

Polymodal Mechanism for TWIK-Related K⁺ Channel Inhibition by Local Anesthetic

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BACKGROUND: Local anesthetics cause reversible block of pain and robustly inhibit TWIK-related K⁺ channel (TREK-1) currents. Before local anesthesia onset, injection of local anesthetics can cause unwanted transient pain. TREK-1 is an anesthetic-sensitive potassium channel that when inhibited produces pain. A disordered C-terminal loop of TREK-1 is thought to contribute to anesthetic sensitivity, but the molecular basis for TREK-1 inhibition by local anesthetics is unknown. Phospholipase D2 (PLD2) is an enzyme that produces phosphatidic acid (PA) required for TREK-1 activation and also binds to the channel's C terminus.

METHODS: Here, we use biophysical and cellular techniques to characterize direct and indirect lipid-mediated mechanism for TREK-1 inhibition (respectively). We characterized direct binding of local anesthetic to TREK-1 by reconstituting the purified channel into artificial membranes and measuring ion flux. We characterized indirect PA-mediated inhibition of TREK-1 by monitoring lipid production in live whole cells using a fluorescent PLD2 product release assay and ion channel current using live whole-cell patch-clamp electrophysiology. We monitored anesthetic-induced nanoscale translocation of PLD2 to TREK-1 channels with super-resolution direct stochastic reconstruction microscopy (dSTORM).

RESULTS: We find local anesthetics tetracaine, lidocaine, and bupivacaine directly bind to and inhibit PLD2 enzymatic activity. The lack of PLD2 activity indirectly inhibited TREK-1 currents. Select local anesthetics also partially blocked the open pore of TREK-1 through direct binding. The amount of pore block was variable with tetracaine greater than bupivacaine and lidocaine exhibiting a minor effect. Local anesthetics also disrupt lipid rafts, a mechanism that would normally activate PLD2 were it not for their direct inhibition of enzyme catalysis.

CONCLUSIONS: We propose a mechanism of TREK-1 inhibition comprised of (1) primarily indirect PLD2-dependent inhibition of lipid catalysis and (2) limited direct inhibition for select local anesthetics through partial open pore block. The inhibition through PLD2 explains how the C terminus can regulate the channel despite being devoid of structure and putative binding sites for local anesthetics. (Anesth Analg 2019;129:973–82)

KEY POINTS

- **Question:** What is the molecular basis for anesthetic inhibition of TWIK-related K⁺ (TREK-1) channels?
- **Findings:** Local anesthetics indirectly inhibit TREK-1 channels by blocking synthesis of an anionic lipid.
- **Meaning:** Any local pain mediated through TREK-1 inhibition is primarily regulated by anionic lipids not direct binding of the anesthetic to the channel.

Local anesthetics are low-affinity hydrophobic molecules that modulate cell excitability in peripheral neurons and are used to reversibly block local pain.^{1,2} Clinically, the most frequent complaint noted with the use of local anesthetics, including tetracaine and lidocaine, is stinging or burning pain associated with the initial

subcutaneous infiltration.³ Current remedies for this initial pain are limited to buffering the pH of the injected solution and optimizing injection techniques. These remedies help but do not eliminate the pain. Understanding the molecular pathways that generate the transient pain has the potential to better eliminate these side effects in patients.

Classically, local anesthetics block voltage-gated sodium (Na⁺) channels resulting in analgesia.^{1,4} However, local anesthetics also block potassium (K⁺) channels.¹ The inhibition of K⁺ channels is important as K⁺ conductance maintains the resting potential of the cell, such that block of K⁺ channels can result in depolarization of neurons and increased pain.^{5,6} TWIK-related K⁺ channel (TREK-1; K_{2P}2.1 gene) is robustly inhibited by local anesthetics and is a known pain channel,^{7–11} making this channel a suitable model to study potential mechanisms involved in the transient pain of local anesthetics. TREK-1 is also regulated by membrane lipids and polyunsaturated fatty acids and belongs to the family of 2-pore domain potassium (K₂P) channels.^{12–14}

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Accepted for publication April 3, 2019.

Funding: This work was supported by a Director's New Innovator Award to S.B.H. (1DP2NS087943-01) from the National Institutes of Health.

The authors declare no conflicts of interest.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (www.anesthesia-analgesia.org).

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DOI: 10.1213/ANE.00000000000004216

Previous studies narrowed TREK-1 sensitivity of local anesthetics to a disordered loop in the channels C-terminal domain (CTD).^{7,15} It has been unclear how this disordered loop could regulate the gating of a channel. Recently, inhaled anesthetics were shown to increase binding of phospholipase D2 (PLD2) to the same C-terminal segment of TREK-1 identified for sensitivity of local anesthetic.¹⁶ Inhaled anesthetics ejected PLD2 from nanoscale lipid domains causing PLD2 to translocate a very small distance to phosphatidylinositol 4,5-bisphosphate (PIP₂) clusters that activated the enzyme (see Supplemental Digital Content, Figure 1A, <http://links.lww.com/AA/C820>).^{16–18} The localized production of phosphatidic acid (PA) by PLD2¹⁹ then indirectly activated TREK-1 by binding to a lipid site in the channels transmembrane domain.^{20,21} Local anesthetics also perturb membranes,^{22–24} raising a second question, why do they inhibit rather than activate TREK-1? Presumably disruption of lipid rafts by local anesthetics would also activate PLD2. Here, we show that despite disrupting lipids, local anesthetics directly bind to and inhibit TREK-1 conductance through inhibition of PLD2 lipid catalysis.

METHODS

Reagents

Local anesthetics tetracaine, lidocaine, and bupivacaine were purchased from Sigma-Aldrich (St Louis, MO). Purified PLD2 from peanut, cabbage, and bacteria (*Streptomyces chromofuscus*) was purchased from Enzo Biochem (Farmingdale, NY) and Sigma-Aldrich, respectively. PLD assay reagent amplex red 10-acetyl-3,7-dihydroxyphenoxazine was purchased from Cayman Chemical (Ann Arbor, MI). Horseradish peroxidase (HRP) and choline oxidase were purchased from VWR (Radnor, PA). All lipids, including 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (C8-PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (POPG), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC or 18:1 PC), were purchased from Avanti Polar Lipids (Alabaster, AL) or Cayman Chemical. Furimazine, a glow-type luminescent substrate for the bioluminescence resonance energy transfer (BRET) assay, was purchased from Promega (Madison, WI). Flux assay fluorophore 9-amino-6-chloro-2-methoxyacridine (ACMA) was purchased from Life Technologies (Carlsbad, CA). Ionophore Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and valinomycin were purchased from Tocris Bioscience (Minneapolis, MN), and stocks were made in ethanol. Methyl- β -cyclodextrin (M β CD) to extract cholesterol from cellular membranes and disrupt lipid rafts was purchased from Sigma-Aldrich.

In Vitro and In Vivo PLD Assay

To confirm direct inhibition of PLD by local anesthetics, mouse PLD2 (mPLD2) was purified from mPLD2 expressing Chinese hamster ovary (CHO) cells. Cells were grown in serum and included Ham's F-12 (Thermo, Waltham, MA). PLD expression was induced by adding 1 μ g/mL tetracycline 1 day before the assay. The PLD from *Streptomyces* species was purchased from VWR. To generate the dose–response curves, partially purified mPLD2 or fully purified bacterial PLD was treated with different concentrations of local anesthetics or a PLD2 inhibitor and then its enzymatic activity was measured by an enzyme-coupled

product release assay using amplex red reagent in 96-well flat culture plates as described previously.²⁵ The assay reaction was initiated by adding 50 μ L of working buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 5 mM CaCl₂, pH 8.0) containing 100 μ M amplex red, 2 U/mL HRP, 0.2 U/mL choline oxidase, and 60 μ M C8-PC with the indicated amount of PLD2 and local anesthetic or PLD2 inhibitor. The assay reaction was performed for an hour at 37°C, and the activity was kinetically measured with a fluorescence microplate reader (Tecan Infinite 200 Pro, Männedorf, Switzerland) at excitation and emission wavelengths of 530 nm and 585 nm, respectively.

