Valproic acid (VPA), or 2-propyl pentanoic acid, was accidentally found to be an effective antiepileptic treatment in the 1960s. The drug is now used to treat around one-third of patients newly diagnosed with epilepsy (24), although its mechanism of action remains unclear (26). VPA is also licensed by the U.S. Food and Drug Administration for the treatment of bipolar disorder and is used for migraine prophylaxis. In addition to these effects, we have previously shown that prolonged VPA treatment attenuates inositol-(1,4,5)-trisphosphate (InsP3) signaling (12, 50, 62) using the social amoeba Dictyostelium discoideum, we show that an acute effect of VPA is the inhibition of chemotactic cell movement, a process partially dependent upon phospholipid signaling. Analysis of this process shows that VPA attenuates the signal-induced translocation of PHCrac-GFP from cytosol to membrane, suggesting the inhibition of phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) production. Direct labeling of lipids in vivo also shows a reduction in PIP and PIP2 phosphorylation following VPA treatment. We further show that VPA acutely reduces endocytosis and exocytosis—processes previously shown to be dependent upon PIP3 production. These results suggest that in Dictyostelium, VPA rapidly attenuates phospholipid signaling to reduce endocytic trafficking. To examine this effect in a mammalian model, we also tested depolarization-dependent neurotransmitter release in rat nerve terminals, and we show that this process is also suppressed upon application of VPA and an inhibitor of phosphatidylinositol 3-kinase. Although a more comprehensive analysis of the effect of VPA on lipid signaling will be necessary in mammalian systems, these results suggest that VPA may function to reduce phospholipid signaling processes and thus may provide a novel therapeutic effect for this drug.

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strate, PIP2. We show that in the presence of VPA, in vivo labeling of phospholipids in Dictyostelium is reduced, in agreement with a VPA-dependent reduction in the production of phosphatidylinositol-(3,4,5)-trisphosphate (PIP3). Since previous studies in Dictyostelium have shown that endocytosis and exocytosis are dependent upon PIP3 production (7, 46, 66), we analyzed the effect of VPA on these functions, and we show that VPA also reduces these processes. Translation of these studies to a mammalian synapse model shows that both VPA and a phosphatidylinositol 3-kinase (PI3K) inhibitor attenuate glutamate release. These data propose a novel mechanism of action of VPA by the modulation of phospholipid signaling.

MATERIALS AND METHODS

Reagents. All compounds used were supplied by Sigma-Aldrich Co., Ltd., apart from latrunculin B, which was from Molecular Probes, and the enhanced chemiluminescence reagent from Amershams. Complete protease inhibitor cocktail was from Roche Ltd. Phosphate buffer (PB) contained 16.5 mM potassium dihydrogen orthophosphate, 3.8 mM dipotassium hydrogen orthophosphate, pH 6.2.

Chemotaxis experiments. Chemotaxis assays, live cell imaging, and imaging analysis were performed as described previously (63). Dictyostelium strain AX2 cells, developed for 6 h, were plated on a four-well chamber for uniformly applied stimulation and a one-well chamber for the microinjector-delivered cyclic AMP (cAMP) stimulation (Nalgene Nunc International, Naperville, IL) and allowed to adhere to the cover glass for 10 min in PB. Live cells were imaged using a Zeiss laser scanning microscope, LSM 510 META, with a 40× objective and numerical aperture of 1.3 or a 63× objective. To monitor PH Crac-GFP, the interference contrast Plan-Neofluar objective. To monitor PH Crac-GFP, the specimens were excited with a laser line at 488 nm for GFP, and fluorescent emission was recorded between 505 and 530 nm. Live cells were imaged during stimulations with a uniform concentration (100 nM) or a gradient of cAMP as previously described (63) in the presence or absence of 1 mM VPA. To quantify cell movement, images were analyzed using the DIAS software package (55).

