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ORIGINAL INVESTIGATION

Up-regulation of $cPLA_2$ gene expression in astrocytes by all three conventional anti-bipolar drugs is drug-specific and enzyme-specific

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Abstract
Rationale Common biological effects by all three conventional anti-bipolar drugs, the lithium ion (Li^+), carbamazepine, and valproic acid, are important because identical effects may provide information about the pathophysiology of affective disorders. It has been reported that chronic treatment with either drug in vivo down-regulates the turnover of arachidonic acid in brain. This reaction is catalyzed by Ca^{2+}-dependent phospholipase A_2 ($cPLA_2$), the expression of which was down-regulated by Li^+ or carbamazepine but not by valproic acid; expression of two other PLA subtypes, $iPLA_2$ and $sPLA_2$ was unaffected. $cPLA_2$ is amply expressed in astrocytes, and in the present study, effects of 1–4 weeks of treatment with clinically relevant concentrations of each of the three anti-bipolar drugs on $cPLA_2$, $iPLA_2$, and $sPLA_2$ mRNA and protein expression were determined in primary cultures of mouse astrocytes by reverse transcription polymerase chain reaction (RT-PCR) and immunoblotting.
Results Two or more weeks treatment with Li^+ concentrations below 2 mM, carbamazepine or valproic acid *up-regulated* mRNA and protein expression of $cPLA_2$, but had no effect on $iPLA_2$ and $sPLA_2$, showing enzyme specificity. The effect occurred more rapidly at higher than lower concentrations but also tended to end after 4 weeks at the higher concentrations. Two millimolar Li^+ caused an initial increase of $cPLA_2$ followed by a decrease after 3 and 4 weeks. Topiramate had no effect, indicating specificity for anti-bipolar drugs.
Conclusions Both up- and down-regulation of $cPLA_2$ gene expression are involved in the mechanisms of action of anti-bipolar drugs; astrocytes are a target for these drugs.

Keywords Astrocytes · Bipolar disorder · Carbamazepine · $cPLA_2$ · Lithium · Valproic acid

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Introduction

Lithium salts (Li^+), valproic acid (VPA), and carbamazepine (CBZ) are the three classical anti-bipolar drugs, which all have to be administered for a couple of weeks before their therapeutic action becomes manifest. Li^+ has been used as a mood stabilizer for more than 50 years (Schou 2001). The anticonvulsants valproate (VPA) and carbamazepine (CBZ) were originally developed as antiepileptic drugs and were later found to be effective in the treatment of bipolar disorder, but this does not apply to all anticonvulsants, as, for example, topiramate has no antimanic effect (Goodnick 2006). Li^+, VPA, and CBZ have nothing in common in chemical structures, and only a few metabolic parameters in the brain are affected similarly by all of them. Shared effects by all three drugs are important because common mechanisms of action may provide information about the pathophysiology of bipolar disorder and facilitate drug development.

Most of the common effects by Li^+, CBZ, and VPA have been observed in cultured astrocytes (Lubrich and van Calker 1999; Wolfson et al. 2000; Hertz et al. 2004), where they all affect uptake of myo-inositol, or in cultured neurons (Williams et al. 2002; Di Daniel et al. 2006), where they affect process dynamics of developing neurons. In intact brain, Rapoport and coworkers have established that after 6 weeks (Li^+ and CBZ) or 30 days (VPA) of treatment of rats with doses leading to therapeutically relevant drug levels, each drug decreases the in vivo turnover of arachidonic acid by deacylation from glycerophospholipids followed by re-acylation (Chang et al. 1996, 2001; Bazinet et al. 2006); this effect is specific for arachidonic acid, as docosahexaenoic acid turnover was not affected in a similar manner (Rapoport and Bosetti 2002; Bazinet et al. 2005, 2006).

The enzyme that specifically hydrolyzes the acyl bond of arachidonic acid from the *sn*-2 position of glycerophospholipids in the cell membrane is Ca^{2+}-dependent phospholipase A_2 ($cPLA_2$). Chronic treatment with Li^+ for 6 weeks decreases the expression of $cPLA_2$ in rat brain, whereas Li^+ has no effect on two other types of PLA_2, secretory PLA_2 ($sPLA_2$) and intracellular PLA_2 ($iPLA_2$) (Rintala et al. 1999; Weerasinghe et al. 2004; Basselin et al. 2005). The down-regulation was documented for the IVA subtype (85 kD) of $cPLA_2$, also called $cPLA_{2a}$, one of the six paralogs of $cPLA_2$ (Ghosh et al. 2006), but other paralogs were not discussed. Similar results were found in rat chronically treated with CBZ (Ghelardoni et al. 2004), whereas chronic treatment with VPA did not influence $cPLA_2$ in the brain in vivo (Chang et al. 2001).

Among the cells that express $cPLA_2$ are astrocytes (Sun et al. 2005), a glial cell type that accounts for 20–30% of the total volume in brain cortex (Williams et al. 1980; Wolff and Chao 2004). Indeed, in the brain in vivo, the majority of $cPLA_{2a}$ in the gray matter is located in astrocytes (Stephenson et al. 2004; Lautens et al. 1998; Balboa et al. 2002), but no information is available about the cellular location of $cPLA_{2b}$ and $cPLA_{2c}$ in the brain. The activity of $cPLA_{2a}$ may be regulated by transmitters, as astrocytes display receptors for a large number of transmitters (Hansson and Rönnbäck 2004; Fiacco and McCarthy 2006), including serotonin and ATP, known to catalyze the formation of free arachidonic acid (AA) from glycerophospholipid in C6 glioma cells (Garcia and Kim 1997) and primary cultures of astrocytes (Xu et al. 2002). In the present study, we have investigated (1) the mRNA expression of $cPLA_{2a}$, $cPLA_{2b}$, and $cPLA_{2c}$ in astrocytes and neurons; (2) the effects of therapeutically relevant concentrations of Li^+, VPA, and CBZ on gene and protein expression of $cPLA_2$, $sPLA_2$, and $iPLA_2$ in primary cultures of mouse astrocytes (to investigate the potential role of astrocytes as a drug target and enzyme specificity of the drug effect), and (3) the effect of topiramate, an anticonvulsant which has no anti-bipolar effect, on gene and protein expression of $cPLA_2$ (to study drug specificity).

Experimental methods

Chemicals for preparation of medium and most other chemicals, including VPA, CBZ and topiramate were purchased from Sigma (St. Louis, MO, USA). Lithium carbonate (Li_2CO_3) was obtained from Shanghai Hengxin Chemical Reagent (Shanghai, China). Santa Cruz Biotechnology (Santa Cruz, CA, USA) provided the first antibody raised against $cPLA_2$ recognizing $cPLA_{2a}$ (85 kDa), $cPLA_{2b}$ (114 kDa), and $cPLA_{2c}$ (61 kDa), and Sigma (St. Louis, MO, USA) supplied the first antibody, raised against β-actin (42 kDa). The second antibody goat anti-mouse IgG HRP conjugate was purchased from Promega (Madison, WI, USA).

