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Phospholipase D2 specifically regulates TREK potassium channels via direct interaction and local production of phosphatidic acid

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Membrane lipids serve as second messengers and docking sites for proteins and play central roles in cell signaling. A major question about lipid signaling is whether diffusible lipids can selectively target specific proteins. One family of lipid-regulated membrane proteins is the TWIK-related K channel (TREK) subfamily of K2P channels: TREK1, TREK2, and TWIK-related arachdonic acid stimulated K$^+$ channel (TRAAK). We investigated the regulation of TREK channels by phosphatidic acid (PA), which is generated by phospholipase D (PLD) via hydrolysis of phosphatidylcholine. Even though all three of the channels are sensitive to PA, we found that only TREK1 and TREK2 are potentiated by PLD2 and that none of these channels is modulated by PLD1, indicating surprising selectivity. We found that PLD2, but not PLD1, directly binds to the C terminus of TREK1 and TREK2, but not to TRAAK. The results have led to a model for selective lipid regulation by localization of phospholipid enzymes to specific effector proteins. Finally, we show that regulation of TREK channels by PLD2 occurs natively in hippocampal neurons.

potassium channels | neuron excitability | alcohol | micro-regulatory domain | K2P2.1

Growing evidence indicates that the trafficking and function of membrane proteins, including ion channels and receptors, can be regulated by both their associated protein and lipid environments. Membrane lipids play a key role in intracellular signal transduction via lipid mediators that act as second messengers and docking sites for proteins. Membrane phospholipids, specifically, function as signaling molecules that are able to exert their effects on membrane proteins dynamically in conjunction with enzymes, such as phospholipases, which alter their phosphate head groups. Phospholipase D (PLD) is an enzyme that catalyzes the hydrolysis of the membrane phospholipid phosphatidylcholine to produce phosphatidic acid (PA). PA, much like phosphatidylinositol-4,5-bisphosphate (PIP2), is a second messenger that is involved in a variety of cellular functions such as cell proliferation, cytoskeleton organization, morphogenesis, and vesicle trafficking (1–3). However, unlike PIP2, following its production by PLD, PA is extremely short-lived and is rapidly converted to DAG by DAG kinase, which raises the question of how PLD activity can effectively regulate cellular processes (4, 5). Notably, primary alcohols can compete with water as a substrate for PLD2, which can lead to the production of phosphatidylethanol (PEtOH) or phosphatidyl-butan-1-ol (P-1-BtOH) rather than PA (6). There are two mammalian isoforms of PLD, PLD1 and PLD2, which share 50% amino acid identity and are both widely expressed in the nervous system (7).

The family of K_{2P} channels serves as a hub for the generation and regulation of a negative resting membrane potential throughout the nervous system. The members of the TWIK-related K channel (TREK) subfamily of K_{2P} channels, TREK1 (K2P2.1), TREK2 (K2P10.1), and the more evolutionarily distant TWIK-related arachdonic acid stimulated K$^+$ channel (TRAAK) (K2P4.1) channel, are widely expressed in the nervous system (8, 9). TREK1 gene knock out produces mice with reduced sensitivity to volatile anesthetics (10), impaired neuroprotection afforded by PUFAs against ischemia (10), and altered pain perception (11). In addition, loss of TREK1 renders mice resistant to depression, suggesting TREK1 as a candidate target for antidepressant medications (12). Although classical methods of genetic knockout or pharmacological approaches have been used for most work on TREK channels, we recently developed the photoswitchable conditional subunit (PCS) method, which allows us to endow endogenous TREK1 channels with light sensitivity. The PCS technique allows for the study of native TREK1 channels without the need for transgenic manipulation or nonspecific pharmacological agents. Previously, we used the TREK1-PCS method to discover a role for TREK1 in mediating the hippocampal $GABA_B$ response (13).

In many cases, regulation of membrane proteins is mediated by the organization of complexes between various proteins and signaling molecules that serve to enhance both the speed and specificity of the regulation (14, 15). For example, TREK1 interacts with AKAP150 (AKAP79), a scaffolding protein, which brings protein kinase A (PKA) into the proximity of TREK1 to

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Significance

Our work provides evidence for a mechanism for the formation of membrane microdomains in which the local concentration of a phospholipid can change independently of the bulk membrane to confer selectivity on membrane protein regulation. We found that, despite the fact that all TWIK-related K channel (TREK) family members are sensitive to phosphatidic acid (PA), only TREK1 and TREK2 are potentiated by phospholipase D2 (PLD2) (which produces PA), but not by PLD1. This surprising specificity is due to the direct binding of PLD2 to TREK. This binding allows a local PA production that tonically activates the channel. Furthermore, we found the local signaling via PA to have a secondary focusing effect for primary alcohols, which inhibit the channel by altering the PA microdomain.

Author contributions: Y.C., J.L., E.Y.I., and G.S. designed research; Y.C., J.L., M.A.K., and G.S. performed research; M.A.K. and F.L. contributed new reagents/analytic tools; Y.C., J.L., and G.S. analyzed data; and Y.C., J.L., E.Y.I., and G.S. wrote the paper.

The authors declare no conflict of interest.

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facilitate specific regulation of the cytoplasmic domain of TREK1 by PKA-mediated phosphorylation (2, 16, 17). In the case of phospholipids, one potential mechanism for specific regulation is spatiotemporal segregation where the local concentrations of specific phospholipids are either dynamically increased or decreased relative to the bulk of the membrane.

