Chronic treatment of astrocytes with therapeutically relevant fluoxetine concentrations enhances cPLA\(_2\) expression secondary to 5-HT\(_{2B}\)-induced, transactivation-mediated ERK\(_{1/2}\) phosphorylation

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Abstract

Introduction We have recently shown that fluoxetine, a serotonin-specific reuptake inhibitor (SSRI), has low micromolar affinity for the 5-HT\(_{2C}\) receptor (but not for 5-HT\(_{2A}\) and 5-HT\(_{2B}\) receptors) in primary cultures of mouse astrocytes. This was determined as phosphorylation (stimulation) of extracellular-regulated kinase 1 and 2 (ERK\(_{1/2}\)) by transactivation-mediated phosphorylation of the epidermal growth factor (EGF) receptor, followed by conventional EGF receptor signaling (Li et al., Psychopharmacology 194:333–334, 2007). Paroxetine has an identical effect. The present study shows that chronic fluoxetine treatment with even higher affinity (EC\(_{50}=0.5–2.0 \, \mu M\)) upregulates Ca\(^{2+}\)-dependent phospholipase A\(_2\) (cPLA\(_2\)), which releases arachidonic acid from the sn-2 position of membrane-bound phospholipid, without effect on secretory PLA\(_2\) (sPLA\(_2\)) and intracellular PLA\(_2\) (iPLA\(_2\)).

Discussion This demonstration replicates the fluoxetine-induced cPLA\(_2\) upregulation in rat brain shown by Rao et al. (Pharmacogenomics J 6:413–420, 2006) and provides the new information that upregulation (1) occurs in astrocytes, (2) is evoked by stimulation of 5-HT\(_{2B}\) receptor, and (3) requires transactivation-mediated ERK\(_{1/2}\) phosphorylation. Similar upregulation of cPLA\(_2\) in intact brain in response to 5-HT\(_{2}\)-mediated signaling by elevated serotonin levels and/or an SSRI during antidepressant treatment may explain the repeatedly reported ability of SSRIs to normalize regional decreases which occur in brain metab-

Keywords Arachidonic acid · Ca\(^{2+}\)-dependent phospholipase A\(_2\) · Epidermal growth factor · Extracellular-regulated kinases 1 and 2 · Serotonin · Unipolar depression

Introduction

Fluoxetine is an antidepressant drug that is generally assumed to exert its therapeutic effect exclusively as a selective serotonin (5-HT) reuptake inhibitor (SSRI) causing an increase in the concentration of 5-HT in the synaptic cleft and thereby enhancing synaptic 5-HT signaling (Benfield et al. 1986). During chronic administration of an SSRI, the 5-HT levels in the brain become increased, at least in most regions (Beyer and Cremers 2008), long before amelioration of mood (Nierenberg et al. 2000), suggesting that changes in signaling in response to either the increased extracellular 5-HT concentration, or perhaps even the SSRI itself, are required for therapeutic effectiveness. In an attempt to clarify such mechanisms, Rapoport and coworkers (Qu et al. 2006; Rao et al. 2006; Lee et al. 2008) recently showed that chronic administration of fluoxetine (10 mg/kg/day) for 21 days leads to stimulation and enhanced mRNA and protein expression of Ca\(^{2+}\)-dependent phospholipase A\(_2\) (cPLA\(_2\)) in the rat brain, but not of...
the two other phospholipases A_2 (secretory PLA\textsubscript{2} [sPLA\textsubscript{2}] and intracellular PLA\textsubscript{2} [iPLA\textsubscript{2}]). After stimulation of one of several different neurotransmitter receptors, including 5-HT\textsubscript{2A/2C} receptors, cPLA\textsubscript{2} releases arachidonic acid from the sn-2 position of membrane-bound phospholipid substrate in neural preparations (Felder et al. 1990; Stout et al. 2002; Qu et al. 2003; Rapoport 2008), including glioma cells (Garcia and Kim 1997). Released arachidonic acid and its metabolites play important roles in brain plasticity (1997) in glioma cells, astrocytes are among the cells that express cPLA\textsubscript{2} (Sun et al. 2004; Li et al. 2007). In the brain cells (Garcia and Kim 1997). Released arachidonic acid therefore, likely that the activation of cPLA\textsubscript{2} observed in the two other phospholipases A\textsubscript{2} (secretory PLA\textsubscript{2} [sPLA\textsubscript{2}] and intracellular PLA\textsubscript{2} [iPLA\textsubscript{2}]). After stimulation of one of several different neurotransmitter receptors, including 5-HT\textsubscript{2A/2C} receptors, cPLA\textsubscript{2} releases arachidonic acid from the sn-2 position of membrane-bound phospholipid substrate in neural preparations (Felder et al. 1990; Stout et al. 2002; Qu et al. 2003; Rapoport 2008), including glioma cells (Garcia and Kim 1997). Released arachidonic acid and its metabolites play important roles in brain plasticity (1997) in glioma cells, astrocytes are among the cells that express cPLA\textsubscript{2} (Sun et al. 2004; Li et al. 2007). In the brain....

