Sodium Valproate Down-regulates the Myristoylated Alanine-Rich C Kinase Substrate (MARCKS) in Immortalized Hippocampal Cells: A Property of Protein Kinase C-Mediated Mood Stabilizers

DAVID G. WATSON, JEANNETTE M. WATTERSON and ROBERT H. LENOX

ABSTRACT

Sodium valproate (VPA) is a short-chain fatty acid with well-established anticonvulsant properties and apparent clinical efficacy in the treatment of bipolar disorder (manic-depressive illness). Little is known regarding the mechanism of action of VPA in the brain that could account for this clinical therapeutic profile. Lithium has been the standard treatment for bipolar disorder, and it is known to be an uncompetitive inhibitor of inositol monophosphatase in the phosphoinositide (PI) signaling cascade at clinically relevant concentrations. Recent studies have provided data in support of a role for protein kinase C and the down-regulation of expression of the myristoylated alanine-rich C kinase substrate (MARCKS) in the long-term therapeutic action of lithium in the brain, which is dependent on both the relative activity of receptor-coupled PI signaling and the concentration of myo-inositol. Our current results demonstrated that valproate induces a concentration- and time-dependent reduction of MARCKS in immortalized hippocampal cells that appears to be independent of both the level of muscarinic receptor-activated PI signaling as well as the concentration of myo-inositol. In CHO-K1 cells transfected with the human m1 muscarinic receptor, unlike lithium, there is no evidence for receptor-mediated accumulation of CMP-PA in the presence of VPA, providing more direct data for its lack of interaction within the PI signaling cascade. The action of VPA on MARCKS occurs within the therapeutic concentrations and time course observed in clinical studies of patients with bipolar disorder. Furthermore, the effect on MARCKS protein is additive in the presence of therapeutic concentrations of both lithium and valproate, consistent with clinical observations regarding the enhanced efficacy of the combination treatment. Finally, in studies examining acute and chronic effects of a variety of psychotropic compounds and VPA structural analogs, it is evident that the property of regulation of MARCKS is shared by the mood-stabilizers lithium and VPA, which may be specific to a class of drugs effective in the treatment of bipolar disorder.

VPA is a short-chain fatty acid that was first discovered to have antiepileptic effects in 1963 and consequently has been used as an anticonvulsant in this country for nearly 20 years (Meunier et al., 1963; Ramsay, 1984). In recent years, VPA has emerged as an effective alternative to lithium in the treatment of acute mania in patients with bipolar disorder (Gerner and Stanton, 1992; McElroy et al., 1992, Bowden et al., 1994). VPA also may have a wider spectrum of efficacy than lithium, with accumulating evidence of its use in atypical (dysphoric/mixed) mania, rapid cycling, and secondary manias, in which lithium appears to be less clinically effective (Calabrese and Delucchi, 1990; Calabrese et al., 1992, 1993; Bowden et al., 1994). However, as with lithium, the mechanisms mediating the therapeutic properties of VPA in the treatment of bipolar disorder are not clearly understood (Post et al., 1992b). Although the therapeutic actions of VPA have been reported to be associated with enhancement of GABAergic transmission (Petty, 1995), these effects are often reported at rather high concentrations and may relate more to the anticonvulsant properties of VPA than to its mood-stabilizing effects (Waldmeier, 1987, Motohashi, 1990). Furthermore, similar to lithium, there is a delay of several days in the onset of clinical action, although loading strategies with VPA have demonstrated antimanic effects at times as early as 3 days (McElroy et al., 1996). Thus, any mechanism postulated for the therapeutic action of these drugs must

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ABBREVIATIONS: CMP-PA, cytidine monophosphate-phosphatidic acid; DAG, diacylglycerol; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; IMPase, myo-inositol-1-monophosphatase; IP, inositol phosphate; MARCKS, myristoylated alanine-rich C kinase substrate; PI, phosphoinositide; PKC, protein kinase C; VPA, valproate (sodium salt); 2-PGA, 2-propylglutaric acid; HVPA, hydroxyvalproic acid.
take this delayed onset into account (Manji et al., 1995). Accumulating evidence from our laboratory as well as others strongly implicates receptor-mediated PI signaling in brain via inhibition of the enzyme IMPase in the mechanism of action of lithium in the brain (Berridge et al., 1982; Godfrey, 1989; Lenox and Watson, 1994), a property apparently not shared by VPA (Vadnal and Parthasarathy, 1995). Moreover, it has become evident that the action of chronic lithium in the brain is mediated through subsequent regulation of PKC and the downstream post-translational modification of selective protein substrates (Lenox, 1987; Manji and Lenox, 1994; Watson and Lenox, 1996).