In this study, we report an inhibitory effect of protracted exposure to primary alcohols on TREK1 and TREK2 channels. We investigated the metabolic pathway involved in this indirect regulation and found that TREK1 and TREK2, but not TRAAK, are specifically potentiated by PLD2, but not PLD1. We also provide evidence that the specificity of this regulation is due to the direct binding of PLD2 to TREK channels. Furthermore, using a catalytically inactive mutant of PLD2 to compete with endogenous PLD2, we were able to reduce TREK current by decreasing the local PA concentration in the vicinity of the channel. We then studied the functional coupling of native TREK1 channels with endogenous PLD2 in hippocampal neurons and found that PLD2-mediated regulation is associated with tonic potentiation of the basal TREK current. These findings demonstrate a previously unidentified mechanism of regulation of an ion channel by direct interaction with a phospholipase that is able to locally modulate the phospholipid composition of the membrane.

Results

TREK1 Is Inhibited by Protracted, but Not Acute, Primary Alcohol Application. TREK channels can be stimulated by phospholipids, including directly applied PA (18), but so far there has been no determination of whether such PA-mediated activation is regulated. Because alcohols target PLD (6) and PLD catalyzes the production of PA (19), we wondered whether alcohol might modulate TREK channels through PLD. A diverse population of potassium channels are directly regulated by ethanol, including BK (20), SK (21), K_V (22), and GIRK (23). We initially investigated the possible regulation of TREK1 by alcohols in a heterologous system. We first tested primary alcohols and found that acute application of either 0.25% butan-1-ol (27 mM) or 0.6% ethanol (104 mM) for ∼1 min did not modify TREK1 current in HEK 293T cells (Fig. 1 *A* and *B*). However, protracted (≥1 h) application of either of these primary alcohols reduced TREK1 current by around 50% (Fig. 1 *C* and *F*) (current densities were 39 ± 5 pA/pF for TREK1, 18 ± 5 pA/pF for TREK1 plus ethanol, $P < 0.05$; and 18 ± 4 pA/pF for TREK1 plus butan-1-ol, $P < 0.05$). We then tested secondary alcohols and found that, unlike ethanol or butan-1-ol, protracted application of 0.25% butan-2-ol did not modify TREK1 current (Fig. 1 *D* and *E*) (current density was 43 ± 6 pA/pF, $P > 0.4$).

We then investigated the potential regulation of native TREK1 current by alcohol in primary cultures of hippocampal neurons. We expressed an engineered TREK1-photoswitchable conditional subunit (TREK1-PCS) to endow light sensitivity to the native TREK1 channels (24) (*SI Appendix, SI Materials and Methods*). As in HEK 293T cells, protracted (≥1 h) application of 0.6% ethanol reduced TREK1 current by around 70% compared with untreated cells (Fig. 1*F*). These results suggest that primary alcohols modulate native and heterologously expressed TREK1 channels via an indirect mechanism, such as a metabolic effect on a second messenger that regulates TREK1.

PLD2-Mediated Potentiation of TREK1 Current Is Reversed by Protracted Primary Alcohol Treatment and the PLD Inhibitor FIPI. We next asked whether the observed effects of alcohol on TREK1 could be mediated by PLD. To address this question, we first set out to determine whether PLD can regulate TREK activity. We tested this hypothesis by coexpressing TREK1 and PLD2 and found that PLD2 coexpression increased TREK1 current by more than fourfold (Fig. 2*A*) (current densities for TREK1 and TREK1 plus PLD2 were 19 ± 2 pA/pF and 86 ± 9 pA/pF, respectively, $P < 0.001$).