Consistent with the observations by Garcia and Kim (1997) in glioma cells, astrocytes are among the cells that express cPLA\textsubscript{2} (Sun et al. 2004; Li et al. 2007). In the brain in vivo, the majority of cPLA\textsubscript{2} expression in gray matter may be in astrocytes (Lautens et al. 1998; Balboa and Balsinde 2002; Sun et al. 2004), although a predominantly neuronal expression was reported by Ong et al. (1999) and Pardue et al. (2003). Astrocytes display receptor subtypes for a large number of transmitters (Hansson and Rönnbäck 2004; Fiacco and McCarthy 2006), including serotonin (Hertz et al. 1979). They express 5-HT\textsubscript{2} receptors (Hansson et al. 1987; Nilsson et al. 1991; Chen et al. 1995; Poblete and Azmitia 1995), including the more recently discovered 5-HT\textsubscript{2B} receptor (Sandén et al. 2000; Kong et al. 2002). This receptor subtype is of special interest in the present context because it is activated in astrocytes by low micromolar concentrations of fluoxetine (Kong et al. 2002), which selectively stimulates this subtype of the 5-HT\textsubscript{2} receptor, but not 5-HT\textsubscript{2A} or 5-HT\textsubscript{2C} receptors (Li et al. 2008b). It is, therefore, likely that the activation of cPLA\textsubscript{2} observed in the brain in vivo may at least partly be an astrocytic phenomenon.

In the present study, we have tested the potential role of astrocytes in the upregulation of cPLA\textsubscript{2} by investigating mRNA and protein expression of cPLA\textsubscript{2a}, the major isoform of cPLA\textsubscript{2} in astrocytes (Li et al. 2007), in mouse astrocytes in primary cultures during chronic incubation with 1 or 10 µM fluoxetine. Consistent with the results by Rao et al. (2006), cPLA\textsubscript{2a} was found to be upregulated, whereas the two other forms of PLA\textsubscript{2}, sPLA\textsubscript{2} and iPLA\textsubscript{2}, were unaffected. Since these cultures express no serotonin transporter (Kong et al. 2002; Li and Peng, unpublished experiments) and any 5-HT present in the serum added to the culturing medium is rapidly metabolized by the high monoxygenase activity in the cells (Yu and Hertz 1982, 1983), the upregulation cannot be caused by inhibition of 5-HT uptake, but must be a direct effect of fluoxetine, i.e., of stimulation of 5-HT\textsubscript{2B} receptors. However, in the brain in vivo, elevated serotonin during treatment with an SSRI would have a similar effect because serotonin potently (a statistically significant stimulation at 0.1 nM) stimulates 5-HT\textsubscript{2B} receptors in astrocytes (Li and Peng, unpublished results).

