

Differential Display PCR Reveals Novel Targets for the Mood-Stabilizing Drug Valproate Including the Molecular Chaperone GRP78

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ABSTRACT

Differential display polymerase chain reaction was used to identify genes regulated by the mood-stabilizing drug valproate (VPA). Four differentially displayed valproate-regulated gene fragments were isolated in rat cerebral cortex after i.p. injection of sodium VPA (300 mg/kg) for 3 weeks, and their expression was confirmed by Northern and slot blot analysis in rat cerebral cortex and C6 glioma cells. Sequencing analysis revealed three previously unidentified cDNA fragments in addition to a sequence with 100% homology with a molecular chaperone, 78-kDa glucose-regulated protein (GRP78). VPA treatment did not increase mRNA expression of 70-kDa heat shock protein, which is a related stress-induced molecular chaperone protein. All four candidate genes, including *GRP78*, showed similar VPA

concentration-dependent increases in mRNA abundance. Another commonly prescribed mood-stabilizing anticonvulsant, carbamazepine, also increased *GRP78* mRNA expression in C6 glioma cells, whereas lithium had no effect at doses up to 2 mM. Immunoblotting revealed that GRP78 protein levels were also increased in C6 glioma cells treated with VPA under the same conditions. Nuclear runoff analysis showed that VPA increased *GRP78* gene transcription. Because GRP78 possesses molecular chaperone activity, binds Ca^{2+} in the endoplasmic reticulum, and protects cells from the deleterious effects of damaged proteins, the present findings suggest that VPA (and possibly carbamazepine) treatment may target one or more of these processes.

The mood-stabilizing drug sodium valproate (VPA) is a branched-chain saturated fatty acid (2-propylpentanoic acid) that possesses anticonvulsant effects in the treatment of epilepsy (Penry and Dean, 1989). Recent evidence has established that VPA has a mood-stabilizing effect; this drug is quickly becoming a first line treatment for bipolar disorder (BD) because it has a broad spectrum of efficacy in patients with this illness (McElroy et al., 1992; Bowden, 1996). Long-term prophylactic treatment with mood-stabilizing drugs is currently recommended for BD. It is believed that chronic treatment with these drugs regulates gene expression (Post, 1992; Hyman and Nestler, 1996). The specific targets of these drugs are, however, poorly understood. Increased Na^+ channel subunit mRNA levels have been reported after VPA treatment (Yamamoto et al., 1997). VPA has also been shown to decrease myristoylated alanine-rich C kinase substrate expression (Lenox et al., 1996). VPA increases activator protein-1 binding in cultured cells (Chen et al., 1997; Asghari et

al., 1998), suggesting that genes containing this consensus sequence in their promoter may be targets of this drug. The available evidence is far from explaining the mechanism of this drug. Therefore, isolation of specific valproate-regulated genes (VRGs) is important, particularly if the products of these genes are found to be relevant to the pathophysiology of BD.

Differential display polymerase chain reaction (PCR) was developed to identify differentially expressed genes and to detect individual mRNA differences in various sets of mammalian cells (Liang et al., 1992). Side by side comparison of RNA samples from different cells allows the identification of differentially expressed genes. Amplified cDNAs can then be used as probes to isolate genes from genomic and cDNA libraries for further molecular characterization. To date, these methods have been successfully applied to isolate differentially expressed genes in cancer (Liang et al., 1992), heart disease (Utans et al., 1994), and diabetes (Nishio et al., 1994). We used this method to isolate differentially expressed genes in rat cerebral cortex after administering VPA for 3 weeks. Cerebral cortex was chosen because this is the region in which abnormalities have been reported in BD patients

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ABBREVIATIONS: CNS, central nervous system; VRG, valproate-regulated gene; GRP78, 78-kDa glucose-regulated protein; HSP70, 70-kDa heat shock protein; VPA, valproate; BD, bipolar disorder; PCR, polymerase chain reaction; SSPE, sodium chloride/sodium dihydrogen phosphate/EDTA; bp, base pairs; CBM, carbamazepine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

and where mood stabilizers have been shown to have numerous effects (Song and Jope, 1992; Li et al., 1993). One of these genes is the 78-kDa glucose-regulated protein (GRP78), a molecular chaperone that participates in protein trafficking and endoplasmic reticulum (ER) calcium homeostasis. We have confirmed the specificity and dose-response relationship of GRP78 regulation by VPA. Additionally, VPA treatment resulted in increased GRP78 protein levels.

