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Mobilization of arachidonate and docosahexaenoate by stimulation of the 5-HT_{2A} receptor in rat C6 glioma cells

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Abstract

In this study, we demonstrate that astroglial 5-HT_{2A} receptors are linked to the mobilization of polyunsaturated fatty acids (PUFA). Stimulation of C6 glioma cells, prelabeled with [^{3}H]arachidonate (AA, 20:4n6) and [^{14}C]docosahexaenoate (DHA, 22:6n3), with serotonin and the 5-$HT_{2A/2C}$ receptor agonist (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) resulted in the mobilization of both [^{3}H] and [^{14}C] into the supernatant of the cell monolayers. The increased radioactivity in the supernatant was mainly associated with free fatty acids. Experiments using inhibitors of phosphoinositide-specific phospholipase C and PLA_2, inhibited the DOI-stimulated mobilization of AA and DHA, suggesting the involvement of both phospholipases. Ketanserin (1 μM), a 5-$HT_{2A/2C}$ receptor antagonist, and MDL 100,907 (*R*(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol) (1 μM), a highly selective antagonist for 5-HT_{2A} receptors, significantly decreased the DOI-stimulated release of AA and DHA. These results indicate that the 5-HT_{2A} receptor is coupled to the mobilization of PUFA. The release of AA and DHA in response to serotonin may represent a mechanism through which astroglia provide these polyunsaturated fatty acids to neurons. © 1997 Elsevier Science B.V.

Keywords: Arachidonate; Docosahexaenoate; Serotonin-2 receptor; Phospholipase A_2; Phospholipase C; C6 glioma cell

1. Introduction

Serotonergic neurons are believed to play a role in depression, anxiety and aggression [23]. They send collaterals to different areas of the brain and therefore control the regulation of other neurotransmitters and hormones. Physiological responses to serotonin (5-hydroxytryptamine; 5-HT) are mediated by receptors that in binding studies have been classified into four main classes: 5-HT_1, 5-HT_2, 5-HT_3 and 5-HT_4 with certain recombinant (e.g. 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$) and orphan receptors (5-HT_7) [19,23]. The signal from the activation of these receptors is transduced by different effectors. For example, 5-HT_{1A} receptors are linked to adenylate cyclase activity while stimulation of 5-HT_{2A} and 5-HT_{2C} (formerly 5-HT_{1C}) receptors increases phosphoinositide (PI) turnover. Hydrolysis of phosphoinositides by stimulation of 5-HT receptors was initially observed in smooth muscle [10,11] and later in rat brain [8]. It is well established that 5-HT_3 receptor is coupled to a cation-selective channel by a G-protein independent process [19].

Astroglia, accounting for approximately 20% or more of the total cell volume in the cerebral cortex display receptors for serotonin, which once activated lead to the mobilization of calcium and the generation of second messengers such as IP_3 and cAMP [14]. In astrocytes it has been shown that arachidonic acid (AA, 20:4n6) is released from membrane phospholipids by phospholipase A_2 (PLA_2) [5,27], although its accumulation may also involve phospholipase C hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP_2) [29]. It has been also reported that 20:4n6 is mainly metabolized by lipoxygenases in cultured astrocytes [15] and there is evidence that arachidonate metabolism may be an early indicator for the secretion of nerve growth factor by astroglial cells [5]. Although serotonin and agonists for the 5-HT_2 receptor induce phosphoinositide hydrolysis [1,3,12] and increases in intracellular

Abbreviations: PLA_2, phospholipase A_2; AA, arachidonic acid; DHA, docosahexaenoic acid; DOI, (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride; MDL 100,907, *R*(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol; $AACOCF_3$, arachidonyl trifluoromethyl ketone; DMEM, Dulbecco's modified Eagle's medium

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calcium [12,18,28], the involvement of serotonin (5-HT) on the mobilization of 20:4n6 in astroglia has not been demonstrated. In addition to arachidonic acid, mammalian brain is highly enriched with docosahexaenoic acid (DHA, 22:6n3) [22]. Although 22:6n3 is considered to be essential for the proper development of brain and retina [22,2], little is known about its mobilization. Astroglia which are in close contact with neurons, may play an important role in providing this fatty acid to neuronal cells.

In this study we investigated the mobilization of both 20:4n6 and 22:6n3 in relation to the activation of 5-HT receptors using a rat glial cell line that retains properties of both astrocytes and oligodendroglia [30] and also express abundant serotonergic type 2A (5-HT_{2A}) receptor [12,18]. Here, we provide evidence for the first time that the stimulation of 5-HT_{2A} receptors is also linked to the mobilization of both 20:4n6 and 22:6n3 fatty acids in astroglia.