An important effect of fluoxetine acting on rodent astrocytes in primary cultures is phosphorylation (activation) of extracellular-regulated kinase 1 and 2 (ERK\textsubscript{1/2}) (Mercier et al. 2004), and phosphorylation of ERK\textsubscript{2} has also been reported in the rat brain concomitant with alleviation of the depressive-like behavior in rats exposed to chronic forced swim stress (Qi et al. 2008). In cultured astrocytes, ERK\textsubscript{1/2} phosphorylation in response to stimulation of G\textsubscript{i/o} or G\textsubscript{q} protein-linked receptors, such as the 5-HT\textsubscript{2B} receptor and the α\textsubscript{2}-adrenergic receptor, is secondary to metalloproteinase-mediated release of an agonist of the epidermal growth factor (EGF) receptor, perhaps mainly heparin-binding EGF (HB-EGF), and resulting activation of the receptor tyrosine kinase of the EGF receptor in a transactivation process (Li et al. 2008a,b). The transactivation is inhibited by GM6001, a metalloproteinase inhibitor (Levy et al. 1998), and by AG1478, an inhibitor of the receptor tyrosine kinase of the EGF receptor (Levitzki and Gazit 1995). For these reasons, the effect of the inclusion of either of these compounds or of the 5-HT\textsubscript{2B} antagonist SB 204741 (Jerman et al. 2001) in the medium during chronic treatment with fluoxetine was studied. In order to examine if the induced ERK\textsubscript{1/2} phosphorylation is required for the upregulation of cPLA\textsubscript{2}, the effect of an inhibitor of ERK phosphorylation, U0126 (Favata et al. 1998), was also examined.

**Methods**

**Materials**

Most chemicals, including fluoxetine and SB 204741 (N-(1-methyl-5-indolyl)-N',3-(methyl-5-isothiazolyl) urea), as well as the first antibody raised against β-actin (42 kDa) were purchased from Sigma (St. Louis, MO, USA). Tyrphostin AG1478 (4-(3-chloroanilino)-6,7-dimethoxyquinazoline), GM6001 (N-[(2R)-2-(hydroximocarbonyl-methyl)-4-methylpentanoyl]-1-tryptophan methylamide), and U0126 (1,4-diamo-no-2,3-dicyano-1,4-bis[2-aminophenyl-ethyl]butadiene) were obtained from Calbiochem, La Jolla, CA, USA. Santa Cruz Biotechnology (Santa Cruz, CA, USA) provided the first antibody raised against β-actin (42 kDa) and the secondary antibody goat antimouse IgG horse radish peroxidase conjugate. ECL Western blotting detection reagents were from Amersham Biosciences, Buckinghamshire, UK.

**Cultures**

Primary cultures of astrocytes were prepared as previously described (Hertz et al. 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 culturing medium was 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 CO₂/air (5%:95%).

Fig. 1 mRNA expression measured by RT-PCR of cPLA₂α in primary cultures of mouse astrocytes chronically treated with fluoxetine. a Cells were treated with 1 and 10 µM fluoxetine for 2 weeks. a1 A representative experiment showing mRNAs for cPLA₂α in the upper row and for TBP, as a housekeeping gene, in the lower row in control cultures and the corresponding results in fluoxetine-treated cultures after 1 day, 3 days, 1, 2, 3, or 4 weeks of treatment. a2 All results are presented as the means ± SEM of scanned ratios between cPLA₂α and TBP (four samples from four different batches of cultures) in control cultures and cultures treated with fluoxetine. *p<0.05 vs. control cultures and cultures treated with 10 µM fluoxetine from the same batch and treatment period; **p<0.05 vs. control cultures from the same batch and treatment period.

