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Incorporation of [^{3}H]Valproic Acid into Lipids in GT1–7 Neurons

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ABSTRACT. Valproic acid (2-propylpentanoic acid, valproate, VPA), an 8-carbon, branched chain fatty acid, is effectively used in the treatment of mania and epilepsy. The biochemical mechanisms by which this drug has its therapeutic effects are not yet established. The purpose of this study was to partially characterize the incorporation of [^{3}H]VPA into phospholipids of GT1–7 neurons, an immortalized hypothalamic cell line. GT1–7 neurons were grown to confluence in culture dishes, and then were incubated with various concentrations of [^{3}H]VPA between 10 and 400 μg/mL for various times up to 20 hr. Total lipids were extracted and phospholipids were separated from neutral lipids using TLC. Our results indicate that [^{3}H]VPA (10 μg/mL) was incorporated into phospholipids of GT1–7 neurons in a time-dependent and saturable manner over 300 min. Subsequent separation of the lipid fraction by TLC indicated that 44.4% of the radioactivity taken up by the cells was incorporated into phospholipids and neutral lipids. One of the phospholipids migrated with a slightly lower R_f value than authentic phosphatidylcholine. Our results show that the incorporation of VPA into phospholipids and glycerides was linear with VPA concentrations from 10 to 400 μg/mL. Finally, we synthesized 1-acyl-2-valproyl-*sn*-glycero-3-phosphocholine and validated its structure with nuclear magnetic resonance and electrospray mass spectrometry to verify the structure of this compound, confirming that this compound is structurally possible. We conclude that VPA is incorporated into lipids in GT1–7 neurons and discuss the possible effects of valproyl phospholipids on neuronal functional properties. BIOCHEM PHARMACOL **56**;2: 207–212, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. valproic acid; phospholipid; lipid; neuron; hypothalamus; GT1–7; epilepsy; mania

VPA§ is a branched, short-chain fatty acid with a wide spectrum of activities against different types of seizures and is the drug of choice for treatment of absence epileptic seizures [1]. Recently, Bowden *et al.* [2] reported that VPA was significantly more effective than a placebo in reducing the symptoms of acute mania and that the efficacy of the divalproex form of valproate appeared to be independent of prior responsiveness to lithium.

The pharmacodynamic and pharmacotherapeutic effects of VPA and the possible mechanisms of action have been studied extensively, but the pharmacodynamic basis of its anticonvulsant and antimanic action remains unknown [1]. It has been suggested that VPA acts through a combination of several mechanisms. VPA increases GABA turnover, reduces the release of the epileptogenic amino acid γ-hydroxybutyrate, and exerts a direct action on ion channels [3]. VPA is known to affect GABA and norepinephrine metabolism through the inhibition of GABA transaminase and aldehyde reductase [4], respectively. Chen *et al.* [5] reported that chronic sodium valproate selectively decreases the α and ε, but not the δ and ζ isoforms of protein kinase C in C6 rat glioma cells. Petty *et al.* [6] reported that plasma GABA predicts acute response to valproate treatment in mania. Also, VPA inhibits the synthesis of major phospholipids [7], decreases the fluidity of brain mitochondrial membranes [8], interferes with embryonic lipid synthesis [9], may affect transport of phospholipid precursors across the cell membrane [10], and inhibits the secretion of triacylglycerols from rat hepatocytes [11].

The results reported here were obtained using GT1–7 neurons, an immortalized neuronal cell line. These adult hypothalamic neurons, developed from tumors in mice [12–14], synthesize and secrete gonadotropin releasing hormone (GnRH) and express several receptor transducing systems, including $GABA_A$ receptors [15–20].

The purpose of this study was to determine whether VPA is incorporated into membrane phospholipids in a neuronal cell line, to determine the time frame and amount of any incorporation, and to identify specific phospholipids into which VPA is incorporated. A long-term goal of this

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§ *Abbreviations:* GABA, γ-aminobutyric acid; PAF, platelet-activating factor; PC-VPA, 1-oleoyl-2-valproyl-*sn*-glycero-3-phosphocholine; TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid; and VPA, valproic acid.