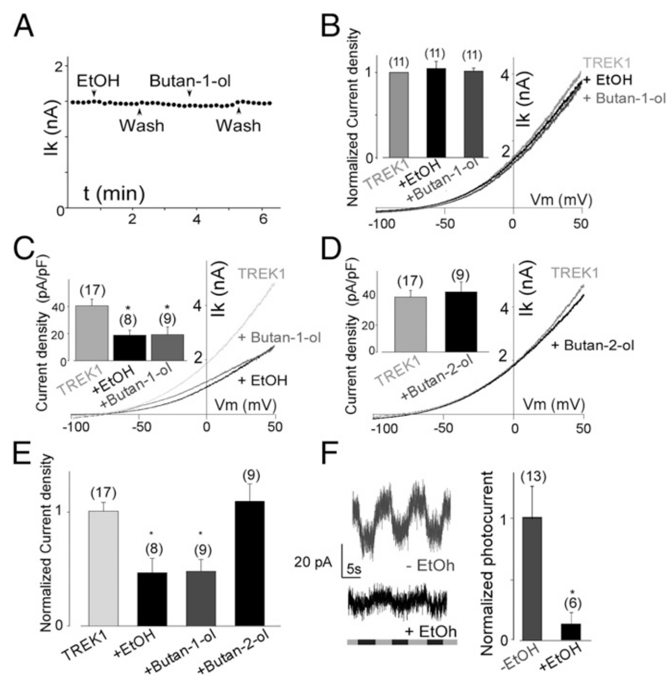

Fig. 1. TREK1 is inhibited by protracted but not acute primary alcohol application. (*A* and *B*) Effect of acute primary alcohol application on TREK1 current. (*A*) Representative example of TREK1 current stability following brief (∼1 min) primary alcohol application in HEK 293T cells. (*B*) Summary of effect of acute primary alcohol application on TREK1 current. Current was elicited by voltage-ramps (from −100 to +50 mV, 1s in duration). (*Inset*) Normalized TREK1 current density after acute primary alcohol application. (*C*) Effect of protracted primary alcohol application on TREK1 current in HEK 293T cells. Current was elicited by voltage-ramps (from −100 to +50 mV, 1s in duration). (*Inset*) Normalized TREK1 current densities after acute primary alcohol application. (*D*) Effect of protracted butan-2-ol application. (*Inset*) TREK1 current densities before and after protracted butan-2-ol application are shown. (*E*) Summary of normalized TREK1 current densities after protracted alcohol application. Student *t* tests (*$P < 0.05$) show the difference between TREK1 and TREK1 after ethanol, butan-1-ol, or butan-2-ol application. The numbers of cells tested are indicated in parentheses. (*F*) Protracted ethanol application decreases native TREK1 photocurrent in hippocampal neurons. (*Left*) Representative examples of TREK1 photocurrent with (black trace) and without (gray trace) protracted (≥1 h) ethanol application. (*Right*) Average normalized TREK1 photocurrent amplitudes with and without protracted ethanol application (≥1 h).

Because the production of PA by PLD2 is inhibited by primary alcohols, we wondered whether protracted treatment with primary alcohols would affect the potentiation of TREK1 by PLD2. In the presence of coexpressed PLD2, protracted application of either ethanol or butan-1-ol reduced TREK1 current by 71% (Fig. 2 *B* and *C*) (current densities were 39 ± 5 pA/pF for TREK1 alone, 139 ± 21 pA/pF for TREK1 plus PLD2, 24 ± 5 pA/pF for TREK1 plus PLD2 plus ethanol, and 22 ± 5 pA/pF for TREK1 plus PLD2 plus butan-1-ol; $P < 0.01$ for TREK1 plus PLD2 vs. TREK1 plus PLD2 plus ethanol and $P < 0.01$ for TREK1 plus PLD2 vs. PLD2 plus butan-1-ol). This inhibition is reversed after washout of ethanol within ∼30 min (*SI Appendix*, Fig. S1). Notably, the current densities observed for TREK1 coexpressed with PLD2 and treated with ethanol or butan-1-ol were not significantly different from the current amplitude for TREK1 expressed alone after primary alcohol incubation (Fig. 2*E*) ($P > 0.4$ for TREK1 plus ethanol vs. TREK1 plus PLD2 plus ethanol and $P > 0.5$ for TREK1 plus butan-1-ol vs. TREK1 plus PLD2 plus butan-1-ol). Consistent with the previous section, the secondary alcohol butan-2-ol failed to modify TREK1 current when coexpressed with PLD2 (Fig. 2*E*) (current density was 127 ± 44 pA/pF, $P > 0.7$). Because primary alcohols, but not secondary alcohols, can

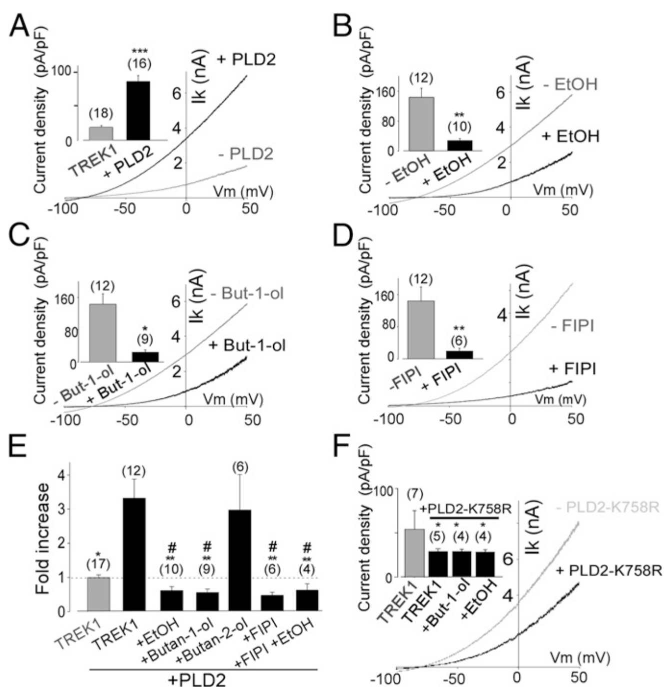

Fig. 2. TREK1 is potentiated by PLD2 in a PA-dependent manner. (*A* and *B*) TREK1 is potentiated by PLD2 coexpression. (*A*) Representative traces showing that PLD2 coexpression increases TREK1 current. (*Inset*) Bar graph showing the TREK1 current densities in the presence or absence of coexpression of PLD2. Student *t* test (***$P < 0.001$) shows the difference between TREK1 and TREK1 coexpressed with PLD2. The numbers of cells tested are indicated in parentheses. (*B–E*) Primary alcohols and FIPI abolish the potentiation of TREK1 current by PLD2. Representative traces showing effects of protracted ethanol (*B*), butan-1-ol (*C*), or FIPI (*D*) application on TREK1 current in the presence of PLD2. (*Insets*) TREK1 current densities before and after treatments. (*E*) Summary of normalized amplitude of TREK1 current in the presence of PLD2 after protracted treatment with alcohol, FIPI, or both. Student *t* tests (*$P < 0.05$, **$P < 0.01$) show the difference between TREK1 coexpressed with PLD2 and after ethanol, butan-1-ol, or butan-2-ol application. Student *t* tests ($^{\#}P < 0.05$) show the difference between TREK1 and TREK1 coexpressed with PLD2 after either ethanol or butan-1-ol or butan-2-ol application. The numbers of cells tested are indicated in parentheses. (*F*) A catalytically inactive mutant of PLD2 (PLD2-K758R) decreases TREK1 current. Representative traces showing that coexpression of PLD2-K758R decreases TREK1 current. (*Inset*) Summary of TREK1 current densities in the presence or absence of coexpressed PLD2-K758R and before and after protracted primary alcohol application are shown. Student *t* tests (*$P < 0.05$) show the difference between TREK1 and TREK1 coexpressed with PLD2-K578R with or without protracted primary alcohol exposure. The numbers of cells tested are indicated in parentheses.