PKC is a calcium-activated, DAG-dependent kinase that exists as 1 of at least 12 structurally related isozymes and it has been implicated in numerous cellular responses associated with regulation of signaling and long-term events, including ion channel and gene regulation (Newton, 1995; Nishizuka, 1995). Numerous studies have suggested a role for PKC in the long-term action of lithium (Lenox, 1987; Manji and Lenox, 1994), and recent data have provided evidence that VPA alters PKC activity as well as the expression of α and ε isozymes (Chen et al., 1994; Manji et al., 1996). Previous studies in our laboratory demonstrated that chronic (but not acute) lithium treatment of rats, resulting in clinically relevant brain concentrations (~1 mM), produces a significant reduction in the PKC substrate MARCKS in the hippocampus, which persists beyond treatment discontinuation (Lenox et al., 1992). This lithium-induced down-regulation of MARCKS has been demonstrated in an immortalized hippocampal cell line (Watson and Lenox, 1996), in which we previously demonstrated that phorbol esters, which directly activate PKC, down-regulate MARCKS protein expression in a PKC-dependent manner (Watson et al., 1994).

MARCKS is a prominent and preferential substrate in the brain for PKC, which by virtue of phosphorylation regulates the cellular localization and activity of this protein. MARCKS binds calmodulin in a calcium-dependent fashion and cross-links filamentous actin, and it has been implicated in cellular processes associated with cytoskeletal restructuring and neuropasticity (e.g., transmembrane signaling and neurotransmitter release) (Aderem, 1992; Blackshear, 1993). Inasmuch as MARCKS may also represent a molecular target for mood stabilizers in the brain (Lenox and Watson, 1994; Watson and Lenox, 1996), we sought to examine the effects of VPA, two VPA analogs, and five unrelated psychotropic agents on MARCKS protein levels in our immortalized hippocampal cell model. Our data provide evidence for a concentration- and time-dependent reduction of MARCKS protein in the immortalized hippocampal cells after exposure to VPA. Data presented here suggest that this property of VPA and lithium is not shared by the other psychotropic drugs examined in this study. We discuss the putative mechanism through which VPA may be exerting its effect and suggest that our findings have significant implications for pursuing drug discovery efforts based on MARCKS regulation as a molecular target for mood stabilization in the brain.

### Methods

**Materials.** Drugs were purchased or provided from sources indicated in table 1. All other reagents and materials were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

**Cell culture.** The immortalized hippocampal cell line HN33.dw was used to assess drug effects on MARCKS protein expression. HN33 cells were kindly provided by Dr. Bruce Wainer (Albert Einstein College of Medicine, Bronx, NY) and were derived from the fusion of primary neurons from the hippocampus of postnatal day 21 mice with the N18TG2 neuroblastoma cell line (Lee et al., 1990). Cells were grown at 37°C in DMEM containing 5% FBS. Culture medium was supplemented with the addition of VPA or other psychotropic drugs as described in table 1. The duration of exposures ranged from 0.5 to 7 days. Drug was delivered in a small volume (~250 μl) in distilled water or 95% ethanol as indicated in table 1. Control cultures grown in parallel were supplemented with vehicle only. Culture medium was changed or cells were passaged every 3 to 4 days, and assays were performed when cells reached 80% to 90% confluence. Cell viability was assessed by trypan blue exclusion both before and after drug exposure. Under control, resting conditions, MARCKS protein is located in both the soluble and membrane fractions, with predominant localization (70–80%) in the soluble fraction.