Primary cultures of astrocytes were prepared as previously described (Hertz et al. 1978, 1998) with minor modifications. The neopallia of the cerebral hemispheres were aseptically isolated, vortexed to dissociate the tissue, filtered through nylon meshes with pore sizes of 80 and subsequently 10 μm, diluted in culture medium, and planted in Falcon Primaria culture dishes. The culture medium was a Dulbecco's medium with 7.5 mM glucose, initially containing 20% horse serum, and the cultures were incubated at 37°C in a humidified atmosphere of CO_2/air (5:95%). The culturing medium was exchanged with fresh medium of similar composition on day 3, and subsequently, every 3–4 days. From day 3, the serum concentration was reduced to 10%, and after the age of 2 weeks, 0.25 mM dibutyryl cyclic AMP (dBcAMP) was included in the medium. Such cultures have been used in our laboratories for more than 25 years (Hertz et al. 1978), and they are highly enriched in astrocytes [>95% purity of glial fibrillary protein (GFAP)- and glutamine synthetase-expressing astrocytes (Hertz et al. 1985)]. Addition of dBcAMP leads to a morphological and functional differentiation as evidenced by the extension of cell processes, increases in several metabolic activities, and expression of voltage sensitive L-channels for calcium (Ca^{2+}) (Hertz et al. 1989; Meier et al. 1991; Zhao et al. 1996). All four drugs were dissolved in phosphate-buffered saline (PBS). Lithium carbonate at final concentrations of 1.0, 0.5, or 0.25 mM (corresponding to Li^+ concentrations of 2, 1, or 0.5 mM), VPA at final concentrations of 1 mM or 100 μM, CBZ at final concentrations of 50 or 25 μM, topiramate at concentration of 100 μM, or PBS (control) was added to the culture medium after 2 weeks of culturing (i.e., at the time the cultures had reached confluency) and was present during continued culturing for another 1, 2, 3, or 4 weeks.

Table 1 Primer sequences used for RT-PCR of mRNA for $cPLA_{2a}$, $cPLA_{2b}$, $cPLA_{2c}$, $iPLA_2$, $sPLA_2$, and TBP

Gene		Sequence	Product size (bp)
$cPLA_{2a}$ [a]	Forward	GCACATTATAGTGGAACACC	430
	Reverse	ACACAGTGCCATGCTGAACC	
$cPLA_{2b}$ [b]	Forward	AAGCCATGTACTGGATGTG	273
	Reverse	TGGATGTAACAAGAAGTGT	
$cPLA_{2c}$ [b]	Forward	CGATTTACCCGAGGAGTGG	329
	Reverse	GCTTCCGAAGTGGGTTATGG	
$iPLA_2$ [b]	Forward	ACCTAGCCGTGGAGCTTGG	291
	Reverse	GCCCTTGGTTGTTTACCTGG	
$sPLA_2$ [c]	Forward	AGCTGACAGCATGAAGGTCCTCC	592
	Reverse	TTCTGGGTGAAGACAGAAGGGCC	
TBP[d]	Forward	CCACGGACAACTGCGTTGAT	236
	Reverse	GGCTCATAGCTACTGAACTG	

[a] From Ivanov et al. 2002
[b] From Lindbom et al. 2001
[c] From Kennedy et al. 1995
[d] From El Marjou et al. 2000

Cerebellar granule neurons were cultured as previously described (Peng et al. 1991) with minor modifications. Briefly, 7-day-old mouse pups were rapidly decapitated and the brains taken out. The cerebella were aseptically separated from the remainder of the brain, and after removal of the meninges, the tissue was cut into cubes of ~0.4 mm side dimensions, exposed to trypsin in a calcium–magnesium-free salt solution, reintroduced into tissue culture medium, passed through nylon sieves, and seeded into polylysine-coated standard 35-mm tissue culture dishes (Wuzhou Medical Plastic Factory, Zhejiang, China), using one cerebellum per culture dish. The cultures were grown in Dulbecco's medium in which the glucose concentration was increased to 30 mM (to eliminate feeding during the culturing) and the K^+ concentration to 24.5 mM, the glutamine concentration was decreased to 0.8 mM and 7% horse serum was added. After 2 days, cytosine arabinoside was added to the medium to a final concentration of 40 μM to curtail the number of astrocytes that develop in the cultures. The cells were used at the age of 7–8 days.

For determination of mRNA expression of different subtypes of PLA_2, a cell suspension was prepared by discarding the culturing medium, adding Trizol to cultures on ice, and scraping the cells off the culture dish. The RNA pellet was precipitated with isopropyl alcohol, washed with 70% ethyl alcohol, and dissolved in 10 μl sterile, distilled water, and an aliquot was used for determination of the amount of RNA (Kong et al. 2002). One microgram of RNA extract was used for RT, which was initiated by a 5-min incubation at 65°C of RNA extract with Random Hexamer at a final concentration of 12.5 ng/μl and deoxyribonucleotide triphosphates (dNTPs) (TaKaRa Biotechnology, Dalian, China) at a final concentration of 0.5 mM. The mixture was rapidly chilled on ice and briefly spun, and 4 μl 5× First-Strand Buffer, 2 μl 0.1 M dithiotreitol and 1 μl RNaseOUT Recombinant RNase Inhibitor (40 U/μl) were added. After the mixture had been incubated at 42°C for 2 min, 1 μl (200 U) of Superscript II (Gibco) was added, and the incubation at 42°C continued for another 50 min. Subsequently, the reaction was inactivated by heating to 70°C for 15 min, and the mixture was chilled and briefly centrifuged.

Polymerase chain reaction (PCR) amplification was performed in a Robocycler thermocycler with 0.2 μM of

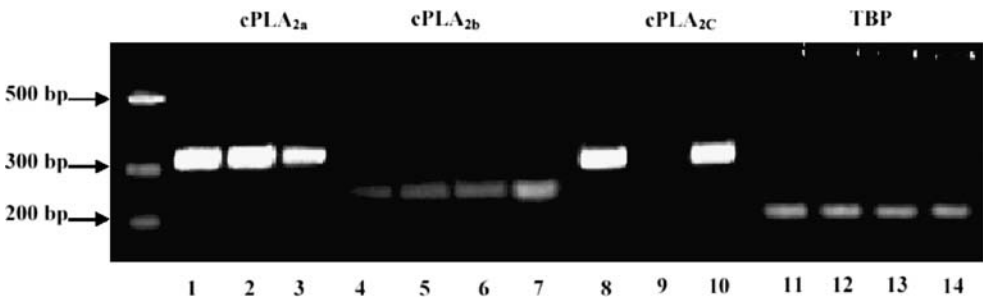

Fig. 1 Expression of $cPLA_{2a}$, $cPLA_{2b}$, and $cPLA_{2c}$ mRNA in primary cultures of astrocytes, with TATA-binding protein (TBP) as a housekeeping gene. The first lane, to the far left, represents a DNA ladder, the next *1* PCR product of $cPLA_{2a}$ from brain, *2* from astrocytes, *3* from cerebellar granule neurons, *4* PCR product of $cPLA_{2b}$ from brain, *5* from astrocytes, *6* from cerebellar granule neurons, *7* from liver, *8* PCR product of $cPLA_{2c}$ from brain, *9* from astrocytes, *10* from cerebellar granule neurons, *11* PCR product of TBP from brain, *12* from astrocytes, *13* from cerebellar granule neurons, and the last, *14*, from liver. One μg of RNA extract was used in RT for all the samples. The size of PCR product of $cPLA_{2a}$ is 430 bp, of $cPLA_{2b}$ 273 bp, of $cPLA_{2c}$ 329 bp, and of TBP 236 bp

sense or antisense and 0.375 U of Taq-polymerase (TaKaRa Biotechnology, Dalian, China) for $cPLA_{2a}$, $cPLA_{2b}$, $cPLA_{2c}$, $iPLA_2$, $sPLA_2$, and TATA-binding protein (TBP), used as a housekeeping gene (Table 1). Brain and liver tissues were used as positive controls of three types of $cPLA_2$. cDNA 2.5, 5, and 10 μg, and 25–45 cycles were tested for each pair of primers. The amount of cDNA used was 5 μg. Thirty cycles were used for $iPLA_2$ and $sPLA_2$, 35 cycles for $cPLA_{2b}$ and $cPLA_{2c}$, and 40 cycles for $cPLA_{2a}$ and TBP. Initially, the template was denatured by heating to 94°C for 2 min, followed by 2-min amplification cycles, each consisting of two 45-s periods and one 60-s period, the first at 94°C, the second at 59°C for $cPLA_{2a}$, 56°C for $cPLA_{2b}$, $cPLA_{2c}$, and $iPLA_2$ or 55°C for $sPLA_2$ and TBP, and the third at 72°C. The final step was extension at 72°C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis, and captured by Fluorchem 5500 (Alpha Innotech, San Leandro, CA, USA).