For the in vivo measurement of PLD2 activity, cultured neuroblastoma 2A (N2A) cells were seeded into 96-well plates ($\approx 5 \times 10^4$ cells per well) and incubated at 37°C overnight to reach confluency. The cells were then differentiated, with serum-free Dulbecco's modified Eagle's medium (DMEM) for a day and washed twice with phosphate-buffered saline (PBS). The PLD reaction was initiated as described in the in vitro assay, but with 100 μ L working solution for 2 hours. For analysis, raw PLD2 activity was determined by subtracting the background activity (reaction buffer with the local anesthetics, but no cells). A regression line and equation were generated to calculate the effect of each drug on PLD2 activity at 10-, 20-, 30-, and 60-minute time points. For the bar graphs, samples were normalized to the control activity at the 60-minute time point.

TREK-1 Electrophysiology

To assess the effects of local anesthetics on the TREK-1 currents, whole-cell current measurements were measured in human embryonic kidney 293t (HEK293t) cells. Cells were transiently transfected with X-tremeGENE 9 DNA transfection reagent (Roche Diagnostics, Basel, Switzerland). Human TREK-1 gene was a gift from Dr Stephen Long. mPLD2 and mutant PLD2-K758R genes were gifts from Dr Michael Frohman. TREK-1 and PLD2 were cotransfected with 1:4 ratio,¹⁹ otherwise a total 1 μ g of DNA was used in the transfection. The transfected HEK293t cells were subjected to standard whole-cell recording using Axopatch 200B amplifier and Digidata 1440A (Molecular Devices, San Jose, CA). Electrode pipettes were made with Borosilicate glass (B150-86-10; Sutter Instrument, Novato, CA) with 4–7 M Ω resistances. The internal solution in the pipette contained (in mM): 140 KCl, 3 MgCl₂, 5 ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 HEPES, 10 tetraethylammonium (TEA), and pH 7.4 (adjusted with KOH). External solution in the bath contained (in mM): 145 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 TEA, and pH 7.4 (adjusted with NaOH). Measured currents were filtered at 2 kHz and sampled at 10 kHz using Clampex 10.3 (Molecular Devices). Currents were elicited by a ramp voltage command (long ramp = –100 mV to +50 mV in 1 second) in the absence and presence of the local anesthetics. Local anesthetics were solubilized in external solution and perfused using a gravity-driven bath application setup.

Expression and Purification of TREK-1

To directly test the effect of local anesthetic on TREK-1 without confounding cellular components, we used detergent-purified proteins in our direct binding experiments. A stable truncated form of zebrafish TREK-1 (amino acids

1–321, corresponding to human TREK-1 GI:14589851) was codon optimized and synthesized for eukaryotic expression (Genewiz, Inc, South Plainfield, NJ).¹² DNA was then inserted into a vector containing a C-terminal fluorescent protein (either green fluorescent protein [GFP] or nano-luciferase [Nluc]) with 10× histidine tag via a short linker (amino acids SNS) followed by a PreScission protease cleavage site (amino acids LEVLFG/GP). The vector was transformed and expressed in *Pichia pastoris* strain SMD1163. Selected colonies were cultured overnight, pelleted in liquid nitrogen, and the cells were disrupted by milling (Retsch, Newton, PA; model MM400, 5 times for 2.5 minutes at 25 Hz). The extracted channels were in dodecyl-β-D-maltoside (DDM) with protease inhibitors. The channel proteins were then purified to homogeneity on a cobalt affinity column followed by size exclusion chromatography (SEC) with a buffer containing 20 mM Tris (pH 8.0), 150 mM KCl, 1 mM EDTA, and 2 mM DDM. All the assembled channel proteins exhibited a predominant monodispersed peak by SEC corresponding to the expected molecular weight (MW) as previously shown.²⁰

TREK-1 Lipid Binding BRET Assay

To help exclude a mechanism based on direct allosteric inhibition of TREK-1 by local anesthetics, we used a PIP₂ BRET assays previously shown to detect allosteric inhibitor binding to TREK-1.²⁰ PIP₂ binding is conformation sensitive and allosteric antagonists inhibit binding of PIP₂ to TREK-1.²⁰ The assays were executed in a 50 μL total reaction volume that contain 1 nM zTREK-1-Nluc, 500 nM boron-dipyrromethene tetramethylrhodamine (BODIPY TMR) fluorescent PIP₂ (FL-PIP₂; Echelon Biosciences, Salt Lake City, UT), local anesthetics, and 1:2000 furimazine (NanoGlo; Promega, Madison, WI), 7 mM DDM in the SEC buffer. The BRET signal was measured in a 384-well plate using Envision Multilabel 2104 plate reader (PerkinElmer, Waltham, MA) set for automatic BRET ratio calculation with dual-emission detection (540 nm for Nluc and 574 nm for FL-PIP₂). The BRET signal by the binding of FL-PIP₂ to TREK-1-Nluc and competition with local anesthetics was calculated by subtracting signal from background fluorescence determined by nonfluorescent C8-PIP₂.

TREK-1 Flux Assay

To characterize the direct effect of local anesthetic binding, we reconstituted purified TREK-1 into proteoliposomes of known lipid and ion composition suitable for testing channel function (ion flux). Flux assays with proteoliposomes were performed as published previously.¹² In brief, 3 μmol lipids (DOPC:POPG molar ratio 85:15) were mixed and dried overnight. The dried lipids were rehydrated with 1 mL buffer (150 mM KCl, 20 mM HEPES, pH 7.4) for 30 minutes, and multilamellar vesicles were formed with vigorous shaking. The unilamellar vesicles were then formed by sonicating the lipid solution in a bath sonicator until the solution became translucent. Lipid vesicles were then destabilized with DDM (3 mM) and used for channel reconstitution (1 protein:100 lipid mass ratio). The DDM detergent was removed using approximately 100 mg of beads BioBeads SM-2 polystyrene beads (Bio-Rad, Hercules, CA). The reconstituted channels in vesicles were pelleted by centrifuging at 250,000g for 45 minutes in Optima XP ultracentrifuge (Beckman Coulter, Brea, CA) with TLA110 rotor. The proteoliposomes were resuspended in 150 μL rehydration buffer, and the reconstitution

was checked by measuring GFP fluorescence on a Tecan Spark 20M plate reader (Tecan, Männedorf, Switzerland). Flux assays were either performed immediately after reconstitution or the proteoliposomes were flash-frozen and stored at –80°C for later experimentation.

To measure the ion flux, proteoliposomes were added to 195 μL of flux assay buffer (150 mM NaCl, 20 mM HEPES, pH 7.4, 2 μM ACMA) in a black 96-well plate (Costar 3915; Corning Inc, Corning, NY). Initial fluorescence readings were taken every 20 seconds for a minute as a baseline with Tecan Spark 20M plate reader. Then potassium flux was initiated using the protonophore CCCP (1 μM final concentration), and the fluorescence signal was read every 20 seconds for 7 minutes. Next, the potassium-selective ionophore valinomycin (20 nM final concentration) was added to terminate the chemical gradient, and fluorescence was read every 20 seconds for 5 minutes. Relative TREK-1 activity was normalized using this equation: $(F_{\text{normalized}} = (F - F_{\text{end}}) / (F_{\text{start}} - F_{\text{end}}))$, where F is arbitrary fluorescence units, F_{start} is the average of measured fluorescence before addition of CCCP and F_{end} is the end point fluorescence after addition of valinomycin. This equation eliminates the fluorescence variations that could arise from the ACMA pipetting error and the intrinsic fluorescence of the compounds. For local anesthetics that affected the liposomes, the anesthetic-treated TREK-1 activity was further normalized to control channel activity accordingly with the valinomycin readings (F_{end}).

Super-Resolution Microscopy

Super-Resolution Microscopy was performed on N2A cells as described previously¹⁷ to detect disruption of lipid nanodomains (lipid rafts; Figure 1A). In brief, cells were grown in 8-well chamber slides (Nunc Lab-Tek chamber slide system; Thermo Scientific, Waltham, MA), washed, and treated with local anesthetics for 10 minutes. The treated cells were fixed (3% paraformaldehyde, 0.1% glutaraldehyde, and drugs for 10 minutes), quenched (0.1% NaBH₄ for 7 minutes), and washed with PBS (3 times). Cells were then permeabilized (0.2% Triton-X 100 in PBS for 15 minutes), blocked using a standard blocking buffer (10% bovine serum albumin [BSA] and 0.05% Triton in PBS for 90 minutes), and stained with anti-PLD2 antibody (Cell Signaling, Danvers, MA) with 1:500 dilution, anti-PIP₂ antibody (Echelon) with 1:130, or cholera toxin B (CTxB) (Life Technologies) with 1:1000 dilution. Alexa 647-conjugated and cyanine 3B (Cy3B)-conjugated secondary antibodies were used to label CTxB/PLD2 and PLD2 or PIP₂, respectively. Cells were postfixed and extensively washed with PBS and water.