b Concentration–response curve for chronic effects of fluoxetine on mRNA expression measured by RT-PCR of cPLA₂α in primary cultures of mouse astrocytes. b1 A representative experiment showing mRNAs for cPLA₂α in the upper row and for TBP, as a housekeeping gene, in the lower row in control cultures and the corresponding results in cultures treated with 1, 3, 10, 30, or 100 µM fluoxetine after 1 week (100 µM), 2 weeks (30 and 10 µM), or 3 weeks (3 and 1 µM) of treatment. b2 All results are presented as the means ± SEM of scanned ratios between cPLA₂α and TBP (three samples [drug-treated cultures] or 15 samples [control] from three different batches of cultures) in cultures treated with fluoxetine minus the corresponding values in control cultures from the same batch and treatment period, i.e., the graph shows the stimulation, which is statistically significant at all fluoxetine concentrations.
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 was included in the medium. Such cultures have been used in our laboratories for more than 30 years, and they are highly enriched in astrocytes (>95% purity of glial fibrillary protein-expressing and glutamine synthetase-expressing astrocytes; Hertz et al. 1985). Addition of dBcAMP leads to a morphological and functional differentiation (Mercier et al. 2004; Hertz et al. 1985; Zhao et al. 1996). For chronic experiments fluoxetine was added to the culture medium after 2 weeks of culturing (i.e., at the time the cultures had reached confluency) and it was present during continued culturing for another 1, 2, 3, or 4 weeks. Since the medium was completely exchanged when the cells were fed fresh medium, there will be no build-up of fluoxetine metabolite(s) except between each feeding and at the expense at the added concentration of fluoxetine.

mRNA expression

For the determination of mRNA expression of different subtypes of PLA_2 by reverse transcription polymerase chain reaction (RT-PCR), 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 the 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 (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 dithiothreitol, 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 0.375 U of Taq polymerase (TaKaRa Biotechnology, Dalian, China)
and 0.2 µM of sense or antisense for cPLA2a (forward, GCACATTATAGTGGAACACC; reverse, ACACAGTGC CATGCTGAACC) (Ivanov et al. 2002); iPLA2 (forward, ACCTAGCCGTGGAGCTTGG; reverse, GCCCTTGG TTGTATACCTGG) (Lindbom et al. 2001); sPLA2 (forward, AGCTGACACATGAAAGGTCTTCC; reverse, TTCTGGGTGAAGACAGAAGGGCC; Kennedy et al. 1995); and TATA box-binding protein (TBP), used as a housekeeping gene (forward, CCACGGACAACTGCGTT GAT; reverse, GGCTCATAGCTACTGAACTG; El-Marjou et al. 2000). The amount of cDNA used was 5 µg (Li et al. 2007). Thirty cycles were used for iPLA2 and sPLA2, and 40 cycles were used for cPLA2a 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 cPLA2a and iPLA2 and 55°C for sPLA2 and TBP, and the third at 72°C. The final step was

Fig. 3 mRNA expression measured by RT-PCR of sPLA2 or iPLA2 in primary cultures of mouse astrocytes chronically treated with 1 and 10 µM fluoxetine. A representative experiment showing mRNAs for iPLA2 in the upper row, for sPLA2 in the middle row, and for TBP, as a housekeeping gene, in the lower row in control cultures and the corresponding results in fluoxetine-treated cultures after 1 day, 3 days, 1, 2, 3, or 4 weeks of treatment. All results are presented as the means ± SEM of scanned ratios between iPLA2 and TBP (2) or sPLA2 and TBP (3; four samples from four different batches of cultures) in control cultures and cultures treated with fluoxetine.
extension at 72°C for 10 min. The PCR products were separated by 1% agarose gel electrophoresis, and captured by Flurchem 5500 (Alpha Innotech, San Leandro, CA, USA). Ratios between PLA$_2$ mRNA and TBP were determined and averaged.