Materials and Methods

Animal Treatment and Cell Culture. Male Sprague-Dawley rats (200–250 g) were injected i.p. with VPA (300 mg/kg) or 0.9% saline (control) once daily for 21 days. Rats were weighed weekly to control for nutritional status, sacrificed by decapitation, and their brains were dissected rapidly in 0.32 M sucrose at 4°C. All areas of cerebral cortex were dissected, pooled for each animal, and used immediately for RNA extraction. Rat C6 glioma cells were grown in a medium containing Dulbecco's modified Eagle's medium and 10% fetal calf serum. Cells were treated at various times in the presence or absence of different concentrations of VPA. Cell viability was greater than 98% as confirmed by trypan blue exclusion and was not different across drug treatments (data not shown).

Differential Display PCR. Total RNA from tissues and cell lines was isolated using Trizol reagent (Gibco Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Chromosomal DNA was digested with RNase-free DNase I (Boehringer Mannheim, Laval, Quebec) for 30 min at 37°C. The concentration of RNA was determined by measuring the absorbance at 260 nm and the purity determined by the 260:280 ratio. Three micrograms of total RNA was subjected to electrophoresis on a denaturing 1% agarose gel to check the integrity of the RNA (18S and 28S rRNA bands).

All reagents used in differential display PCR were obtained from GenHunter Corporation (Nashville, TN) except *Taq* polymerase, which was obtained from Perkin-Elmer Corp. (Branchburg, NJ). DNA-free RNA was transcribed to cDNA with reverse transcriptase using three different anchored primers: oligo(dTG), oligo(dTA), and oligo(dTC), respectively. Primers for PCR reaction were one of eight arbitrary primers and one of oligo(dTG), oligo(dTA), or oligo(dTC). The PCR reaction mixture (20 μ l) contained 2 μ M of each primer, 25 μ M dNTP, 0.5 μ M [α -³⁵S]dATP (1200 Ci/mmol) and 1 U of *Taq* polymerase. The PCR reaction (40 cycles) consisted of sequential incubations for 30 s at 94°C, for 2 min at 40°C, and for 30 s at 72°C, with a final extension for 5 min at 72°C. The amplified cDNAs were then separated on 6% denaturing polyacrylamide gel. Gels were dried and exposed to Kodak X-Omat film. Differentially displayed cDNAs were then recovered from the gel and reamplified by PCR using the same primers. The reamplified cDNAs were extracted from a 1.5% agarose gel using a QIAEX Gel Extraction Kit (Qiagen Inc., Chatsworth, CA); the protocol was provided by the manufacturer.

DNA Cloning and Sequence Analysis. Differentially displayed PCR products were ligated into a pNotA/T7 vector at 25°C for 30 min with T4 DNA ligase (5 Prime-3 Prime, Inc., Boulder, CO). Competent bacterial cells were transformed with the ligated construct. Plasmid DNA from subclones containing inserts were then purified using a Wizard Miniprep DNA purification system (Promega Biotec, Madison, WI). Inserts were sequenced by the dideoxy chain-termination method (Sanger et al., 1977). The sequencing products were run by electrophoresis on a 6% polyacrylamide-urea gel and identified by autoradiography. Sequences thus derived were compared for homology to the sequences present in the current GenBank database through the National Center for Biotechnology Information with the BLASTN program (Altschul et al., 1990).

Northern Hybridization and Slot Blot Analysis. Total RNA from rat cerebral cortex or C6 glioma cells was applied to nylon membranes using either a slot blot apparatus or by capillary transfer

after electrophoresis in 1% agarose/formaldehyde gel, and ultraviolet-cross-linked. Membranes were then prehybridized at 42°C for 3 h in 5 \times sodium chloride/sodium dihydrogen phosphate/EDTA (SSPE), 50% formamide, 5 \times Denhardt's, 1.0% SDS, 5% dextran sulfate, and salmon sperm DNA (100 μ g/ml) and then hybridized at 42°C overnight in this buffer with differentially displayed cDNA probes derived as described above. cDNA probes were labeled with [α -³²P]dCTP by using a random prime labeling method (Feinberg and Vogelstein, 1983). Membranes were then washed at room temperature for 45 min in 2 \times SSPE and 0.1% SDS twice and at 58°C for 15 min in 0.1 \times SSPE and 0.1% SDS twice. Membranes were exposed overnight at -80°C to Kodak X-Omat film with an intensifying screen.

