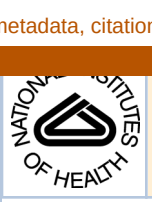

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Propylisopropylacetic acid (PIA), a constitutional isomer of valproic acid, uncompetitively inhibits arachidonic acid acylation by rat acyl-CoA synthetase 4: a potential drug for bipolar disorder

Hiren R. Modi[1,*], **Mireille Basselin**[1], **Ameer Y. Taha**[1], **Lei O. Li**[2], **Rosalind A. Coleman**[2], **Meir Bialer**[3], and **Stanley I. Rapoport**[1]

[1]Brain Physiology and Metabolism Section, National Institute on Aging, National Institutes of Health, Bethesda, MD, USA

[2]Department of Nutrition, University of North Carolina, Chapel Hill, NC, USA

[3]Department of Pharmaceutics, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel

Abstract

Background—Mood stabilizers used for treating bipolar disorder (BD) selectively downregulate arachidonic acid (AA) turnover (deacylation-reacylation) in brain phospholipids, when given chronically to rats. *In vitro* studies suggest that one of these, valproic acid (VPA), which is teratogenic, reduces AA turnover by inhibiting the brain acyl-CoA synthetase (Acsl)-4 mediated acylation of AA to AA-CoA. We tested whether non-teratogenic VPA analogues might also inhibit Acsl-4 catalyzed acylation, and thus have potential anti-BD action.

Methods—Rat Acsl4-flag protein was expressed in *E. coli*, and the ability of three VPA analogues, propylisopropylacetic acid (PIA), propylisopropylacetamide (PID) and N-methyl-2,2,3,3-tetramethylcyclopropanecarboxamide (MTMCD), and of sodium butyrate, to inhibit conversion of AA to AA-CoA by Acsl4 was quantified using Michaelis-Menten kinetics.

Results—Acsl4-mediated conversion of AA to AA-CoA *in vitro* was inhibited uncompetitively by PIA, with a K_i of 11.4 mM compared to a published K_i of 25 mM for VPA, while PID, MTMCD and sodium butyrate had no inhibitory effect.

Conclusions—PIA's ability to inhibit conversion of AA to AA-CoA by Acsl4 *in vitro* suggests that, like VPA, PIA may reduce AA turnover in brain phospholipids in unanesthetized rats, and if so, may be effective as a non-teratogenic mood stabilizer in BD patients.

Keywords

bipolar disorder; valproate; arachidonic acid; acyl-CoA synthetase 4; mood stabilizer; Acsl4; brain; MTMCD; N-methyl-2,2,3,3-tetramethylcyclopropanecarboxamide; PIA;

[*]Corresponding author: Hiren R. Modi, Ph.D. Brain Physiology and Metabolism Section, National Institute on Aging, National Institutes of Health, Bldg. 9, Rm. 1S126; 9000 Rockville Pike, Bethesda, MD 20892. modihr@mail.nih.gov; Tel: 301 594 5076; Fax: 301 402 0074.

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propylisopropylacetic; PID; propylisopropylacetamide; rat; butyrate; inhibition; uncompetitive; enzyme; anticonvulsant

Introduction

Valproic acid (VPA; 2-propylpentanoic acid; di-n-propylacetic acid, Figure 1), an eight-carbon, branched side-chain dicarboxylic acid, is an anticonvulsant that also is FDA-approved to treat bipolar disorder (BD) [1, 2]. However, VPA can produce unwanted clinical side effects, including hepatotoxicity, weight gain and metabolic disturbances [3–7]. It also is teratogenic, because it inhibits the chromatin-modifying enzyme, histone deacetylase [8, 9]. As such, it poses a significant fetal risk in pregnant women taking the drug [10], thus justifying the need for a non-teratogenic yet equipotent mood-stabilizer that may act by the same mechanism as VPA. Identifying a pharmacological brain target of VPA with regard to BD could lead to the rational development of effective VPA-like compounds with fewer side effects, including teratogenicity.