A Zeiss Elyra PS1 microscope, equipped with Andor iXon 897 EMCCD camera (Carl Zeiss, San Diego, CA), was used for super-resolution microscopy with an oil-immersed 63× objective lens in total internal reflection fluorescence (TIRF) mode. Images were acquired by Zen 10D software (Carl Zeiss, San Diego, CA) with an exposure time of 18 ms per acquisition, and total 10,000 frames were collected. Alexa Fluor-labeled CTxB-labeled domains were excited with a 642 nm laser and Cy3B-labeled PIP₂ domains were excited with a 561 nm laser in a photo-switching buffer (1% β-mercaptoethanol, 0.4 mg glucose oxidase, and 23.8 μg catalase [oxygen scavengers], 50 mM Tris, 10 mM NaCl, and 10% glucose at pH 8.0). An autocorrelative algorithm²⁶ or tracking immobile fiducial marker (TetraSpeck beads) was applied to correct the sample

drift. The obtained localization coordinates were converted to be compatible to Vutara SRX software (version 5.21.13; Bruker, Billerica, MA) by an Excel macro and then raft sizes and the cross-correlation were determined by the default cluster analysis package in the Vutara SRX software. The raft sizes were calculated applying density-based spatial clustering of applications with noise (DBSCAN) algorithm with a search radius (ϵ) of 100 nm for clusters consisting of at least 10 localizations.¹⁷ The analysis model consists of well-defined distribution, and statistical interpretation is more useful for analyzing the localizations presented here because the methods generate a population of clusters rather than the information on a per-cluster basis. The relative raft size values were determined by measuring the full width half maximum (FWHM) of the clusters and reported as 95% confidence interval ($n = 5-7$ cells per staining and imaging condition).

Statistical Analyses

All the data calculations and plots were performed using Prism6 (GraphPad software; Graphpad, San Diego, CA) or Microsoft Excel. Biochemical experiments were done 3–4 times to ensure reproducibility. To ensure reproducible super-resolution imaging, a labeling experiment was performed at least twice and at least 2 cells were analyzed per labeling experiment. All the experimental samples were performed in random orders when on treatments or

microscopy to avoid experimental bias. Statistical significance was evaluated using analysis of variance (ANOVA) with post hoc Dunnett test, 2-tailed t tests, parametric or nonparametric wherever appropriate. Data are presented as the mean and the error bars with standard deviation (SD) or 95% confidence interval as appropriate. Significance is indicated by * $P \leq .05$, ** $P \leq .01$, *** $P \leq .001$, and **** $P \leq .0001$.

RESULTS

Effect of Local Anesthetics on PLD2 Catalysis

Phosphatidylcholine (PC) is a hydrophobic phospholipid with a positively charged headgroup similar to local anesthetics² (Supplemental Digital Content, Figure 1B–D, <http://links.lww.com/AA/C820>). PC is the substrate of PLD2. The binding of PLD2 to the same region of TREK-1 that harbors lidocaine sensitivity,¹⁹ combined with the shared chemical properties of PC with lidocaine, led us to hypothesize that local anesthetics could bind to and inhibit PLD2 catalysis.

We previously developed a product release assay to monitor PC hydrolysis by PLD2 in the membranes of live cells.¹⁷ The assay works by measuring the release of PLD2 product choline in real time. During the assay, choline freely exits the cell, where it is converted into a fluorescent signal. The assay then measures the fluorescent signal in the culture medium of live cells (Figure 1). Using this assay, we found local anesthetics tetracaine (0.15 mM), lidocaine (10 mM),

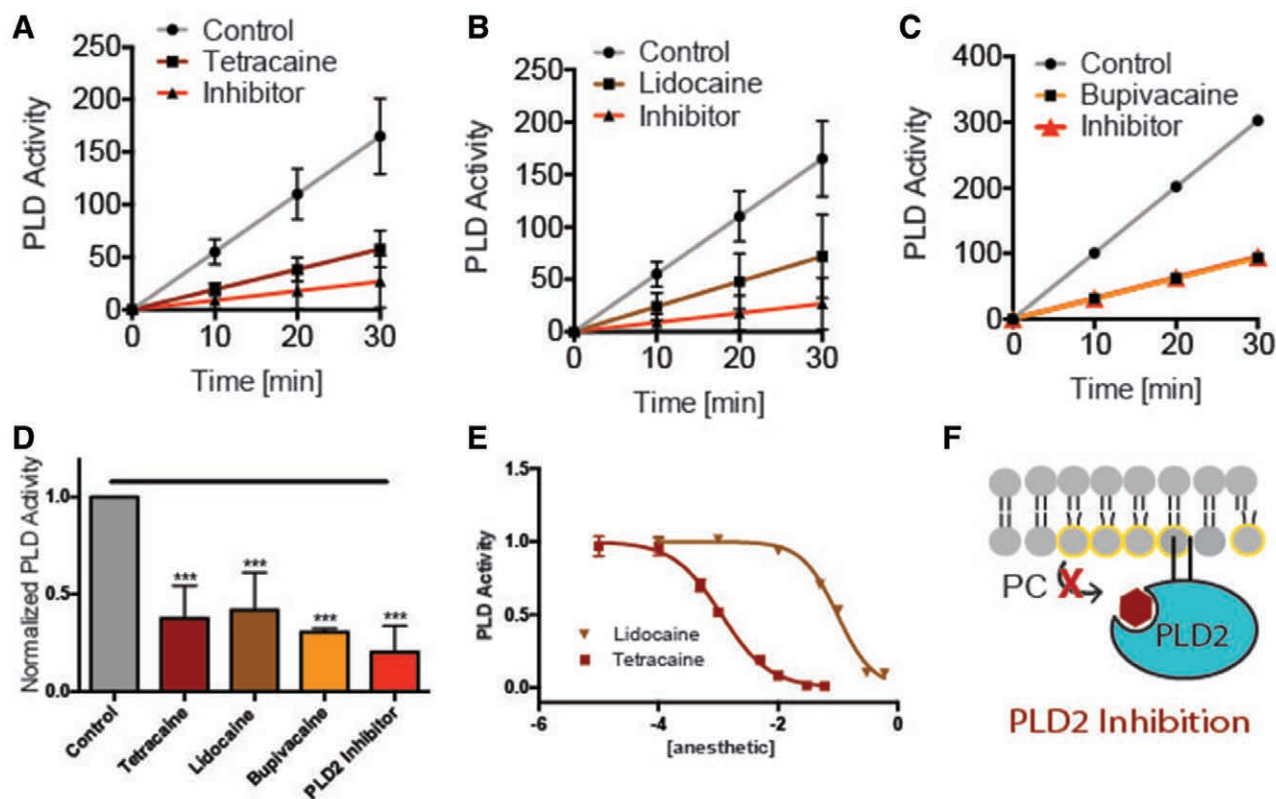


Figure 1. Local anesthetics inhibit phospholipase D2 (PLD2) enzyme activity. A–C, Live cell fluorescent PLD2 product release assays are shown for N2A cells after treatment with local anesthetic. Tetracaine (150 μ M) (A), lidocaine (10 mM) (B), and bupivacaine (200 μ M) (C) inhibit most PLD2 activity in neuroblastoma (N2A) cells similar to a specific inhibitor (2.5–5 μ M) ($n = 4$, mean [standard deviation (SD)]). D, Summary of normalized activity of PLD2 after local anesthetics treatment shown in A–C at 30 min ($n = 4$, mean [SD]). *** $P \leq .001$. E, Dose–response curves showing local anesthetic inhibition of partially purified mouse PLD2 ($n = 3-4$, mean [SD]). PLD was assayed as in A–C. F, Proposed model showing PLD2 inhibition by local anesthetic. Local anesthetic is shown bound to the catalytic pocket of PLD2 (cyan) inhibiting (red X) the hydrolysis of the PLD2 substrate phosphatidylcholine (PC) and blocking the production of phosphatidic acid (PA).