serve as alternative substrates in PLD-catalyzed transphosphatidylation to produce phosphatidylalcohols instead of PA, these results suggest that the inhibition of TREK1 by primary alcohols is mediated by inhibition of the production of PA by PLD2. To confirm that the effect of alcohol on TREK1 is directly linked to inhibition of PA production, we used the recently developed, specific PLD inhibitor 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) (25). Incubation for 1 h with FIPI reduced TREK1 plus PLD2 current by 76% to a level similar to primary alcohol incubation (Fig. 2 *D* and *E*) (current density was 19 ± 4 pA/pF for TREK1 plus PLD2 plus FIPI; $P < 0.01$ for TREK1 plus PLD2 vs. TREK1 plus PLD2 plus FIPI). Furthermore, coapplication of primary alcohol and FIPI did not show an additional inhibitory effect, indicating that both treatments may work through the same mechanism (Fig. 2*E*) (current density was 24 ± 6 pA/pF; $P > 0.4$ for TREK1 plus PLD2 plus FIPI vs. TREK1 plus PLD2 plus FIPI plus EtOH and $P > 0.6$ for TREK1 plus PLD2 plus EtOH vs. TREK1 plus PLD2 plus FIPI plus EtOH). In addition, FIPI reduced TREK1 current densities to similar levels with or without PLD2 coexpression as was also observed for primary alcohol treatment (*SI Appendix*, Fig. S2). These results strongly support the idea that PLD2 potentiates TREK1 channel activity through production of PA.

PLD2-Mediated Potentiation of TREK1 Requires Basic Residues in the TREK1 C Terminus. Our results so far indicate that regulation of TREK1 channels by PLD2 depends on the production of PA by PLD2. To further test this idea, we turned our attention to the portion of the TREK1 channel that is known to be essential for PA regulation, and where PA has been conjectured to bind (18). Stimulation of TREK1 by PA depends on five positively charged residues in the TREK1 carboxyl-terminal domain (Ctd) and the negative charge of the phosphate group of PA. This modulation can be eliminated by mutation of the positively charged residues to produce "TREK1-pentaA."

To test whether the ability to sense phospholipids is required for TREK1 to be potentiated by PLD2, we examined the effect of PLD2 coexpression on TREK1-pentaA. Unlike in wild-type TREK1, TREK1-pentaA was not potentiated by PLD2 coexpression (*SI Appendix*, Fig. S3). Together with the suppression of PLD2 modulation of TREK1 by primary alcohols and FIPI, this result argues that enzymatic production of PA by PLD2 is required for stimulation of TREK1.

A Catalytically Inactive Mutant of PLD2 Decreases TREK1 Current. Our findings that protracted exposure to primary alcohols and FIPI reduces TREK1 current in cells transfected with only TREK1, that the magnitude of this suppression is far greater when PLD2 is coexpressed, and that the current that remains after primary alcohol or FIPI treatment is the same whether or not PLD2 is coexpressed suggest that endogenous PLD2 tonically stimulates TREK1 and that this stimulation is suppressed by primary alcohols or FIPI. To test whether TREK1 is regulated by endogenous PLD2, we coexpressed a catalytically inactive mutant of PLD2 (PLD2-K758R) (26, 27). Coexpression of PLD2-K758R significantly decreased the TREK1 current (Fig. 2*F*) (current densities were 51 ± 7 pA/pF and 28 ± 3 pA/pF for TREK1 and TREK1 plus PLD2-K758R, respectively; $P < 0.05$). This suppression was similar to that elicited by protracted application of primary alcohols and FIPI (Fig. 1 and *SI Appendix*, Fig. S2). Moreover, protracted primary alcohol application did not further inhibit TREK1 current when PLD2-K758R was coexpressed (Fig. 2*F*) (current densities were 30 ± 4 pA/pF for TREK1 plus PLD2-K758R plus ethanol, $P > 0.6$; and 29 ± 4 pA/pF for TREK1 plus PLD2-K758R plus butan-1-ol, $P > 0.7$), which is consistent with a dependency of primary alcohol regulation of TREK on PLD2.

The ability of the overexpressed catalytically inactive form of PLD2 to prevent endogenous wild-type PLD2 from stimulating TREK1 could be explained by competition for localization to the vicinity of TREK1. We therefore examined this possibility by asking whether the channel and enzyme directly associate.