**Chinese hamster ovary cells (CHO-K1) stably transfected with the PI-linked human muscarinic m1 receptor subtype.**

### Table 1

<table>
<thead>
<tr>
<th>Drug (source)</th>
<th>Effective plasma concentration in humans</th>
<th>Concentrations tested</th>
<th>Vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium valproate (Sigma P-4543; Abbott A-44089.5)</td>
<td>50–125 μg/ml = 0.30–0.75 mM (seizure control)</td>
<td>0.01, 0.03, 0.06, 0.1, 0.3, 0.6, 1.0 mM</td>
<td>dH2O</td>
</tr>
<tr>
<td>2-Propylglutaric acid (Abbott A-49999.0)</td>
<td></td>
<td>0.6, 1.5, 3.0 mM</td>
<td>dH2O</td>
</tr>
<tr>
<td>Hydroxyvalproate, sodium salt (Abbott A-49822.5)</td>
<td></td>
<td>0.6, 1.5, 3.0 mM</td>
<td>dH2O</td>
</tr>
<tr>
<td>Lithium Cl (Sigma L-8895)</td>
<td>0.5–1.25 mM (mood stabilizer and antimanic)</td>
<td>1.0 mM</td>
<td>dH2O</td>
</tr>
<tr>
<td>Carbamazepine (Sigma C-4024)</td>
<td>8–12 μg/ml = 33–50 μM (seizure control)</td>
<td>10, 25, 100 μM</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Fluoxetine HCl (Lilly 111040L/06803)</td>
<td>Varies</td>
<td>50, 200, 1000 ng/ml</td>
<td>dH2O</td>
</tr>
<tr>
<td>Haloperidol (Sigma H-1512)</td>
<td>4–20 ng/ml (antipsychotic)</td>
<td>5, 20, 100 ng/ml</td>
<td>dH2O</td>
</tr>
<tr>
<td>Diazepam (Sigma D-0899)</td>
<td>300–400 ng/ml (anxiolytic)</td>
<td>50, 200, 1000 ng/ml</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Morphine sulfate (Sigma M-8777)</td>
<td>&gt;600 ng/ml (seizure control)</td>
<td>5, 20, 100 ng/ml</td>
<td>dH2O</td>
</tr>
</tbody>
</table>

* Benet and Williams (1990).
dr. Mark Brann (University of Vermont, Burlington, VT). This cell line has been effectively used to examine the effects of lithium on the phosphatidylinositol cycle and CMP-PA accumulation (Atack et al., 1993). CHO-K1 (Ham1) cells were grown in inositol-free Ham's F-12/DMEM (1:1) supplemented with 5% FBS.

**MARCKS quantification by Western blotting.** Harvested cells were homogenized in a buffer containing 20 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM PMSF, 2 mM DTE and 10 μg/ml aprotinin. Cells were sonicated to disrupt cell membranes, and the soluble and pellet fractions were separated by centrifugation. The homogenate was centrifuged at 100,000 x g, and the soluble fraction was collected. The pellet was resuspended in buffer containing 0.1% Triton X-100 and solubilized for 30 min. Solubilized fractions were then centrifuged at 50,000 x g, and the supernatant containing the solubilized membrane protein was collected. Samples were adjusted by addition or dilution to 0.05% Triton X-100. Equal amounts of soluble and membrane cell protein (50–125 μg), as determined by the Bradford method, were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) in a BioRad (Her cules, CA) Trans-Blot electrophoresis apparatus at 100 V for 2 hr using Towbin’s buffer [25 mM Tris, pH 8.3, 192 mM glycine and 20% (v/v) methanol]. A polyclonal MARCKS antibody was prepared in TS IgG/avidin-conjugated peroxidase/DAB system described previously (Watson and Lenox, 1996) or the Pierce (Rockford, IL) enhanced chemiluminescence system. Western blots were quantified using NIH Image 1.47 software for densitometric analysis. The interassay coefficient of variation for MARCKS quantitation was calculated to be <15% for both soluble and membrane fractions.

**CMP-PA assay.** The effects of VPA and lithium on CMP-PA accumulation were measured in the CHO-K1 (Ham1) cell line as described previously (Lenox and Watson, 1994). Cells were prelabeled with [3H]cytidine for 60 min before the addition of LiCl (5 mM) or VPA (2.5 mM), with or without additional inositol (10 mM). After 10 min, carbachol (1 mM) was added, and the incubation continued for the times indicated. Incubation was terminated with chloroform/methanol, and phases were split by the addition of chloroform and water. After centrifugation, the bottom layer was removed, washed with methanol/1 M HCl and then dried down and counted for radioactivity by liquid scintillation spectrometry.