One suggested target of VPA, as well as of the other FDA-approved mood stabilizers, lithium, carbamazepine and lamotrigine, is the brain arachidonic acid (AA, 20:4n–6) cascade [11–13]. This suggestion is based on evidence that VPA as well as the other mood stabilizers, when given chronically to rats to produce therapeutically relevant plasma concentrations, downregulate markers of the brain AA cascade [11–13]. Since markers of the cascade are upregulated in the postmortem BD brain, in association with excitotoxicity, neuroinflammation, apoptosis and synaptic loss [14–16], dampening by the drugs of the brain AA cascade may contribute to their efficacy in BD [12, 13].

AA can be released from membrane phospholipid by an AA-selective calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$) in response to excitotoxicity or inflammation associated with microglial activation and increased cytokine production [17–21], and these neuropathological processes are found in BD [14–16]. AA also is liberated as a second messenger at post-synaptic neuronal membranes during neurotransmission *via* dopaminergic D_2 receptors, muscarinic $M_{1,3,5}$, serotonergic 5-$HT_{2A/2C}$ and glutamatergic N-methyl-D-aspartate receptors, all of which are coupled to $cPLA_2$. Neurotransmission involving these receptors is disturbed in BD [13, 22–24]. After being hydrolyzed from the stereospecifically number-2 position of membrane phospholipid by a PLA_2, a portion of the released AA is converted into pro-inflammatory lipid mediators including prostaglandin (PG)E_2 and multiple other bioactive metabolites [11, 25], whereas the majority (~97%) is reincorporated into phospholipid via the serial actions of Acsl and acyltransferase.

When given chronically to rats to produce therapeutically relevant plasma levels, lithium and carbamazepine, in addition to VPA, downregulated turnover (deacylation-reacylation [26]) of AA but not of docosahexaenoic acid (DHA, 22:6n-6) or palmitic acid (16:0) in brain phospholipid [27–30]. Downregulation of AA turnover by lithium and carbamazepine was associated with decreased brain expression of $cPLA_2$ IVA via reduced activity of one of its transcription factor, activator protein-2. Chronic VPA did not affect this enzyme or transcription factor, but its effect has been ascribed to uncompetitive inhibition of brain acyl-CoA synthetase (Acsl, long-chain-fatty-acid--CoA ligase, E.C.6.2.1.3) 4, which preferentially converts unesterified AA to acyl-CoA compared to other long chain fatty acids, palmitic acid or DHA [31–33]. This was demonstrated by kinetic studies on a rat brain microsomal fraction, and by using recombinant Acsl4. Rat tissue contains at least 5 ACSL genes (ACSL1, ACSL3, ACSL4, ACSL5 and ACSL6v1 and ACSL6v2 splice variants) [34], and the protein product of ACSL4, Acsl4, preferentially acylates AA [32, 33] and is found in cell mitochondria, peroxisomes, microsomes and endoplasmic reticulum (http://

www.genecards.org/cgi-bin/carddisp.pl?gene=ACSL4&search=ACSL4). Acsl4 is the rate-limiting enzyme that regulates AA reincorporation into brain phospholipid within the AA deacylation-reacylation cycle [35, 36].

ACSL4 is highly expressed in newborn and adult mouse brain, especially in granule cells of the dentate gyrus and the pyramidal cell layer of CA1 in the hippocampus, and the granular cell layer and Purkinje cells of the cerebellum [37]. Additionally, a deficiency of the ACSL4 gene has been associated with X-linked mental retardation, microcephaly and other congenital malformations in humans [38, 39]. The Alport syndrome with intellectual disability is a contiguous gene deletion syndrome involving several genes on Xq22.3 including ACSL4 [40].

Using recombinant plasmids for the main ACSL's found in rat brain (ACSL3, ACSL4, ACSL6v1 and ACSL6v2), we reported that VPA selectively and uncompetitively inhibited incorporation of AA into AA-CoA by Acsl4 [32]. VPA did not equally reduce activation of palmitate or DHA to their acyl-CoAs, consistent with observations on rat brain microsomal extracts [31, 41]. There also was no inhibitory effect of lithium on AA conversion to AA-CoA [32].

