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How can the mood stabilizer VPA limit both mania and depression?

Lili Cheng,[a] Michael Lumb,[a] László Polgár,[b] and Anne W. Mudge[a,*]

[a]*MRC Laboratory for Molecular Cell Biology and Departments of Biology and Pharmacology, UCL, Gower Street London, WC1E 6BT, UK*
[b]*Institute of Enzymology, Hungarian Academy of Sciences, PO Box 7, H-1518 Budapest 112, Hungary*

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The mood stabilizing drugs commonly used to treat bipolar disorder–lithium, valproic acid (VPA), and carbamazepine (CBZ)–limit the frequency of swings to either manic or depressive states. We previously showed that these drugs all have a common action on cultured neurons, which can be reversed by the addition of either inositol or specific inhibitors of the enzyme prolyl oligopeptidase (PO). Inhibition of PO activity is reported to enhance phosphoinositide (PIns) signaling consistent with the suggestion that mood stabilizers inhibit PIns signaling. We now report that VPA directly inhibits recombinant PO activity, which would have the opposite effect on PIns signaling. This unexpected result suggests a model that could explain the dual action of VPA in stabilizing mood: We propose that euthymic mood is dependent on stable PIns signaling and that VPA may limit mood swings to mania by decreasing PIns signaling, and that it may limit mood swings to depression by inhibiting PO and thus increasing PIns signaling.

Introduction

Bipolar disorder is ranked by the World Health Organisation as the sixth leading cause of disability worldwide. It is characterized by severe mood swings to both mania (Type 1) or hypomania (Type 2) and depression. The mood-stabilizing drugs commonly used to treat this illness are often very effective in controlling symptoms, but in many cases intolerable side-effects make them unacceptable. The therapeutically relevant molecular targets of the mood-stabilizing drugs are not known. Identification of them should provide specific targets for the development of more effective and specific drugs with fewer side-effects: In addition, understanding how the drugs work should shed light on the underlying causes of bipolar disorder (Gould et al., 2004; Williams et al., 2002). Several lines of evidence suggest that neuronal PIns signaling plays a key role in mood control (Agam et al., 2002; Berridge et al., 1989; O'Donnell et al., 2000; Silverstone et al., 2002; van Calker and Belmaker, 2000; Williams et al., 2002). We showed that the three mood stabilizers–lithium, VPA, and CBZ–each decrease the collapse and increase the spread area of growth cones of cultured neurons: inositol addition reversed the drug effects suggesting that each drug caused inositol depletion and/or inhibit PIns signalling (Williams et al., 2002). Inhibitors of the enzyme PO also reversed the effects of the mood stabilizers (see below). To date, the PIns signaling pathway is the only one shown to be targeted by all three mood stabilizers (van Calker and Belmaker, 2000; Williams et al., 2002). Lithium is known to directly inhibit the enzymes that recycle inositol from inositol phosphates (InsPs)–inositol monophosphatase (IMPase) and inositol polyphosphatase (IPPase) (Berridge et al., 1989; Gould et al., 2004)–and VPA indirectly inhibits the enzyme responsible for InsP synthesis—myo-inositol phosphate synthase (MIP-synthase) in yeast (Agam et al., 2002; Ju et al., 2004): the mechanism for inositol depletion by CBZ (Williams et al., 2002) is unknown, but CBZ is reported to inhibit the cyclic adenosine monophosphate (cAMP) second messenger system (Gould et al., 2004). Lithium also inhibits glycogen synthase kinase 3 (GSK3) (Gould et al., 2004; Klein and Melton, 1996), and VPA also inhibits histone deacetylase (HDAC) (Gould et al., 2004; Phiel et al., 2001): these and other signaling pathways affected directly or indirectly by the mood stabilizers (Gould et al., 2004) are not common to all, however, and so seem less likely to be crucial for the control of mania, although they probably contribute to other drug effects including neuroprotection and structural changes in the brain. These alternate drug targets have been discussed in several recent reviews (Coyle and Duman, 2003; Gurvich and Klein, 2002; Harwood and Agam, 2003; Klein and Melton, 1996).