and bupivacaine (0.2 mM) all robustly inhibit PLD2 activity in live N2A neuroblastoma and C2C12 myoblast cells (Figure 1A–D; Supplemental Digital Content, Figure 2A–C, <http://links.lww.com/AA/C820>). The same assay previously showed PLD activation with inhaled anesthetics.¹⁶

To confirm direct binding of local anesthetic to the enzyme (Figure 1F), we tested inhibition of partially purified mPLD2 in a dose-dependent manner. Tetracaine and lidocaine directly inhibited PLD2 with an half maximal inhibitory concentration (IC₅₀) of 1.0 ± 0.1 and 96.8 ± 4.4 mM, respectively (Figure 1E). We further confirmed direct binding of anesthetic to PLD with a purified enzyme from a bacterial source (bPLD). The bPLD retains full activity when purified to homogeneity. Consistent with direct binding, bPLD enzymatic activity was inhibited by local anesthetics similar to mPLD2 1.3 ± 0.2 and 139.5 ± 10.8 mM for tetracaine and lidocaine, respectively (Supplemental Digital Content, Figure 2D–E, <http://links.lww.com/AA/C820>). These IC₅₀ values are well below the range of estimated anesthetic concentration based on partitioning of the anesthetic into a lipid membrane (Supplemental Digital Content, Figure 1C, <http://links.lww.com/AA/C820>).

Open Pore Block of TREK-1 by Local Anesthetics

Like sodium channels, potassium channels conduct a single positive charge. Hence similar to the early speculation on the mechanism of sodium channels,^{1,4,27,28} local anesthetics could inhibit K⁺ channels by open pore block. The most definitive experiment to show a direct functional interaction of a ligand with a channel is to purify and functionally reconstitute the channel into lipids of known composition.

We observed direct inhibition of functionally reconstituted TREK-1 by all local anesthetics but with dramatically different degrees. Lidocaine (1 mM) only slightly inhibited TREK-1 (~10%) while bupivacaine (0.2 mM) more so (~40%) and tetracaine (0.15 mM) the most at 60% (Figure 2A). TREK-1 was reconstituted into liposomes comprising 18:1 PC with 18:1 phosphatidylglycerol (PG) (85:15 mol% ratio) and assayed with local anesthetic using an ion flux assay (Supplemental Digital Content, Figure 3A, <http://links.lww.com/AA/C820>).²⁰ The direct inhibition by local anesthetics differs from inhaled anesthetics which do not bind to or alter TREK-1 activity in the same assay.¹⁶

The observed direct inhibition could be from (1) open pore block or (2) binding to an allosteric site that inhibits

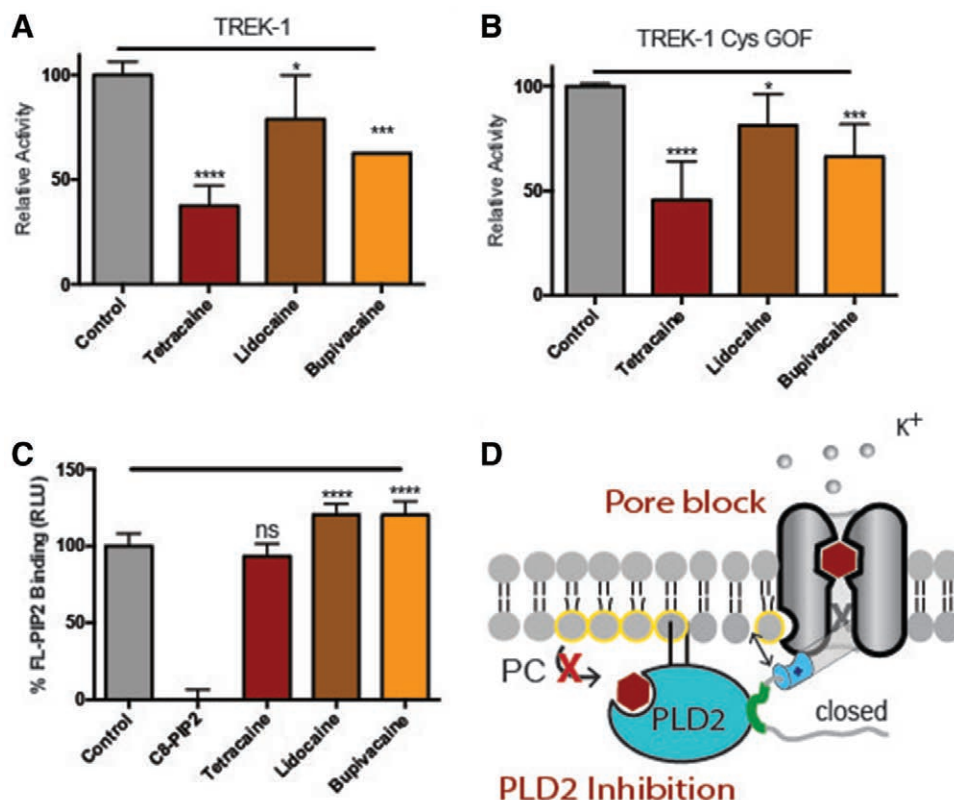


Figure 2. Open pore block of TWIK-related K⁺ channel (TREK-1) by local anesthetics. A, Normalized ion flux showing local anesthetics partially inhibit purified TREK-1 reconstituted into 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) proteoliposomes with anionic lipid 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac glycerol) (DOPG). Tetracaine (150 μM), 1 mM lidocaine, and 200 μM bupivacaine were applied (n = 4–6, mean [standard deviation {SD}]). B, Blocking of the TREK-1 gain of function (GOF) cysteine mutant (TREK-1 Cys) by local anesthetics with the same concentrations as in (A) (n = 4–7, mean [SD]). C, Bioluminescence resonance energy transfer (BRET) assay showing competition of soluble 1,2-dioctanoyl-sn-glycero-3-phosphocholine (C8) phosphatidylinositol 4,5-bisphosphate (PIP₂; 100 μM) and local anesthetics with fluorescent PIP₂ (FL-PIP₂) probe (500 nM) (n = 8, mean [SD]; relative light units [RLU], not significant [ns]). D, Cartoon contrasting direct open pore block of TREK-1 with indirect PLD2 inhibition by anesthetic. PLD2 inhibition leads to depletion of local phosphatidic acid (PA). Without PA, TREK-1 anionic lipid binding site (cyan cylinder) is unoccupied and the gating helix is extended (double arrow) leaving TREK-1 (gray cylinders) in a closed conformation (closed pore, gray X). A local anesthetic is also shown partially blocking the pore of TREK-1. PLD2 is bound to the C terminus of TREK-1 through a PLD2 binding site (green segment). *P ≤ .05; ***P ≤ .001; ****P ≤ .0001; ns P > .05. GOF indicates gain of function; PC, phosphatidylcholine.

the channel. To distinguish these 2 mechanisms, we utilized a previously reported pair of cysteine mutants (cysTREK-1) that lock TREK-1 in the open state with a disulfide bond.^{20,29} If TREK-1 is locked in the open state and local anesthetics still inhibit, then local anesthetic likely binds in the open pore region of TREK-1.

Figure 2B shows that tetracaine and bupivacaine inhibit most ion flux of functionally reconstituted cysTREK-1 in a manner almost identical to WT TREK-1. This confirms a component of inhibition can happen through open pore block for select local anesthetics (Figure 2D). However, lidocaine only slightly inhibited TREK-1 consistent with his the previous study that showed the C terminus accounts for all detectable lidocaine's inhibition.⁷

If tetracaine binds to the open pore of the channel, it may also increase the affinity of ligands that bind the open state. Signaling lipids like PA or PIP₂ are thought to bind to a conformation close to the open state of the channel.^{13,20} We tested the effect of local anesthetics on the affinity of PA and PIP₂ binding to detergent-purified TREK-1 using a conformational sensitive fluorescent lipid binding assay.²⁰ Lidocaine and bupivacaine significantly increased PIP₂ binding to TREK-1 suggesting that pore block may have an allosteric effect on TREK-1, although tetracaine did not (Figure 2C; Supplemental Digital Content, Figure 3B, <http://links.lww.com/AA/C820>).