In view of VPA's clinical teratogenic and hepatotoxic side-effects (see above), and of evidence that it reduces AA turnover in rat brain *in vivo* and uncompetitively inhibits recombinant Acsl4 *in vitro*, we thought it of interest to test whether non-teratogenic VPA structural analogues also would inhibit conversion of AA to AA-CoA by Acsl4 *in vitro*, as potential new agents with fewer side effects than VPA for treating BD. To do this, we used *in vitro* Michaelis-Menten kinetics to test inhibition of Acsl4 by the VPA analogues, propylisopropylacetic acid (PIA, 2-isopropylpentanoic acid), propylisopropylacetamide (PID), and N-methyl-2,2,3,3-tetramethylcyclopropanecarboxamide (MTMCD) (Figure 1). They were chosen because they do not inhibit histone deacetylase at relevant clinical doses tested in mice [42] and should not be teratogenic [43, 44], and because their published pharmacokinetic and anticonvulsant profiles suggest *in vivo* bioactivity and brain penetration [43, 45]. Each has eight carbon atoms in its chemical structure, like VPA. PID is an amide derivative, MTMCD is an amide cyclopropyl derivative, and PIA is a constitutional isomer of VPA (Figure 1). We also used sodium butyrate as a negative control. Butyrate is a 4-carbon analog of VPA that does inhibit histone deacetylase [42].

Briefly, we found that Acsl4-mediated conversion of AA to AA-CoA was inhibited uncompetitively by PIA, with a inhibitory constant K_i less than reported for VPA [32]. PID, MTMCD or butyrate had no inhibitory action. An abstract of part of this work has been published [46].

Materials and Methods

Reagents

[1-^{14}C]AA (50 mCi/mmol) was purchased from Moravek Biochemicals (Brea, CA). Unlabeled AA, sodium butyrate, coenzyme A, and ATP were purchased from Sigma (St. Louis, MO). Racemic PIA was obtained from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program (Research Triangle Park, NC). PID and MTMCD were synthesized according to published procedures [47].

Preparation of bacterial lysate

Recombinant plasmids for rat liver ACSL4-Flag were expressed in *E. coli* strain BL21-codonPlus (DE3)-RIL [48]. As a negative control, the same strain, transformed with the empty vector, was used under identical conditions. Recombinant Acsl-Flag proteins were

induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at A600=1.0. *E. coli* were grown in Terrific Broth medium supplemented with carbenicillin (final concentration 50 μg/ml) at 37°C and shaken at 206 rpm for 6 h. Cells were harvested by centrifugation at 4000 g for 20 min in a Sorval (Newton, CT) SA-600 rotor at 4°C after the 6-h induction period. The cell pellet was resuspended in a buffer containing 10 mM HEPES (pH 7.8) and 0.5 mM EDTA, and sonicated on ice with six 10-s bursts each followed by a 10-s rest, using a cell disruptor sonicator (Heat Systems Ultrasonics, Farmingdale, NY) at setting 4. Lysate aliquots were stored at −80°C for enzyme assay. Protein concentrations were determined by the Bradford method [49].

As reported earlier [32], we demonstrated using Western blotting and a specific anti-Flag M2 monoclonal antibody, that the enzyme preparation that we are studying was a single Acsl 4 isoenzyme, whereas the empty control showed no immunostaining.

Acsl4 activity assay

Acsl4 activity was measured using 1–3 μg protein as previously described [32]. The assay medium contained 175 mM Tris-HCl pH 7.4, 8 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM ATP, 0.25 mM CoA, 0.01 mM EDTA, and 5 μM [^{14}C]AA in 0.5 mM Triton X-100, and increasing concentrations of unlabeled AA in a total volume of 200 μl. PIA (0, 5, 10 or15 mM in ethanol), PID (10 mM in water) or MTMCD (10 mM in water), was added directly to the reaction mixture during inhibition assays. The drug controls consisted of the respective vehicle without the drug. As an additional negative control, sodium butyrate (a short-chain VPA analog) was added to the reaction mixture at 60 mM [32]. Assays were performed at 37°C for 5 min with shaking. The reaction was started by adding 15 μl bacterial lysate to the reaction mixture, and was terminated by adding 1 ml Dole's Reagent (isopropanol:heptane: 1M H_2SO_4, 80:20:2, by vol). In a preliminary experiment, the pH of reaction mixtures spiked with VPA and sodium butyrate at concentrations of 60 mM was measured using a pH meter. The pH (7.4) remained constant at these drug concentrations.