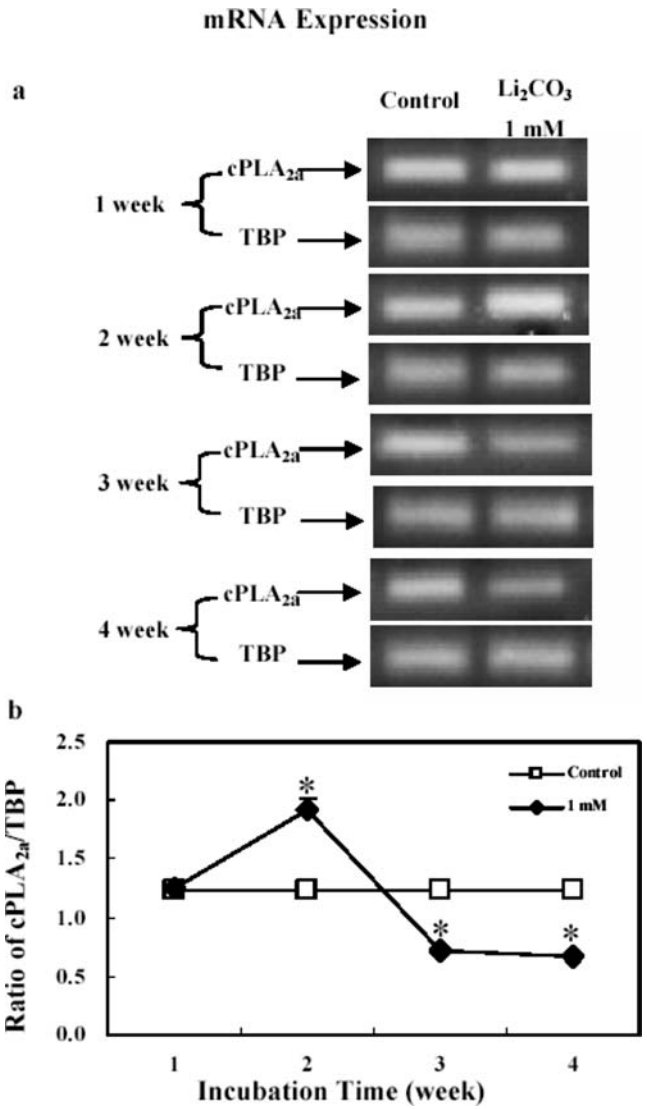

Fig. 2 mRNA expression measured by RT-PCR of $cPLA_{2a}$ in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 1 mM lithium carbonate (2 mM lithium). **a** A representative experiment showing mRNAs for $cPLA_{2a}$ in the upper row and for TBP, as a house-keeping gene, in the lower row, in control cultures and the corresponding results in lithium-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means ± SEM of scanned ratios between $cPLA_{2a}$ and TBP (five samples from five different batches of cultures) in control cultures and cultures treated with 1 mM lithium carbonate. $P<0.05$: * vs control cultures from the same batch and treatment period

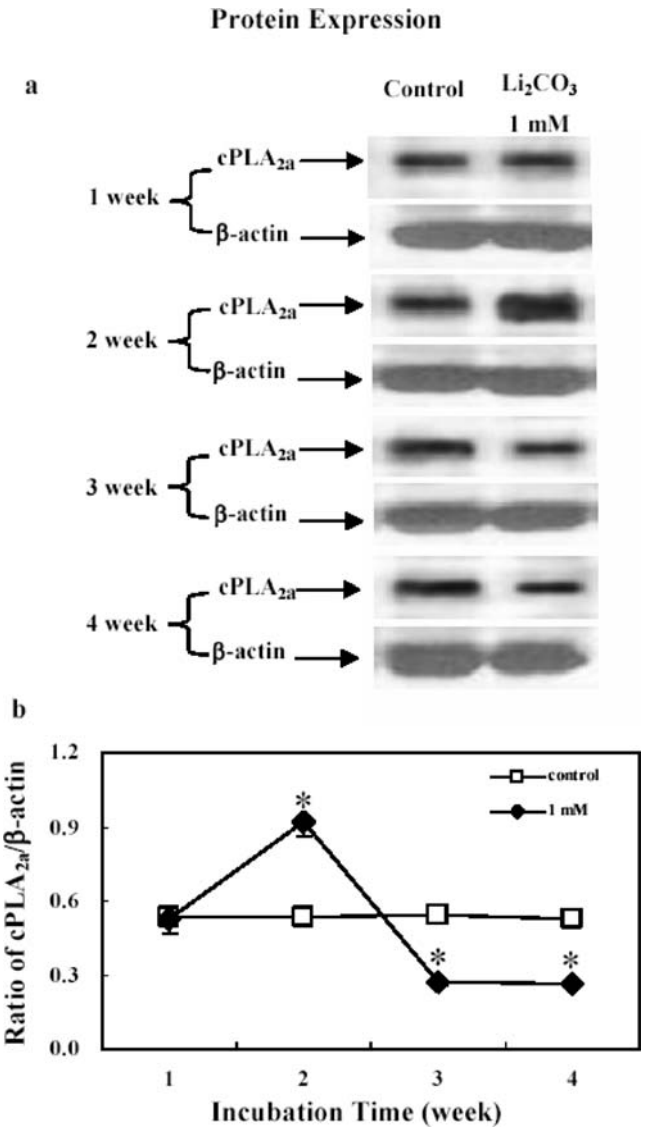

Fig. 3 Protein expression measured by immunoblotting of $cPLA_{2a}$ (85 kDa) in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 1 mM lithium carbonate (2 mM lithium). **a** A representative experiment showing proteins for $cPLA_{2a}$ in the upper row and for β-actin, as a house-keeping protein, in the lower row, in control cultures and the corresponding results in lithium-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means ± SEM of scanned ratios between $cPLA_{2a}$ and β-actin (four samples from four different batches of cultures) in control cultures, cultures treated with 1 mM lithium carbonate. $P<0.05$: * vs control cultures from the same batch and treatment period

For determination of protein expression of $cPLA_2$, the cells were washed with ice-cold phosphate-buffered saline (PBS) containing 7.5 mM glucose, scraped off the dishes, and harvested in 0.5 ml of ice-cold buffer A [0.25 M sucrose, 10 mM HEPES, the phosphatase inhibitors alpha-mercaptoethanol (10 mM) and phenylmethyl sulfonyl fluoride (1 mM), and 1 mM sodium orthovanadate, pH 7.4] and homogenized to make a whole cell lysate. The protein content was determined in the homogenates by the Bradford method (Bradford 1976), using bovine serum albumin as the standard. Samples containing 50 μg protein were applied on slab gels of 10% polyacrylamide. After transfer to nitrocellulose membranes, the samples were blocked by 5% skimmed milk powder in TBS-T (30 mM Tris–HCl, 125 mM NaCl, 0.1% Tween 20) for 1 h. The nitrocellulose membranes were incubated with the first