VPA, lithium, and CBZ are all effective antimanic drugs, with only limited efficacy for acute bipolar depression (Goodwin, 2003). The defining (and puzzling) characteristics of the mood stabilizing drugs, however, are that they limit the frequency of mood swings to mania without affecting normal mood (euthymia) and both lithium and VPA can also limit mood swings to depression (Goodwin, 2003). In addition, antipsychotic drugs such as olanzapine are used to control acute mania and recently the anticonvulsant drug lamotrigine was shown to be effective in

* Corresponding author. Fax: +44 207 679 7805.
E-mail address: a.mudge@ucl.ac.uk (A.W. Mudge).
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controlling bipolar depression (Calabrese et al., 2002; Goodwin, 2003). The question of how mood stabilizers such as lithium and valproate might function to control both poles of mood is rarely discussed, however, when considering the relevant therapeutic targets for the drugs. In this study, we address the issue of how VPA might limit swings to both mania and depression by controlling both the highs and the lows of PIns signaling via a mechanism involving the enzyme PO.

PO is a serine protease that belongs to a family of structurally-related proteases (Polgar, 2002). PO cleaves prolyl bonds of small (<3 kDa) peptides, whereas the prolyl bonds of proteins are not cleaved because the β-propeller structure of PO restricts access of larger peptides or protein to the active site (Fulop et al., 1998). This preference for small peptides initially focussed attention on neuropeptides as the natural substrates for PO, but because PO is a cytoplasmic enzyme, neuropeptides are unlikely to be substrates because they are not free in the cytoplasm. The intracellular substrate for PO is still not known, but there is evidence suggesting that PO may inhibit the PIns signaling pathway, but again, the mechanism is not known: Deletion of the PO gene in the slime-mold *Dictyostelium* (Williams et al., 1999) and inhibition of PO activity in an astrocytoma cell line (Schulz et al., 2002) results in increased intracellular basal levels of inositol 1, 4, 5-trisphosphate ($InsP_3$) (~2-fold) together with an enhanced response to agonists that stimulate PIns signaling (Schulz et al., 2002). The ability of PO inhibitors to mimic the effect of inositol on the growth cones is consistent with PO being a negative regulator of the PIns signaling pathway in neurons (Williams et al., 2002) and it raised the question of what part PO plays in drug action and mood control. PO is widely distributed in the body but the highest concentration is in the frontal cortex where there are both soluble and membrane-bound forms: the latter are enriched in synaptic structures (Irazusta et al., 2002; O'Leary et al., 1996). PO had been linked previously to mood and stress-related disorders because blood levels of PO in these illnesses differ from levels in control subjects (Maes et al., 1995, 1998), although the significance of these blood studies is still unclear. In addition, PO inhibitors have been tested in Phase 1 trials for their ability to enhance cognition in elderly subjects (Morain et al., 2000). In the present study, we tested whether mood stabilizers or other psychoactive drugs can directly affect PO activity. We find that VPA directly inhibits recombinant PO activity with a K_i of ~1 mM, which is close to the therapeutic blood levels of 0.3–0.7 mM. This result, together with our previous work with mood stabilizing drugs, suggests a model for the ability of VPA to limit swings to both mania and depression.

Results

We first expressed recombinant human PO (rhPO) in bacteria, purified it using a histidine tag, and tested enzyme activity using the synthetic PO substrate Z-Gly-Pro-AMC. As shown in Fig. 1a, VPA inhibited the activity of rhPO with an IC_{50} of 1–2 mM. In contrast, several other psychoactive drugs tested did not significantly affect PO activity at doses consistent with their therapeutic blood levels (10–50 μM): the inactive drugs included the mood stabilizers, lithium and CBZ, the antidepressants, desipramine and fluoxetine, and the antipsychotic, olanzapine (Figs. 1a, b).