Unesterified fatty acids were extracted using two 2-ml heptane washes, and acyl-CoA radioactivity was measured by liquid scintillation counting. As a negative control, Acsl enzyme activity of the *E. coli* cell lysate lacking a gene coding for ACSL-Flag was measured with AA as substrate as described above. The results were corrected for blanks (samples without cell lysates added and samples analyzed in the absence of fatty acids). The negative control (empty vector) activity were compared with Acsl4 to make sure that the signal to noise ratio was adequate between the test and negative control at each concentration of AA.

Analysis and Statistics

Initial reaction velocity V was plotted against AA concentration for each PIA analogue concentration I_o, and the plots were fitted by least squares to a hyperbolic Michaelis–Menten model using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). K_m (μM) and V_{max} (nmol/min/mg protein) were calculated by the following equation, in which V is reaction velocity (nmol acyl-CoA formed/min/mg enzyme protein, e.g. nmol/min/mg protein) at a given AA substrate concentration, S (μM)

$$V=\frac{V_{\max}S}{K_m+S} \quad (1)$$

The model in which the substrate (i.e. AA) inhibits the reaction velocity can be described as [50],

$$V=\frac{V_{\max}S}{K_m+S\left(1+\frac{S}{K_s}\right)} \quad (2)$$

A model that involves both substrate inhibition and uncompetitive inhibition by the inhibitor I_o can be represented as,

$$V=\frac{V_{\max}S}{K_m=S\left(1+\frac{S}{K_s}+\frac{I_o}{K_i}\right)} \quad (3)$$

where K_i is the enzyme inhibition constant.

Data were plotted as a function of inhibitor concentration I_o, for PIA, PID and MTMCD or sodium butyrate, and the enzyme inhibition constant (K_i) was derived from the ascending part of the plot. Lineweaver–Burke plots of 1/V vs. 1/S in the presence of different inhibitor concentrations were plotted [50].

Selection of model

To determine which inhibition model best described the data, we utilized the Akaike Information Criterion (AIC) [51],

$$\mathrm{AIC}=2k-2\ln(L) \quad (4)$$

where k = number of parameters and L = maximized value of the likelihood function of the model. For small sample sizes, the AIC is corrected and is given as AICc [52],

$$\mathrm{AICc}=N\times\ln(\mathrm{ss}/N)+2K+\frac{2K(K+1)}{N-K-1} \quad (5)$$

where ss is the sum of squares from the fit, N is the number of experimental observations and K is the number of parameters in the model. As the goodness of fit of a model to the measured data improves, the value of AIC declines. Therefore, AICc is a formal method to evaluate model quality and simplicity.

The probability that the model is correct can be determined by the following equation, where Δ is the difference between AIC scores [52]

$$\mathrm{Probability}=\frac{e^{-5\Delta}}{1-e^{-5\Delta}} \quad (6)$$

For this study with AA as a substrate, the lowest AICc was found for the “uncompetitive inhibition” model, as reported for VPA [32, 50].

Data are presented as mean ± S.D. Linear regression analyses for obtaining K_m, V_{max}, K_i and other parameters were made using GraphPad Prism Version 5.0 (GraphPad Software,).

Results

As previously described, Acsl4-mediated conversion of AA to AA-CoA showed substrate inhibition [32]. The kinetics of the Acsl4-mediated reactions using AA as a substrate without an inhibitor followed a simple Michaelis-Menten model, with pooled mean K_m and V_{max} of 4.12 ± 0.56 μM, and 132.6 ± 8.81 nmol/min/mg (n = 3) respectively, among the

different experiments. These values are comparable to previously reported means of 4.98 ± 1.41 μM and 143 ± 11.1 nmol/min/mg, respectively, for Acsl4 [32].

PIA inhibited AA to AA-CoA conversion by Acsl4 with a K_i of 11.44 ± 1.28 mM (n = 3) (Figure 2A). When calculating the Lineweaver-Burke plots in Figure 2B, we considered substrate AA concentrations only in the rising phase of the V vs. [AA] curves, from 0 to 35 μM AA, since at higher AA concentrations the enzyme showed substrate inhibition (Figure 2A). Inhibition by PIA, determined by graphical analysis of the Lineweaver-Burke plots showing parallel slopes, was consistent with an uncompetitive inhibition mechanism (Figure 2B) [50]. The difference between AICc values for the uncompetitive and noncompetitive enzyme inhibition models was 3.491 (Eq. 5), which means that the probability that the uncompetitive model was correct, was 85%, compared to 15% for the noncompetitive model (Eq. 6).