Nuclear Runoff. Nuclear runoff assays were performed as previously described (Lofquist et al., 1995). Briefly, C6 cells were treated or untreated with VPA at 1 mM for 1 week. The cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40). Several microliters of cell lysate were examined with a hemacytometer on a phase-contrast microscope to determine whether the cells were free of membrane components. Nuclei were collected by centrifugation. The nuclei were resuspended in 100 μ l of glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA). Runoff transcription was initiated by adding 100 μ l of reaction buffer [(10 mM Tris-Cl, pH 8.0, 5 mM MgCl₂, 0.3 M KCl, 1 mM dithiothreitol, 40 units/ml RNasin, 1 mM ATP, 1 mM GTP, 1 mM CTP, and 25 μ l of [α -³²P]UTP (3000 Ci/mmol)] at 30°C for 30 min. DNA was digested by adding 1 μ l of 20,000 U/ml RNase-free DNase, whereas protein was digested by adding 10 μ l of proteinase K (20 mg/ml). Newly transcribed RNA was purified by 25:24:1 buffered phenol/chloroform/isoamyl alcohol, followed by precipitation with isopropyl alcohol. The membrane with cDNAs applied by slot blotting was then prehybridized at 42°C for 3 h in 5 \times SSPE, 50% formamide, 5 \times Denhardt's, 1.0% SDS, and salmon sperm DNA (100 μ g/ml), and was hybridized at 42°C overnight in this buffer with the ³²P-labeled newly transcribed RNA. After hybridization, membranes were then washed at room temperature for 45 min in 2 \times SSPE and 0.1% SDS twice and at 58°C for 15 min in 0.1 \times SSPE and 0.1% SDS twice. Membranes were exposed overnight at -80°C to Kodak X-Omat film with an intensifying screen.

Immunoblotting Analysis. Cells were washed in PBS (100 mM NaCl, 100 mM Na₂HPO₄/NaH₂PO₄, pH 7.5) and then suspended in cell lysis buffer (150 mM NaCl, 15 mM Mg₂Cl, 1 mM EGTA, 50 mM HEPES-KOH, 10% glycerol, 1% Triton X-100, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride and 2 μ M benzamidine. After gentle agitation for 30 min at 4°C, cell lysates were centrifuged for 10 min at 12,000g. Supernatants were used for immunoblotting analysis. The protein concentration was determined according to the method of Bradford (1976).

Five micrograms of protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with a 12% acrylamide gel for 1 h at 120 V. Proteins were transferred to polyvinylidene difluoride membranes at 100 V for 1 h, blocked in 3% milk-PBS (30 min, 22°C), and incubated overnight at 22°C with a monoclonal antisera for GRP78 (StressGen Biotechnologies Corp., Victoria, BC) at a 1:5000 dilution. Blots were then incubated with secondary antibody, goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:2000 in blocking buffer for 1 h at 22°C. Immunoreactive bands were detected with the enhanced chemiluminescence system (Amersham, Arlington Heights, IL). The blots were subsequently exposed to Kodak X-Omat.

Analysis and Interpretation of Data. All sequence data were aligned with sequences available in GenBank. Data from slot blot, nuclear runoff, and immunoblotting studies were obtained by densitometric analysis of autoradiograms using the Northern Exposure program from ImagExperts, Inc. (Oakville, Ontario, Canada). Results were expressed as a percentage of control. Changes in gene expression after drug treatment were expressed as the mean \pm S.E.M. from three separate experiments. Statistical significance of

differences between means was determined by Student's *t* tests or one-way ANOVA.

Results

Identification of Candidate VRGs by Differential Display PCR. With differential display PCR, six cDNA PCR products were differentially expressed in the rat cerebral cortex between controls and rats treated for 3 weeks with VPA at 300 mg/kg i.p.. The six differentially expressed cDNA PCR products were then excised from the 6% denaturing polyacrylamide gel and reamplified with the same primers used in differential display PCR. These six candidate VRGs were purified and ligated into a pNo-TA/T7 cloning vector. The differentially expressed candidate VRG cDNA inserts were sequenced and named: VRG1 [112 base pairs (bp)], VRG2 (106 bp), VRG3 (146 bp), VRG4 (105 bp), VRG5 (62 bp), and VRG6 (386 bp). VRG1 and VRG3–6 are previously unidentified cDNA fragments and showed no significant homology (<85%) to any entry in existing nucleic acid databases available through the National Center for Biotechnology Information. VRG2, however, displayed 100% homology to the *GRP78*, which is a stress-induced molecular chaperone. Figure 1 shows an autoradiograph of VRG2 demonstrating increased expression after VPA treatment. The cDNA fragments of candidate VRGs were radioactively labeled for Northern blot hybridization. Total RNA from cerebral cortex of rats treated for 3 weeks with VPA at 300 mg/kg i.p. and from control animals were used for Northern blot analysis. VRG1, VRG2, VRG4, and VRG6 were verified to be differentially expressed by Northern blot analysis (Fig. 2), whereas no expression of VRG3 or VRG5 was detected in rat brain.