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research is to test the hypothesis that valproyl-phospholipids modify neuronal activity and that this modification plays a role in the antiepileptic and antimanic-depressive effects of the drug.

MATERIALS AND METHODS

Culture dishes were obtained from Fisher Scientific. Culture media were products of Life Technologies. All other reagents were purchased from the Sigma Chemical Co. [^{3}H]VPA, sodium salt in 80% ethanol, 1 mCi/mL, 15 Ci/mmol, was purchased from ARC. TLC plates were purchased from Whatman.

Cell Culture of GT1–7 Neurons

GT1–7 neurons were plated at a density of approximately 5×10^6 cells/dish in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies No. 12800, glucose 4.5 g/L) supplemented with 5% horse serum, 5% fetal bovine serum, penicillin (50 IU/mL), and streptomycin (50 μg/mL). Cells were grown to confluence in 100-mm-diameter culture dishes at 37° in an atmosphere of 95% air/5% CO_2 for approximately 5 days.

Incorporation of [^{3}H]VPA into Lipids of GT1–7 Neurons and Extraction of Lipids

GT1–7 neurons were incubated with various concentrations of [^{3}H]VPA (1 μCi/mL) for various times (see legends to figures for details of specific experiments). The medium was removed and cells were washed twice with 5 mL of ice-cold normal saline. Next, 3 mL of methanol and 1.2 mL of water were added and the cells were scraped from the plates. This procedure was then repeated to ensure the removal of all the cells. The scraped fractions were transferred to glass tubes containing 3 mL of chloroform. After vortexing, 3 mL of chloroform and 3 mL of water were added to the mixture to achieve phase separation [21]. The lower phase, containing total lipids, was transferred to another tube and evaporated under nitrogen to dryness. Lipids were dissolved in chloroform:methanol (4:1).

Lipid Fractionation by TLC and Measurement of Radioactivity

Total lipids were subjected to TLC (K6 Whatman Silica Gel Plates) using two solvent systems: 1) chloroform: acetone (9:5) for the separation of total phospholipids from various neutral lipids and VPA; and 2) chloroform:methanol:water (65:35:7) for the separation of individual phospholipids. Lipids were visualized on the plates using TNS spray [22]. Phospholipids were located with ammonium molybdenum spray. Radioactivity in each lane was measured using a thin-layer scanner coupled with a data system (BioScan System 200 Imaging Scanner, BioScan). Additionally, fractions were scraped from the plates, and placed in vials with 10 mL of Beckman Ready Protein+ scintillation fluid; then radioactivity was quantitated using a liquid scintillation counter (Beckman LS7500) [23].

Synthesis and Structure Verification of Valproylphosphatidylcholine

VPA was converted to valproyl chloride by treatment overnight at room temperature with 0.35 mL of freshly distilled thionyl chloride in a calcium chloride and NaOH desiccator. Unreacted thionyl chloride was evaporated at 80° under nitrogen. A 10-μL aliquot of the resultant valproyl chloride was added to a mixture of 1-oleoyl-2-lysoglycerophosphocholine (5 mg) and *N*,*N*′-dimethylaminopyridine (17.5 mg) in dry chloroform (0.1 mL) [24]. The mixture was stirred at room temperature for 1.5 hr, and the PC-VPA formed was purified using preparative TLC with three different solvent systems: a basic solvent system containing chloroform:methanol:28% ammonium hydroxide (65:35:8, by vol.), a chloroform:acetone (9:5, v/v) system, and a neutral solvent system containing chloroform:methanol:water (65:35:7, by vol.).

The chromatographic profile of the synthesized compound was assessed using the three solvent systems described above and an acidic solvent system that contained chloroform:methanol:acetic acid:water (65:35:8:4, by vol.).