Phospholipid labeling. A saponin-based cell permeabilization protocol for Dictyostelium was adapted from one for mammalian cells (17). Dictyostelium strain AX2 cells were developed for 6 h as previously described (5), transferred to still dishes (2.5 cm), allowed to settle to give a confluent monolayer in PB, and allowed to adhere to the cover glass for 10 min in PB. Live cells were imaged using a Zeiss laser scanning microscope, LSM 510 META, with a 40× objective and numerical aperture of 1.3 or a 63×, 1.4 numerical aperture oil differential interference contrast Plan-Neofluar objective. To monitor PH Crac-GFP, the specimens were excited with a laser line at 488 nm for GFP, and fluorescent emission was recorded between 505 and 530 nm. Live cells were imaged during stimulations with a uniform concentration (100 nM) or a gradient of cAMP as previously described (63) in the presence or absence of 1 mM VPA. To quantify cell movement, images were analyzed using the DIAS software package (55).

Endocytic trafficking. The uptake of particles and fluid, as well as exocytosis, was measured in AX2 cells as described previously (16, 33, 45).

Synaptosome preparation and glutamate release. Isolated nerve terminals (synaptosomes) were prepared from the cerebral cortices of 2-month-old male Sprague-Dawley rats by homogenization, differential centrifugation, and Percoll gradient purification as described elsewhere (52). Glutamate release from synaptosomes (0.1 to 0.2 mg of total protein) resuspended in 1 ml of HEPES-buffered incubation medium (140 mM NaCl, 5 mM KCl, 5 mM NaHCO3, 1 mM MgCl2, 1.2 mM Na2HPO4, 10 mM glucose, and 20 mM HEPES, pH 7.4) was assayed by online fluorimetry (21, 40, 53). CaCl2 (1 mM) and drugs were added as indicated at the start of incubation at 37°C in a spectrophotometer chamber. Ca2+ and drugs were added as indicated at the start of incubation at 37°C in a spectrophotometer chamber. Ca2+ and drugs were added as indicated at the start of incubation at 37°C in a spectrophotometer chamber. Ca2+ and drugs were added as indicated at the start of incubation at 37°C in a spectrophotometer chamber. Ca2+ and drugs were added as indicated at the start of incubation at 37°C in a spectrophotometer chamber. Ca2+ and drugs were added as indicated at the start of incubation at 37°C in a spectrophotometer chamber.

Acceleration of VPA by the modulation of phospholipid signaling.

RESULTS

VPA inhibits chemotactic cell movement. The social amoeba Dictyostelium has been used to elucidate signaling pathways involved in cell movement (11, 14, 63) and to examine the cellular targets of bipolar disorder treatments (12, 62). The latter work demonstrated that VPA blocks the aggregation of cells into fruiting bodies. To examine this process in more detail, we analyzed the movement of aggregation-competent cells in a chemoattractant (cAMP) gradient (see the movie in Fig. S1 in the supplemental material). Cell movement towards the cAMP source is pulsatile, with periods of rapid migration followed by a transient slowing (Fig. 1A). Exposure to VPA at 1 mM, a concentration close to clinical plasma levels in treated patients of 0.3 to 0.6 mM (23), blocked chemotaxis within 5 min (Fig. 1B). This result was of particular interest since the analysis of this rapid effect may help in understanding the acute mechanism by which intravenous VPA can stop seizures (within 20 min of administration) (64).

VPA inhibits signal-induced translocation of PHCrac-GFP. The receptor-mediated regulation of phosphatidylinositol signaling plays an important role in the chemotactic response (49), and this pathway is highly conserved in eukaryotic cells. In Dictyostelium, cAMP binds to the G-protein-coupled receptor cAR1 and induces the dissociation of heterotrimeric G-proteins into Go and Gβγ subunits (19, 20), leading to the activation of PI3K, which converts phosphatidylinositol(4,5)-bisphosphate (PIP2) to PIP3 on the inner plasma membrane (14, 48). Function of this pathway is necessary for chemotaxis under defined conditions (29). PIP3 mediates cellular responses by recruiting proteins with pleckstrin homology (PH) domains, such as CRAC (cytosolic regulator of adenyl cyclase) and Akt/PKB (protein kinase B), to the plasma membrane (36, 41). It has been shown that cAMP-induced PI3K activation results in an increase in PIP3 levels that can be monitored by the membrane translocation of PHCrac-GFP (29, 42, 63), and in Dictyostelium, translocation can be used to measure PIP3 generation (29). To examine the effect of VPA on the cAMP-induced signal transduction pathway leading to PIP3 generation, we visualized the membrane translocation of PHCrac-GFP (Fig. 2A and B) in living cells upon cAMP stimulation. Following uniform cAMP stimulation, a transient increase in PIP3 generation caused a transient PHCrac-GFP