**Protein expression**

For the determination of protein expression of cPLA$_2$, the cells were washed with ice-cold phosphate-buffered saline containing 7.5 mM glucose, scraped off the dishes, and
An artifact has occurred; the text is not legible.
upon ERK1/2 phosphorylation, and Mercier et al. (2004) showed fluoxetine-mediated upregulation of cPLA₂ activity and expression in intact brain by demonstrating similar cPLA₂ upregulation by fluoxetine in astrocytes, a brain cell type that constitutes about 20% in gray matter (Hertz 2008) and may be the site of most of its cPLA₂ expression (Lautens et al. 1998; Balboa and Balsinde 2002; Sun et al. 2004). They provide no information whether neurons may respond in a similar manner, but attempts to treat neurons chronically with fluoxetine failed, because the neuronal cultures did not tolerate the treatment well (Li and Peng, unpublished experiments).

The present results also extend the findings by Rapoport and coworkers by showing (1) that the increased cPLA₂ expression is contingent upon activation of the 5-HT₂B receptor; (2) that it depends upon metalloproteinase-mediated shedding of an EGF receptor ligand and subsequent stimulation of the receptor tyrosine kinase of this receptor; and (3) that EGF receptor-mediated ERK₁/2 phosphorylation is indispensable. The linkage between fluoxetine-mediated ERK₁/2 phosphorylation and cPLA₂ upregulation in the cultured astrocytes is not known. On one hand, Rao et al. (2006) concluded that several transcription factors known to regulate cPLA₂ gene expression were not significantly changed by chronic fluoxetine administration in the brain in vivo. However, nuclear AU-rich element/poly(U)-binding/degradation factor-1 RNA-stabilizing protein was significantly increased, suggesting that chronic fluoxetine increases brain cPLA₂ gene expression post-transcriptionally by increasing stabilization of cPLA₂ mRNA. A mainly post-transcriptional regulation of cPLA₂ mRNA has also been reported in rat mesangial kidney cells (Tay et al. 1994). On the other hand, expression of other genes like cfos and fosB (Li et al. 2008b) is upregulated by fluoxetine in cultured astrocytes chronically treated with fluoxetine, contingent upon ERK₁/2 phosphorylation, and Mercier et al. (2004) showed that fluoxetine caused a rapid upregulation of glial-derived nerve factor, which was inhibited by U0126. Moreover, chronic treatment with imipramine, which also increases cPLA₂ gene expression in the brain, does increase activator protein-2α (Lee et al. 2008).

The two concentrations of fluoxetine (1 and 10 µM) used in this study are slightly higher and lower, respectively, than the $K_ι$ values found for fluoxetine and its primary metabolite, norfluoxetine, at the human 5-HT₂B receptor by Rothman et al. (2000), although other authors found lower affinity of this receptor. The lower concentration is comparable to a combined concentration of fluoxetine and norfluoxetine in plasma of fluoxetine-treated individuals of 1–2 µM (Bolo et al. 2000; Henry et al. 2005; Ferguson and Hill 2006). However, both compounds are heavily protein-bound, and in the cerebrospinal fluid, the concentration is at least ten times lower (Martensson et al. 1989; Liu et al. 2005). In the present experiments in which 10% horse serum was present in the medium during the chronic treatment, the protein binding amounted to 36±2.0% at 10 µM fluoxetine (Zhang et al., unpublished experiment) and it might be higher at a lower concentration. Thus, at the EC₅₀ concentration for the chronic effect of fluoxetine, its free concentration (after 3 weeks of treatment at most 64% of 0.5 µM) is virtually similar to the plasma concentration in patients treated with therapeutic doses of fluoxetine. However, the acute effect of fluoxetine is considerably less potent, since an EC₅₀ of ~5 µM can be deduced from the data presented by Li et al. (2008b). Further supporting the concept that chronically administered fluoxetine and related drugs at therapeutically relevant concentrations may activate 5-HT₂B receptors is the observation that virtually identical affinities for 5-HT₂B receptors have been found during acute and chronic treatment with paroxetine (Li and Peng, unpublished experiments), another antidepressant supposed to act exclusively by inhibition of the neuronal serotonin transporter. One reason for the increased potency of fluoxetine (and paroxetine) during chronic treatment is probably that the 5-HT₂B receptor in astrocytes upregulates during chronic treatment with fluoxetine (Li and Peng, unpublished experiments).