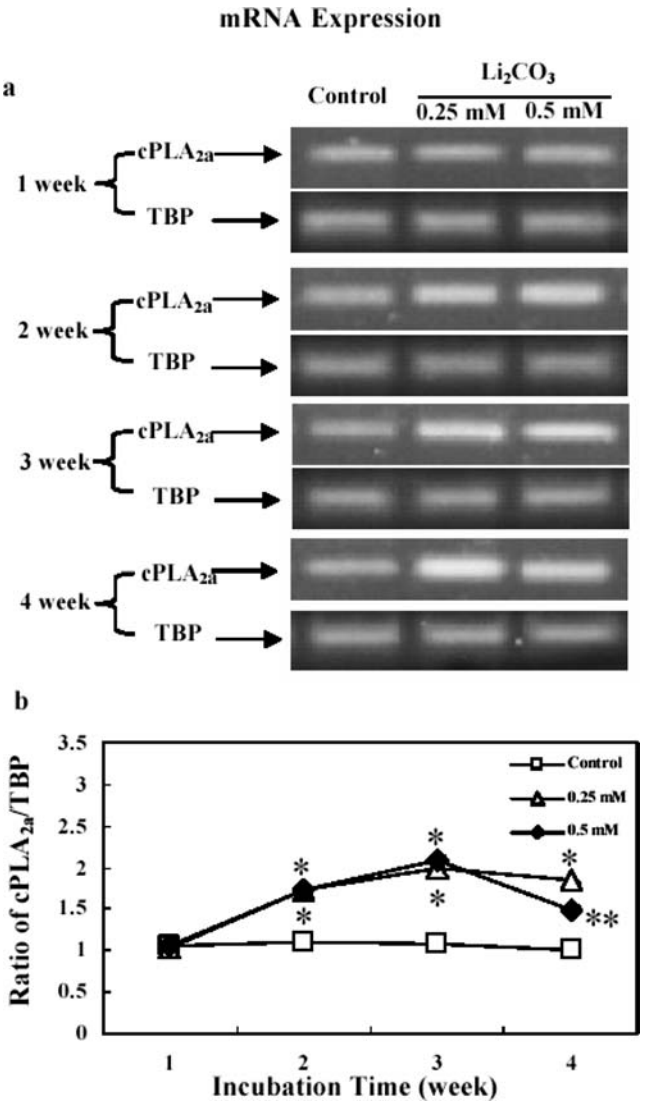

Fig. 4 mRNA expression measured by RT-PCR of $cPLA_{2a}$ in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 0.5 or 0.25 μM lithium carbonate (1 and 0.5 mM lithium). **a** A representative experiment showing mRNAs for $cPLA_{2a}$ in the upper row and for TBP, as a house-keeping gene, in the lower row, in control cultures and the corresponding results in lithium-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means ± SEM of scanned ratios between $cPLA_{2a}$ and TBP (four samples from four different batches of cultures) in control cultures, cultures treated with 0.5 mM lithium carbonate, and cultures treated with 0.25 mM lithium carbonate. $P<0.05$: * vs control cultures from the same treatment period; ** vs control cultures and cultures treated with 0.5 mM lithium carbonate from the same batch and treatment period

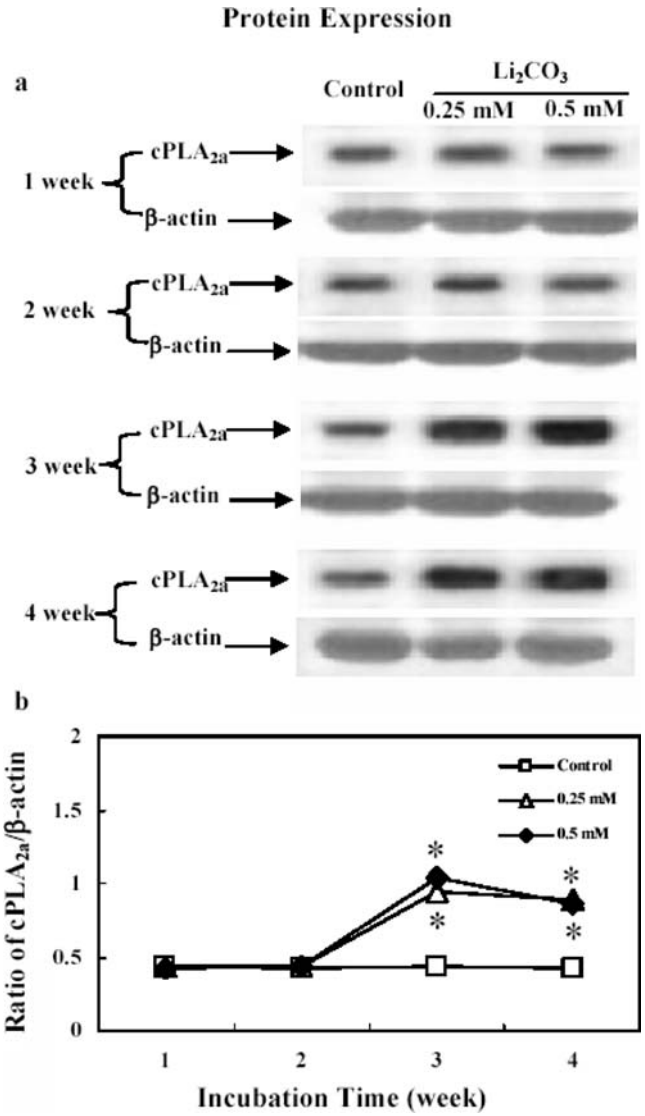

Fig. 5 Protein expression measured by immunoblotting of $cPLA_{2a}$ (85 kDa) in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 0.5 or 0.25 mM lithium carbonate (1 and 0.5 mM lithium). **a** A representative experiment showing proteins for $cPLA_{2a}$ in the upper row and for β-actin, as a house-keeping protein, in the lower row, in control cultures and the corresponding results in lithium-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means ± SEM of scanned ratios between $cPLA_{2a}$ and β-actin (four samples from four different batches of cultures) in control cultures, cultures treated with 0.5 mM lithium carbonate, and cultures treated with 0.25 mM lithium carbonate. $P<0.05$: * vs. control cultures from the same batch and treatment period

antibody, specific to either $cPLA_2$ at 1 × 1,000 dilution or β-actin (used for housekeeping) at 1 × 4,000 dilution for 2 h at room temperature. After washing, specific binding was detected by goat-anti-mouse horseradish peroxidase-conjugated secondary antibody at 1 × 1,000 dilution. Staining was visualized by ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK), followed by exposure to film (FuJi Photo Film, Tokyo, Japan). The results were collected by Fluorchem imaging system. Band density was measured with Window AlphaEaseTM FC 32-bit software.

Ratios between $cPLA_2$ mRNA or protein and the respective housekeeping gene or protein (TBP and β-actin) were determined and averaged. All drug-treated cultures were compared to controls from the same batch at the same age in culture. The differences between multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD)