To characterize the inhibition of PO by VPA, we performed a detailed analysis of the Michaelis–Menten kinetic parameters using

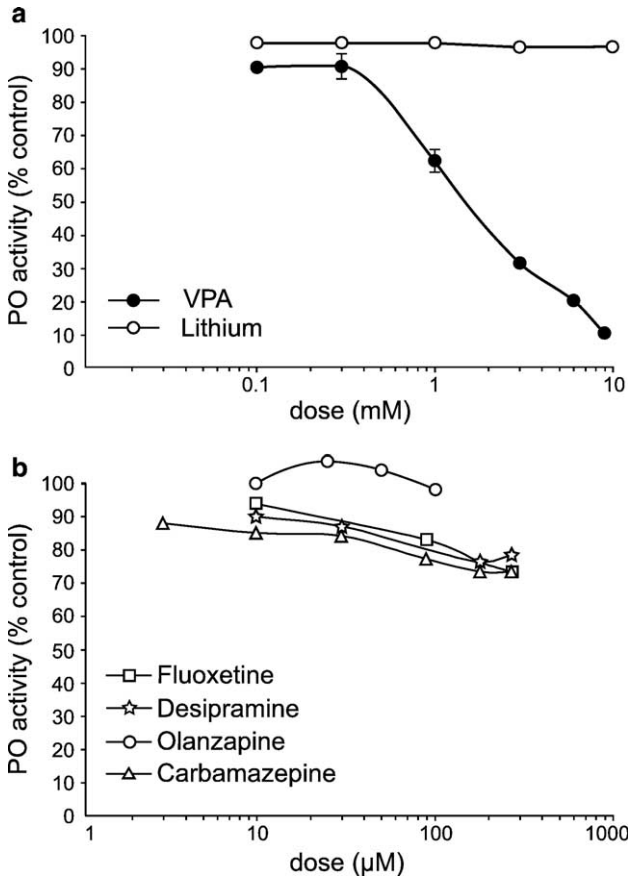
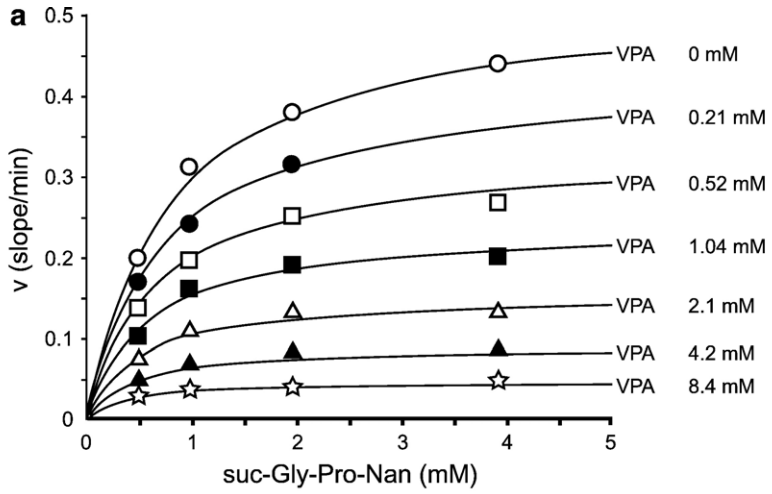
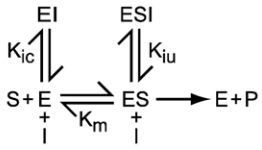


Fig. 1. (a) VPA inhibition of rhPO. PO activity was measured using Z-Gly-Pro-AMC substrate at 30 μM (K_m). Relative activity of PO in the presence of VPA is shown as a percent of activity in the absence of VPA. Lithium tested in the range of 0.1–10 mM had no significant effect on PO activity. (b) Relative activity of rhPO with the drugs CBZ, desipramine, fluoxetine, and olanzapine. PO activity was measured as in (a) but in a lower concentration range (μM) and activity was compared with controls with a matched concentration of DMSO.