FIG. 1. VPA blocks chemotactic movement in wild-type Dictyostelium cells. (A) Quantification of speed for chemotaxing cells moving towards a source of cAMP in a pulsatile way, alternately increasing and decreasing speed. (B) Addition of VPA (1 mM) to cells (arrow) acutely blocks cell movement within 5 min. Three typical experiments are shown in panels A and B. See also the movie in Fig. S1 in the supplemental material.
membrane translocation, which peaked at about 5 s (Fig. 2C), corresponding to the temporal signal-induced production of PIP₃ by PI3K. Treatment of cells with VPA (1 mM, 10 min) blocked this membrane translocation, suggesting that VPA reduces PIP₃ production (Fig. 2C). Since VPA is used therapeutically at plasma concentrations of 0.3 to 0.6 mM, we repeated these experiments by treating the cells with 0.5 mM VPA for 10 min and monitored PH₃Crac-GFP cytosolic depletion (Fig. 2D). This treatment reduced the rate and magnitude of the PH₃Crac-GFP translocation without totally abolishing it. The block in PH₃Crac-GFP translocation was not caused by an inhibitory effect on protein translocation, since movement of an unrelated fusion protein, yellow fluorescent protein (YFP)-actin-binding domain (YFP-ABD) (6), to the F-actin-rich cortex was not altered following VPA treatment (see the movie in Fig. S2 in the supplemental material). We previously showed that treatment with VPA (1 mM, 10 min) affected neither cAMP-induced cAR1 phosphorylation nor cAMP-triggered dissociation of Go2Gβγ (5), thus suggesting that VPA reduces PIP₃ production downstream of cAR1/G-protein activation. Treatment of cells with LY294002 (a PI3K inhibitor) phenocopied the effect of VPA on PH₃Crac-GFP membrane translocation, consistent with the idea that VPA reduces PIP₃ production (29).

Since we have shown that VPA blocks chemotaxis by inhibiting cell movement in a chemotactic gradient, it is possible that VPA blocks motility but not cAMP gradient sensing. Analysis of this effect has employed latrunculin-treated cells, exposed to a cAMP gradient, giving rise to PH₃Crac-GFP translocation to the membrane in a polarized manner (41, 48). To examine whether VPA treatment inhibits the gradient-sensing ability of the cells, we visualized dynamics of PH₃Crac-GFP translocation in VPA- and latrunculin-treated cells directionally exposed to cAMP. Without VPA treatment, PH₃Crac-GFP translocated to the front of the cell in a biphasic pattern (Fig. 2E).
2E), as has been previously shown (63). In contrast, treatment of cells with 1 mM VPA for 10 min blocked the formation of both peaks of PH\textsubscript{C\textalpha{}}-GFP translocation (Fig. 2E). These results suggest that VPA blocks the gradient sensing ability of cells by inhibiting both the first peak of PIP\textsubscript{3} production and the amplification of this signal shown in the second peak.