The structure of PC-VPA was verified with electrospray mass spectrometry using a Finnigan Quadruple MAT SSQ700 with an Analytica Bradford electrospray system at a voltage of −3500 V for positively charged molecules. The purified compound was solubilized in chloroform:methanol (4:1, v/v) at a concentration of 1.5 mg/mL. An aliquot was diluted 20-fold in the same solvent and a few microliters injected into the spectrometer. The flow rate was 2 μL/min. NMR was performed using a Varian NMR Unity Plus, 300 MHz.

RESULTS

GT1–7 neurons were incubated with 10 μg/mL of [^{3}H]VPA (1 μCi/mL) for 5, 15, 30, 90, and 300 min. Total lipids were extracted and subjected to TLC using a solvent system (chloroform:acetone, 9:5) in which total phospholipids remained at the origin. VPA, $R_f \cong 0.5$, and neutral lipids (mono-, di-, and triglycerides) migrated into the plates and were separated as shown in the representation of a TLC plate in Fig. 1A. We performed a radioactivity scan of lane f of the TLC plate, which contained the extract from the GT1–7 neurons that had been incubated with 10 μg [^{3}H]VPA/mL for 300 min. [^{3}H]VPA was incorporated into phospholipids as well as into neutral lipids (Fig. 1B).

The TLC plates were next exposed to a second solvent system (chloroform:methanol:water, 65:35:7) for separation of individual major phospholipid classes. Neutral lipids migrated near the solvent front with the second solvent system. Our results indicate that radioactivity was present at R_f positions that were consistent with migration of

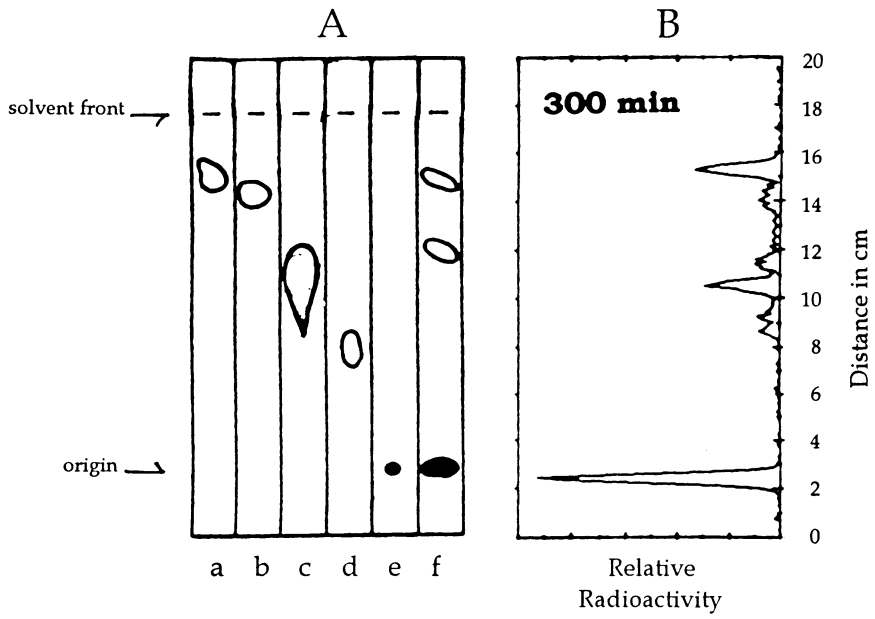

FIG. 1. Incorporation of [^{3}H]VPA into lipids in GT1–7 neurons. GT1–7 neurons were grown in culture dishes, and then were incubated for 300 min with 10 μg/mL of [^{3}H]VPA as described in Materials and Methods. Total lipids were extracted from GT1–7 neurons with the Bligh–Dyer procedure [21] and then were subjected to TLC using a chloroform: acetone (9:5, v/v) solvent system that separated phospholipids from VPA and other neutral lipids. Panel A is a representation of the TLC plate for the 300-min incubation. All lipids were located by staining with TNS (open and solid spots). Phospholipids were located by staining with ammonium molybdenum (solid spots). Key: (a) triglycerides, (b) diglycerides, (c) VPA, (d) monoglycerides, (e) phosphatidylcholine, and (f) GT1–7 neuronal extract. Panel B is a radioactivity scan of lane f of the TLC plate for the 300-min incubation with VPA.