Dose-Response Relationship of VRG Expression. To further investigate the effect of VPA on the differentially expressed candidate VRGs (VRG1, VRG2, VRG4, and VRG6), we determined VPA dose-response curves for these genes. First, VRG1, VRG2, VRG4, and VRG6 were confirmed to be differentially expressed by Northern blot analysis in C6 glioma cells treated with 1 mM VPA for 1 week. We used C6

cells to study the dose-response effect on *GRP78* because 1) C6 cells are commonly used to study the mechanism of mood stabilizers including VPA, and 2) *GRP78* is known to be expressed in this cell line (Brostrom et al., 1991; Chen et al., 1996, 1997). As shown in Fig. 2, all four genes were increased by VPA in C6 glioma cells as in rat cerebral cortex. Next, C6 glioma cells were exposed to VPA at different concentrations of 0, 0.25, 0.5, and 1 mM for 1 week and VRG1, VRG2, VRG4, and VRG6 mRNAs were quantitated by slot blot hybridization. These concentrations represent serum levels commonly used to treat patients (Bowden et al., 1996). The results indicated that VPA increased VRGs' mRNA expressions in a dose-dependent manner and that VPA dose-response curves for these genes showed similar patterns (Fig. 3).

Effects of VPA on *GRP78* and Heat Shock Protein 70 (*HSP70*) mRNA Levels. *HSP70* is a related stress-induced molecular chaperone. To study the specificity of VPA on *GRP78* mRNA expression and to further verify the effect of VPA on *GRP78*, *HSP70* and *GRP78* mRNA levels were measured with Northern blot analysis using *HSP70* and *GRP78* coding region sequences as probes in rat cerebral cortex and C6 glioma cells. Seven hundred sixty-seven-bp *GRP78* and 495-bp *HSP70* cDNA probes were generated by reverse transcription-PCR. The *GRP78* upstream primer (5'-CCACCG-TAACAATCAAGGTC-3') and downstream primer (5'-CACGTGAGCAACTGCTAATG-3'), which are located in regions +276 to +295 and +1024 to +1043 of the *GRP78* gene, and *HSP70* upstream primer (5'-GGTGCTGACCAAGATGAAG-3') and downstream primer (5'-CAGAGAGTCGATCTCCAGG-3'), which are located in regions +543 to +561 and +1020 to +1038 of *HSP70* gene were used, respectively, for PCR amplification. *GRP78* and *HSP70* cDNA probes from PCR were confirmed by sequencing analysis. As shown in Fig. 4, VPA increased *GRP78*, but not *HSP70*, mRNA in both rat cerebral cortex and C6 glioma cells. VPA at 1 mM also showed time-dependent increases of *GRP78* mRNA after treatment for 1 and 5 h, and 1, 3, 5, and 7 days. After 7 days of treatment with 1 mM VPA, the drug was subsequently withdrawn from cells to determine the reversibility of *GRP78* induction and rule out cell death as a result

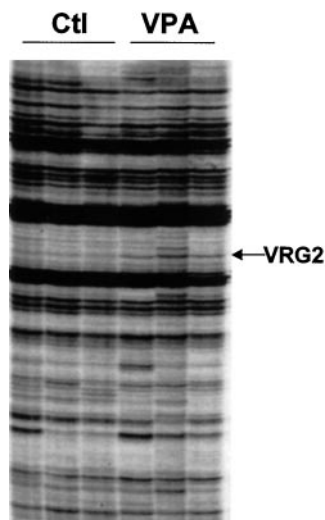


Fig. 1. Differential display of mRNA in control (Ctl) and sodium VPA (300 mg/kg i.p. for 3 weeks)-treated rats. Each lane represents tissue from one animal. A sample of 0.2 μ g of total RNA from each animal was used for differential display PCR. Arrows point to the differentially displayed product VRG2.

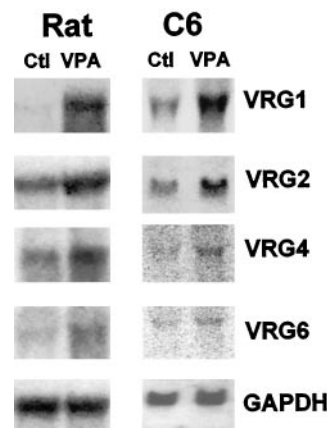


Fig. 2. Northern blot analysis of candidate VRGs in rat cerebral cortex and C6 glioma cells. Total RNA was isolated from rat cerebral cortex after 3 weeks of sodium VPA (300 mg/kg i.p.) treatment or from rat C6 glioma cells after 1 week of 1-mM VPA compared with untreated control (Ctl) tissues. Twenty-five micrograms of RNA was used for Northern blot analysis using probes prepared from products of differential display (VRG1, VRG2, VRG4, and VRG6) or the housekeeping gene *GAPDH*.

of VPA treatment. As shown in Fig. 5, the *GRP78* mRNA level was decreased from $65 \pm 9\%$ to $16 \pm 8\%$ at 6 h, and to $6 \pm 8\%$ at 24 h after VPA withdrawal.