Activation of cAR1/G-proteins leads to a recruitment of PI3K from the cytosol to the membrane, increasing the amount of membrane-bound active PI3K and therefore PIP\textsubscript{3} production and thus playing a role in amplifying a cAMP gradient in highly polarized chemotaxing cells (19, 48). To examine whether VPA affects PI3K membrane translocation, we pulsed cells containing a PI3K-GFP construct with cAMP and measured PI3K-GFP translocation in the presence of VPA. Cells without VPA treatment showed transient membrane localization of PI3K-GFP, peaking at around 10 s after cAMP stimulation (Fig. 2F). Treatment of cells with 1 mM VPA for 10 min prior to stimulation blocked this translocation (Fig. 2F). However, as previously mentioned, VPA does not reduce the translocation of a marker for filamentous actin, YFP-ABD, suggesting that VPA treatment does not generally affect protein redistribution (see the movies in Fig. S2 in the supplemental material). Thus, the positive feedback loop that amplifies the quantification of phospholipid turnover. Strong labeling of PIP and PIP\textsubscript{2} was observed under control conditions (Fig. 3A). PIP\textsubscript{3} labeling was not detected due to the comparatively small amount of this compound in the cell, and the addition of exogenous cAMP (10 \(\mu\)M) showed no quantifiable change in phospholipid labeling (data not shown). Under these conditions, VPA (1 mM) produced a significant (\(P > 0.01\)) reduction in both PIP and PIP\textsubscript{2} turnover (Fig. 3B). Treatment of cells with LY294002 (50 \(\mu\)M) also caused a significant reduction in lipid turnover (\(P > 0.005\)) (Fig. 3B). These results show that acute VPA treatment causes a reduction in lipid phosphorylation. Although this may be due to reduced kinase or increased phosphatase levels, these results are consistent with a reduction in PIP\textsubscript{3} production, as suggested by PH\textsubscript{C\textalpha{}}-GFP translocation studies.

**VPA treatment and inhibition of PI3K activity attenuate in vivo phospholipid phosphorylation.** To investigate a potential change in phospholipid turnover caused by VPA in *Dictyostelium*, we developed cells for 5 h by pulsing with cAMP (5), pretreated cells for 5 min with defined drugs, permeabilized cells, and labeled lipids with \([\gamma\text{32P}]\text{ATP}\) for 5 min. Extraction of lipids and separation by thin-layer chromatography allowed the quantification of phospholipid turnover. Strong labeling of PIP and PIP\textsubscript{2} was observed under control conditions (Fig. 3A). PIP\textsubscript{3} labeling was not detected due to the comparatively small amount of this compound in the cell, and the addition of exogenous cAMP (10 \(\mu\)M) showed no quantifiable change in phospholipid labeling (data not shown). Under these conditions, VPA (1 mM) produced a significant (\(P > 0.01\)) reduction in both PIP and PIP\textsubscript{2} turnover (Fig. 3B). Treatment of cells with LY294002 (50 \(\mu\)M) also caused a significant reduction in lipid turnover (\(P > 0.005\)) (Fig. 3B). These results show that acute VPA treatment causes a reduction in lipid phosphorylation. Although this may be due to reduced kinase or increased phosphatase levels, these results are consistent with a reduction in PIP\textsubscript{3} production, as suggested by PH\textsubscript{C\textalpha{}}-GFP translocation studies.

**VPA affects endocytosis and exocytosis.** The inhibition of cAMP-induced PH\textsubscript{C\textalpha{}}-GFP translocation and the reduction in phospholipid phosphorylation by VPA in *Dictyostelium* suggests it causes a reduction in phospholipid signaling, and this signaling pathway has been shown to affect vesicle trafficking in this model (7, 46, 65). In order to identify whether VPA may affect this process, we examined the acute effect of VPA on phagocytosis, pinocytosis, and exocytosis. To measure phagocytosis, we added VPA (1 or 3 mM) to growing cells and immediately recorded the uptake of labeled yeast particles over time (Fig. 4A). Treatment of cells with 1 mM VPA strongly reduced phagocytosis, while 3 mM VPA completely abolished particle uptake. Fluid-phase uptake was measured by quantifying the uptake of a soluble fluorescent marker (Fig. 4B). VPA (1 mM) caused an immediate, substantial reduction in fluid uptake, suggesting that this process is more sensitive to VPA than is phagocytosis. This concurs with previous work, as mutants lacking PI3K 1 and 2, or wild-type cells treated with a PI3K inhibitor, showed strongly blocked pinocytosis (46, 65) but only partially reduced phagocytosis (65). Since cellular components involved in endocytosis are likely to be necessary also for exocytosis, we characterized the effect of VPA on exocytosis. In these experiments, cells were allowed to take up fluorescent fluid-phase marker until saturation in the absence of VPA. Subsequent challenge with 1 mM VPA substantially delayed the release of fluorescence, compared with untreated cells (Fig. 4C). Similarly, the release of marker from cells loaded with endocytic tracer is also delayed in PI3K1/2 double mutants (7), as well as in PI3K inhibitor-treated cells (46). These data suggest that VPA acts to reduce endocytic trafficking through the attenuation of the phospholipid signaling.