phosphatidylcholine, phosphatidylethanolamine, sphingolipids, and possibly phosphatidylinositols (Fig. 2B). The migration of phospholipids in the control and sample lanes was visualized with ammonium molybdenum, and the presence of radioactivity was measured to indicate the presence of incorporated [^{3}H]VPA. A scan of radioactivity for a control sample of [^{3}H]VPA is shown in Fig. 2C.

Figure 3 describes the time frame of the incorporation of radioactivity into phospholipids as the percentage of total lipids that were extracted from the GT1–7 neurons into the organic fraction of the Bligh-Dyer procedure [21]. The incorporation of radioactivity, presumably associated with [^{3}H]VPA, into phospholipids of GT1–7 neurons proceeded in a time-dependent fashion over 20 hr (Fig. 3). The incorporation was saturated by about 5 hr when the cells were incubated with 10 μg/mL of [^{3}H]VPA. Approximately 50% of the incorporated radioactivity was associated with the phospholipid fraction. The results in Fig. 2 suggest that

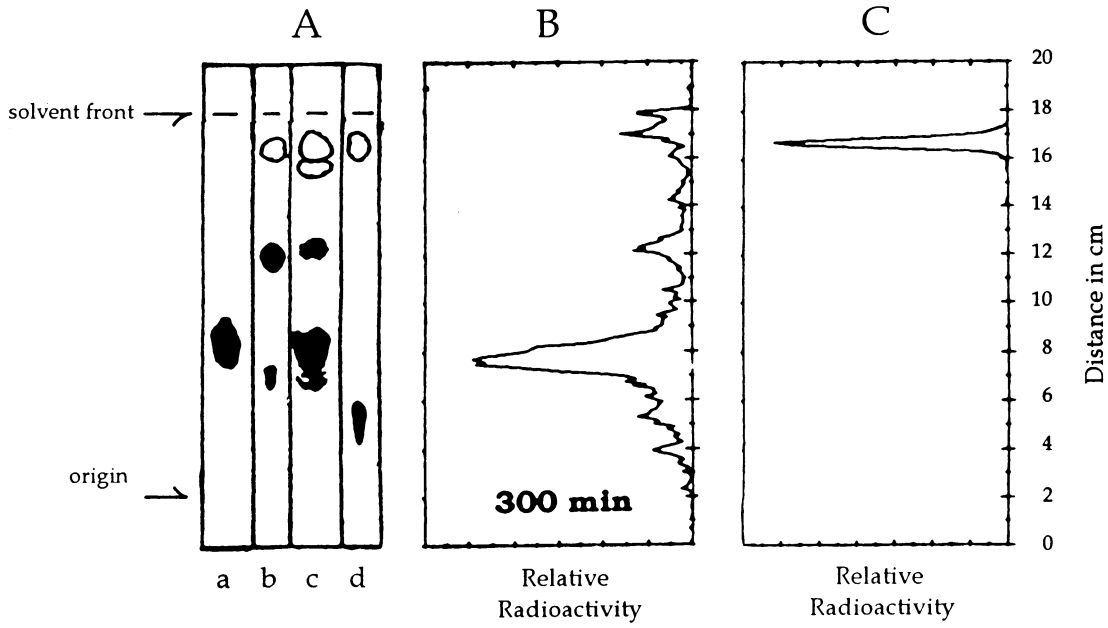

FIG. 2. Incorporation of [^{3}H]VPA into phospholipids in GT1–7 neurons. The TLC plate shown in Fig. 1 was developed further using a second solvent system, chloroform:methanol:water (65:35:7, by vol.), which was designed to separate individual phospholipids. All lipids were located by staining with TNS (open and solid spots). Phospholipids were located by staining with ammonium molybdenum (solid spots). Panel A is a representation of the TLC plate. The lanes contained: (a) phosphatidylcholine, (b) sphingomyelin, phosphatidylethanolamine, VPA (in order from origin to solvent front), (c) GT1–7 neuronal extract, and (d) lysophosphatidylcholine, VPA. Panel B is a radioactivity scan of lane c of the TLC plate. Panel C is a radioactivity scan of lane d in which [^{3}H]VPA was tested.