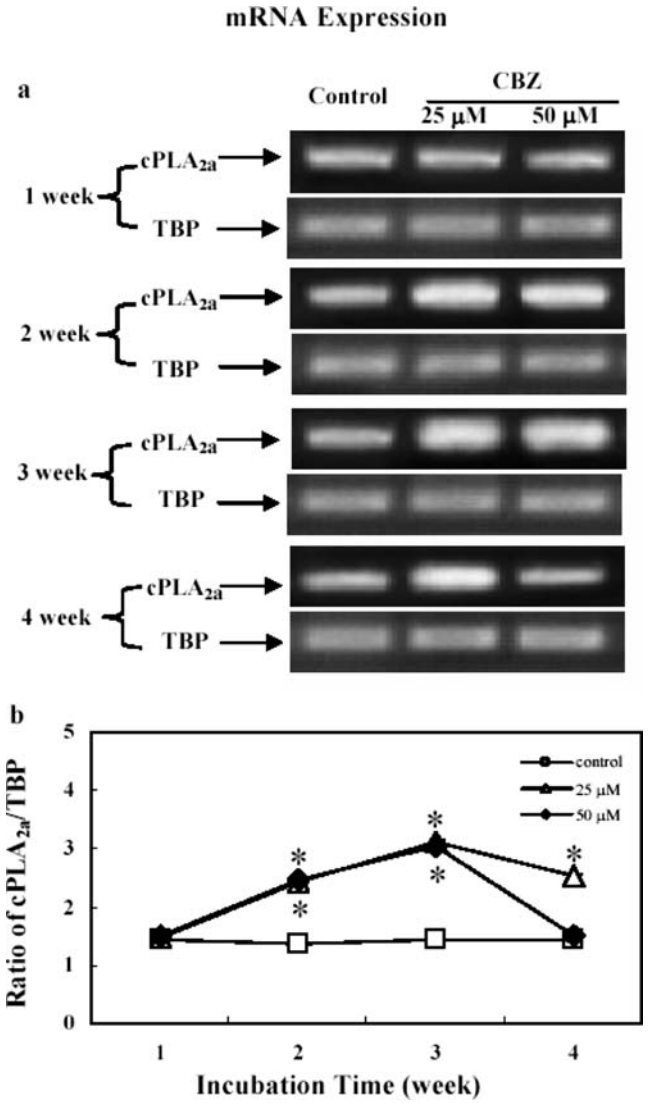

Fig. 6 mRNA expression measured by RT-PCR of $cPLA_{2a}$ in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 50 or 25 μM CBZ. **a** A representative experiment showing mRNAs for $cPLA_{2a}$ in the upper row and for TBP, as a house-keeping gene, in the lower row, in control cultures and the corresponding results in CBZ-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means±SEM of scanned ratios between $cPLA_{2a}$ and TBP (four samples from four different batches of cultures) in control cultures, cultures treated with 50 μM CBZ, and cultures treated with 25 μM CBZ. $P<0.05$: * vs. control cultures from the same batch and treatment period

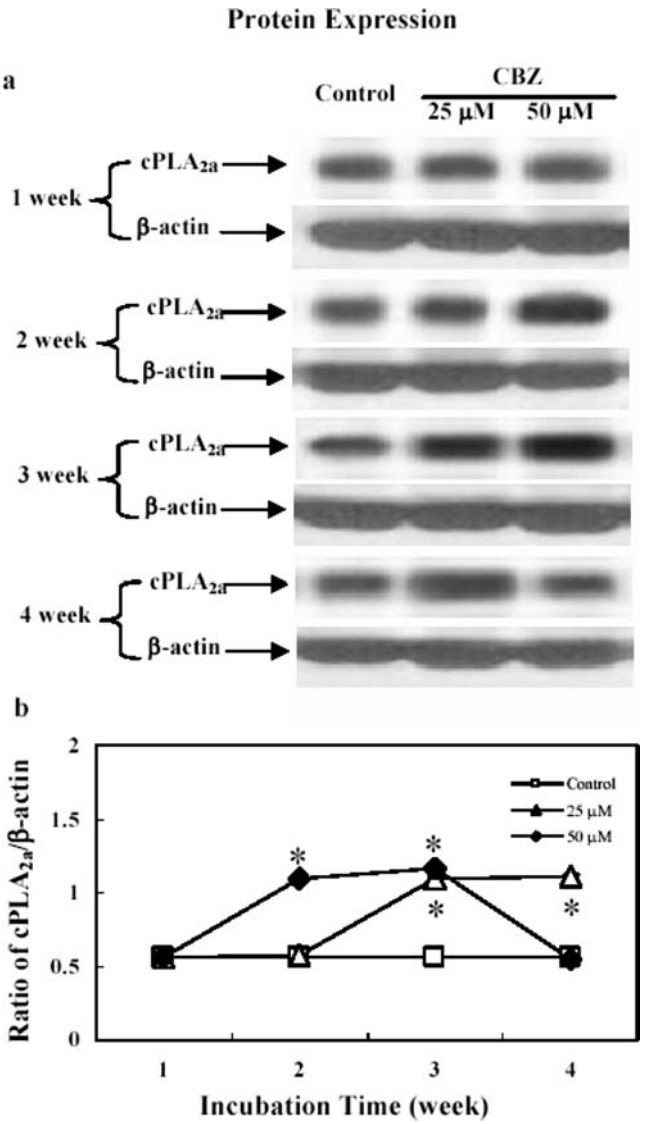

Fig. 7 Protein expression measured by immunoblotting of $cPLA_{2a}$ (85 kDa) in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 50 μM or 25 μM CBZ. **a** A representative experiment showing proteins for $cPLA_{2a}$ in the upper row and for β-actin, as a house-keeping protein, in the lower row, in control cultures and the corresponding results in CBZ-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means ± SEM of scanned ratios between $cPLA_{2a}$ and β-actin (four samples from four different batches of cultures) in control cultures, cultures treated with 50 μM CBZ, and cultures treated with 25 μM CBZ. $P<0.05$: * vs control cultures from the same batch and treatment period

multiple comparison test for unequal replications. The level of significance was set at $p<0.05$.

Results

Expression of cPLA$_2$ Figure 1 shows that the expression of mRNA for cPLA$_{2a}$ in astrocytes equals that in brain tissue, whereas less cPLA$_{2a}$ is present in neurons. Much smaller amounts of mRNA for cPLA$_{2b}$ was found in brain or the cultured cells, compared to that in liver, and cPLA$_{2c}$ was expressed in brain and in neurons, but not in astrocytes. A similar result was found in Western blot that predominantly cPLA$_{2a}$, an 85-kDa band was recognized by the antibody

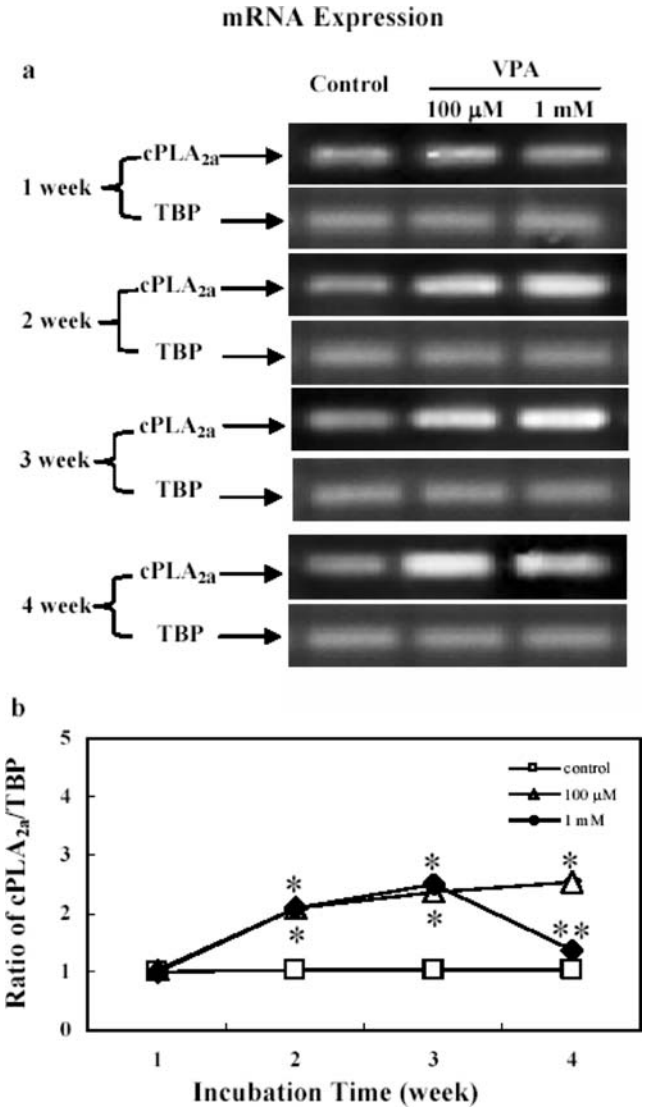

Fig. 8 mRNA expression measured by RT-PCR of cPLA$_{2a}$ in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 1 mM or 100 μM VPA. **a** A representative experiment showing mRNAs for cPLA$_{2a}$ in the upper row and for TBP, as a house-keeping gene, in the lower row, in control cultures and the corresponding results in VPA-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means ± SEM of scanned ratios between cPLA$_{2a}$ and TBP (four samples from four different batches of cultures) in control cultures, cultures treated with 1 mM VPA, and cultures treated with 100 μM VPA. $P<0.05$: * vs. control cultures from the same treatment period; ** vs control cultures and cultures treated with 100 μM VPA from the same batch and treatment period