pure recombinant porcine PO (rpPO) with two synthetic substrates, Suc-Gly-Pro-Nan (Fig. 2a) or Z-Gly-Pro-Nap (data not shown), as previously described: these substrates show differences in their pH dependence. For both substrates, VPA decreased V_{max} in the dose range 0.2–8 mM (see Fig. 2a): note that there is substantial inhibition of PO activity at 0.5 mM VPA, which is within the standard range for therapeutic blood levels, namely 0.3–0.7 mM. When fitted with the equation describing pure non-competitive inhibition, we obtained a K_i of ~1 mM for both substrates. We obtained better fits with the equation describing mixed inhibition, however, indicating that the inhibition possessed a significant competitive component with both substrates (Figs. 2b, c). This analysis implies that VPA interferes with both the catalytic activity of the enzyme and the binding of substrate to the active site. The inhibition of PO by VPA was specific for this enzyme because VPA, even at a dose of 12 mM, did not inhibit oligopeptidase B (Fig. 3), which cleaves dibasic residues and also belongs to the PO family of serine peptidases—all of which are structurally and mechanistically related (Polgar, 2002). VPA's specificity for PO over oligopeptidase B, together with the competitive inhibitory component, suggests that VPA binding may specifically affect the proline-binding site of PO, which would provide an explanation of



b



c

	A	A + 150 mM KCl	B + 150 mM KCl
K_m (mM)	0.77 ± 0.05	1.14 ± 0.05	0.011 ± 0.002
K_{ic} (mM)	1.81 ± 0.43	1.51 ± 0.20	2.7 ± 0.7
K_{iu} (mM)	0.87 ± 0.06	0.92 ± 0.07	0.7 ± 0.1

A: substrate Suc-Gly-Pro-Nan
B: substrate Z-Gly-Pro-Nap

Fig. 2. VPA inhibition of rpPO. (a) Initial rates (v) of enzyme activity were monitored versus substrate Suc-Gly-Pro-Nan and VPA concentrations as shown. Results were analyzed using the multidimensional equation for mixed inhibition: $v = V_{max}S / [K_m(1 + I/K_{ic}) + (1 + I/K_{iu})\ S]$ where I (VPA) is the second independent variable and K_{ic} and K_{iu} are the competitive and uncompetitive components of the dissociation constant, V_{max} and K_m are the Michaelis–Menten parameters. The data were fitted by nonlinear regression. (b) Mechanism for mixed inhibition. (c) Table shows the calculated values for K_m, K_{ic} and K_{iu} with either Suc-Gly-Pro-Nan (A) or Z-Gly-Pro-Nap (B) as substrate, in the presence or absence of 150 mM KCl as indicated.

why a short, branched-chain fatty acid has specificity as a drug. Analysis of the structure of PO crystallized with VPA bound should reveal the precise location of VPA binding {see Fulop et al. (1998)}.

The direct inhibition of PO by VPA was at first surprising: because PO inhibitors reversed the action of VPA on growth cones (Williams et al., 2002), we had predicted that if VPA affected PO activity either directly or indirectly, then the effect would be to activate PO rather than inhibit it. We therefore tested whether we could find conditions where the mood stabilizers could have opposite effects on the behavior of the neurons. Indeed, we found that lithium, CBZ, and VPA can have opposite effects on the morphology of neuronal growth cones, depending on the levels of intracellular c-AMP (see below). We tested cAMP effects for three reasons: first, the cyclic-AMP-response-element-binding protein (CREB) is thought to play a major role in depression (Nestler et al., 2002) and is regulated by $InsP_3$ signaling as well as by cAMP (Lonze and Ginty, 2002), suggesting that both these second messengers may be crucial for mood control. Second, cAMP modulates synaptic transmission (Nagy et al., 2004), as does $InsP_3$ (Nagase et al., 2003; Nahorski et al., 2003). Third, a rise in intracellular cAMP switches the response of growth cones to the signal protein semaphorin from attractive to repellent (Nishiyama et al., 2003).