2. Materials and methods

2.1. Materials

[^{3}H]Arachidonic (100 Ci/mmol) and [1-^{14}C]docosahexaenoic acids (50 mCi/mmol) were obtained from New England Nuclear (Wilmington, DE). Dulbecco modified Eagle's medium (DMEM), fetal bovine serum and other tissue culture reagents were purchased from Gibco Life Technologies (Grand Island, NY). Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL). (±)-2,5-Dimethoxy-4-iodoamphetamine hydrochloride (±-DOI hydrochloride) and ketanserin tartrate were purchased from Research Biochemicals Incorporated (Natick, MA). Serotonin and mepacrine were obtained from Sigma Chemical Co. (St. Louis, MO). MDL 100,907 (*R*(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol) was a generous gift from Dr. Eckelman at NIH. Silica gel plates 60 were obtained from Analtech (Newark, DE). Neomycin was obtained from Serva (Heidelberg, Germany). Arachidonoyl trifluoromethyl ketone ($AACOCF_3$) was obtained from Biomol (Plymouth, PA). The receptor agonists and inhibitors were prepared in phosphate buffered saline solution (PBS).

2.2. Cell culture conditions

The rat C6 glioma cell line was obtained from the American Type Cell Culture (ATCC CCL-107) and maintained in a culture medium consisting of DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were grown in 20 ml of this culture medium in 75-cm^2 Corning culture flasks in a humidified environment of 5% CO_2, 95% air at 37°C. The medium was changed every 3–4 days.

2.3. [^{3}H]AA and [^{14}C]DHA labeling of C6 glioma cells

For each experiment the cells were subcultured in 6-well (35 mm diameter) plates (Costar) in a initial density of 3.9×10^5 cells/well and incubated under the culture conditions described above. The incubation medium was then removed and the cells were further incubated in the medium containing 0.5% fetal bovine serum and 0.5 μCi each of [^{3}H]AA and [^{14}C]DHA for another 24 h. The final concentration of each fatty acid was adjusted to 10 μM using unlabeled fatty acids. Fatty acids were added in a small volume of ethanol (final concentration $< 0.2\%$). Vitamin E (40 μM) was also added as an antioxidant [34]. Alternatively, the cell monolayers were separately labeled with either [^{3}H]AA or [^{14}C]DHA in order to compare the results with those from double labeling studies.

2.4. Agonist-stimulated release of fatty acids

After incubation with radiolabeled fatty acids, the culture medium was removed and the cells were rinsed with 1 ml of DMEM containing 0.2% bovine serum albumin and 20 mM HEPES, pH 7.3, in order to remove unincorporated label. Before adding the agonists, the cells were equilibrated for 15 min in 1 ml of this latter medium at 37°C. Cells were then stimulated with the indicated concentrations of serotonin (5-HT) or DOI for 10 min at 37°C. When the effect of antagonists or inhibitors was studied, they were added 15 min prior to stimulation. The incubation was terminated by removing the supernatant medium and placing it on ice. After centrifugation to remove unattached cells, aliquots were counted by liquid scintillation. Results were obtained using either disintegrations per minute (dpm) or percentage calculated against total cellular radioactivity.

2.5. Lipid analysis

Lipids from the cells and the supernatant were extracted separately by the method of Bligh and Dyer [4]. The lipid extracts were resuspended in a small volume of chloroform and applied to TLC plates. The lipids present in the supernatant were resolved by a solvent system, consisting of hexane/ether/formic acid (90:60:4). Cell lipids were separated by a modification of the two-step thin layer chromatography method of Smith and Waite [25]. The plates were chromatographed halfway up in a phospholipid system containing chloroform/methanol/glacial acetic acid/water (100:75:7:4), taken out and dried briefly under a stream of nitrogen. The plates were then chromatographed again completely in a solvent system containing hexane/diethylether/formic acid (90:60:4). Lipid classes were identified by comparison with standards visualized by exposure to iodine. Radioactivity for each of the selected regions was determined by liquid scintillation.

2.6. Statistical analysis

Statistical analysis was performed using the Student's *t*-test.