To estimate the relative contributions of tetracaine's PLD2 inhibition versus open pore block, we assayed TREK-1 currents in the presence of a catalytically dead PLD2 mutant (PLD2_K758R [xPLD2]) with patch-clamp

electrophysiology. Tetracaine (150 μ M) caused an $\approx$ 50% reduction of TREK-1 current in the presence of xPLD2 (Figure 3A–C). Assuming all the inhibition in the presence of xPLD2 arises from open pore block, we estimate that 59.4% of tetracaine inhibition comes from pore block and 40.6% from PLD2/PA signaling (Figure 3D–F).

Raft Disruption (Monosialotetrahexosylganglioside and PIP₂ Domains) by Tetracaine and PLD2 Translocation

Local anesthetics are known to perturb model membranes and the lipids that surround channels.^{24,30–34} Within the membrane, lipids can partition laterally into clusters or domains of saturated and unsaturated lipids often called liquid ordered (Lo) and liquid disordered (Ld) phases, respectively.^{35,36} The Lo phase is enriched in sphingomyelin (eg, monosialotetrahexosylganglioside [GM1]) and referred to as lipid rafts or GM1 domains (see Supplemental Digital Content, Figure 1, <http://links.lww.com/AA/C820>). The Ld phase is comprised of unsaturated lipids and includes polyunsaturated signaling lipids like PA and PIP₂; the latter can cluster with proteins separate from GM1 domains.^{17,37}

We asked if local anesthetics perturb GM1 or PIP₂ domains, and if so, do they release PLD2 from the GM1 domain? To observe the effect of the local anesthetic tetracaine on the GM1 lipid rafts in cellular membranes, we used direct stochastic optical reconstruction microscopy (dSTORM)^{26,38,39} of N2A neuroblastoma cells. For this study, we define rafts or domains as heterogeneity of lipids or

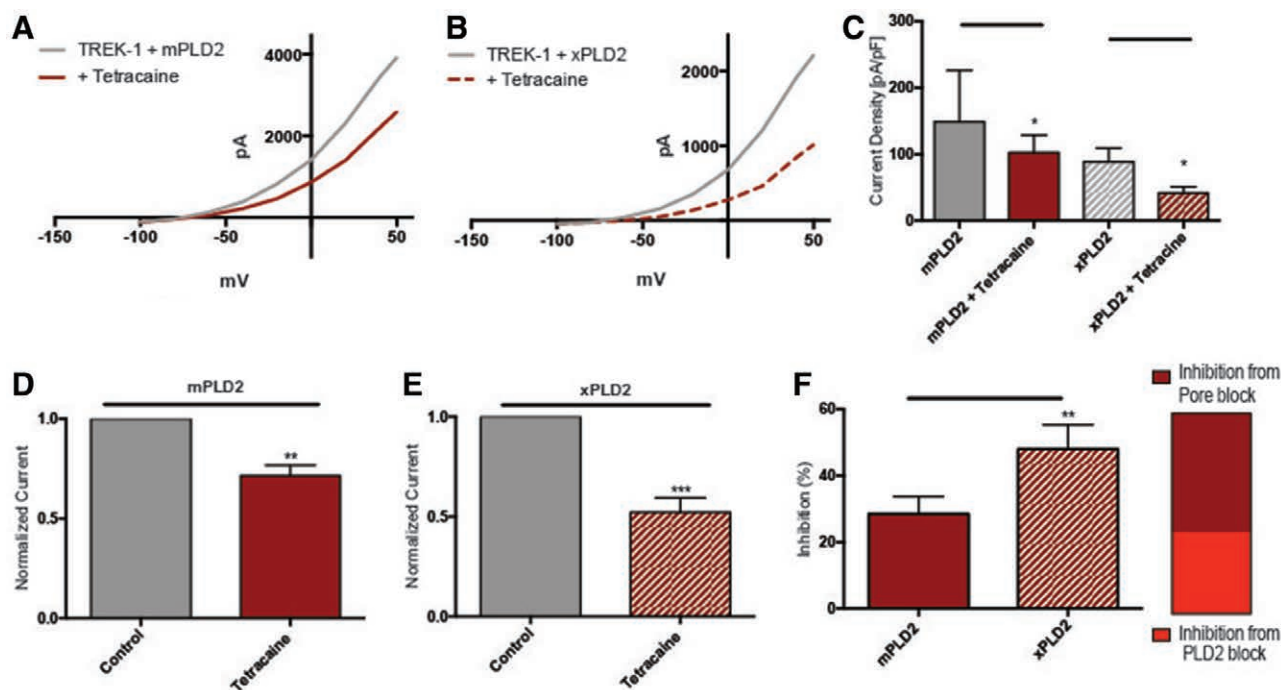


Figure 3. Contribution of channel block to tetracaine inhibition of TWIK-related K⁺ channel (TREK-1). Whole-cell TREK-1 currents measured in human embryonic kidney cells. A, B, Representative current-voltage relationships (I-V curves) showing the inhibition of TREK-1 current by tetracaine (100 μ M) in the presence of both mouse phospholipase D2 (mPLD2) (A) and a catalytically inactive mutant of PLD2 (xPLD2 = PLD2_K758R) (B). Inhibition of PLD2-independent current is presumed to be due to channel block. C, Current density at 0 mV showing the inhibition by tetracaine ($n = 4-5$, mean [standard deviation {SD}]). D, E, Normalized current showing $\approx$ 30% of current inhibition mPLD2 were coexpressed (D) or $\approx$ 50% of current inhibition with xPLD2 (E). F, Bar graph showing xPLD2 causes 20% more TREK-1 inhibition than mPLD2. Bar in the right panel shows estimated current inhibition through the direct pore block (59.4%) or the PLD2 inhibition (40.6%) after partial pore block. * $P \leq .05$; ** $P \leq .01$; *** $P \leq .001$.