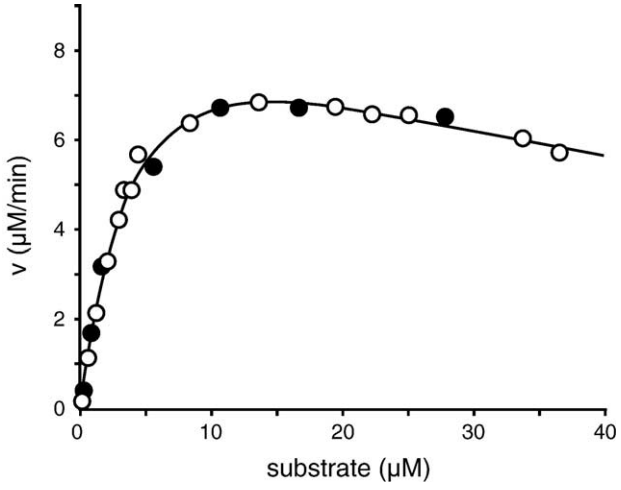


Fig. 3. Oligopeptidase B is not inhibited by VPA. Initial rates (v) were monitored using variable concentrations of the substrate Abz-Thr-Arg-Arg-|-Phe(NO_2)-Ser-Leu-NH_2 as shown. This substrate binds in an alternative unreactive mode, so that the data were fitted by nonlinear regression with a modified Michaelis–Menten equation, $v = V\ [S] / (K_m + [S] + [S]^2/K_{is})$ where K_{is} is the inhibition constant for the second substrate. The filled points were determined in the presence of 12 mM VPA.

We treated cultured sensory neurons with a non-hydrolyzable analogue of cAMP, cpt-cAMP, in order to increase the levels of cAMP-dependent protein phosphorylation. Cpt-cAMP had the same effect as the mood stabilizers on the dynamic behavior of the growth cones (Williams et al., 2002), decreasing the number of collapsed growth cones as previously reported (Fig. 4). When we added the mood stabilizers together with cpt-cAMP, however, the effects were not additive-instead lithium, VPA and CBZ had the opposite effect and reversed the cpt-cAMP-induced collapse. Importantly, the PO inhibitor also reversed the effect of cpt-cAMP on growth cones, consistent with the possibility that VPA reversed the effects of cpt-cAMP on growth cones by inhibiting PO activity. Because cpt-cAMP changed the neuronal response to lithium and CBZ (Fig. 4), but these drugs did not inhibit PO activity directly, it may be that there are additional regulators of PIns signaling that are

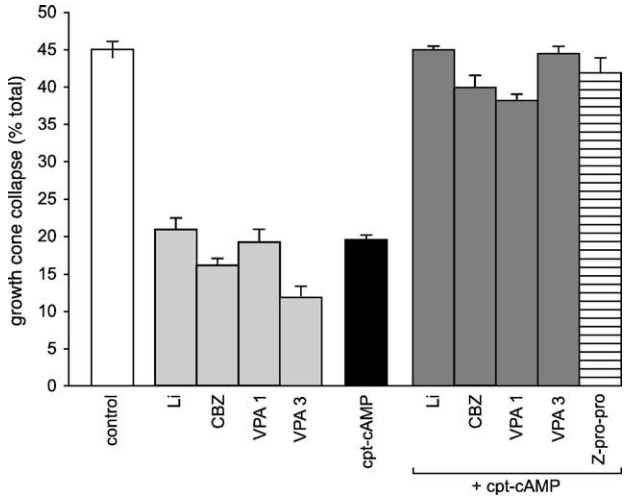


Fig. 4. Cpt-cAMP changes the response of growth cones to mood stabilizers. Sensory neuron explants were treated with 3 mM lithium, 3 mM VPA, 20 μM CBZ, or the PO inhibitor Z-Pro-Prolinal (133 μM) in the presence or absence of 200 μM cpt-cAMP as indicated. The number of collapsed growth cones was counted as a percent of total (n = 6).

drug-sensitive and that result in increased PIns signaling as does VPA inhibition of PO.

Discussion

We report that the proteolytic enzyme PO is a direct molecular target for the mood-stabilizing drug, VPA. The K_i for direct inhibition of PO by VPA is ~1.0 mM, which is compatible with the surprisingly high therapeutic blood level of 0.3–0.7 mM that is routinely used to treat bipolar disorder (Taylor et al., 2001). Moreover, VPA accumulates inside cells and it may also be more effective in inhibiting PO action on its native but unknown substrate. (For comparison, the IC_{50} for VPA inhibition of HDAC is 0.4 mM and the K_is for lithium inhibition of IMPase and GSK3 are 0.8 mM and 2 mM, respectively.) Because PO is involved in some way in the regulation of the PIns signaling pathway (Schulz et al., 2002; Williams et al., 1999), this result adds further support to the suggestion that the PIns signaling pathway is involved in the therapeutic action of the mood stabilizing drugs and that defects in the mechanisms that regulate PIns signaling may underlie bipolar disorder. This result also suggests that other small molecule inhibitors of PO may be useful in the treatment of bipolar disorder.