**VPA treatment and inhibition of PI3K activity reduces neurotransmitter release.** Since our data suggest that, in *Dictyostelium*, VPA reduces the phospholipid-dependent signaling and vesicle release, we examined an effect of VPA and PI3K inhibition in the modulation of synaptic signaling. We thus assessed the effect of VPA and a PI3K inhibitor on depolarization-dependent neurotransmitter glutamate release from isolated rat cerebrocortical nerve terminals (synaptosomes), an established model for assessing presynaptic function (54). In this model, VPA dose dependently inhibited 4-aminopyridine-evoked glutamate release from synaptosomes (Fig. 5), as measured by online fluorimetry (40). VPA at 1 mM showed a slight
decrease in glutamate release (19%) and, at 3 mM and 10 mM, VPA produced clear decreases of glutamate release of 39% and 59%, respectively. High VPA concentrations were necessary in this model to overcome high membrane content of the synaptic preparation, which tends to sequester lipophilic agents like VPA. This effect was reproduced using the PI3K inhibitor LY294002, giving a 57% suppression of glutamate release at a concentration shown to affect glutamate release in the same manner as wortmannin (100 μM) (9). This effect was not through modulation of GABA levels, as the established GABA-transaminase inhibitor vigabatrin caused no significant reduction (Fig. 5).

**DISCUSSION**

In this work we have identified a novel acute effect of VPA in blocking movement in chemotaxing Dictyostelium cells (Fig. 1). We showed this effect occurs with a block in the production of PIP₃, as measured by PH₃_crac-GFP translocation (Fig. 2), whereby high cellular concentrations of VPA strongly reduced signal-induced PIP₃ production while lower concentrations slowed and attenuated this process. We also showed that VPA exposure acutely reduces phospholipid phosphorylation (Fig. 3), consistent with a reduction in the production of PIP₃ caused by the drug. We further showed that VPA modulates Dictyostelium endo- and exocytosis, processes previously associated with PIP₃ production (7, 46, 65). VPA concentrations used in these experiments are around therapeutic limits, since plasma concentrations of VPA are 0.3 to 0.6 mM (31) and chronic treatment of animal models can give rise to brain concentrations of 1.0 to 1.5 mM (2). We further showed that in a mammalian synaptic model for vesicle release—a key process in seizure occurrence—both VPA and a block in phospholipid signaling caused by PI3K inhibition significantly reduced glutamate release.

Results presented in this paper suggest that VPA causes an acute reduction in PIP₃ production; however, it remains unclear how VPA reduces the level of this critical signaling lipid. This effect could be caused by a variety of methods, including reduced supply of the PIP₃ precursor PIP₂, direct or indirect inhibition of phosphatidylinositol kinase enzymes (e.g., PI3K), or direct or indirect activation of phosphatidylinositol phosphatase enzymes (e.g., PTEN). The coordinate decrease in phosphorylated PIP and PIP₂ formation suggests that VPA’s effect on PIP₃ production may be caused by an effect on an enzyme responsible for phosphorylation or dephosphorylation of multiple phospholipid targets (as we have shown for PI3K inhibition). Further work will examine the role of phosphatidylinositol kinases (3’ and 5’) and phosphatases as targets for VPA. It is also of interest that sequence variations have been found in 5’ kinase (intron 9 PIP5K2A) that have been linked with bipolar disorder and schizophrenia (57). These results contrast with work in cerebellar granule cells, whereby VPA...
functions in a neuroprotective role by the activation of the PI3K/PKB signaling pathway (38).

It remains a formal possibility that the reduction in PH \textsubscript{Crac} encompasses GF permeability is caused by reduced movement of the receptor complex, not the production ofPIP\textsubscript{2}. This seems unlikely, though, as another fluorescently tagged construct was not grossly affected in cellular localization by VPA treatment. Another possibility, as shown by Luo et al. (30), is that InsP\textsubscript{7/8} is not grossly affected in cellular localization by VPA treatment. Nonetheless, the coordinate reduction in PIP and PIP\textsubscript{2} production caused by VPA, and the common effect of VPA and the PI3K inhibitor, strongly suggest the observed results occur via a VPA-catalyzed reduction in phospholipid phosphorylation (and PIP\textsubscript{3} production).