**Data analyses.** For MARCKS protein, statistical analyses were conducted on data derived from densitometric scans of Western blots. Results are expressed as the percentage of control. Differences between treatment conditions were determined using analysis of variance. The factors analyzed were drug concentration, treatment duration, and cell fraction (soluble and membrane). Post hoc comparisons were conducted using Fisher's LSD. In each case, the minimum acceptable level for statistical significance was P < 0.05. For drug concentration experiments (tables 2 and 3, see fig. 2), statistical comparisons were made vs. the therapeutic dose of VPA (0.6 mM). For time course analyses (see fig. 1), statistical comparisons were made to the exposure of shortest duration (1 day).

**Results**

**Effect of VPA on MARCKS protein expression.** HN33 cells were exposed continuously to 0.6 mM VPA for periods ranging from 12 hr to 7 days. This concentration is equivalent to 100 ng/ml, which is within the reported therapeutic range for the mood-stabilizing action of VPA (50–125 ng/ml). After chronic VPA exposure, a slight-to-moderate increase in morphological differentiation was observed in HN33 cells exposed to concentrations higher than 0.1 mM. Additionally, after long-term (>3 days) exposure, cellular growth rates were notably slower for HN33 cells exposed to VPA concentrations of >0.6 mM. Cells grown in 0.6 mM VPA for up to 7 days appeared healthy and viable, with cell viability exceeding 98% in all cultures examined.

**MARCKS protein levels in both soluble and membrane fractions were measured by Western blotting and are expressed as percentage of control (fig. 1). Chronic exposure (≥3 days) produced a statistically significant decrease in MARCKS, whereas acute exposure (12 hr to 1 day) produced no significant change. The decrease in MARCKS was most prominent in the membrane fraction, beginning at day 3 (55% of control) and continuing through day 7 (39% of control). The soluble fraction, in contrast, produced a more modest decrease (22% reduction) at day 3 and appeared to plateau thereafter, with a 27% reduction observed at 7 days. The differential effect on the cellular fractions (soluble vs. membrane) was statistically significant after 3 to 7 days of VPA exposure (P < 0.05).

VPA was administered at concentrations ranging from 0.001 to 1.0 mM for 1 to 3 days. Statistically significant concentration- and time-dependent effects were observed. At the therapeutic concentration, 0.6 mM, a significant reduction in MARCKS protein expression was observed at 3 days but not at 1 day (table 2). On acute exposure (1 day), a

**TABLE 2**

<table>
<thead>
<tr>
<th>[VPA] (mM)</th>
<th>Soluble</th>
<th>Membrane</th>
<th>Day 1</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>N.D.</td>
<td></td>
<td>112.0 ± 12.4</td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>101.3 ± 6.9</td>
<td>104.2 ± 4.1</td>
<td>103.3 ± 14.4</td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>98.8 ± 12.5</td>
<td>108.2 ± 6.1</td>
<td>110.6 ± 18.4</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>105.0 ± 7.2</td>
<td>107.0 ± 5.7</td>
<td>110.0 ± 18.4</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>100.8 ± 4.0</td>
<td>104.6 ± 3.5</td>
<td>102.2 ± 7.8</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>81.0 ± 8.6</td>
<td>97.8 ± 5.8</td>
<td>89.2 ± 13.1</td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>85.0 ± 4.5</td>
<td>93.4 ± 5.3</td>
<td>75.3 ± 7.5</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>74.1 ± 9.5</td>
<td>85.3 ± 6.6</td>
<td>77.8 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>73.4 ± 5.3</td>
<td>82.4 ± 6.9</td>
<td>73.8 ± 7.2</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.

* Cells were exposed to VPA for 1 or 3 days, and MARCKS protein levels were determined by Western blot analysis.

* Not determined.

* Significantly different from day 3 (soluble) 0.6 mM VPA; P < .005.

* Significantly different from day 3 (membrane) 0.6 mM VPA; P < .005.
reduction of MARCKS expression was observed in the soluble fraction of cells exposed to only VPA concentrations as high as 1 mM (see table 2) or greater (data not shown). In contrast, after chronic (3-day) exposure, down-regulation of MARCKS protein in both soluble and membrane fractions was observed at all concentrations above 0.03 mM (fig. 2, table 2).