PLD2, but Not PLD1, Specifically Regulates TREK1 Through Direct Interaction. Having found that PLD2 modulates TREK1, we asked whether a related phospholipase D, PLD1, has the same effect. Whereas coexpression of PLD2 significantly increased TREK1 current (Fig. 3 *A* and *B*) (current densities were 168 ± 28 pA/pF for TREK1 plus PLD2 versus 41 ± 8 pA/pF for TREK1 alone; $P < 0.001$), coexpression of PLD1 had no effect on TREK1 current (Fig. 3 *A* and *B*) (current density was 58 ± 9 pA/pF for TREK1 plus PLD1; $P > 0.6$).

We asked whether the ability of PLD2, but not PLD1, to stimulate TREK1 could be accounted for by direct association of only PLD2 with the TREK1 channel. PLD2 was coimmunoprecipitated with TREK1, but PLD1 was not (Fig. 3*C*). As a control, in the absence of TREK1 expression, anti-TREK1 antibodies did not precipitate PLD2 (Fig. 3*C*). Furthermore,

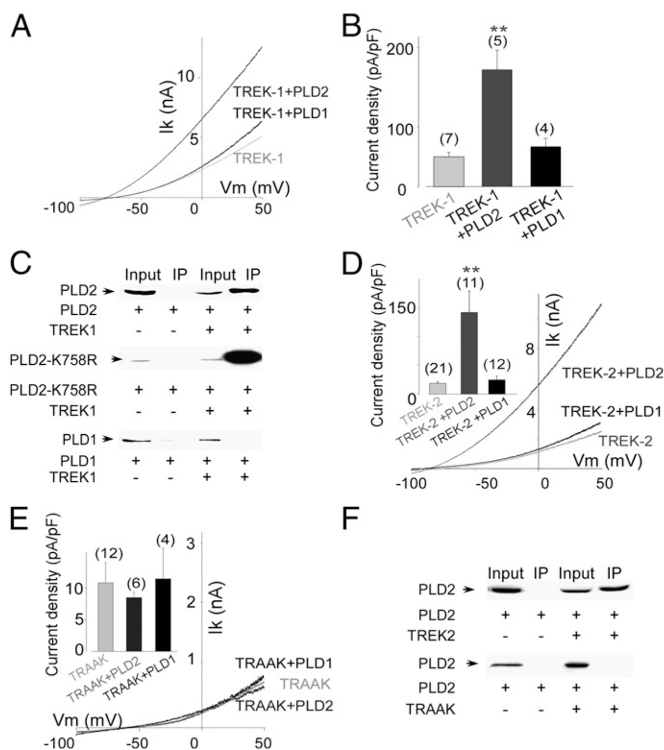

Fig. 3. PLD2, but not PLD1, specifically regulates TREK1 and TREK2 but not TRAAK. (*A*–*C*) Unlike PLD2, PLD1 does not regulate TREK1. (*A*) Representative traces showing the effects of PLD1 and PLD2 coexpression on TREK1 current. (*B*) Summary of TREK1 current in the presence or absence of either PLD1 or PLD2. Student *t* tests (***P* < 0.01) show the difference between TREK1 and TREK1 coexpressed with PLD2 or PLD1. The numbers of cells tested are indicated in parentheses. (*C*) Coimmunoprecipitation of PLD2 (*Top*), PLD2-K758R (*Middle*), or PLD1 (*Bottom*) by anti-TREK1 antibodies from transfected HEK 293T cells. (*D*–*F*) PLD2 specifically regulates TREK1 and -2, but not TRAAK. (*D*) PLD2 coexpression potentiates TREK2 current whereas PLD1 does not. (*Inset*) TREK2 current densities in the presence or the absence of either coexpressed PLD2 or PLD1. (*E*) PLD2 or PLD1 coexpression does not alter TRAAK current. (*Inset*) TRAAK current densities in the presence or absence of coexpressed PLD2 or PLD1. (*F*, *Upper*) Coimmunoprecipitation of PLD2 by anti-TREK2 antibodies from transfected HEK 293T cells. (*Lower*) Coimmunoprecipitation of PLD2 by anti-TRAAK antibodies from transfected HEK 293T cells.

using immunocytochemistry, we found that TREK1 and PLD2 colocalize in HEK 293T cells (*SI Appendix*, Fig. S4*A*), but TREK1 and PLD1 do not (*SI Appendix*, Fig. S4*B*). In addition, PLD2-K758R is also able to coimmunoprecipitate with TREK1 (Fig. 3*C*), confirming the hypothesis that it competes with endogenous PLD2 to decrease TREK1 current (Fig. 2*F*). Taken together, these results indicate that PLD2 interacts with TREK1 but that PLD1 does not and that this observation explains the exclusive modulation of the channel by PLD2.