PID and MTMCD did not inhibit Acsl4-mediated conversion of AA to AA-CoA, with inhibitor concentrations as high as 10 mM (Figures 3A and 3B). As an additional control, we measured Acsl4 activity in the presence of sodium butyrate, the 4-carbon analog of VPA that also inhibits histone deacetylase [42]. As reported [32], sodium butyrate did not inhibit Acsl4 activity at a concentration of 60 mM (Figure 3C).

Discussion

We examined inhibition of the conversion of AA to AA-CoA by rat recombinant Acsl4 *in vitro* by each of three non-teratogenic VPA analogues, PIA, PID and MTMCD, and of sodium butyrate, a 4-carbon teratogenic analogue, using our previously published method [53]. Similar to VPA, PIA inhibited Acsl4 conversion by an uncompetitive acylation mechanism, whereas PID, MTMCD or butyrate had no measurable inhibitory effect. PIA inhibited Acls4 activity with a K_i of 11.4 mM, half the reported K_i of 25 mM for VPA [32]. An uncompetitive pattern of inhibition using Michaelis-Menten kinetics was consistent with the parallel Lineweaver-Burke plots of Figure 2B, and was demonstrated to have a high probability compared with other mechanisms using the Akaike Information Criterion (AIC). Uncompetitive inhibition implies that PIA binds to the Acsl4-AA substrate complex at a different binding site than does substrate AA, and causes a conformational change that reduces enzyme activity and conversion rate [50]. A similar model was derived for inhibition by VPA of Acsl4 activity [32].

Acsl4 mediated conversion of AA to AA-CoA also showed substrate inhibition (Figure 2A), with best-fit values for K_m and V_{max} of 4.12 ± 0.56 μM, and 132.6 ± 8.81 nmol/min/mg, respectively, comparable to values of 4.98 ± 1.41 μM and 143.3 ± 11.1 nmol/min/mg, respectively, that were reported previously [32].

PIA inhibited recombinant Acls4 activity with a K_i of 11.4 mM. In comparison, VPA inhibited AA acylation by recombinant Acsl4 *in vitro* at a K_i of 25 mM, about twice that of PIA, suggesting that PIA would be more effective in vivo on an equi-concentration basis. Rat brain PIA concentrations have not been reported, although penetration occurs, based on its anticonvulsant effects in rats [43], whereas the mean brain VPA concentration is estimated as 1.0 – 1.5 mM after VPA administration at a therapeutically relevant dose (200 mg/kg, i.p.) that selectively reduces AA turnover in rat brain phospholipid [27, 32, 54–56]. The discrepancy between the *in vitro* concentration required for Acsl4 inhibition and the estimated mean therapeutic brain level for VPA was reconciled by evidence that VPA can accumulate, *via* a short-chain fatty acid transporter, within cellular mitochondria, microsomes and other organelles in which Acsl4 also is found [37, 39, 57–59]. Similar considerations may apply to PIA, which also is a short chain fatty acid. For both PIA and

VPA, their *in vitro* kinetic inhibition constant for Acsl4 may differ from the actual *in vivo* value, since it may depend on bath conditions such as pH, temperature, salt and ATP concentrations, and on the absence of fatty acid transport proteins that are present *in vivo* [60]. At clinical therapeutic levels, VPA can be hepatotoxic, and it can be teratogenic in pregnant women because it inhibits histone deacetylase [8, 9, 43]. PIA is less teratogenic than VPA. It is not teratogenic at 3.6 mmol/kg in mice compared to marked teratogenicity of VPA at this dose, but its teratogenicity at higher doses remains to be further evaluated [43, 61, 62], and it does not inhibit histone deacetylase *in vitro* [42]. Although PID and MTMCD have equal or better anticonvulsant activity in the rat than does VPA [43, 45], neither compound inhibited Acsl4 in this study. These differences distinguish between anticonvulsant activity and anti-BD activity of these drugs, and suggest that they have different mechanisms of action in each of the two disorders. Similarly, the clinically useful anticonvulsants, topiramate and gabapentin, do not measurably affect rat brain AA metabolism [63–66].