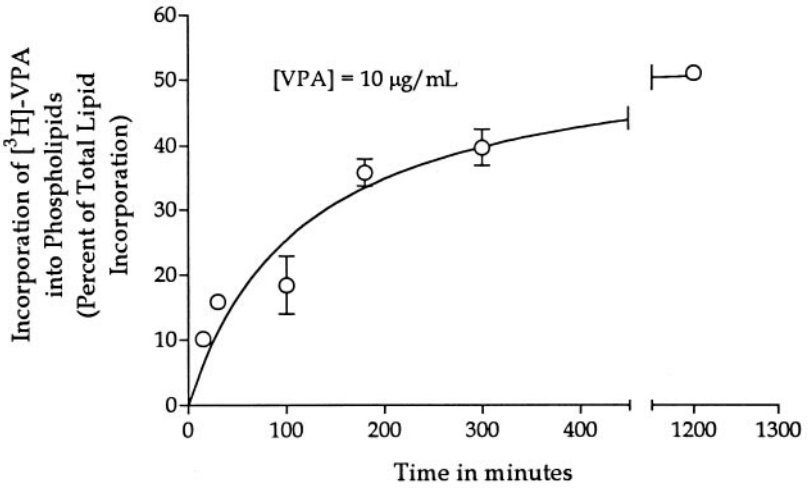

FIG. 3. Time course of incorporation of [^{3}H]VPA into total phospholipids in GT1–7 neurons. This experiment was performed similarly to the experiments described in the legends to Figs. 1 and 2. The cells were incubated for 5, 15, 30, 90, 300, and 1200 min with 10 μg/mL of [^{3}H]VPA, and then were washed to remove extracellular radioactivity. Lipids were extracted from the cells and analyzed by TLC as described in Materials and Methods. The incorporation of [^{3}H]VPA into phospholipids is expressed as a percentage of the radioactivity associated with the total lipid fraction, which was approximately 15,000–20,000 dpm at each time point. Each symbol represents the mean and SEM for two experiments in duplicate.

[^{3}H]VPA was incorporated into phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin. The remainder of the radioactivity in the organic fraction of the Bligh-Dyer procedure was probably free VPA and neutral lipids (Fig. 2, lane b, near the solvent front).

The data in Fig. 4 indicate that the incorporation of [^{3}H]VPA into neutral lipids and phospholipids in GT1–7 neurons increased in a linear fashion with concentrations of VPA up to 400 μg/mL. In this experiment, the cells were incubated with VPA for 180 min. This linear relationship was observed for total uptake of VPA into GT1–7 neurons and for incorporation into phospholipids as well as di- and triacylglycerols. The fraction labeled di- and triacylglycerols could also include small amounts of other neutral lipids. A comparison of the slopes of the linear regressions indicates that 20.6 and 23.8% of the [^{3}H]VPA taken up by the cells was incorporated into phospholipid and neutral lipid (di- and triglycerols) fractions, respectively.