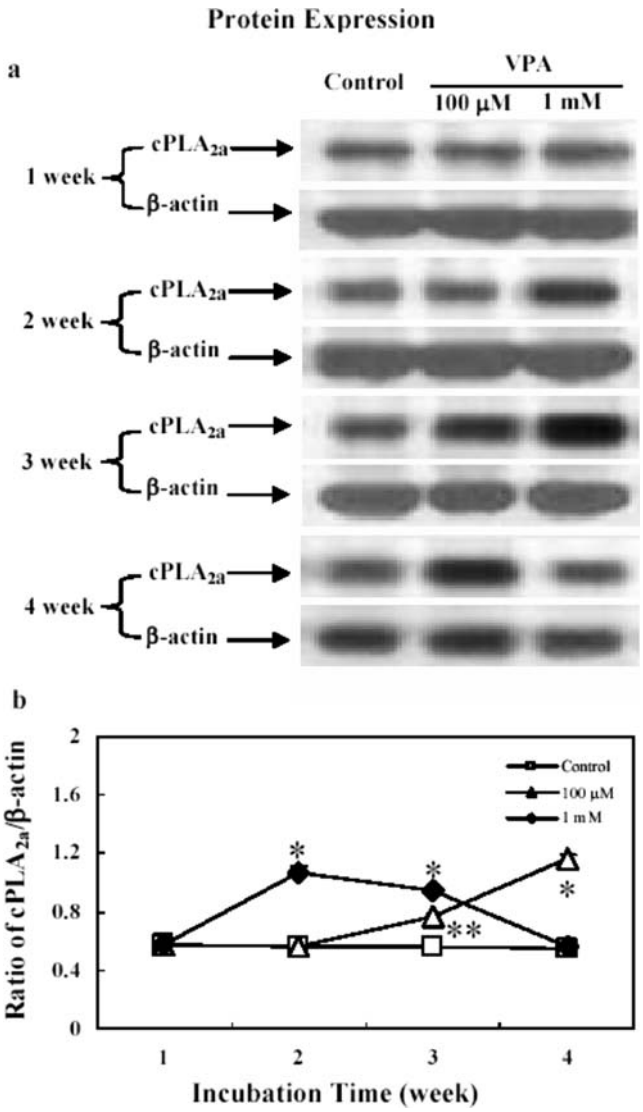

Fig. 9 Protein expression measured by immunoblotting of cPLA$_{2a}$ (85 kDa) in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 1 mM or 100 μM VPA. **a** A representative experiment showing proteins for cPLA$_{2a}$ in the upper row and for β-actin, as a house-keeping protein, in the lower row, in control cultures and the corresponding results in VPA-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means ± SEM of scanned ratios between cPLA$_{2a}$ and β-actin (four samples from four different batches of cultures) in control cultures, cultures treated with 1 mM VPA, and cultures treated with 100 μM VPA. $P<0.05$: * vs control cultures from the same batch and treatment period; ** vs control cultures and cultures treated with 1 mM VPA from the same batch and treatment period

against $cPLA_2$ in astrocytes (results not presented). In the following, only drug effects on this paralog will be discussed, but this does not exclude that other $cPLA_2$ paralogs could also be affected by treatment with anti-bipolar drugs.

Effects of Li^+ As shown in Fig. 2a, 2 weeks of treatment with 1 mM lithium carbonate (2 mM Li^+) induced an up-regulation of mRNA expression of $cPLA_{2a}$ in astrocytes, whereas 1 week of treatment had no effect and treatment for 3 or 4 weeks led to a down-regulation. The up-regulation

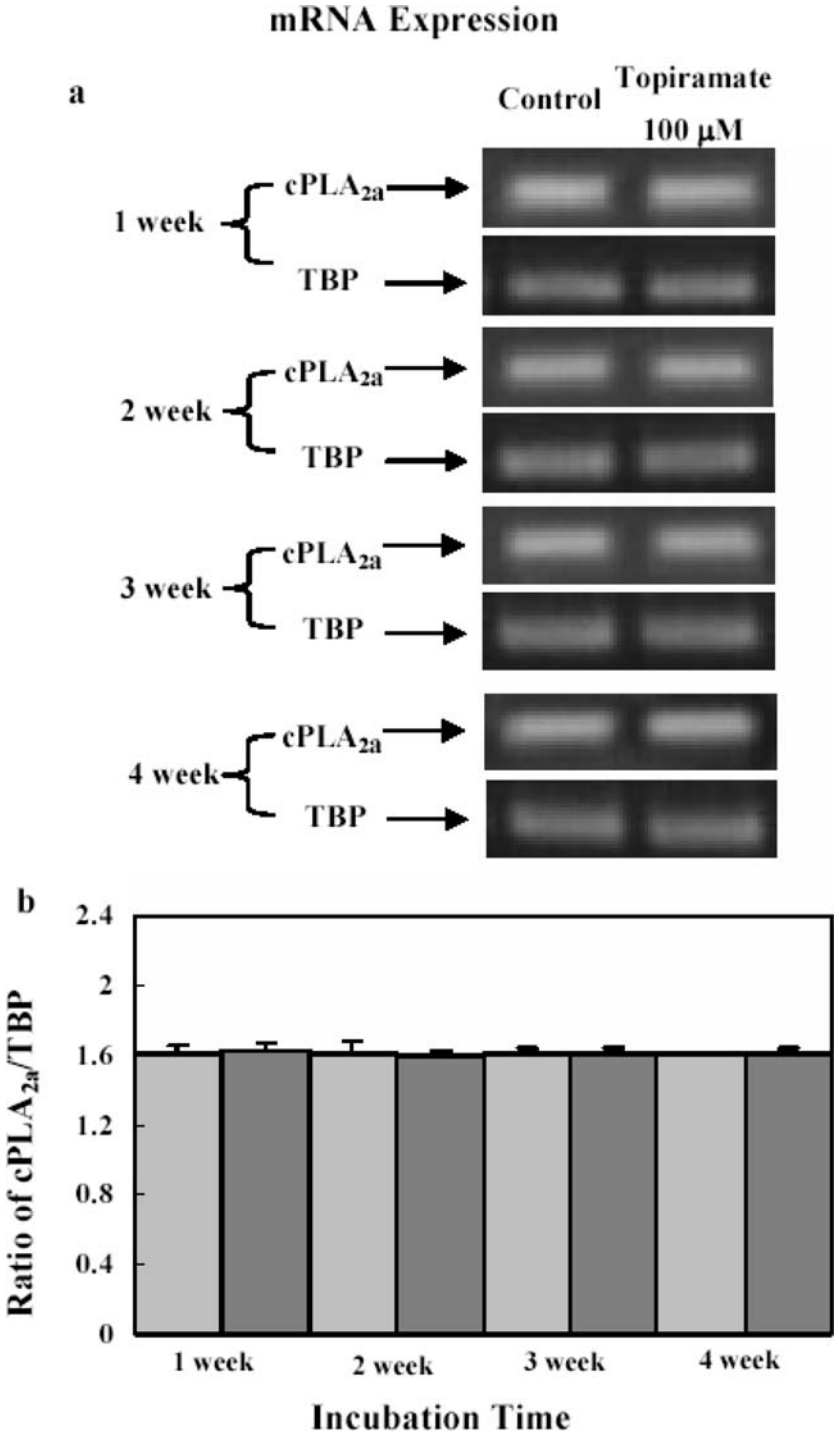

Fig. 10 mRNA expression measured by RT-PCR of $cPLA_{2a}$ in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 100 μM topiramate. **a** A representative experiment showing mRNAs for $cPLA_{2a}$ in the upper row and for TBP, as a house-keeping gene, in the lower row, in control cultures and the corresponding results in topiramate-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means ± SEM of scanned ratios between $cPLA_{2a}$ and TBP (four samples from four different batches of cultures) in control cultures and cultures treated with 100 μM topiramate

was reflected in the averaged densitometrically determined ratios between expression of $cPLA_{2a}$ and of TBP by a statistically significant increase from the control after 2 weeks follow by a decrease after 3 and 4 weeks (Fig. 2b). Protein expression of $cPLA_{2a}$, determined by an 85-kDa band in Western blotting with β-actin as house-keeping protein (Fig. 3a), was also unaffected by 1 week of treatment with 1 mM lithium carbonate, significantly increased after 2 weeks of treatment, and significantly reduced after 3 and 4 weeks of treatment (Fig. 3b).