Direct inhibition of PO by VPA was surprising for three reasons: First, we found previously that both PO inhibitors and inositol reverse the effects of VPA and other mood stabilizers indicating that VPA depletes inositol or inhibits the PIns signaling pathway (Williams et al., 2002). Second, VPA lowers inositol levels in the brain (O'Donnell et al., 2000; Silverstone et al., 2002). Third, inhibition of PO activity increases basal $InsP_3$ in astrocytomas (Schulz et al., 2002). We had predicted therefore that VPA might activate PO rather than inhibit it. The unexpected direct inhibition of PO by VPA suggests that, in some circumstances, VPA could increase PIns signaling and, perhaps, inositol availability, the opposite of the effects described previously (Agam et al., 2002; O'Donnell et al., 2000; Williams et al., 2002). Although surprising, our results again point to the PIns signaling pathway as being key for mood control. More importantly, this unexpected effect of VPA suggests an explanation for the dual function of VPA in limiting both depression and mania. Moreover, our experiment showing that the neuronal response to VPA varies with the level of cAMP-dependent phosphorylation in the neurons also suggests that ongoing activity in the brain may alter the response to the drugs, thus allowing either the positive or negative regulation of PIns signaling to predominate, but this suggestion needs further exploration.

The efficacy of drugs in the control of mood seems likely to involve drug regulation of synaptic transmission in relevant brain circuits. Although the PIns signaling pathway is not required for basal synaptic transmission, the intracellular second messengers $InsP_3$ and diacylglycerol (DAG) enhance synaptic transmission and are required for some types of synaptic plasticity (Brose and Rosenmund, 2002; Nahorski et al., 2003), as is the second messenger, cAMP (Nagy et al., 2004). The second messengers can have both acute and long-term effects mediated by changes in gene expression (Lonze and Ginty, 2002). Moreover, given its role in synaptic plasticity, PIns metabolism is likely to be under stringent local control in specific synapses. Both lithium and VPA can decrease inositol levels in brain (O'Donnell et al., 2000; Silverstone et al., 2002), although such global changes are difficult to interpret because PIns metabolism differs between cell types, with glial cells having higher levels than neurons (van Calker and Belmaker, 2000). In addition, PIns signaling is spatially restricted within single neurons (Delmas et al., 2004; Nahorski et al., 2003).

Inositol levels in the brains of manic subjects are reported to be higher than in controls, although the studies so far are limited in subject numbers (Davanzo et al., 2001; Silverstone et al., 2002). In contrast several studies have reported that brain inositol levels are low in depression (Barkai et al., 1978; Frey et al., 1998; Shimon et al., 1997). Interestingly, euthymic subjects treated with either lithium or VPA have normal inositol levels compared with controls, suggesting that the mood stabilizers may return inositol levels and PIns signaling to an optimum compatible with normal mood (Silverstone et al., 2002) (see Fig. 5). In addition, raising the levels of brain inositol improves the mood of depressed subjects in pilot studies (Chengappa et al., 2000; Levine et al., 1995), and inositol acts like an antidepressant in animal studies (Einat and Belmaker, 2001; Einat et al., 1999). These observations, together with our results showing the possibility of dual effects of VPA on PIns signaling, suggest a model for the dual action of this mood stabilizer (see Fig. 5). They also suggest that both the manic and the depressive phases of bipolar disorder may involve defects in the PIns signaling pathway.