MARCKS protein levels in both the soluble and membrane fractions of cells exposed to 0.03 mM VPA were determined to be significantly different than in cells exposed to 0.6 mM VPA. Based on these results, the estimated EC50 value for VPA on MARCKS protein down-regulation in both soluble and membrane fractions is ~0.06 mM.

Effect of valproate analogs on MARCKS protein expression. MARCKS protein levels in HN33 cells were measured after exposure to each of two VPA analogs, HVPA and 2-PGA, for 3 or 7 days (table 3). Cells were exposed to concentrations of 0.6, 1.5 and 3.0 mM. Unlike VPA, which produced noticeable changes in cell morphology at 0.6 mM, these analogs produced no such change at this or higher concentrations. Figure 3 shows a representative Western blot of MARCKS expression in both soluble and membrane fractions after exposure to each VPA analog for 7 days (2-PGA is shown in lanes 4–6 and HVPA in lanes 7–9). For 2-PGA, in the soluble fraction, no significant reduction in MARCKS protein was evident, even at concentrations 5 to 10 times higher than the minimal effective concentration of VPA. However, in the membrane fraction, a reduction in MARCKS protein was observed in cells exposed to 1.5 to 3.0 mM HVPA. At the highest concentration tested (3.0 mM; five times the therapeutic level of VPA), a 30% to 65% reduction in MARCKS protein was observed after 3 to 7 days of exposure.

Effect of combined VPA and LiCl exposure on MARCKS protein expression in HN33 cells. HN33 cells were exposed to 0.6 mM VPA and/or 1 mM LiCl/1 mM car-

### Table 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>Time</th>
<th>Concentration</th>
<th>MARCKS protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
<td></td>
<td>Soluble</td>
</tr>
<tr>
<td>2-Propylglutaric acid</td>
<td>3</td>
<td>0.6 mM</td>
<td>100 ± 05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 mM</td>
<td>101 ± 04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0 mM</td>
<td>98 ± 05</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.6 mM</td>
<td>111 ± 02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 mM</td>
<td>101 ± 03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0 mM</td>
<td>97 ± 05</td>
</tr>
<tr>
<td>Hydroxyvalproic acid</td>
<td>3</td>
<td>0.6 mM</td>
<td>92 ± 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 mM</td>
<td>100 ± 09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0 mM</td>
<td>103 ± 08</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.6 mM</td>
<td>89 ± 05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 mM</td>
<td>90 ± 04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0 mM</td>
<td>86 ± 08</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M.

*a* Cells were exposed to VPA analogs for 3 or 7 days, and MARCKS protein levels were determined by Western blot analysis.

*b* Significantly different from day 3 (membrane) 0.6 mM HVPA, P < .01.

*c* Significantly different from day 7 (membrane) 0.6 mM HVPA, P < .05.
Effects of carbachol and myo-inositol on the VPA-induced down-regulation of MARCKS. To examine the effects of carbachol and myo-inositol on MARCKS protein expression, carbachol for a period of 3 days to assess the effects of VPA, lithium combination on MARCKS protein expression and distribution. Results are presented in figure 4. A greater reduction of MARCKS protein was observed in both soluble and membrane fractions of cells exposed to both VPA and lithium/carbachol than was produced by either VPA or lithium/carbachol alone. The enhanced reduction in MARCKS protein produced by the combination of VPA and lithium/carbachol at these concentrations suggests its effects to be additive. The reduction in MARCKS protein produced by the combined treatment of VPA and lithium/carbachol was greater in the membrane fraction than in the soluble fraction.

Fig. 4. Effect of combined VPA/LiCl treatment on MARCKS expression. HN33 cells were exposed to 0.6 mM VPA and/or LiCl/carbachol (1 mM/1 mM) in inositol-free DMEM. After 3 days of continuous exposure, cells were collected and homogenized, and soluble and membrane fractions were prepared. MARCKS protein levels were determined by Western blot analysis and expressed as the percentage of MARCKS in untreated cell cultures. Data are mean ± S.D. values for at least three determinations. * P < .01 compared with both 0.6 mM VPA and 1 mM LiCl/carbachol values. [ ] Soluble, [ ], membrane.