We synthesized PC-VPA from lysophosphatidylcholine and valproyl chloride in the presence of dimethylaminopyridine and dry chloroform. VPA and thionyl chloride were used for the synthesis of valproyl chloride, which was then purified using TLC with three different solvent systems. The structure of PC-VPA was proven by NMR and electrospray mass spectrometry (data not shown). As expected, the electrospray-MS spectra indicated that the phosphatidylcholine with a valproyl residue gave intense signals of $[M + H]^+$ at m/z 648.3 and of $[M - 144.2 + H]^+$ at m/z 504.1. The molecular weight of VPA is 144.2. Two characteristic fragment ions of choline phospholipids were observed at m/z 184 ($[phosphocholine]^+$) and 104.1 ($[choline]^+$). The NMR results confirmed those obtained with electrospray MS (data not shown).

We synthesized PC-VPA both to establish that this compound was stereospecifically possible and to aid in the identification of compounds into which [^{3}H]VPA was incorporated. Our results show that the R_f value for synthesized PC-VPA was identical to one of the phospholipids in the GT1–7 extract into which the radioactivity was incorporated. This result suggests that PC-VPA was synthesized by GT1–7 neurons during our experiments.

DISCUSSION

Our results show that VPA was incorporated into lipids of GT1–7 neurons in a time-dependent manner that reached equilibrium within about 5 hr at 10 μg/mL. Incorporation into total lipids, phospholipids, and neutral lipids was linear

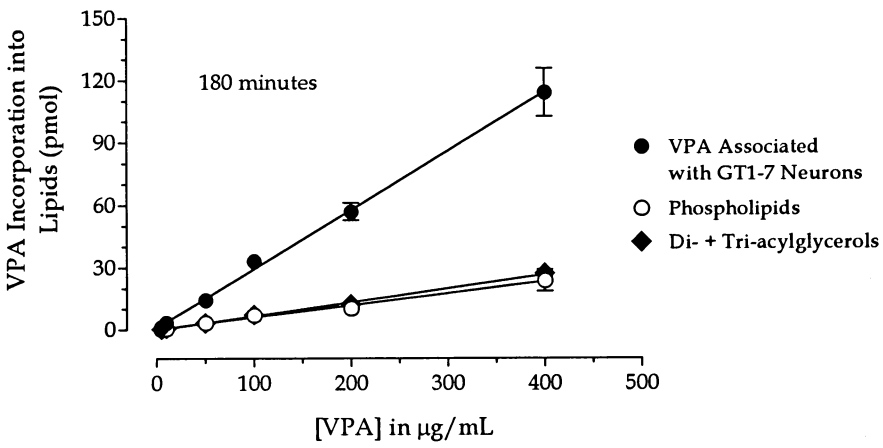

FIG. 4. VPA concentration curve for incorporation of VPA into lipids of GT1–7 neurons. This experiment was performed similarly to the experiments described in the legends to Figs. 1 and 2. The cells were incubated for 180 min with 10, 50, 100, 200, and 400 μg/mL of [^{3}H]VPA, and then were washed to remove extracellular radioactivity. Lipids were extracted from the cells and analyzed by TLC. The incorporation of VPA into the phospholipid (open circles) and neutral lipid (solid diamonds) fractions is expressed as picomoles incorporated. Also reported are picomoles of VPA taken into the cells (solid circles). Each symbol represents the mean and SEM for two experiments in duplicate.

with the concentration of VPA. We accomplished the first synthesis of PC-VPA, thereby establishing that this compound is stereospecifically possible and stable. The R_f value for PC-VPA was identical to the R_f value for one of the principal phospholipids into which [^{3}H]VPA was incorporated, supporting the likelihood that PC-VPA is formed in neuronal membranes with valproate administration.