The potency of SB 204741 as an inhibitor is also lower acutely than chronically, since the IC₅₀ during acute exposure can be estimated to be ~200 nM (Li et al. 2008b). Assuming normal receptor binding kinetics, permitting the use of the equation $K_i=IC_{50}(1+L/K)$ where $L$ is the concentration of fluoxetine at which SB 204741 was tested (10 µM) and $K$ is the EC₅₀ for fluoxetine (chosen as 1.0 µM during chronic exposure as a compromise between the value after 1 and 3 weeks of treatment), the $K_i$ for SB 204741 can accordingly be determined to be ~4.5 nM during chronic treatment with fluoxetine and ~67 nM during acute exposure (when EC₅₀ was ~5.0 µM and IC₅₀ 200 nM). The latter value is in reasonably close agreement with a value of 200 nM determined by Jerman et al. (2001) for human 5-HT₂B receptors expressed in SH-SY5Y cells.

5-HT₂B receptors are widespread in the human (Kursar et al. 1994; Baez et al. 1995; Bonhaus et al. 1995; Schmuck et al. 1996) and rodent (Choi and Maroteaux 1996; Bonaventure et al. 2002) central nervous system, but their function is mainly unknown. A very interesting function is
its requirement for hyperlocomotion and 5-HT release induced by the "club drug" 3,4-methylenedioxymethamphetamine (MDMA), but this is supposed to be a presynaptic effect probably as an agonist of 5-HT 2B receptors (Setola et al. 2003), caused by reversal of the serotonin transporter and release of serotonin from serotonergic nerve terminals (Doly et al. 2008). For this reason, the suggestion was made that 5-HT2B antagonists might be useful in preventing MDMA use and toxicity. The use of 5-HT2B antagonists would, however, also prevent serotonin-mediated and fluoxetine-mediated cPLA2 upregulation. It, therefore, becomes important to look into potential consequences of cPLA2 upregulation. Release of arachidonic acid from astrocytes is stimulated by adenosine triphosphate (Strokin et al. 2003), a "gliotransmitter" (Haydon and Carmignoto 2006), and may exert both autocrine and paracrine effects. In primary cultures of astrocytes, exposure to arachidonic acid strongly stimulates both glucose metabolism (Yu et al. 1993) and glycogenolysis (Sorg et al. 1995), and astrocytes account for ~30% of glucose metabolism in the brain (reviewed by Hertz 2008). Stimulation of glucose metabolism by arachidonic acid may be important in the pathophysiology of depressive illness and its pharmacological treatment. Glucose metabolism in the brain is reduced in many regions, primarily in the frontotemporal parts, in patients suffering from unipolar depression (Little et al. 1996, 2005; Videbech 2000; Rasgon et al. 2008), with a correlation between the degree of hypometabolism and severity of the illness (Kimbrell et al. 2002) and normalization following treatment with an SSRI (Buchsbaum et al. 1997; Mayberg et al. 2000; Meyer et al. 2001; New et al. 2004). That arachidonic acid may play a role in determining cerebral glucose metabolism in vivo can be seen from the observation of a rectilinear correlation between rate of cerebral glucose utilization in many of the same regions affected by depression and plasma concentration of arachidonic acid in depressed patients (Elizabeth Sublette et al. 2009). Moreover, remission from major depression during treatment with a monoamine uptake inhibitor may be associated with a

![Graph showing upregulation of cPLA2 protein expression by chronic treatment with fluoxetine](image)
decreased glucose metabolism in the midbrain prior to the onset of treatment (Milak et al. 2009). Accordingly, fluoxetine effects on astrocytes may well have a direct bearing on the pathophysiology of major depression.

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