Effect of Other Mood Stabilizers such as Lithium and Carbamazepine on *GRP78* mRNA Abundance. To further investigate possible similarities between VPA and the two other mood stabilizers commonly used to treat BD, we determined the effect of therapeutically relevant concentrations of lithium chloride (LiCl) and carbamazepine (CBM) on *GRP78* gene expression by slot blot analysis. As shown in Fig. 6, 0.5 to 2 mM LiCl and 0.025 and 0.05 mM CBM had no effect on *GRP78* gene expression. VPA at 0.5 mM and 1.0 mM significantly increased *GRP78* gene expression by $31.6 \pm 6.2\%$ ($p < .05$) and $50.5 \pm 8.9\%$ ($p < .05$), respectively. CBM at 0.1 mM also increased *GRP78* gene expression by $25.2 \pm 5.3\%$ ($p < .05$). There was no difference in the level of hybridization of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) compared to control cells after any of these treatments.

Effect of VPA on *GRP78* Gene Transcription. To determine whether the changes in the *GRP78* mRNA level is contributed to by the induction of *GRP78* transcription, we used nuclear runoff analysis to measure the effect of VPA on transcription of the *GRP78* gene. Five micrograms of empty plasmid DNA or plasmid DNA with *GRP78* cDNA insert was blotted to nylon membranes. C6 glioma cells were treated with and without VPA at 1 mM for 1 week. Nuclei were isolated from control and VPA-treated cells. Newly transcribed RNAs from nuclei of control and VPA-treated cells were hybridized with *GRP78* cDNA and empty plasmid DNA blots. As shown in Fig. 7, VPA treatment induced a significant increase of *GRP78* gene transcription in C6 glioma cells.

Effect of VPA on *GRP78* Protein Level. To determine whether the changes in the *GRP78* mRNA level contribute to *GRP78* translation, we measured the effect of VPA on the

GRP78 protein level using immunoblotting analysis. Figure 8 shows that VPA treatment of C6 glioma cells for 1 week at therapeutically relevant concentrations (0.25–1.0 mM) (Bowden et al., 1996; Chen et al., 1996, 1997; Asghari et al., 1998) increased the *GRP78* protein level in a dose-dependent manner.

Discussion

Differential display PCR revealed four differentially expressed gene products in rat cerebral cortex after i.p. injection with VPA (300 mg/kg) for 3 weeks. Although the identity of three of these gene products is presently unknown, the expression of a cDNA with 100% homology to the gene coding for the molecular chaperone *GRP78* was markedly increased after VPA treatment. The expression of this cDNA in rat cerebral cortex was confirmed by Northern blot hybridization. Quantitative slot blot analysis demonstrated that VPA increased the expression of these candidate genes in a dose-dependent manner at therapeutically relevant concentrations in rat C6 glioma cells. These results strongly suggest that specific genes, including the molecular chaperone *GRP78*, are regulated by VPA treatment. Furthermore, *GRP78* mRNA expression was also increased by treatment with the closely related mood-stabilizing anticonvulsant carbamazepine, but not lithium chloride. These results are consistent with the current notion that psychotropic drug treatment may act through long-term adaptational changes in the central nervous system (CNS) to bring about their therapeutic effects (Post, 1992; Hyman and Nestler, 1996).

GRP78 is induced as part of the stress response in eukaryotic cells (Brostrom and Brostrom, 1998). This gene product functions in endoplasmic reticulum (ER) glycoprotein trafficking, possesses molecular chaperone activity, and protects cells from the deleterious effects of damaged proteins. *GRP78* is constitutively expressed in the ER, and transcription of the