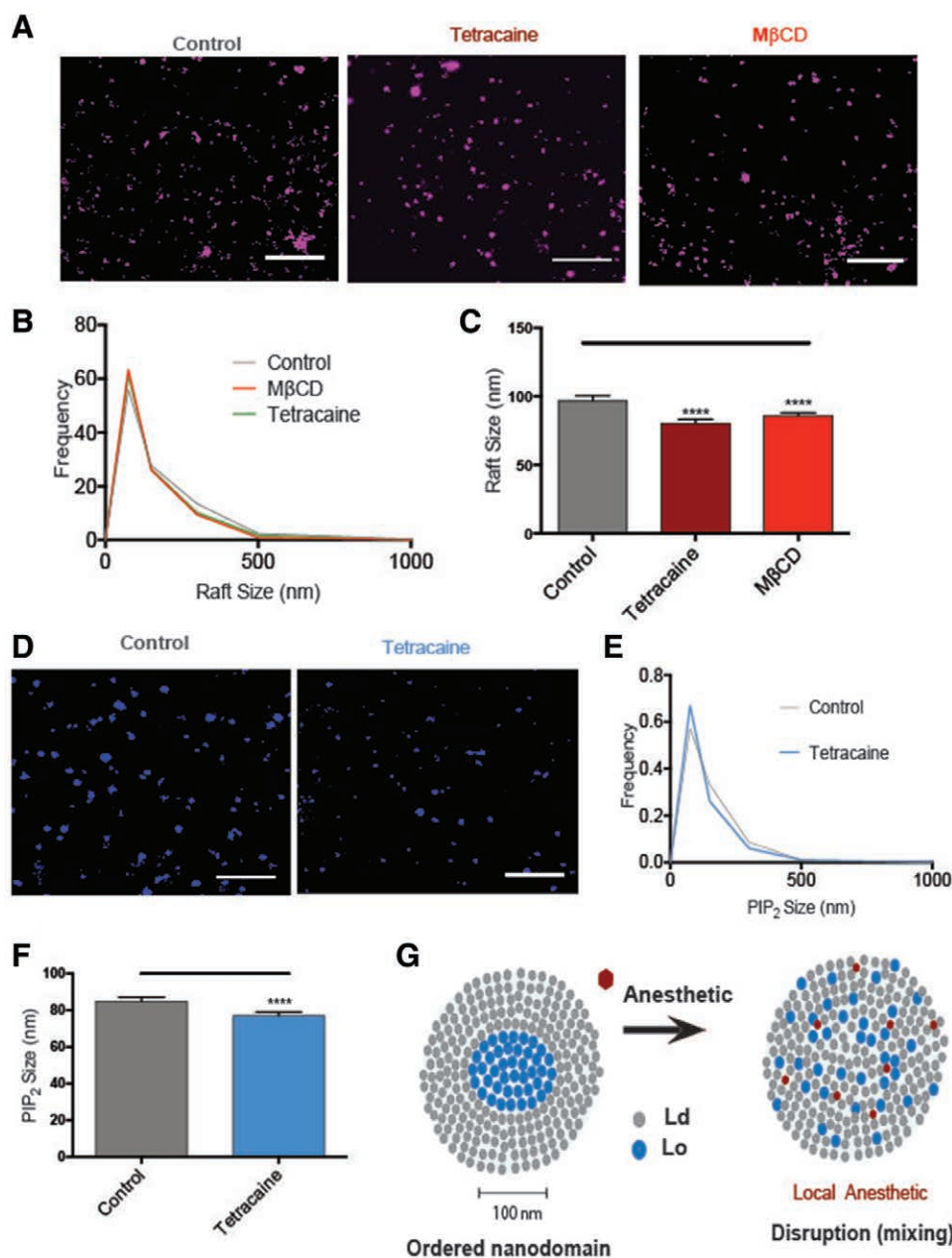


Figure 4. Tetracaine disrupts monosialotetrahexosylganglioside (GM1) and phosphatidylinositol 4,5-bisphosphate (PIP₂) domains in neuroblastoma (N2A) cell membrane. A, Super-resolution direct stochastic optical reconstruction microscopy (dSTORM) images showing the lipid raft (GM1 domain) disruption by tetracaine (150 μ M) and methyl- β -cyclodextrin (M β CD; 100 μ M) in N2A cells (Scale bars: 1 μ m). B, Size distribution comparing the frequency of raft sizes between tetracaine and M β CD-treated cells ($n = 5$). C, Histogram of the mean raft sizes quantified by the cluster analysis ($n = 2201$ – 3406 , mean [95% confidence interval (CI)]). D, dSTORM images showing the effect of tetracaine 150 μ M on PIP₂ domains in N2A cells. (Scale bars: 1 μ m). E, Frequency distribution of sizes comparing the control cells to the tetracaine ($n = 7$ – 8 , mean [95% CI]). F, Average PIP₂ domain sizes ($n = 1991$ – 2128). G, Schematic representation of domain disruption by tetracaine. Local anesthetic (maroon hexagon) causes the lipid domain components to mix with the disordered lipids (gray). **** $P \leq .0001$.

lipid-protein mixtures with no regard to an absolute size. Hence, GM1 domains are simply a subset of the membrane where the GM1 lipids are in higher concentration than expected for a perfectly homogeneous mixture of lipids.

We found 150 μ M tetracaine, a concentration that completely inhibited TREK-1 channels in atrial myocytes,¹⁰ decreased the apparent diameter (Figure 4A–C; Supplemental Digital Content, Figure 4E, F, <http://links.lww.com/AA/C820>), and area (Supplemental Digital Content, Figure 4A, B, <http://links.lww.com/AA/C820>) of GM1 domains in N2A cellular membranes similar to the M β CD, a positive control. M β CD sequesters cholesterol from the membrane to cause GM1 domain disruption.⁴⁰ Analysis of the distance between domains (Ripley radius) suggests that tetracaine reduces the number of observed large domains into small ones causing the disruption of rafts

in the membrane (Supplemental Digital Content, Figure 4G, H, <http://links.lww.com/AA/C820>).¹⁷

We also investigated the effect of tetracaine on the distribution and relative size of PIP₂ domains. Tetracaine (150 μ M) decreased the diameter (Figure 4D–F) and area (Supplemental Digital Content, Figure 4C, D, <http://links.lww.com/AA/C820>) of PIP₂ domains, similar to its effect on GM1 domains. This result agrees with the previous report that local anesthetics reduce the amount of PIP₂ in cellular membrane.⁴¹

Last, the disruption should release PLD2 from GM1 domains and activate the enzyme.¹⁷ If the enzyme is indeed released, this would confirm that the direct inhibition of the enzyme is the mechanistic reason for local anesthetic inhibition of TREK-1. As expected, we found disruption of rafts by tetracaine or M β CD control causes translocation of PLD2

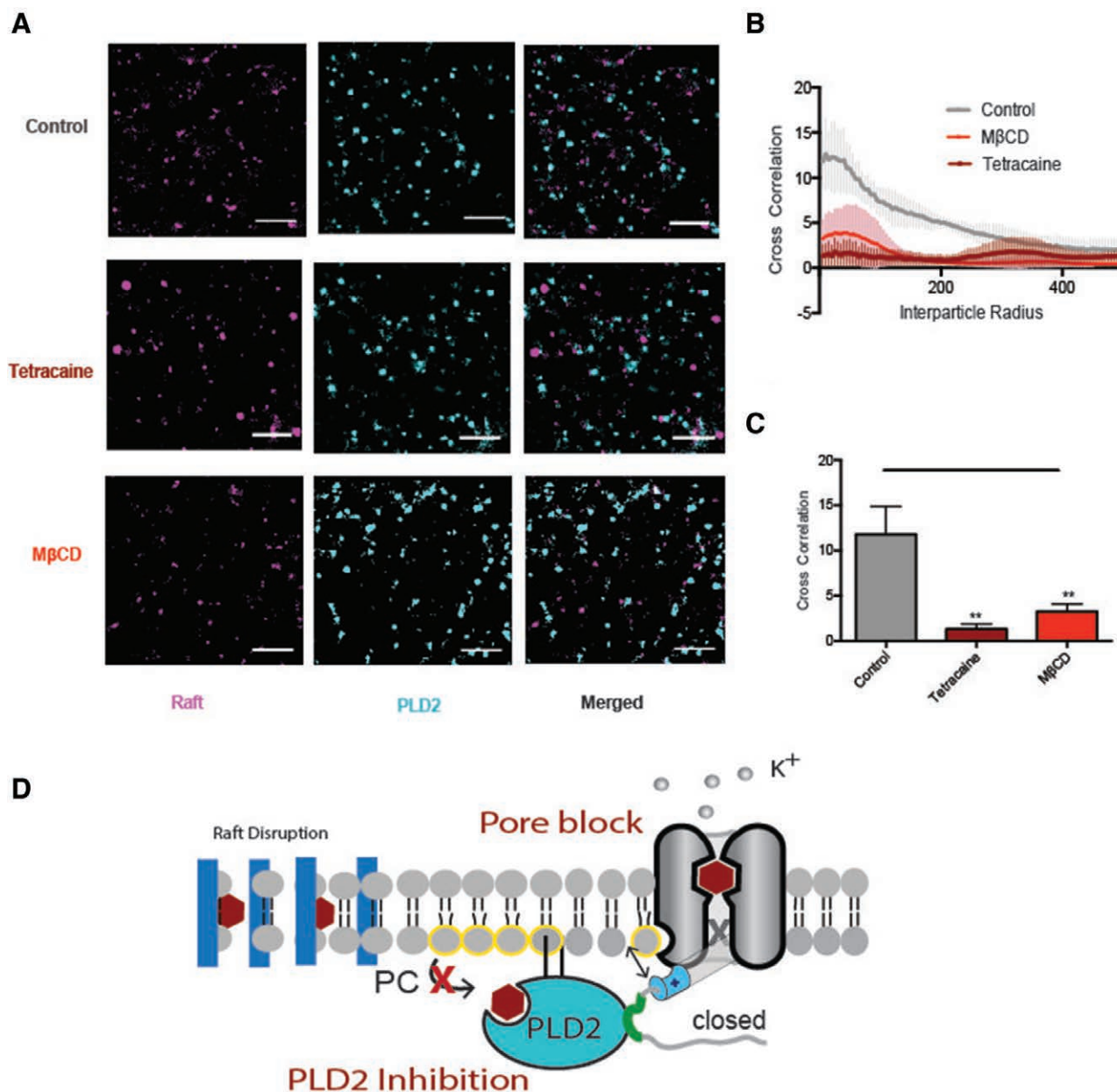


Figure 5. Tetracaine dissociates phospholipase D2 (PLD2) from monosialotetrahexosylganglioside (GM1) domains. A, Representative super-resolution images (dSTORM) of lipid raft (cholera toxin B [CTxB] labeled) and PLD2 before treatment (Control) and after treatment with tetracaine (150 μ M), and methyl- β -cyclodextrin (M β CD) 6 (100 μ M) in neuroblastoma (N2A) (scale bars = 1 μ m). B, Average cross-correlation functions ($C(r)$) showing a decrease in PLD2 association with ordered GM1 domains after treatment with local anesthetic or M β CD. C, Bar graph comparing $C(r)$ at $r = 5$ nm ($n = 4-6$, mean [standard deviation {SD}]). $**P \leq .01$. D, Graphic depicting the summary of the polymodal mechanism for TWIK-related K⁺ channel (TREK-1) inhibition by local anesthetic. PLD2 catalysis and TREK-1 inhibition are labeled as described in Figures 1F and 2D. Disrupted GM1 domains are shown as blue bars. Disruption of rafts would normally activate PLD2 by substrate presentation, but direct block of PLD2 by local anesthetic inhibits the enzyme aided by partial pore block of the channel's conduction pathway. PC indicates phosphatidylcholine.

out of GM1 domains (Figure 5A–C). Because raft disruption normally increases PLD2 activity¹⁷ and PLD2 positively modulates TREK-1,¹⁹ the direct inhibition of PLD2 likely accounts for TREK-1 inhibition by local anesthetics.