PLD2 Is Able to Potentiate TREK2, but Not TRAAK. Having observed that PLD2 interacts with and regulates TREK1 but that PLD1 does not, we asked whether the interaction and modulation extend to other members of this K_{2P} subfamily of channels. To test this possibility, we coexpressed PLD2 with TREK2 and the more distantly related TRAAK channel. TREK2 and TRAAK, like TREK1, are lipid and mechano-gated and display the same PA-sensitivity as TREK1 (18) (*SI Appendix*, Fig. S5). PLD2 coexpression significantly increased TREK2 current (Fig. 3*D*) (current densities were 24 ± 3 pA/pF for TREK2 alone and 144 ± 29 pA/pF for TREK2 plus PLD2; *P* > 0.01) but did not significantly affect TRAAK current (Fig. 3*E*) (current densities were 8 ± 3 pA/pF for TRAAK alone and 8 ± 3 pA/pF for TRAAK plus PLD2; *P* > 0.8). Consistent with these results, PLD2 was coimmunoprecipitated with TREK2 (Fig. 3*F*), but not with TRAAK (Fig. 3*F*). As a control, in the absence of TREK2 expression, anti-TREK2 antibodies did not precipitate PLD2 (Fig. 3*F*), confirming the specificity of the assay. In addition, immunocytochemistry of PLD2 and TREK2 showed colocalization (*SI Appendix*, Fig. S4*C*) whereas TRAAK and PLD2 showed no overlap (*SI Appendix*, Fig. S4*D*). Furthermore, we found that PLD1 coexpression did not affect TREK2 current (*SI Appendix*, Fig. S6*E*) (current densities were 31 ± 11 pA/pF for TREK2 alone and 39 ± 21 pA/pF for TREK2 plus PLD1; *P* > 0.9 for TREK2 alone vs. TREK2 plus PLD1). These results suggest that PLD2 interacts with, and thus regulates, TREK2, which is closely related to TREK1, but not the more distantly related TRAAK.

To further test the idea that direct interaction between enzyme and channel is necessary for the channel to be potentiated, we forced an interaction between TRAAK and PLD2 by fusing the proteins to one another to produce a PLD2-TRAAK tandem (Fig. 4*A*). PLD2-TRAAK showed significantly increased current compared with TRAAK alone (Fig. 4*B*) (current densities were 5.5 ± 2 pA/pF for TRAAK alone and 51 ± 13 pA/pF for PLD2-TRAAK; *P* < 0.01). Similarly to TREK1 and TREK2, protracted application of ethanol reduced the current density of PLD2-TRAAK to the amplitude observed for TRAAK alone, indicating that PLD2-mediated production of PA is required for the potentiation observed with PLD2-TRAAK (*SI Appendix*, Fig. S8). These results are consistent with the notion that anchoring of PLD2 to the channel enables it to regulate the channel's activity via its local enzymatic activity.

We next investigated to which part of the channel PLD2 binds. We hypothesized that this interaction may take place in the Ctd because this region is the major part of TREK channels that is accessible to the cytosol and this domain is highly conserved

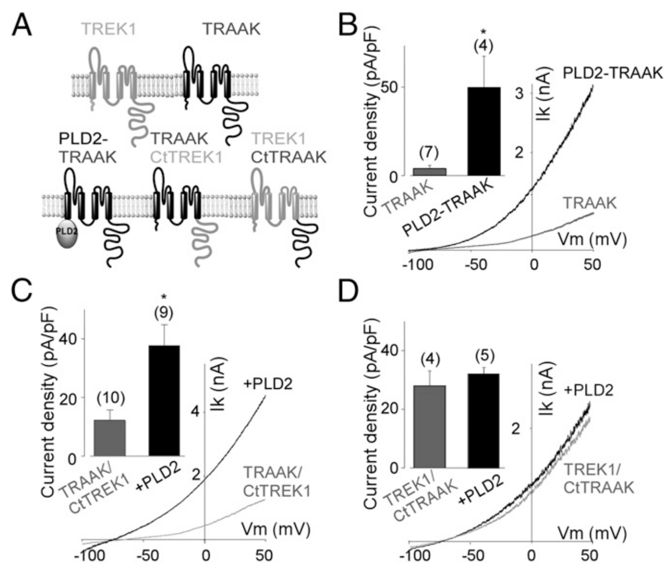

Fig. 4. Binding of PLD2 to the cytoplasmic tails of TREK1 and TREK2 enables specific regulation. (*A*) Schematic representing the different constructs used. (*B*) N-terminal fusion of PLD2 to TRAAK potentiates TRAAK current. (*Inset*) Comparison of TRAAK and PLD2-TRAAK current densities. (*C* and *D*) The specificity of PLD2 regulation of TREK1 is mediated by the cytoplasmic tail of TREK1. (*C*) PLD2 coexpression potentiates current from a chimeric TRAAK/Ct-TREK1 channel containing the core region of TRAAK fused to the cytoplasmic Ctd of TREK1. (*Inset*) TRAAK/Ct-TREK1 current densities in the presence or absence of coexpressed PLD2. (*D*) PLD2 coexpression does not alter current from a chimeric TREK1/Ct-TRAAK channel containing the core region of TREK1 fused to the cytoplasmic Ctd of TRAAK. (*Inset*) TREK1/Ct-TRAAK current densities in the presence or absence of coexpressed PLD2. Student *t* tests (**P* < 0.05) show the difference between PLD2 potentiation of PLD2-TRAAK, TRAAK/CtTREK1 or TREK1/CtTRAAK. The numbers of cells tested are indicated in parentheses.