Investigating a role for a VPA-catalyzed reduction in phospholipid turnover that modulates synaptic function showed that both VPA and PI3K inhibition reduced glutamate release in purified mammalian synaptic preparations. The acute nature of this effect is of importance, as the intravenous administration of VPA inhibits epileptic seizures within 20 min (64), and few studies have identified short-term effects of the drug (26). The modulation of phospholipid signaling also provides an attractive and sensible mechanism for the function of branched short chain fatty acids, such as VPA. In GT1-7 neurons, the drug has been shown to be incorporated into lipids (51), although the resulting valproyl-phospholipid has not been identified, nor is its function understood. Incorporation of VPA into the lipid bilayer, either free or as a phospholipid, may modify the normal function of membrane-bound signaling components.

The demonstration of phospholipid signaling and specifically the function of PIP\textsubscript{3}-dependent processes, has been widely linked to potential epilepsy-related targets. For example, epileptic seizures have traditionally been thought to result from changes in ion channel activity, and PI3K (59) and phospholipid signaling (32) have both been implicated in the regulation of ion channels. Conversely, the deletion of the enzyme PTEN, the PI3K antagonist, causes seizures in mice (1), and Storey et al. (58) suggested that altered PI3K signaling might contribute to the pathophysiology of epilepsy.

The acute nature of the block in PIP\textsubscript{3} production suggests that the observed effects of VPA are not caused by InsP\textsubscript{3} depletion, which requires >5-h treatment in Dictostelium (unpublished data) or mammalian neurons (12, 62). However, phospholipase C functions to regulate stimulation-induced production of InsP\textsubscript{3} from PIP\textsubscript{2}, and its activity can be directly regulated by PIP\textsubscript{3} (44). Thus, a potential inhibition of both PIP\textsubscript{2} and PIP\textsubscript{3} production by VPA treatment may suppress an overactive InsP\textsubscript{3} signaling cascade, hence proposing a mechanism by which VPA functions, through phospholipid modulation, in the inositol depletion hypothesis.

Although VPA's role as an anticancer agent may be through HDAC inhibition (4), our data also suggest two additional potential mechanisms. Recently, VPA may act by directly attenuating PIP\textsubscript{3} production, which is elevated in many cancer types (22). In support of this, Denlinger et al. (10) recently found that the combined inhibition of PI3K and HDAC induced lung cancer cell apoptosis in vitro and in vivo. Secondly, it may suppress tumor metastasis (4) by inhibiting chemotaxis, and VPA has also been shown to inhibit chemotaxis in basic fibroblast growth factor-induced human umbilical vein endothelial cell migration (37) and to reduce individual neural crest cell migration (13). It is also interesting that the role of PIP\textsubscript{3} is increasingly being recognized in cell migration (28). The attenuation of synaptosomal glutamate release by VPA through the modulation of phospholipid signaling may also play a therapeutic role for this drug. Alterations in the release of glutamate and other neurotransmitters have been widely associated with seizures and as targets for antiepileptic treatments. Elevated glutamate release has been shown in the hippocampus of rats following pentylentetrazol kindling (27) as a seizure model, and VPA reduced glutamate release in this system by an unknown mechanism. VPA also suppresses status epilepticus induced by 4-aminopyridine in the hippocampus (34) and 4-aminopyridine-induced excitatory synaptic activity (35). Our results, therefore, suggest a potential mechanism for these VPA effects—through phospholipid modulation to alter vesicle release. This latter process is also targeted by the new epilepsy treatment levetiracetam (31).

To conclude, our data identify a mechanism of action of valproic acid via the attenuation of phospholipid signaling and downstream phospholipid-dependent endocytic trafficking in the biomedical model system Dictostelium. We further show that both VPA and a PI3K inhibitor attenuate glutamate release in a mammalian synaptic model. Although further work will be necessary in mammalian systems, these results propose a novel role for valproic acid in the attenuation of phospholipid-dependent signaling which may provide a new insight into the therapeutic function of the drug.

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