3. Results

In order to examine the release, the cells were first labeled with [^{14}C]DHA and [^{3}H]AA. Fig. 1 shows the time course of incorporation of radioactivity into C6 glioma cells incubated with 0.5 μCi (10 μM) each of both [^{3}H]AA and [^{14}C]DHA from 5 to 72 h. Fig. 1A shows that [^{14}C]DHA appeared to incorporate into C6 glioma slightly more than [^{3}H]AA. Both PUFA were readily incorporated into the lipids of glioma cells after 24 h, and the incorporation did not increase thereafter, with approximately 90–95% of the incorporated radioactivity found in phospholipids. The time course of the radioactivity distribution among phospholipids is shown in Fig. 1B and C. After 5 h of incubation, 41% of the total cell radioactivity from [^{3}H]AA was in choline phospholipids, which then decreased progressively to 31% and 19% after 10 and 24 h, respectively (Fig. 1B). Concomitantly, [^{3}H] label in ethanolamine phospholipids increased from 15% to 21% and then 26% after 5, 10 and 24 h of incubation, respectively. [^{3}H]AA incorporated into inositol phospholipids reached the maximum at 10 h, and then the radioactivity decreased. Serine phospholipids steadily increased and reached 25% after 48 h of incubation. The time course of [^{14}C]DHA incorporation in phospholipids classes (Fig. 1C) was different from that of [^{3}H]AA. After 5 h [^{14}C]DHA was incorporated in choline and ethanolamine phospholipids (31% and 22.2%, respectively). After 24 h the radioactivity in choline phospholipids decreased with a concomitant increase of the label in serine phospholipids reaching 39.6% of total cell radioactivity. The radioactivity in serine phospholipid continued to increase to 43.7% after 72 h. A small portion of [^{3}H]AA and [^{14}C]DHA was esterified in neutral lipids (4–10%) after 24 h. Since the level of radioactivity stabilized in most phospholipids pools after 24 h, we used the cells labeled at least 24 h for subsequent experiments.

Fig. 2 shows that serotonin at 10^{-7} and 10^{-6} M was able to stimulate the mobilization of radioactivity into the supernatant. This concentration range is close to that previously reported to stimulate the low affinity 5-HT receptor [18,19]. Serotonin at 1 μM concentration significantly promoted the accumulation of the [^{3}H] and [^{14}C] in the supernatant by 27% and 14%, respectively. Experiments using cell monolayers labeled separately with either [^{3}H]AA or [^{14}C]DHA rendered similar results (data not shown). Since C6 glioma cells abundantly express serotonin type-2A receptors [12,18], we investigated the involvement of this receptor subtype in the mobilization of AA and DHA using DOI, a well known 5-HT$_{2A/2C}$ receptor agonist. DOI at concentrations as low as 10^{-8} M increased the accumulation of [^{3}H] and [^{14}C] radioactivity by more than 50% above the basal (Fig. 2). The higher response to DOI than to 5-HT may be due to the fact that serotonin stimulates multiple receptor subtypes which can exert the opposite effect on fatty acid release while DOI targets 5-HT$_{2A/2C}$ receptors.