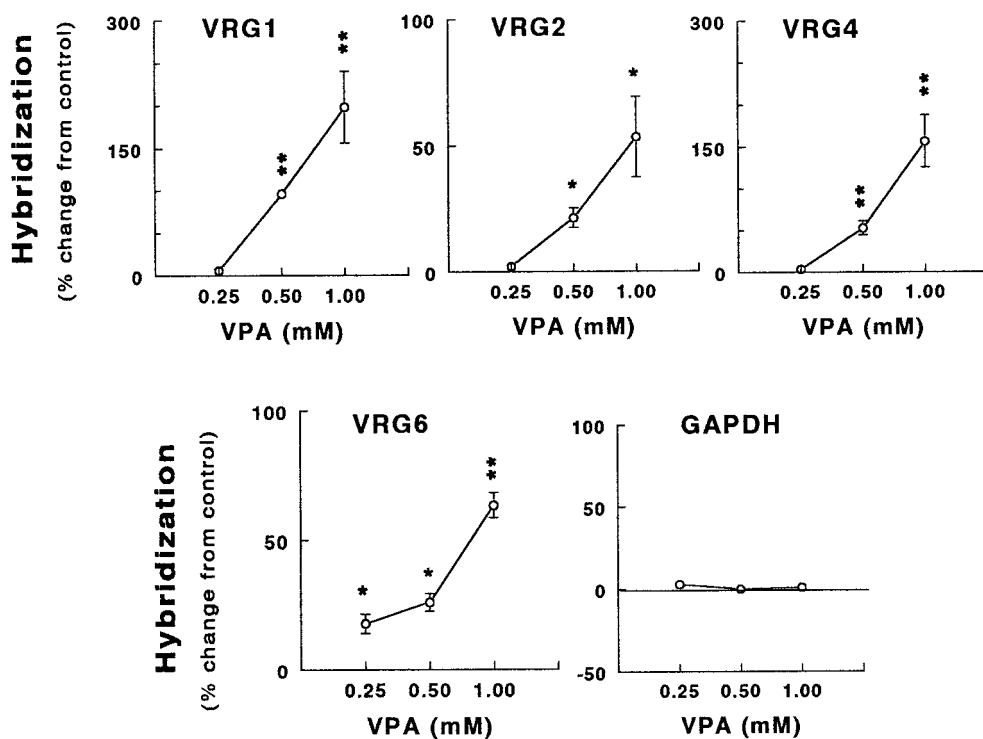


Fig. 3. The effects of different doses of VPA on candidate VRG expression. Rat C6 glioma cells were grown for 1 week with 0.25, 0.5, and 1 mM VPA. Control cells (Ctl) were treated identically but without addition of drug. Total RNA was analyzed by slot blot hybridization using [α - 32 P]dCTP-labeled probes prepared from products of differential display (VRG1, VRG2, VRG4, and VRG6) or the housekeeping gene *GAPDH*. Autoradiograms were quantitated by densitometry. Results are the mean \pm S.E.M. of triplicate determinations. * $p < .05$ and ** $p < .01$ significantly different from controls by ANOVA.

GRP78 gene is elevated in response to malformed proteins and treatments that interfere with protein glycosylation, protein trafficking, and ER storage of calcium (Kim et al., 1987; Wooden et al., 1991; Little et al., 1994). The molecular chaperone activities of GRP78 make it critical to ER functioning. GRP78 assists in the folding of the newly synthesized proteins and the acquisition of their correct tertiary and quater-

nary structure (Bole et al., 1986; Lee, 1987). Malformed protein binds to GRP78 ultimately to be disposed of by nonlysosomal proteolytic process (Bonifacino and Lippincott-Schwartz, 1991; Werner et al., 1996).

Our results indicate that chronic VPA treatment increases both GRP78 mRNA and protein levels at therapeutically relevant concentrations in rat C6 glioma cells. Although the functional relevance of these changes is not yet known, the fact that VPA increased GRP78 protein levels suggests that these changes may be relevant to the pharmacological action of VPA. It has been previously reported that classical inducers of stress proteins such as thapsigargin lead to 6-fold or higher induction of *GRP78* in various cell lines (Miles et al., 1994; Hsieh et al., 1996). In the present study, VPA induced *GRP78* more modestly than thapsigargin, suggesting that the effect of VPA on *GRP78* may be due to a different mechanism. Thapsigargin depletes ER Ca^{2+} , which in turn inhibits ER protein processing and cellular protein synthesis. These changes after thapsigargin treatment secondarily lead to *GRP78* induction, which may protect the cell against damage or cell death that would ultimately occur after ER Ca^{2+} depletion. Our results suggest that *GRP78* induction by VPA is not the result of ER damage or insult. Indeed, withdrawal of VPA after 1 week of treatment resulted in a return to baseline of *GRP78* mRNA levels, which occurs as early as 6 h.

There is increasing interest in the role of GRP78 in CNS disease and injury. GRP78 expression is induced after CNS injuries such as seizures, global ischemia, and acute trauma (Lowenstein et al., 1994), and in neurodegenerative diseases like Alzheimer's (Hamos et al., 1991). *GRP78* induction by chronic VPA treatment may play a neuroprotective role by clearing malformed proteins. Recently, chronic lithium treatment at therapeutically relevant concentrations was found to protect cultured rat cerebellar, cerebral cortical, and hippocampal neurons against glutamate-induced excitotoxicity (Nonaka et al., 1998). In our study, chronic lithium treatment did not alter *GRP78* gene expression, suggesting that these drugs may both have neuroprotective effects, but these effects occur through different pathways.