Discussion

VPA is a broad-spectrum antiepileptic whose exact mechanism of anticonvulsant action is unclear. Although its acute efficacy was originally thought to be in part a result of its reported effect on inhibitory GABA neurotransmission, there is evidence for activity on excitatory amino acids as well as ion channels in the brain, particularly the voltage-dependent Na⁺ type (Chapman et al., 1982; Loscher, 1993). In recent years, VPA has emerged as an effective treatment for acute mania and may well possess prophylactic properties in the management of patients with bipolar disorder (Bowden et al., 1996).
Unlike its acute efficacy in the treatment of seizure disorder, VPA, like lithium, requires time for onset of action in the treatment of acute mania, suggesting that its therapeutic action in the treatment of bipolar disorder is mediated via effects on long-term processes in the brain.

Bipolar disorder is a chronic affective disorder that affects 1% of the U.S. population (Goodwin and Jamison, 1990). Although lithium has been a first-line treatment for the acute and prophylactic management of bipolar disorder, it is estimated that almost half of patients either fail to respond to lithium or experience such problematic side effects that compliance becomes an issue and patients are unable to continue treatment with lithium (Goodwin and Jamison, 1990; Goldberg et al., 1995; Lenox and Manji, 1995). The clinical significance of noncompliance is enhanced given the elevated risk of episode recurrence and potential for response refractoriness on lithium discontinuation (Suppes et al., 1991; Post et al., 1992a; Faedda et al., 1993). Alternative treatment strategies over the past few years have focused on anticonvulsants based on a "kindling" model of bipolar disorder, and VPA has recently been approved by the Food and Drug Administration for the treatment of acute mania.

Numerous studies, including our own, over the past several years have confirmed that receptor-coupled PIP$_2$ hydrolysis, which generates two second messengers (IP$_3$, which mobilizes intracellular calcium, and DAG, which activates PKC), is an important site for the acute action of lithium in the brain (for reviews, see Berridge, 1989; Rana and Hokin, 1990; Lenox and Manji, 1995). Lithium, by virtue of an interaction with one of two Mg$^{2+}$ enzyme binding sites, is an uncompetitive inhibitor of IMPase, which catalyzes the breakdown of the inositol monophosphates and the generation of free inositol (Pollack et al., 1994; Atack et al., 1995). Because the brain has limited access to inositol other than that derived from recycling of the inositol monophosphates and the affinity of PI synthase is relatively low, it was suggested by Berridge et al. (1989) that a major physiological consequence of the action of lithium is derived through a depletion of free inositol required to recombine with CMP-PA to replenish the PI signaling pool. Thus, lithium might be expected to be most effective in systems undergoing the highest rate of receptor-mediated PIP$_2$ hydrolysis. As a consequence of inositol depletion in the presence of lithium, receptor-mediated accumulation of CMP-PA in both cell models and brain has been used by our
laboratory as well as others as a sensitive index of intracellular inositol deficiency during activation of receptor-coupled PLC pathways (Kennedy et al., 1990; Stubbs and Agranoff, 1993; Jenkinson et al., 1994; Lenox and Watson, 1994). Thus, the action of chronic lithium may stem initially from its inhibition of inositol recycling through the receptor-mediated hydrolysis of PIP2 and ultimately may be explained by its indirect action in accumulating DAG (Lenox, 1987, 1988) and subsequent change in the activation of PKC isozymes, altering the phosphorylation or expression, or both, of key phosphoprotein substrates.

Such an hypothesis is strongly supported by our data in the CHO-K1 cells, in which we observed a significant accumulation of CMP-PA after exposure of cells to lithium in the presence of muscarinic receptor activation, which is prevented in the presence of excess myo-inositol (Lenox and Watson, 1994). These data support previously reported findings in the CHO-K1 cell model. However, in the presence of VPA at concentrations as high as 2.5 mM, we found no evidence for an accumulation of CMP-PA even in the presence of receptor activation, consistent with the lack of effect of VPA on IMPase activity.