In contrast to the biphasic change of $cPLA_{2a}$ expression during exposure to 1 mM lithium carbonate, lithium carbonate at concentrations of 0.5 and 0.25 mM (i.e., 1.0 and 0.5 mM Li^+) caused only an up-regulation of mRNA expression of $cPLA_{2a}$ (Fig. 4a). At both concentrations, the mRNA levels were increased after 2, 3, and 4 weeks of exposure to Li^+, but after 4 weeks of treatment, the response was smaller with 0.5 mM lithium carbonate than with 0.25 mM (Fig. 4b). The $cPLA_{2a}$ protein was also increased but the response was slower than the mRNA increase, as it was not seen until after 3 and 4 weeks (Fig. 5).

Effects of CBZ The effects of 25 or 50 μM CBZ on mRNA expression of $cPLA_{2a}$ were almost indistinguishable from those of 0.25 and 0.5 mM lithium carbonate. Two weeks of drug treatment induced a significant up-regulation of the expression, which persisted at 3 weeks (Fig. 6). At 50 μM CBZ, there was no significant difference between treated cultures and control cultures after 4 weeks, whereas the mRNA expression of $cPLA_{2a}$ remained increased during exposure to 25 μM CBZ. A similar difference was observed in the expression of the $cPLA_{2a}$ protein after 4 weeks, but after 2 weeks, an increase was only observed at 50 μM CBZ (Fig. 7).

Effects of VPA The results with 1 mM and 100 μM VPA were similar to those with CBZ. Again, there was no effect after 1 week of treatment, but both concentrations caused an up-regulation of $cPLA_{2a}$ mRNA after 2 and 3 weeks treatment, whereas the up-regulation after 4 weeks persisted only at 100 μM (Fig. 8). One millimolar VPA also up-regulated $cPLA_{2a}$ protein after 2 and 3 weeks of treatment but not after treatment for 4 weeks, whereas 100 μM VPA caused a small, but significant up-regulation after 3 weeks and a larger up-regulation after 4 weeks of treatment (Fig. 9).

Lack of effects by topiramate Treatment with 100 μM topiramate, a drug that is ineffective as an anti-bipolar medication had no effect on either $cPLA_{2a}$ mRNA or $cPLA_{2a}$ protein during up to 4 weeks of treatment (Figs. 10 and 11).

Lack of effects by anti-bipolar drugs on $iPLA_2$ and $sPLA_2$ Li^+, AVP, and CBZ at concentrations affecting $cPLA_{2a}$ mRNA had no effect on the expression of either $iPLA_2$ (Table 2) or $sPLA_2$ (Table 3). Nor did topiramate have any effect (Tables 2 and 3).

Discussion

Drug effects In agreement with observations in the brain in vivo (Rintala et al. 1999; Weerasinghe et al. 2004),

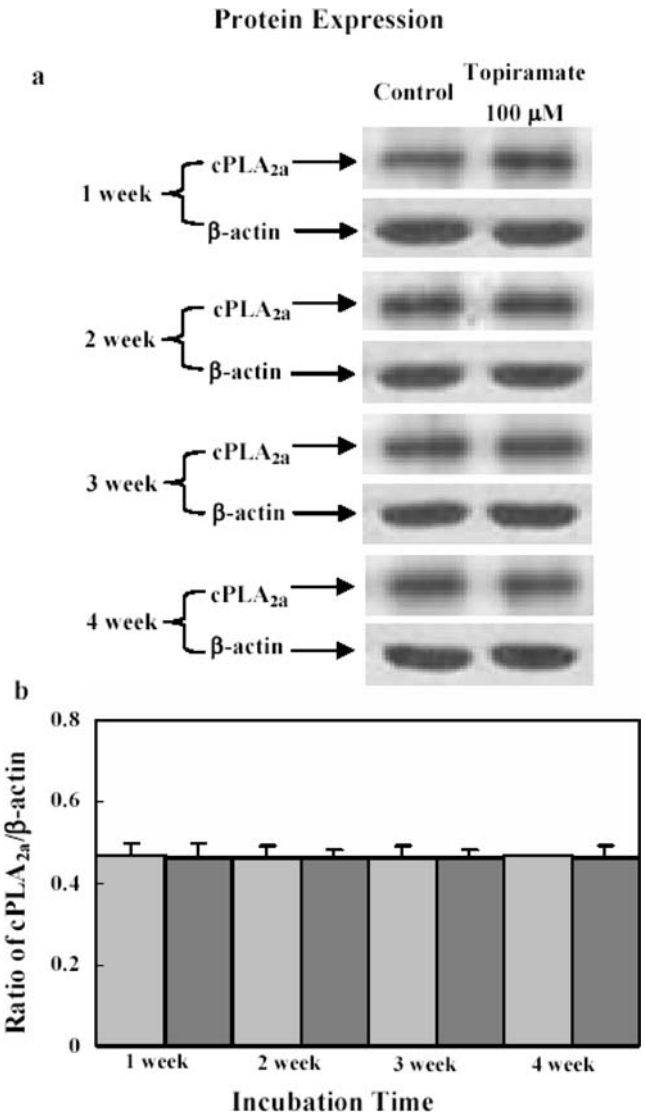

Fig. 11 Protein expression measured by immunoblotting of $cPLA_{2a}$ (85 kDa) in primary cultures of mouse astrocytes treated for 1 to 4 weeks with 100 μM topiramate. **a** A representative experiment showing proteins for $cPLA_{2a}$ in the upper row and for β-actin, as a house-keeping protein, in the lower row, in control cultures and the corresponding results in topiramate-treated cultures after 1, 2, 3, or 4 weeks of treatment. **b** All results are means ± SEM of scanned ratios between $cPLA_{2a}$ and β-actin (four samples from four different batches of cultures) in control cultures and cultures treated with 100 μM topiramate

Table 2 Expression of mRNA of $iPLA_2$ in astrocytes chronic treated with PBS (control), Li_2CO_3, CBZ, VPA, or Topiramate

Treatment		1 week	2 week	3 week	4 week
Li_2CO_3	Control	1.01±0.02	1.05±0.02	1.07±0.01	1.10±0.02
1 mM	1 mM	1.02±0.02	1.00±0.01	1.07±0.01	1.07±0.01
Li_2CO_3 0.25	Control	1.96±0.01	1.96±0.01	1.97±0.01	1.970±0.01
or 0.5 mM	0.25 mM	1.97±0.01	1.95±0.01	1.97±0.01	1.96±0.01
	0.5 mM	1.97±0.01	1.96±0.01	1.97±0.01	1.96±0.01
CBZ	Control	1.72±0.01	1.70±0.01	1.70±0.01	1.70±0.01
	25 μM	1.71±0.01	1.71±0.01	1.70±0.01	1.71±0.01
	50 μM	1.70±0.01	1.71±0.01	1.71±0.01	1.70±0.01
VPA	Control	1.53±0.01	1.53±0.01	1.53±0.01	1.53±0.01
	100 μM	1.54±0.01	1.53±0.01	1.53±0.01	1.53±0.01
	1 mM	1.54±0.01	1.54±0.01	1.53±0.01	1.54±0.01
Topiramate	Control	1.35±0.02	1.35±0.01	1.35±0.02	1.34±0.01
	100 μM	1.34±0.01	1.36±0.01	1.34±0.02	1.34±0.02