It has recently been shown that GRP78 not only participates in protein trafficking but is an important Ca^{2+} -binding

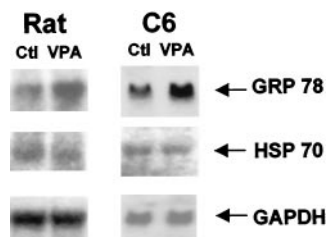


Fig. 4. Northern blot analysis of *GRP78* and *HSP70* in the rat cerebral cortex and C6 glioma cells. Total RNA was isolated from rat cerebral cortex after 3 weeks of VPA (300 mg/kg i.p.) and from C6 glioma cells grown for 1 week with 1 mM VPA and untreated control (Ctl) tissues. Total RNA was used by Northern blot analysis using probes prepared from *GRP78* cDNA, *HSP70* cDNA, or the housekeeping gene *GAPDH*.

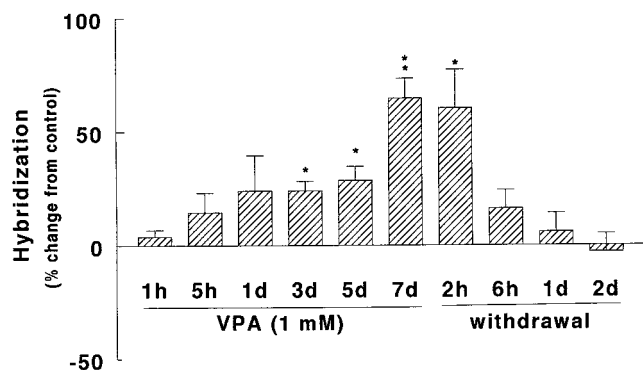


Fig. 5. Time course and withdrawal of VPA on *GRP78* gene expression. C6 glioma cells were treated with 1 mM VPA for 1 and 5 h (h), and 1, 3, 5, and 7 days (d). VPA was withdrawn at 2 h, 6 h, 1 day, and 2 days after a 1-week treatment with VPA at 1 mM. Control cells were treated identically but without addition of drug. Autoradiograms were quantitated by densitometry. Results are the mean \pm S.E.M. of triplicate determinations. * $p < .05$ and ** $p < .01$ significantly different from control by ANOVA.

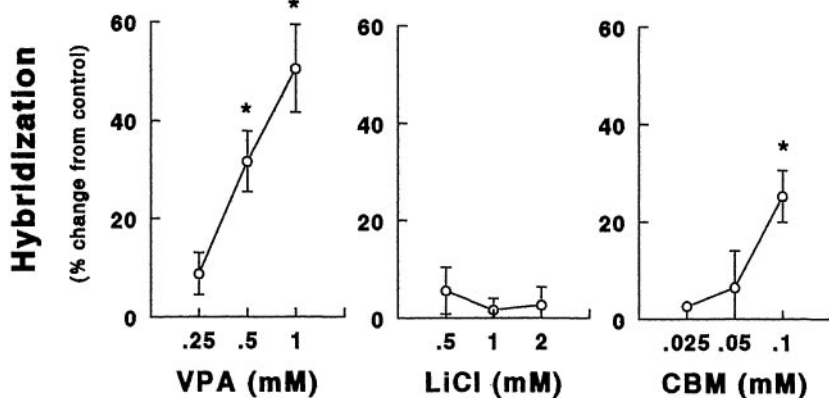
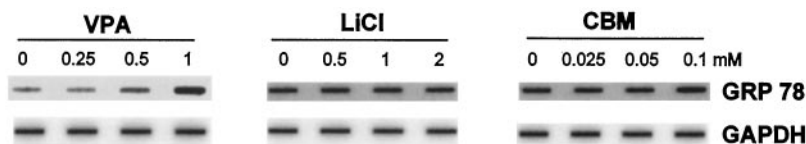


Fig. 6. Effects of sodium VPA, LiCl, and CBM on *GRP78* gene expression. C6 glioma cells were grown for 1 week with VPA at concentrations of 0.25, 0.5, and 1 mM; LiCl at 0.5, 1, and 2 mM; CBM at 0.025, 0.05, and 0.1 mM. Control cells were treated identically but without addition of drug. Total RNA was analyzed by slot blot hybridization using [α - 32 P]dCTP-labeled probe of *GRP78* or the housekeeping gene *GAPDH*. Autoradiograms were quantitated by densitometry. Results are the mean \pm S.E.M. of triplicate determinations. * $p < .05$ significantly different from control by ANOVA.

protein in the ER (Lièvreumont et al., 1997). Overexpression of exogenous hamster GRP78 in HeLa cells induced increases of the ER Ca^{2+} storage capacity (Lièvreumont et al., 1997). Therefore, GRP78 may play an important role in the control of the ER luminal Ca^{2+} homeostasis. Increased expression of the molecular chaperone GRP78 after VPA suggests that this drug may regulate Ca^{2+} homeostasis. Consistent with this hypothesis is the fact that VPA has been shown to inhibit 5-hydroxytryptamine-induced increase of intracellular free calcium in C6 glioma cells (Yamaji et al., 1996). Lithium was also found to inhibit *N*-methyl-D-aspartate receptor-mediated calcium influx (Nonaka et al., 1998). Because increased intracellular calcium levels have consistently been reported in blood cells obtained from patients with BD (Dubovsky et al., 1989; Emamghoreishi et al., 1997), a novel target for VPA that could regulate intracellular Ca^{2+} levels is of potential importance.