Previous studies in our laboratory have demonstrated a significant reduction in the expression of a prominent phosphoprotein substrate for PKC in brain, MARCKS, on exposure to chronic lithium (Lenox et al., 1992; Watson and Lenox, 1996). These findings have been observed in both rat hippocampus and immortalized hippocampal cells in culture. This alteration in MARCKS protein expression persists beyond discontinuation of lithium administration and is not observed after acute lithium exposure. In more recent studies, we demonstrated that phorbol esters down-regulate MARCKS protein in neuronally derived cell populations dependent on the activation of PKC isozymes (Watson et al., 1994). Most recently, we demonstrated in the same immortalized hippocampal cell model that MARCKS down-regulation by chronic therapeutic levels of lithium is dependent on both the relative concentration of inositol and the state of receptor activation, conferring the selective action of lithium in brain where inositol is limiting (Watson and Lenox, 1996). Furthermore, preliminary studies in our laboratory have provided evidence that the lithium-induced down-regulation of MARCKS protein is accompanied by a corresponding reduction in MARCKS mRNA of similar magnitude (Watson and Lenox, 1997). These data are consistent with accumulating data from other laboratories that have provided convincing evidence for a role of PKC in mediating the long-term effects of lithium exposure and the destabilization of MARCKS mRNA (Brooks et al., 1991, 1992; Lindner et al., 1992; Manji and Lenox, 1994).

In the present study, we examined the effects of VPA on MARCKS protein expression in immortalized hippocampal cells. HN33 cells were formed by the somatic cell hybridization of primary hippocampal cells and N18TG2 neuroblastoma cells, and they exhibit morphological, cytoskeletal and electrophysiological features characteristic of hippocampal neurons in culture (Lee et al., 1990). Exposure of HN33 cells to VPA at concentrations up to 1 mM produced no apparent cytotoxicity; however, a dose-dependent increase in cell doubling time was noted. This effect was reversible on removal of the drug (data not shown). These findings are similar to
reported effects of VPA on C6 glioma cells, in which VPA exhibited a reversible antiproliferative effect that was not attributable to cytotoxicity (Martin and Regan, 1991). In this study, VPA was found to arrest cells at the postmitotic mid-G1 phase of the cell cycle. In our present study, the effects of VPA on MARCKS protein expression appear to be unrelated to its antiproliferative effect because MARCKS protein levels were reduced at VPA concentrations well below the concentrations that produced the altered growth rates.

The data presented here demonstrate that VPA exposure elicits both a time- and concentration-dependent reduction in MARCKS protein expression in an immortalized hippocampal cell line. Similar findings have been observed in C6 glioma cells (H. K. Manji, personal communication). In our studies, the most robust effect of VPA on MARCKS was observed at concentrations corresponding to the therapeutically effective range of the drug (0.3–0.75 mM, or 50–125 µg/ml). Also consistent with clinical observations (Bowden et al., 1994), in which effects of VPA are observed only after chronic administration, a significant reduction of MARCKS protein in both soluble and membrane fractions of HN33 cells was not observed after acute (1-day) VPA administration. The down-regulation of MARCKS appears to be maximal at day 3, as no further significant reduction was observed on continued exposure (5–7 days). The effect of VPA on MARCKS was most pronounced in the membrane fraction of these cells. This finding is in contrast to lithium, which exerts a preferential down-regulation of MARCKS in the soluble fraction (Watson and Lenox, 1996). Although the significance of this finding is as yet unclear, the differences observed in the patterns of MARCKS down-regulation between these two drugs may be a function of differences in the pathways of PKC activation, as discussed below. This is of particular interest in light of the findings that concomitant exposure of the immortalized hippocampal cells to both VPA and lithium appears additive on MARCKS down-regulation at the therapeutic concentrations of both drugs. These data are in agreement with clinical studies that show VPA/lithium combination therapy to have greater therapeutic efficacy for treatment of bipolar disorder than either agent administered individually (Calabrese and Delucchi, 1989; Hayes, 1989).

Muscarinic receptor activation of PI signaling potentiates the down-regulation of MARCKS, and the addition of myo-inositol, even within the micromolar range, can both prevent and reverse the lithium-induced reduction in MARCKS protein expression (Watson and Lenox, 1996). These findings clearly implicate a role for the receptor-coupled PI signaling pathway in the mechanism of action of chronic lithium. In contrast, addition of carbachol or inositol (even at millimolar concentrations) to the culture medium had no effect on the VPA-induced reduction in MARCKS, consistent with the lack of effect of VPA on receptor-coupled accumulation of CMP-PA observed in the CHO-K1 cells. Thus, despite the shared property of lithium and VPA in down-regulating the expression of MARCKS, these data suggest that the mechanism through which this occurs may involve, at least in part, alternative pathways.