In proposing the 'inositol depletion' hypothesis for the action of lithium in the control of mood, Berridge et al. (1989) likened the action of lithium to that of a car seat belt where the restraining force only comes into play when there is pressure on the belt. This model, however, only accounts for euthymia and the control of mood in one direction, most likely mania. We propose instead that the control of mood is more like the action of a sound compressor, which limits extremes by attenuating high and amplifying low volumes to keep music at an optimal level. By analogy with such a sound compressor, the inhibitory effects of the three mood stabilizers on PIns signaling that we described previously (Williams et al., 2002) may act to limit the highs, while the direct inhibition of PO by VPA may act to limit the lows of PIns signaling, thereby maintaining PIns signaling in mood-related circuits within the range required for mood stability. (It is also possible that there are other targets of lithium–not PO–that oppose

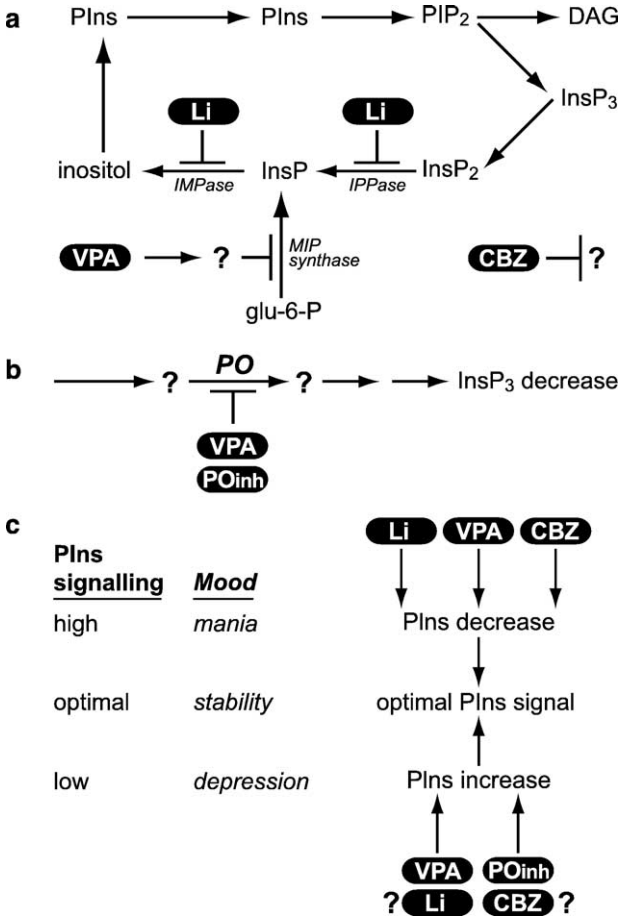


Fig. 5. (a) Diagram showing enzymes in the PIns signaling pathway. The second messengers $InsP_3$ and DAG are generated from phosphatidylinositol 4,5-bisphosphate (PIP_2) and cytoplasmic inositol phosphates are recycled back to phosphoinositides (PIns, PIP and PIP_2) in the membrane. Enzymes inhibited by lithium (Berridge et al., 1989) and possibly VPA (Agam et al., 2002) are indicated by the bars. (b) PO decreases PIns signaling in cells by an unknown mechanism (Schulz et al., 2002; Williams et al., 1999). (c) Model for how PIns signaling defects could be involved in both mania and depression and how VPA and other mood stabilizers may dampen swings in PIns signaling and thus stabilize mood in bipolar disorder.

its PIns inhibitory effects and that could explain lithium's ability to limit depression as we suggest for VPA's inhibition of PO).

In summary, our finding of this new molecular target for VPA, together with our previous study (Williams et al., 2002), strengthens the hypothesis that bipolar disorder involves defects in a regulatory system that acts to dampen swings to either high or low PIns signaling. They suggest that searches for new therapies for bipolar disorder would do well to focus on control elements in the PIns signaling pathway, including PO, which may prove to be a target for drugs that limit the depressive phase of this devastating illness.

Experimental methods

Recombinant PO

To produce recombinant human PO (hrPO) protein, we obtained an IMAGE clone containing the human PO gene (CM276) from HGMP, Hinxton Hall, Cambridge, UK. The coding region was PCR'd and cloned into the bacterial expression vector pTrcHis2 (Invitrogen). The C-terminally His-tagged protein was expressed and purified using the QiaExpress purification system (Qiagen). Recombinant porcine PO rpPO) and oligopeptidase B were prepared and assayed as described previously (Juhasz et al., 2002; Szeltner et al., 2000).