In comparing the structures of VPA and the three analogues used in this study (Figure 1), a free carboxylic group (Figure 1) would appear necessary for Acsl4 inhibition. Thus, PIA's effect was absent when the hydroxyl group of its carboxylic acid moiety was replaced by an amino group (PID and MTMCD). Furthermore, since butyrate did not inhibit Acsl4, a chain of longer than four carbons appears necessary for inhibition.

X-ray crystallography might help to establish structure-activity relations for inhibition of Acsl4 by identifying a common site for PIA and VPA binding. At present, X-ray crystallography-derived structures for mammalian Acsl enzymes are unavailable, although one has been published for the distantly related Acsl from *Thermus thermophilus* HB8. The fatty acid binding pocket of this latter enzyme is at its N-terminus [67].

Brain AA metabolism and turnover are upregulated in animal models of neuroinflammation and excitotoxicity [68–70], and AA metabolic markers are elevated in association with these neuropathological processes in the postmortem BD brain [14–16]. Because lithium, carbamazepine and VPA downregulate brain AA turnover and other AA metabolic markers in rat brain [21, 28, 30, 71–75], their therapeutic efficacy in BD may depend on suppressing the upregulated brain AA cascade of that disease. It remains to be determined whether the observed inhibition by PIA of AA to AA-CoA conversion by recombinant Acsl4 *in vitro* corresponds to its ability to also reduce metabolic markers of the AA cascade *in vivo* [27, 76], which would lend more justification to initiating a clinical trial with PIA in BD.

In conclusion, we have identified PIA as a new uncompetitive Acsl4 inhibitor, similar to VPA. PIA has a lower K_i than does VPA, it does not inhibit histone deacetylase, and it is not teratogenic up to a dose of 3.6 mmol/kg in mice [42, 43, 61, 62]. Thus, PIA may be of interest for treating BD. Showing this also would argue that Acsl4 is a reasonable target for developing new mood stabilizers to treat BD. However, further *in vivo* experiments are required to claim that PIA would decrease AA turnover in rat brain phospholipids like VPA, lithium and carbamazepine, which would justify the need for a clinical trial.

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Abbreviations

AA	arachidonic acid
Acsl	acyl-CoA synthetase
BD	bipolar disorder
MTMCD	N-methyl-2,2,3,3-tetramethylcyclopropanecarboxamide
PIA	propylisopropylacetic acid
PID	propylisopropylacetamide
VPA	valproic acid

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Highlights

- Valproic acid's constitutional isomer, PIA, uncompetitively inhibit Acsl4
- K_i of PIA is 11.4 mM, compared to a published K_i of 25 mM for valproic acid.
- Like VPA, PIA may reduce AA turnover in brain phospholipids in unanesthetized rats
- If so may be effective as a non-teratogenic mood stabilizer in BD patients.
- Justification for designing new Acsl4 inhibitors as potential less toxic drugs for treating bipolar disorder.

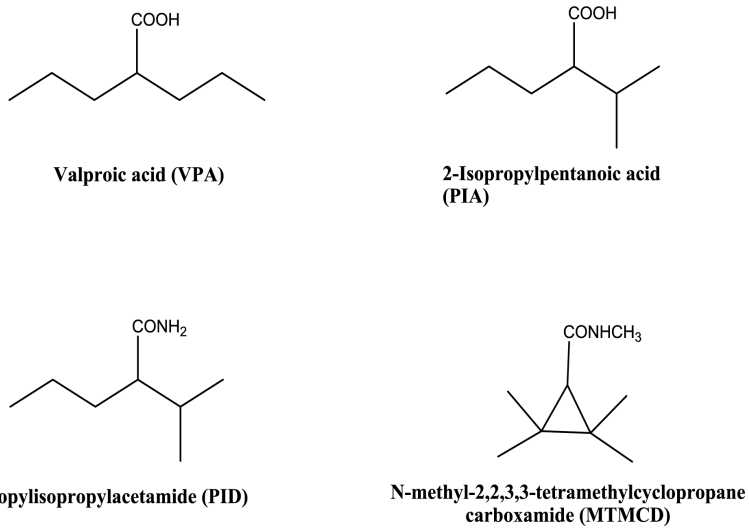

Figure 1.
Structures of VPA, PIA, PID and MTMCD.

