1 [87] showing the two step PLD2 model can account for both activation and inhibition of TREK-1 in specific cases.

As far as the authors are aware, no other models (apart from those described above) of ion channel activation have considered a multistep process for anesthesia or mechanosensation. Whether the properties found in TREK-1 extend to other lipid regulated channels is a question needing further investigation. Anesthetics have a minimal effect on the bulk (disordered) membranes [112] which has been the source of debate in the field. But these studies have not adequately considered the numerous potential lipid intermediaries. Many lipid modifying enzymes are similar to palmitoylated PLD2 and associate with the membrane; some of them undoubtedly bind disordered loops on channels producing local changes in lipid concentration. Any lipid molecule locally produced will have a very high local concentration and a potent effect on an associated channel or other protein. The local high concentration would likely mean a weak binding interaction making the identification of these putative lipid activators challenging.

The existence of stable domains based on lipid partitioning is not necessary for a disruption/mixing mechanism to function in the cell [44]. Much of the lipid heterogeneity could be based on gradients resulting from local production and small reductions in lateral mobility similar to local production of PA near TREK-1. This type of transient signaling through nanoclusters is thought to control cell growth and differentiation [44]. Furthermore, phase separation could simply alter the kinetics of localized signaling rather than form stable long lived domains. Recently phase separation has been observed for the soluble proteins in the cytosol [113] suggesting soluble ligands may also form gradients and activate ion channels through local high concentration. If the cell utilizes phase separation in the cytosol it seems likely the same properties would be utilized in the lipid membrane.

M β CD is not found natively in cells, but in the immune system apolipoproteins modulate raft function [114–116] and apolipoproteins are known to shuttle cholesterol between cells [117], a function that would require both the removal and addition of cholesterol to the membrane. Apolipoproteins could endogenously regulate PLD2 through cholesterol dependent palmitoyl-clustering similar to M β CD. One would expect increased cholesterol to decrease the effect of anesthetics and mechanical disruption, but this requires further study.

The broader implication of anesthetic and force induced de-clustering of palmitoylated proteins remain to be investigated. All cells must sense force [118–120] not just specialized sensory cells. The widespread production of GM1 lipids and expression of PLD2 suggest de-clustering may be the foundation of a suitable mechanism for detecting mechanical force in many unspecialized cells. There are many important palmitoylated proteins including most alpha subunit G-proteins [121] and numerous ion channels [68], many of which are anesthetic sensitive [15,122].

Declaration of competing interests

Authors declare no conflicts of interests.

Transparency document

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