Each value represents the mean ± SEM.

prolonged chronic treatment with 2 mM Li^+ induced a down-regulation of $cPLA_{2a}$ in primary cultures of astrocytes. However, after 2 weeks of treatment, there was an up-regulation, a response not observed in the brain in vivo. A change in the direction of a functional response in astrocytes during prolonged treatment with a psychoactive drug has precedence, as we have previously reported that long-term treatment (2–3 weeks) of astrocyte cultures with fluoxetine, which has $5\text{-}HT_{2B}$ receptor agonist activity, causes an up-regulation of the glycogenolytic response to renewed administration of fluoxetine, whereas short-term treatment (1 week) abolishes the fluoxetine-induced hydrolysis of glycogen (Kong et al. 2002).

Lower concentrations of lithium carbonate led within the period studied only to an up-regulation, but it is possible that longer exposure times might have resulted in a down-regulation, similar to that reported in the rat brain by Rintala et al. (1999), who used a treatment period of 6 weeks. Unfortunately, these authors did not report results after shorter exposure to Li^+. The concept that the length of the treatment period may affect the response is supported by the findings with carbamazepine and valproic acid where the effect of higher concentrations (50 μM; 1 mM) peaked after 2–3 weeks of treatment and was abolished or greatly reduced after 4 weeks, whereas that to the lower concentration (25 μM; 100 μM) was pronounced after

Table 3 Expression of mRNA of $sPLA_2$ in astrocytes chronic treated with PBS (control), Li_2CO_3, CBZ, VPA, or Topiramate

Treatment		1 week	2 week	3 week	4 week
Li_2CO_3	Control	1.02±0.01	1.03±0.01	1.03±0.01	1.04±0.01
1 mM	1 mM	1.02±0.01	1.02±0.01	1.03±0.01	1.02±0.01
Li_2CO_3 0.25	Control	1.02±0.01	1.00±0.01	1.00±0.01	1.01±0.01
or 0.5 mM	0.25 mM	1.00±0.01	1.03±0.01	1.00±0.01	1.02±0.01
	0.5 mM	1.01±0.01	1.02±0.01	1.00±0.01	1.03±0.01
CBZ	Control	1.20±0.01	1.20±0.01	1.21±0.01	1.20±0.01
	25 μM	1.20±0.01	1.21±0.01	1.21±0.01	1.20±0.01
	50 μM	1.22±0.01	1.21±0.01	1.21±0.01	1.20±0.01
VPA	Control	1.84±0.01	1.84±0.01	1.83±0.01	1.83±0.01
	100 μM	1.84±0.01	1.84±0.01	1.84±0.01	1.84±0.01
	1 mM	1.83±0.01	1.84±0.01	1.84±0.01	1.83±0.01
Topiramate	Control	2.00±0.01	2.01±0.01	2.07±0.01	2.04±0.01
	100 μM	2.02±0.03	2.05±0.01	2.02±0.01	2.03±0.01

Each value represents the mean ± SEM.

4 weeks, but also developed more slowly. Thus, with all three drugs, high concentrations resulted in a rapid up-regulation, which within 4 weeks was abolished, greatly reduced, or even transformed to a down-regulation, whereas lower concentrations evoked a slower response and no reduction of the stimulation after 4 weeks of treatment. In contrast, topiramate, which has no anti-bipolar effect had no effect on $cPLA_{2a}$ expression, and mRNA expression of $iPLA_2$ and $sPLA_2$ was unaltered by Li^+, VPA, and CBZ. *Thus, the observed effects (up-regulation and down-regulation) show both enzyme specificity and drug specificity, and it was in a systematic fashion dependent upon drug concentration and the length of the treatment period*. The enzyme specificity is identical to that reported in the brain in vivo after administration of either Li^+ or carbamazepine (Rintala et al. 1999; Weerasinghe et al. 2004; Ghelardoni et al. 2004; Basselin et al. 2005), but no published data seem to be available about the effect of topiramate on $cPLA_{2a}$ expression in intact brain. Also, in contrast to the present observations, VPA was found not to affect $cPLA_2$ expression in the brain in vivo (after 30 days of treatment), although it had the same effect on turnover of arachidonic acid as the other two anti-bipolar drugs (Chang et al. 2001).

The concentrations used in the present study are probably pharmacologically relevant. Thus, Soares et al. (2001) found that Li^+ brain concentrations varied from 0.23 to 0.55 mEq/l at a plasma Li^+ concentration of 0.7 mM, a concentration within the lower range of the therapeutically relevant level (Sproule 2002), and Moore et al. (2002) reported a brain/plasma ratio of 0.9. Accordingly, by studying the range 0.5–2.0 mM Li^+ the pharmacologically relevant concentration range has probably been included (Sproule 2002).

Similar considerations apply to the concentrations used of VPA and CBZ. A therapeutically relevant plasma concentration of VPA is 0.6 mM, but Vajda et al. (1981) observed that the level of VPA in CSF was only 7.6–25.0% of its plasma concentration (50–100 μg/ml), i.e., 30–150 μM, and a brain–serum ratio of 15% was also reported by Wieser (1994). Again, the pharmacologically relevant concentration range is likely to have been covered by using a low concentration (100 μM) and a high concentration (1 mM), the results of which only varied in the time course. The concentration ratio between brain and plasma of CBZ has been determined as 1.4–1.6 in epileptic patients (Friis et al. 1978), but the protein binding of this drug makes estimates of the free concentration in the incubation medium (which contains 10% serum) and cells uncertain. The higher CBZ concentration used in the current study is, however, close to the mean plasma CBZ concentration of 53.6±5.2 μmol/l in the study by Ghelardoni et al. (2004) and to the highest concentration in the therapeutic range reported in bipolar patients, 17–51 μmol/l (Petit et al. 1991; Bialer et al. 1998).

Relevance of astrocytes In agreement with previous observations (Stephenson et al. 2004; Lautens et al. 1998; Balboa et al. 2002), $cPLA_{2a}$ was expressed to a large degree in astrocytes, whereas less expression was observed in neurons. In contrast, $cPLA_{2c}$ was mainly expressed in neurons. In the present context, it is of interest that $cPLA_{2a}$ is the only PLA_2 that has specificity for phospholipid substrates containing arachidonic acid (Ghosh et al. 2006). Arachidonic acid interferes in a complex manner with the free cytosolic concentration of calcium ions ($[Ca^{2+}]_i$) in astrocytes (Sergeeva et al. 2003; Yang et al. 2005; Alloisio et al. 2006). This is important because astrocytic $[Ca^{2+}]_i$ has signaling functions both within individual cells, during propagation of Ca^{2+} waves across the astrocytic syncytium, and in interactions between astrocytes and neurons (Cornell-Bell et al. 2004; Haas et al. 2006; Scemes and Giaume 2006; Fiacco and McCarthy 2006). Moreover, the released arachidonic acid can be further oxidized to eicosanoids, converted to endocannabinoids or re-acylated in the membrane, a process that in the past has been quantitatively greatly underestimated (Purdon et al. 2002). Among the eicosanoids, PGE2 may be of special interest, as it can be formed in astrocytes (Hewett 1999), and an enzyme catalyzing its terminal synthesis is decreased in the frontal and temporal cortex of bipolar patients, with a trend towards normalization by medication (Maida et al. 2006).

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