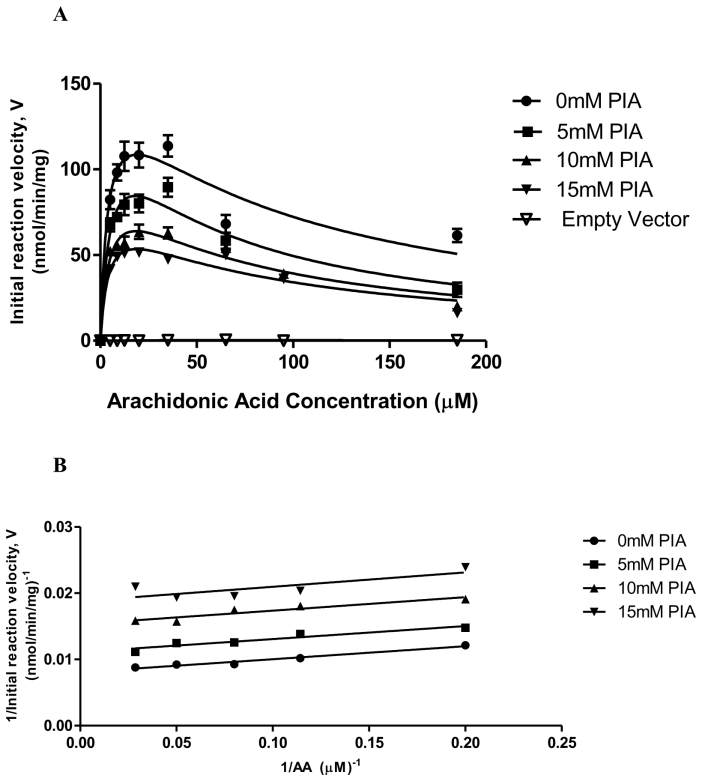

Figure 2.

(2A) Initial reaction velocity (V, nmol/min/mg protein) of Acsl-4 plotted against increasing AA concentration [S] in the presence of 0, 5, 10, or 15 mM PIA [I]. Empty vector contains no Acsl enzyme, and shows no activity.

(2B) Typical Lineweaver-Burke plot of the reciprocal of enzyme activity (1/V) against the inverse of substrate concentration, 1/[S] (1/[AA]), with AA concentration range limited to from 0 to 35 μM (see Results). The plot is typical of 3 experiments as indicated in text. Parallel plots are characteristic of uncompetitive inhibition [50] put in correct reference

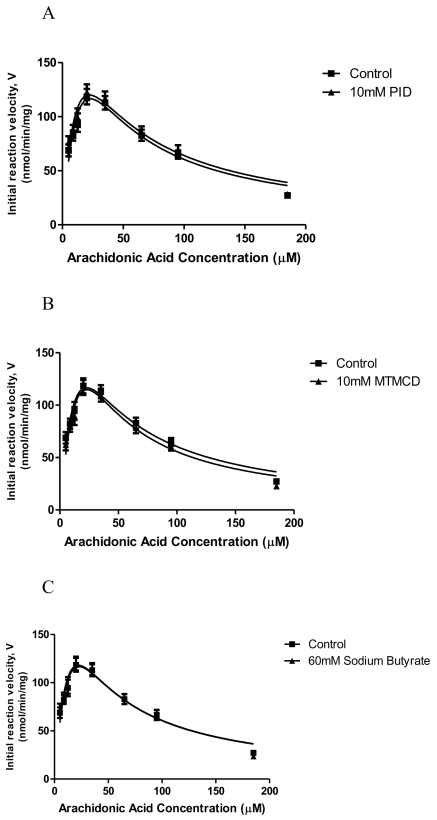

Figure 3.

Initial reaction velocity V of Acsl-4 plotted against increasing AA concentration [S] in the presence of (3A) 0 mM and 10 mM PID, (3B) 0 mM and 10 mM MTMCD, and (3C) 0 mM and 60 mM sodium butyrate.