PO enzyme activity assays

All synthetic substrates were from Bachem, Switzerland. Enzyme assays with brain extracts and rhuPO were done in 150 mM potassium phosphate buffer, pH 7.2 at 37°C with the substrate benzyloxycarbonyl-Gly-Pro-7-amido-4-methylcoumarin (Z-Gly-Pro-AMC) (O'Leary et al., 1996). Enzyme activity with rpPO and the substrate succinyl-Gly-Pro-4-nitroanilide (Suc-Gly-Pro-Nan) was measured at 25°C in 75 mM Mes-Tris buffer, pH 7.0, using 19.5 nM enzyme and VPA in the range of 0.2–8mM; in some experiments 150 mM KCl was added to the buffer to mimic the intracellular salt composition. For activity studies with the substrate Z-Gly-Pro-2-naphthylamide (Z-Gly-Pro-Nap), 0.42 nM enzyme was used in buffer with 0.125% acetonitrile and 10 nM BSA. Initial rates (v) were monitored with either Suc-Gly-Pro-Nan or Z-Gly-Pro-Nap in the absence or presence of the inhibitor, VPA. Results were analyzed using the multidimensional equation for mixed inhibition: $v = V_{max}S/[K_m(1 + I/K_{ic}) + (1 + I/K_{iu})S]$, where I (VPA) is the second independent variable and K_{ic} and K_{iu} are the competitive and uncompetitive components of the dissociation constant, V_{max} and K_m are the Michaelis–Menten parameters. The data were fitted by nonlinear regression using the *GraFit* software (Erythacus Software, Ltd., Staines, UK).

Oligopeptidase B enzyme activity assays

The internally quenched fluorescence substrate Abz-Thr-Arg-Arg-|-Phe(NO_2)-Ser-Leu-NH_2 was prepared by solid phase synthesis. The reactions were monitored fluorometrically at 25°C using 0.19 nM enzyme and excitation and emission wavelengths of 337 nm and 420 nm, respectively, as previously described (Juhasz et al., 2002).

Drugs

Stock solutions of drugs were prepared as follows: lithium, VPA and the c-AMP analogue chlorophenylthio-cAMP (cpt-cAMP) were dissolved in water, while CBZ, desipramine, fluoxetine, and olanzapine were each dissolved in DMSO; solvent controls were used appropriately. Drugs were purchased from the following companies: lithium, VPA, and CBZ (Sigma), fluoxetine (Tocris), cpt-cAMP (Calbiochem), Z-Pro-Pro-aldehyde-dimethyl acetal (Z-Pro-Prolinal, Bachem). Olanzapine was generously provided by GlaxoSmithKline (Harlow, UK).

Growth cone assay

Newborn rat dorsal root ganglia were plated on laminin-coated coverslips and cultured in DMEM/F12 medium with the addition of defined additives (Cheng and Mudge, 1996). Cytosine arabinoside (5×10^{-6}M) was added to kill dividing non-neuronal cells and 50 μg ml^{-1} of NGF (7S-form; Alomone Laboratories) was added to promote neuron survival and axonal

outgrowth. Neurons were grown for 24 h in a 5% CO_2 incubator at 37°C before the addition of drugs. After a further 18 h, the sensory neuron axons that extend from the ganglia were loaded with the cytoplasmic dye Calcein (Molecular Probes) and fixed as described previously (Williams et al., 2002). The number of collapsed growth cones was counted using a Zeiss fluorescence microscope and expressed as a percentage of total growth cones: at least 300 growth cones were counted in duplicate in each experiment and the experiment was repeated three times with similar results. Time-lapse movies showing the dynamic behavior of the axonal growth cones in the presence or absence of mood stabilizers or PO inhibitors can be viewed on http://www.ucl.ac.uk/lmcb//anne/Mudge/MechanismOfMoodStabilizers.html.

Acknowledgments

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