HSP70 is another molecular chaperone that is induced by stress. *HSP70* mRNA levels were not regulated by VPA treatment, suggesting specific regulation of *GRP78* by VPA. It has been shown that the stress response is associated with the

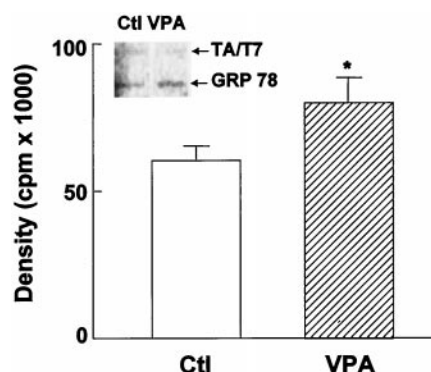


Fig. 7. Effect of sodium VPA on *GRP78* transcription. Nuclei were isolated from C6 glioma cells after treatment without (Ctl) or with 1 mM VPA. Equal amounts (cpm) of ^{32}P -labeled nuclear runoff RNA were hybridized to slot blots containing 5 μg of *GRP78* plasmid cDNA and the empty plasmid vector pNoTA/T7 DNA (TA/T7). Results are the mean \pm S.E.M. of triplicate determinations. * $p < .05$ significantly different from control by ANOVA.

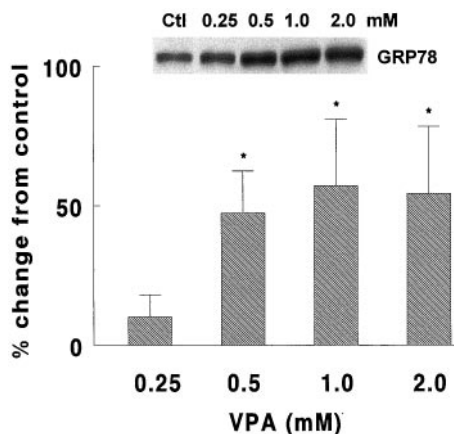


Fig. 8. Effects of VPA on *GRP78* protein levels. C6 glioma cells were treated with VPA at 1 mM for 1 week. Control cells (Ctl) were treated identically but without addition of drugs. Five micrograms of protein was analyzed by immunoblotting analysis using a *GRP78* antisera. Autoradiograms were quantitated by densitometry. Results are the mean \pm S.E.M.. * $p < .05$ significantly different from control by ANOVA.

independent induction of two groups of evolutionarily conserved proteins, HSPs and glucose-regulated proteins (GRPs). *HSP70* induction is fostered by damaged or aberrant cytosolic, nuclear, and mitochondrial proteins caused by thermal or chemical insult, whereas the accumulation of abnormally or poorly processed proteins within the lumen of the ER causes *GRP78* to be synthesized (Brostrom and Brostrom, 1998). Increased *GRP78*, but not *HSP70*, expression after VPA treatment suggests that this drug may specifically regulate protein processing linked to *GRP78* induction.

The increase of *GRP78* mRNA expression by VPA appears to occur in part at the level of transcription based on the results from the nuclear runoff assay. The dose-response curves for *GRP78* and the other candidate VRGs are highly similar, suggesting that VPA could regulate these genes through a common mechanism. Indeed, it is possible that there is a VPA-responsive sequence in the promoter of these genes similar to that found for ethanol (Hsieh et al., 1996). Although VPA has been found to increase AP-1 DNA binding activity (Chen et al., 1997; Asghari et al., 1998), there is no known AP-1 site in the *GRP78* promoter, suggesting the potential importance of other elements (Resendez et al., 1988). The identity of a VPA responsive *cis*-acting element and isolation of *trans*-acting factors need to be further investigated. The role of the other unidentified candidate VRGs induced by VPA remains to be determined. Although all of the VRGs isolated here show induction at therapeutically relevant VPA concentrations, further studies are needed to characterize the therapeutic importance of these changes in gene expression.

In conclusion, chronic VPA treatment resulted in the increased expression of several mRNAs in rat cerebral cortex and C6 glioma cells, one of which has been identified as the molecular chaperone *GRP78*. The results of the present study demonstrate the potential utility of differential display PCR to identify the targets of psychotropic drugs and suggest potentially important and previously unidentified targets for VPA.

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