DISCUSSION

Taken together our data show local anesthetics inhibit TREK-1 indirectly, primarily through inhibition of PLD2 enzymatic activity and the subsequent local depletion of PA from the

membrane. Many channels are regulated by anionic signaling lipids, and the block of PLD2 could affect other channels through their lipid regulatory sites.¹³ In support of a role for PLD in pain, knockdown of PLD shifts the sensitivity of flies to mechanical stimulation and electric shock (N. Petersen, PhD, Departments of Molecular Medicine and Neuroscience, The Scripps Research Institute, Jupiter, FL, unpublished data, 2019). The activation of TREK-1 and subsequent depolarization is also thought to activate Na_v channels, and because

Na_v channels undergo “use-dependent” inhibition, TREK-1 depolarization could eventually facilitate the use-dependent inhibition of Na_v channels by local anesthetics.¹¹

Similar to local anesthetics, ethanol also causes transient pain during subcutaneous injections.⁴² And similar to local anesthetics, ethanol reacts with PLD2. But rather than inhibit the enzyme, ethanol produces an unnatural lipid metabolite phosphatidylethanol (PEtOH). The PEtOH binds to and inhibits TREK-1.⁴³ Ethanol also modulates transient receptor potential V1 (TRPV1) by shifting its sensitivity to temperature.⁴² Whether PEtOH is the key intermediary of TRPV1 has not been tested, but TRPV1 is a pain channel regulated by anionic lipids.⁴⁴ PLD2, while broadly applicable, is likely only one of many factors leading to pain, evident by the fact that propofol activates PLD2¹⁶ yet produces pain during injection.⁴⁵

Clinically, lidocaine and tetracaine are injected at a concentration of 0.5%–2% (≈20–85 mM) and 0.5%–1% (≈20–40 mM), respectively. Due to lipid partitioning, the concentration in the membrane theoretical calculations are much higher—>1 M for a 1% solution of lidocaine (see Supplemental Digital Content, Figure 1C, <http://links.lww.com/AA/C820>) suggesting that the ratio of membrane to anesthetic dictates the local concentration.⁴⁶ PLD2 is a membrane-associated enzyme and hence is expected to interact with local anesthetics near the membrane (Supplemental Digital Content, Figure 1A, <http://links.lww.com/AA/C820>). The IC₅₀ values calculated for tetracaine and lidocaine are 1.0 and 98 mM, respectively (Supplemental Digital Content, Figure 2D, E, <http://links.lww.com/AA/C820>), hence even a small amount of partitioning is sufficient to completely inhibit the enzyme in vivo. The fact that deletion of the CTD rendered TREK-1 completely insensitive to lidocaine confirms the relevance of these concentrations in cultured cells.⁷ We also saw little direct channel block by lidocaine (Figure 2A) in agreement with PLD2 dominating TREK-1 inhibition for that anesthetic. However, we cannot rule out local anesthesia affecting TREK-1 through changes in membrane thickness.⁴⁷

Local anesthetic disruption of rafts could affect other palmitoylated proteins residing in the lipid rafts. Our data show tetracaine disruption affects the composition, size, and longevity of lipid-ordered domains in the plasma membrane. The absolute size of rafts can vary depending on how the clusters are analyzed, but the comparison of treated and untreated provides a reliable estimation of a disruptive effect. Signaling enzymes, receptors, ion channels, and other regulatory proteins that localize into lipid raft microdomains must also be affected. Their function and downstream signaling undoubtedly contributes to the overall effect of local anesthetics in vivo. Although because local anesthetics disrupt rafts rather than stabilize them, disruption is not the feature that distinguishes local from inhaled anesthetic function of TREK-1 channels.

By using a purified system and TREK-1 locked in the open state, we assume the observed effect is pore block, but we cannot rule out the lipid regulatory site as an allosteric site for the local anesthetics especially because the cysteines are located near the putative lipid regulatory site in the channel.^{12,48} Local anesthetics weakly inhibit other members of the K₂P family which share both a similar pore but may also share lipid regulatory sites.⁴⁹

In conclusion, we show local anesthetic exerts a poly-modal effect on TREK-1 channels (Figure 5D). Our work provides evidence that the local anesthesia occurs through a combinatory action rather than a single effect on the channel. Our study also provides new insights on the mechanism of local anesthetics that could facilitate the development of better therapeutic approaches in anesthesia and chronic pain based on lipid signaling. ■

ACKNOWLEDGEMENTS

We thank Michael Frohman from Stony Brook for the mouse phospholipase D (PLD) and mutant PLD complementary DNA (cDNA), Steven Long from Memorial Sloan Kettering for human TWIK-related K⁺ channel (TREK-1), Guangwei Du from UT Health Science Center for PLD expressing cells, Rod Mackinnon from the Rockefeller University for the pichia yeast expression vector (PICZ) vectors, Andrew S. Hansen for PLD experiments, multiple aspects of experimental design and discussion, and Yul Young Park for the electrophysiology experimentation.

DISCLOSURES

Name: Mahmud Arif Pavel, PhD.

Contribution: This author helped design the overall study; write the manuscript; perform the super-resolution imaging, bioluminescence resonance energy transfer (BRET) assays, and ion flux experiments; and contribute the electrophysiology.

Name: Hae-Won Chung, PhD.

Contribution: This author helped the initial inhibition of phospholipase D (PLD) by local anesthetics, perform the purified PLD study, and contribute the electrophysiology.

Name: E. Nicholas Petersen, PhD.

Contribution: This author helped the phospholipase D (PLD) assays and electrophysiology.

Name: Scott B. Hansen, PhD.

Contribution: This author helped write the manuscript and oversee the interpretation of data.

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REFERENCES

1. Lirk P, Hollmann MW, Strichartz G. The science of local anesthesia: basic research, clinical application, and future directions. *Anesth Analg*. 2018;126:1381–1392.
2. Becker DE, Reed KL. Local anesthetics: review of pharmacological considerations. *Anesth Prog*. 2012;59:90–101; quiz 102–103.
3. Martin S, Jones JS, Wynn BN. Does warming local anesthetic reduce the pain of subcutaneous injection? *Am J Emerg Med*. 1996;14:10–12.
4. Butterworth JF, Strichartz GR. Molecular mechanisms of local anesthesia: a review. *Anesthesiology* 1990;72:711–734.
5. Alloui A, Zimmermann K, Mamet J, et al. TREK-1, a K⁺ channel involved in polymodal pain perception. *EMBO J*. 2006;25:2368–2376.
6. Vivier D, Bennis K, Lesage F, Ducki S. Perspectives on the two-pore domain potassium channel TREK-1 (TWIK-related K⁺ channel 1). A novel therapeutic target? *J Med Chem*. 2016;59:5149–5157.
7. Nayak TK, Harinath S, Nama S, Somasundaram K, Sikdar SK. Inhibition of human two-pore domain K⁺ channel TREK1 by local anesthetic lidocaine: negative cooperativity and half-sites saturation kinetics. *Mol Pharmacol*. 2009;76:903–917.
8. Punke MA, Licher T, Pongs O, Friederich P. Inhibition of human TREK-1 channels by bupivacaine. *Anesth Analg*. 2003;96:1665–1673.
9. Shin HW, Soh JS, Kim HZ, et al. The inhibitory effects of bupivacaine, levobupivacaine, and ropivacaine on K2P (two-pore domain potassium) channel TREK-1. *J Anesth*. 2014;28:81–86.
10. Zhang H, Shepherd N, Creazzo TL. Temperature-sensitive TREK currents contribute to setting the resting membrane potential in embryonic atrial myocytes. *J Physiol*. 2008;586:3645–3656.