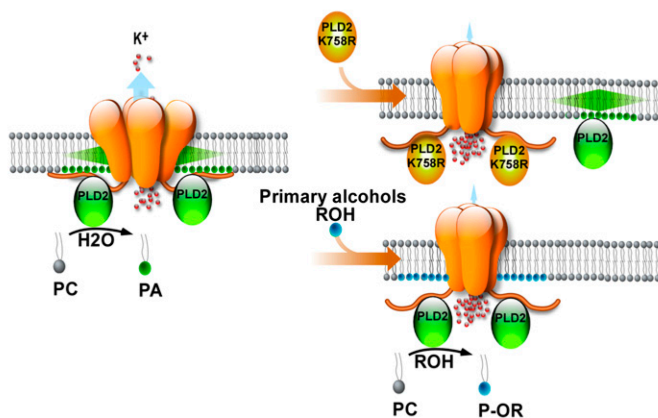

Fig. 5. Model of channel regulation by PLD2. PLD2 is associated with TREK1 and creates a microdomain rich in PA (the gradient of PA is represented by a green arrow) that activates the channel at rest. PLD2-K758R displaces the endogenous PLD2, which reduces the local PA concentration near the channel and therefore reduces TREK channel activity. Alternatively, primary alcohols (ROH) compete with water in the catalytic site of PLD2, which also leads to a reduction of the local PA concentration near the channel and causes a decrease in TREK channel activity.

between TREK1 and -2 (9). To test this hypothesis, we designed chimeras between TRAAK and TREK1 to see whether we could transfer PLD2 sensitivity to TRAAK. The Ctd of TRAAK was replaced by the corresponding Ctd of TREK1 to form TRAAK/Ct-TREK1, and the Ctd of TREK1 was replaced by the corresponding Ctd of TRAAK to form TREK1/Ct-TRAAK (Fig. 4*A*). Unlike wild-type TRAAK, TRAAK/Ct-TREK1 was sensitive to PLD2 (Fig. 4*C*) (current densities were 12 ± 3 pA/pF and 36 ± 7 pA/pF for TRAAK/Ct-TREK1 and TRAAK/CtTREK1 plus PLD2, respectively; $P < 0.05$). However, TREK1/Ct-TRAAK was not sensitive to PLD2 coexpression (Fig. 4*D*) (current densities were 26 ± 4 pA/pF and 29 ± 2 pA/pF for TREK1/Ct-TRAAK and TREK1/Ct-TRAAK plus PLD2 respectively; $P > 0.8$). These results indicate that the specificity of PLD2 depends on the TREK1 Ctd because the Ctd specifically binds to PLD2 (Fig. 5).

PA Regulates Native TREK1 Channels Through Physical Coupling Between TREK1 and Endogenous PLD2 in Hippocampal Neurons. TREK channels are natively expressed in the hippocampus where they contribute to the response to $GABA_B$ receptor activation and are inhibited by protracted primary alcohol application (Fig. 1*B*). Accordingly, we wondered whether hippocampal TREK1 channels are regulated by endogenous PLD2 and whether this measurement can explain their alcohol sensitivity. To investigate this regulation in the native hippocampal TREK1 channels, we coexpressed the catalytically inactive mutant of PLD2, PLD2-K758R, along with the TREK1-PCS (13).

In cultured hippocampal neurons transfected with the TREK1-PCS, alternating illumination between 380 nm and 500 nm modulated the resting membrane potential by 4.3 ± 0.9 mV (Fig. 6*A*). Coexpression of PLD2-K758R decreased this voltage change significantly (Fig. 6 *B* and *E*) (1.3 ± 0.2 mV; $P < 0.01$). Consistent with this voltage change decrease, at a holding potential of −20 mV, TREK1-PCS transfected neurons had photocurrents of 20 ± 4 pA (Fig. 6*C*) and coexpression of PLD2-K758R reduced the photocurrents to 4.8 ± 1.7 pA (Fig. 6 *D* and *F*) ($P < 0.01$) as was observed for protracted EtOH application (Fig. 1*B*). These results show that, in hippocampal neurons, native TREK1 and PLD2 coassemble and that this coassembly leads to a tonic increase in TREK1 activity.

Discussion

We report a novel mechanism for the specific regulation of ion channels by an enzyme that generates signaling lipids. We found that the K_{2P} potassium channels TREK1 and TREK2 are potentiated by the phospholipase PLD2, which produces the charged signaling phospholipid PA from phosphatidylcholine. Surprisingly, PLD2 was unable to regulate the related TRAAK channel despite the fact that TRAAK responds to PA in a similar manner to TREK1 and TREK2. Furthermore, we found that PLD1, which catalyzes the same reaction as PLD2, has no effect on TREK1 or TREK2, indicating that this regulation is specific to the subtype of enzyme and not based on a bulk effect on plasma-membrane composition.

We found that the specific regulation that we observed by only one of the PLDs on two of the three PA-sensitive K2P channels could be explained by selective colocalization. First, TREK1 and TREK2 directly interact with PLD2, but TRAAK does not, explaining the selective activation of the TREKs by PLD2. Second, PLD1 does not interact with TREK1, explaining its lack of effect. Third, fusion of the PLD2 to TRAAK renders TRAAK responsive to PLD2. Finally, replacement of the TRAAK Ctd with that of TREK1 endowed TRAAK with sensitivity to PLD2 whereas replacement of the TREK1 Ctd with that of TRAAK eliminated the sensitivity of TREK1 to PLD2. These results suggest that PLD2 specificity is due to direct interaction of PLD2 with either TREK1 or TREK2 via the Ctd of the channel. This interaction appears likely to be direct, but we cannot fully exclude the possibility of the presence of an adaptor protein that allows PLD2 and TREK to interact which each other. One possible adaptor, which is endogenously expressed at low levels in HEK293 cells, is AKAP79. However, we found that AKAP79 does not play a role in this regulation because shRNA, which targets AKAP79 (28), did not modify TREK1 regulation by PLD2 (*SI Appendix*, Fig. S10). We propose that, by complexing with TREK1 or TREK2, PLD2 is able to alter the local