Previous studies have failed to identify the incorporation of VPA into phospholipids in the brain. Aly and Abdel-Latif [25] did not detect incorporation of [^{3}H]VPA into brain phospholipids 30 min after the i.p. administration of the drug to rats. Becker and Harris [26] found no evidence for the accumulation of valproyl-CoA in rat brain 30 min after the i.p. administration of valproate, although it occurred in liver tissue. Perhaps the 30-min time period of these experiments was insufficient for measurable incorporation because of the time required for distribution of VPA into the brain and of other factors such as enzyme turnover rate that could further delay the incorporation. In this study, with direct application of VPA to the GT1–7 neurons in culture, the time required for saturation of VPA incorporation was approximately 5 hr at a concentration of 10 μg/mL. At 30 min, only about 30–40% of maximal incorporation had been achieved (Fig. 3). It is possible that an even longer time is necessary for detectable synthesis of valproyl-CoA or incorporation of VPA into rat brain phospholipids.

Becker and Harris [26] reported that VPA did not change cerebral acetyl-CoA content and that no valproyl-CoA was found in brain (as mentioned above), hippocampal prisms, or brain mitochondria. These data indicate that the precursor of VPA incorporation into phospholipids did not accumulate at detectable levels in these experiments. Nevertheless, the activation of fatty acids of three and more carbons has been shown in rat brain homogenates [27]. Although we did not measure valproyl-CoA itself, our experiments suggest that valproyl-CoA could be formed in GT1–7 neurons, so it is not clear at this time why the levels were not detectable in Becker's experiments.

The possibility of the incorporation of VPA into lipids as a potential mechanism for its biochemical and physiological effects is heuristically interesting. It has been established that most of the hydrophobic lipid moieties of neuronal membranes are very long chain, highly unsaturated, and, in some cases, hydroxylated residues. Each lipid class contains characteristic molecular species. The contribution of these compounds to the functional properties of neuronal membranes such as excitability or transmembrane receptor-mediated signal conduction is not well elucidated. In general, biological membranes contain species of phospholipids that possess hydrocarbon chains of similar length at the *sn*-1 and *sn*-2 positions. However, asymmetric molecular species with acyl moieties of widely differing lengths have been observed. PAF is an example of an asymmetric phospholipid with a wide spectrum of biological activities [28]. Also, asymmetric phospholipids with a short chain, *sn*-2 positioned monocarboxylyl, dicarboxylyl, or hydroxy-monocarboxylyl groups have been isolated from a bovine brain lipid extract, apparently formed by lipid peroxidation [24].

The function of membrane-associated enzymes may be altered by the presence of asymmetric phospholipids. For example, when rat hepatocyte plasma membranes were enriched with semisynthetic phosphatidylcholines with short acyl chain moieties of 10–12 carbons at the *sn*-2 position, the activity of endogenous membrane phospholipase A_2 was decreased while the activity of acyl-CoA: lysophospholipid acyl transferase was increased [29]. Introduction of VPA, a branched short chain fatty acid, into a membrane phospholipid reduces the symmetry of the resultant molecule. The incorporation of adequate amounts of an asymmetric phospholipid into neuronal membranes thus may alter membrane structure and, consequently, function. Also, Bolaños and Medina [7, 30] have reviewed the effects of valproate on metabolism in the CNS. They concluded that valproate interfered with brain lipid synthesis and altered the structure of neuronal membranes. These findings may be explained, in part, by the incorporation of VPA into phospholipids, but this possibility was not discussed in these reports.

The linear relationship between the concentration of VPA and the incorporation into lipids in GT1–7 neurons was unexpected, because it was not a saturable process. Our data show that the incorporation of [^{3}H]VPA into phospholipids was linear over the concentrations of VPA tested, 5–400 μg/mL. This range of concentrations was chosen because the concentration of VPA in plasma that is associated with therapeutic antiepileptic and antimanic responses is 50–100 μg/mL. Studies in laboratory animals establish that the pharmacokinetics of plasma and brain levels of VPA correspond [1]. It may be that a critical level of valproyl-containing lipids in biological membranes exists for alteration of signal transduction pathways critical to VPA's pharmacodynamic properties fundamental for its antiepileptic or antimanic properties. Studies to characterize the rate of turnover and the effects of valproyl-phospholipid incorporation on membrane function are required to test for and understand this possibility.

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