11. Kindler CH, Yost CS, Gray AT. Local anesthetic inhibition of baseline potassium channels with two pore domains in tandem. *Anesthesiology*. 1999;90:1092–1102.
12. Brohawn SG, del Marmol J, MacKinnon R. Crystal structure of the human K2P TRAAK, a lipid- and mechano-sensitive K⁺ ion channel. *Science*. 2012;335:436–441. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3329120&tool=pmcentrez&rendertype=abstract>. Accessed January 26, 2012.
13. Hansen SB. Lipid agonism: the PIP2 paradigm of ligand-gated ion channels. *Biochim Biophys Acta*. 2015;1851:620–628. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25633344>. Accessed February 6, 2015.
14. Chemin J, Patel AJ, Delmas P, Sachs F, Lazdunski M, Honore E. Regulation of the mechano-gated K2P channel TREK-1 by membrane phospholipids. *Curr Top Membr*. 2007;59:155–70.
15. Patel AJ, Honoré E, Lesage F, Fink M, Romey G, Lazdunski M. Inhalational anesthetics activate two-pore-domain background K⁺ channels. *Nat Neurosci*. 1999;2:422–426.
16. Pavel MA, Petersen EN, Lerner RA, Hansen SB. Studies on the mechanism of general anesthesia. *bioRxiv*. 2018. Available at: <http://biorxiv.org/content/early/2018/05/04/313973.abstract>. Accessed May 4, 2018.
17. Petersen EN, Chung H-WW, Nayeibosadri A, Hansen SB. Kinetic disruption of lipid rafts is a mechanosensor for phospholipase D. *Nat Commun*. 2016;7:13873.
18. Weinrich M, Worcester DL. The actions of volatile anesthetics: a new perspective. *Res Pap Acta Cryst*. 2018;74:1169–1177.
19. Comoglio Y, Levitz J, Kienzler MA, Lesage F, Isacoff EY, Sandoz G. Phospholipase D2 specifically regulates TREK potassium channels via direct interaction and local production of phosphatidic acid. *Proc Natl Acad Sci USA*. 2014;111:13547–13552. Available at: <http://www.pnas.org/cgi/doi/10.1073/pnas.1407160111>. Accessed October 23, 2014.
20. Cabanos C, Wang M, Han X, Hansen SB. A soluble fluorescent binding assay reveals PIP2 antagonism of TREK-1 channels. *Cell Rep*. 2017;20:1287–1294.
21. Chemin J, Patel AJ, Duprat F, Lauritzen I, Lazdunski M, Honoré E. A phospholipid sensor controls mechanogating of the K⁺ channel TREK-1. *EMBO J*. 2005;24:44–53. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=544907&tool=pmcentrez&rendertype=abstract>. Accessed February 7, 2015.
22. Tsuchiya H, Mizogami M. Interaction of local anesthetics with biomembranes consisting of phospholipids and cholesterol: mechanistic and clinical implications for anesthetic and cardiotoxic effects. *Anesthesiol Res Pract*. 2013;2013:297141.
23. Vemparala S, Saiz L, Eckenhoff RG, Klein ML. Partitioning of anesthetics into a lipid bilayer and their interaction with membrane-bound peptide bundles. *Biophys J*. 2006;91:2815–2825.
24. Högborg CJ, Lyubartsev AP. Effect of local anesthetic lidocaine on electrostatic properties of a lipid bilayer. *Biophys J*. 2008;94:525–531.
25. Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal Biochem*. 1997;253:162–168.
26. Mlodzionoski MJ, Schreiner JM, Callahan SP, et al. Sample drift correction in 3D fluorescence photoactivation localization microscopy. *J Opt EXPRESS*. 2011;19:15009–15019.
27. Scheuer T. Local anaesthetic block of sodium channels: raising the barrier. *J Physiol*. 2007;581:423.
28. Tikhonov DB, Zhorov BS. Mechanism of sodium channel block by local anesthetics, antiarrhythmics, and anticonvulsants. *J Gen Physiol*. 2017;149:465–481.
29. Brohawn SG, Campbell EB, MacKinnon R. Physical mechanism for gating and mechanosensitivity of the human TRAAK K⁺ channel. *Nature*. 2014;516:126–130. Available at: <http://www.nature.com/doi/10.1038/nature14013>. Accessed December 3, 2014.
30. Mojumdar EH, Lyubartsev AP. Molecular dynamics simulations of local anesthetic articaine in a lipid bilayer. *Biophys Chem*. 2010;153:27–35.
31. Becker DE, Reed KL. Essentials of local anesthetic pharmacology. *Anesth Prog*. 2006;53:98–108; quiz 109–110.
32. Lundbaek JA, Birn P, Hansen AJ, et al. Regulation of sodium channel function by bilayer elasticity: the importance of hydrophobic coupling. Effects of micelle-forming amphiphiles and cholesterol. *J Gen Physiol*. 2004;123:599–621.
33. Tsuchiya H, Ueno T, Mizogami M, Takakura K. Do local anesthetics interact preferentially with membrane lipid rafts? Comparative interactivities with raft-like membranes. *J Anesth*. 2010;24:639–642.
34. Mildner J, Wnętrzak A, Dynarowicz-Latka P. Cholesterol and cardiolipin importance in local anesthetics-membrane interactions: the Langmuir Monolayer study. *J Membr Biol*. 2019;252:31–39.
35. Coskun Ü, Simons K. Cell membranes: the lipid perspective. *Structure*. 2011;19:1543–1548. Available at: <http://linking-hub.elsevier.com/retrieve/pii/S0969212611003649>. Accessed November 9, 2011.
36. Lingwood D, Simons K. Lipid rafts as a membrane-organizing principle. *Science*. 2010;327:46–50. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20044567>. Accessed October 31, 2013.
37. Wang J, Richards DA. Segregation of PIP2 and PIP3 into distinct nanoscale regions within the plasma membrane. *Biol Open*. 2012;1:857–862. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3507238&tool=pmcentrez&rendertype=abstract>. Accessed April 4, 2014.
38. Betzig E, Patterson GH, Sougrat R, et al. Imaging intracellular fluorescent proteins at nanometer resolution. *Science*. 2006;313:1642–1645.
39. Hess ST, Girirajan TPK, Mason MD. Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. *Biophys J*. 2006;91:4258–4272.
40. Mohammad S, Parmryd I. Cholesterol depletion using methyl- β -cyclodextrin. *Methods Mol Biol*. 2015;1232:91–102.
41. Raucher D, Sheetz MP. Phospholipase C activation by anesthetics decreases membrane-cytoskeleton adhesion. *J Cell Sci*. 2001;114:3759–3766.
42. Trevisani M, Smart D, Gunthorpe MJ, et al. Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. *Nat Neurosci*. 2002;5:546–551.
43. Chung HW, Petersen EN, Cabanos C, et al. A molecular target for an alcohol chain length cutoff. *J Mol Biol*. 2019;431:196–209.
44. Julius D. TRP channels and pain. *Annu Rev Cell Dev Biol*. 2013;29:355–384. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24099085>. Accessed March 24, 2014.
45. Tan CH, Onsiong MK. Pain on injection of propofol. *Anaesthesia*. 1998;53:468–476.
46. Herold KF, Sanford RL, Lee W, Andersen OS, Hemmings HC. Clinical concentrations of chemically diverse general anesthetics minimally affect lipid bilayer properties. *Proc Natl Acad Sci USA*. 2017;114:3109–3114.
47. Nayeibosadri A, Petersen EN, Cabanos C, Hansen SB. A membrane thickness sensor in TREK-1 channels transduces mechanical force. *SSRN Electron J*. 2018. Available at: <https://www.ssrn.com/abstract=3155650>. Accessed April 8, 2018.
48. Dong YY, Pike ACW, Mackenzie A, et al. K2P channel gating mechanisms revealed by structures of TREK-2 and a complex with Prozac. *Science*. 2015;347:1256–1259.
49. Kindler CH, Yost CS. Two-pore domain potassium channels: new sites of local anesthetic action and toxicity. *Reg Anesth Pain Med*. 2005;30:260–274.