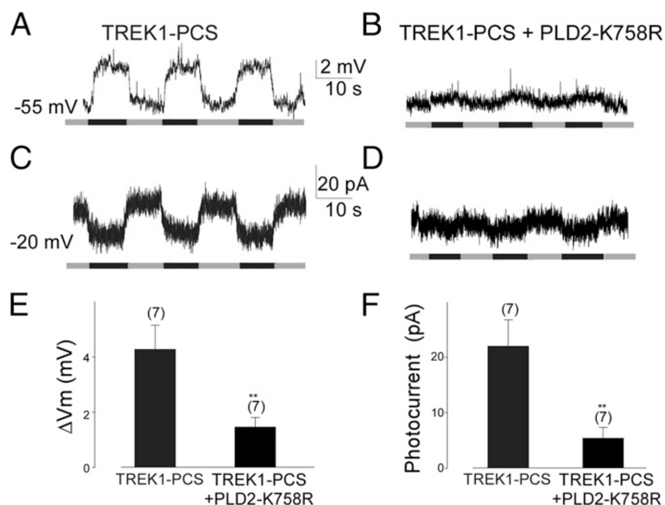

Fig. 6. PLD2-K758R decreases endogenous TREK1 current in hippocampal neurons. (*A*) Representative current-clamp recording from cultured hippocampal neurons expressing TREK1-PCS shows light modulation of membrane potential. The 380-nm light (in black) leads to channel blockade and depolarization whereas 500 nm light (in gray) unblocks the channels. (*B*) Same as in *A* for neurons coexpressing TREK1-PCS and PLD2-K758R. (*C*) Representative whole-cell voltage-clamp recording from hippocampal neurons expressing TREK1-PCS. (*D*) Same as in *C* for neurons expressing TREK1-PCS and PLD2-K758R. (*E*) Average resting membrane potential modulation induced by alternating illumination of neurons with 500 nm and 380 nm. (*F*) Average photocurrent induced by alternating illumination of neurons with 500 nm and 380 nm. Student *t* tests (**$P < 0.01$) show the difference between TREK1-PCS and TREK1-PCS coexpressed with PLD2-K758R. The numbers of cells tested are indicated in parentheses.

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concentration of PA in a microdomain around the channel to stimulate channel activity (Fig. 5). This mechanism is consistent with a short half-life of PA in the plasma membrane (5). In our model, the regulation of TREK channels by PLD2 is specific not because of the enzymatic product, but because the protein–protein interaction between enzyme and channel allows the channel to be directly coupled to the enzymatic production of PA in a way that is independent of the bulk concentration of PA. Independent pools of phospholipids that govern distinct functions in microdomains have been proposed before (29, 30). Our work provides an illustration of such a case and a mechanism by which it can result in specific regulation of a subset of K_{2P} potassium channels.

In addition to regulation by PLD2, we demonstrated that protracted, but not acute, application of primary alcohols inhibits TREK1 and TREK2. We provide evidence that this effect is mediated by PLD2 rather than direct interaction between alcohols and the channel, in contrast to what has been shown for GIRK channels (31). When exposed to primary alcohols such as ethanol or butan-1-ol, PLD2 catalyzes the production of the biologically inactive phospholipids PEtOH or P-1-BtOH rather than PA. Consistent with the hypothesis that regulation of TREK by alcohols is mediated by removal of tonic stimulation by PLD2, PLD2 is insensitive to secondary alcohols, which were also unable to regulate TREK1 or TREK2. We confirmed this mechanism by showing that primary alcohols also prevent the potentiation effect of PLD2 and that a catalytically inactive mutant of PLD2 is unable to potentiate TREK1 and renders TREK1 insensitive to protracted alcohol treatment. This mechanism may be a general means by which ethanol can induce long-term physiological changes by changing the phospholipid composition of the membrane.

Given the previously demonstrated cross-talk between the $GABA_B$ receptor, GIRK, and TREK and the established role of $GABA_B$ receptors in treatment of alcohol addiction, we wanted to address the possibility that regulation of TREK1 by PLD2, which mediates the effects of ethanol in heterologous systems, occurs natively in mammalian neurons. We tested this in hippocampal neurons using the TREK1-PCS technique (32). We found that displacement of the native, wild-type PLD2 by the catalytically inactive mutant PLD2-K578R decreased the endogenous TREK1 depolarization or current by fourfold as was also observed for protracted ethanol application. Based on this measurement, we concluded that, at rest, more than 75% of the hippocampal TREK1 current is associated with potentiation of TREK1 by PA. This result indicates that the regulation of TREK1 by PLD2 occurs natively in neurons of the hippocampus and may be important for mediating some of the effects of protracted ethanol consumption and its reversal by $GABA_B$ receptors (33).

In conclusion, association of specific isoforms of an enzyme that generates signaling lipids with select ion channel subtypes can confer specific regulation of those channels. We conjecture that this specificity is due to the high local concentration of the active lipid product near its site of action on the channel, resulting in strong regulation of the bound channel, whereas channels that do not interact with the enzyme are not regulated because the bulk concentration of that lipid does not reach levels that are high enough to act at a distance.

Materials and Methods

Standard molecular biological, biochemical, and electrophysiological techniques were used as described previously (13) and in *SI Appendix*. Briefly, HEK 293T cells were transiently cotransfected using Lipofectamine 2000 (Invitrogen). Electrophysiology was performed 24–72 h after transfection for HEK 293T and 3–6 d after transfection for hippocampal neurons.

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