There does, however, seem to be evidence for a role of PKC regulation in the action of both lithium and VPA. Chen et al. (1994) reported that chronic VPA exposure of C6 glioma cells significantly reduced PKC activity in both membrane and soluble fractions, as well as the expression of PKC isoforms α and ε in intact cells, a finding similar to that observed after chronic lithium administration in the same cell model (Manji et al., 1993). Subsequent studies revealed PKC-induced alterations in multiple components of the beta-adrenergic receptor-coupled cAMP-generating system, including beta-1 adrenergic receptor, Gα, and adenyl cyclase, each of which is phosphorylated by PKC (Chen et al., 1996). These data suggest that both VPA and lithium down-regulate MARCKS via a PKC-dependent mechanism. On the other hand, because the action of lithium appears to be mediated via the PI signaling pathway, unlike that of VPA, there may be a differential pattern of activation of the multiple PKC isoforms and their temporal expression that may mediate different pathways to the down-regulation of MARCKS expression and thereby confer different clinical therapeutic properties. Such studies are ongoing in our laboratory. Indeed, we recently observed that the expression of MARCKS protein in two separate murine macrophage cell lines is unaltered after chronic exposure to therapeutic levels of VPA (0.6 mM, 3–7 days; data not shown). These data suggest that the action of VPA on MARCKS protein expression could well be cell or tissue specific, or both, to the central nervous system and could be mediated in part by the expression pattern of the PKC isoforms specific to the cell types.

Two structural analogs of VPA, which are significantly less potent as anticonvulsants in comparison to VPA (Chapman et al., 1982), were studied. Both analogs are known metabolites of VPA, although they are produced through minor metabolic pathways and represent a minimal percentage of total brain plasma concentration. Neither HVPA nor 2-PGA is effective in preventing pentylenetetrazol-induced seizures in mice, and neither agent has been tested clinically for efficacy in the treatment of bipolar disorder. In our studies, HVPA produced a statistically significant effect in down-regulating membrane-associated MARCKS but at a concentration well above that observed for VPA. Comparatively, the 2-PGA analog possessed little potency in down-regulating MARCKS in either the membrane or cytosolic fraction. Future analysis of structural differences among these VPA compounds may prove useful in identifying structurally related fatty acids that may be more or less efficacious not only in down-regulation of MARCKS but also potentially as mood stabilizers.

Exposure of immortalized hippocampal cells to structurally and functionally diverse psychotropic agents, including fluoxetine, haloperidol, diazepam, carbamazepine and morphine, did not result in a down-regulation of MARCKS, even at doses that were well above the therapeutic range. Of particular note, carbamazepine, an anticonvulsant that has also been used in the treatment of mania in patients with bipolar disorder, did not have an effect on MARCKS regulation. It is of interest that carbamazepine has not been shown to be as effective overall as lithium or VPA across clinical studies, particularly in a prophylactic role as a mood stabilizer (Keck et al., 1992), nor has it been approved by the Food and Drug Administration for this application. In addition, we have now determined that imipramine, a tricyclic antidepressant structurally similar to carbamazepine, produces no significant down-regulation of MARCKS protein expression (data not shown). Of interest, carbamazepine does appear to have effects within the adenylyl cyclase cascade, a property shared by lithium at higher concentrations (Mork et al., 1992;
Manji et al., 1995), which may contribute to its clinical properties.

Based on our current and previous studies, regulation of MARCKS expression in brain appears to be a putative target for the action of mood stabilizers in the brain that may underlie the prophylactic efficacy that serves to stabilize this illness over time. Both chronic VPA and lithium share this property at clinically relevant concentrations to the exclusion of all other psychotropics agents examined to date. Uncovering potential common molecular targets in the brain for these drugs under conditions that reflect both effective therapeutic concentrations and the time course of clinical action will provide a unique window into the pathophysiology/neurobiology of bipolar disorder. Furthermore, such findings will offer us opportunities for the design and development of the next generation of mood stabilizers, compounds that will possess broad-spectrum efficacy, minimal side effects and widened therapeutic index.

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