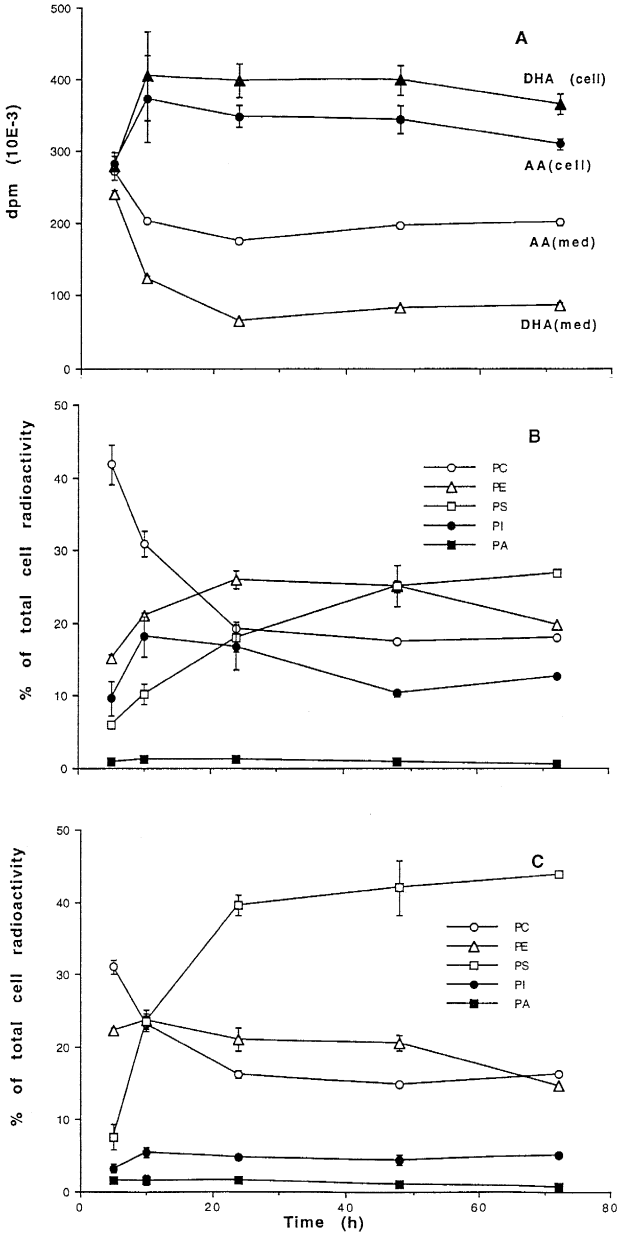

Fig. 1. A: incorporation of [^{3}H]AA and [^{14}C]DHA into C6 glioma cells. Cells were incubated with [^{3}H]AA (circle) and [^{14}C]DHA (triangle) (0.5 μCi each, 10 μM) in media containing 0.5% FBS for the indicated times; total cell (closed symbols) and medium radioactivity (open symbols). B, C: incorporation of [^{3}H]AA (B) and [^{14}C]DHA (C) into major phospholipids classes; phosphatidylcholine (open circle), phosphatidylethanolamine (open triangle), phosphatidylserine (open square), phosphatidylinositol (closed circle), phosphatidic acid (closed square). Values are shown as the percentage of total radioactivity incorporated into cells and are the mean of three determinations from a representative experiment.

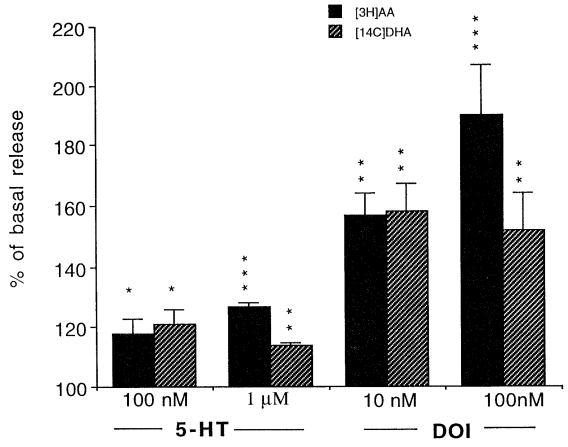

Fig. 2. Effect of 5-HT and 5-HT_2 receptor agonist and on the release of [^{3}H] and ^{14}C from C6 glioma cells. Confluent cultures of C6 glioma cells prelabeled with [^{3}H]AA (closed bars) and [^{14}C]DHA (hatched bars) (10 μM, 0.5 μCi/well, 24 h) were washed and incubated in the presence of the indicated concentrations of serotonin (5-HT) or DOI for 10 min. At the end, an aliquot of the incubation medium was counted for radioactivity. Data are expressed as the percentage calculated against the basal (unstimulated) release and are the mean ± S.D. of triplicate cultures from a representative experiment. The experiment was repeated three times and produced similar results. Statistically significant increases in comparison to the control group by Student's *t*-test are indicated: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

The lipid composition of the supernatants was subsequently characterized. As shown in Table 1, the radioactivity from [^{3}H]AA in the supernatant was found in both diacylglycerol (DG) and fatty acids (FA) fractions in basal and DOI-stimulated cells. Unlike AA, the radioactivity from [^{14}C]DHA found in the supernatant was mostly associated with free fatty acids under basal as well as DOI-stimulated conditions. In both cases, the observed increase in radioactivity by DOI was due to the accumulation of fatty acids, suggesting a role of phospholipase A_2. HPLC analysis with a radioactivity detector confirmed that radioactivity in the free fatty acid fraction from the supernatant of stimulated cells was mostly recovered (> 90%) in a peak having the same retention time as authentic [^{3}H]AA and [^{14}C]DHA. When these cells were pretreated with mepacrine (100 μM) and arachidonyl trifluoromethyl ketone (AACOCF3, 50 μM), inhibitors of PLA_2 [21,29], the DOI-stimulated mobilization of polyunsaturated fatty acids was abolished (Table 2), indicating that PLA_2 is indeed involved in their release. Neomycin has been shown to bind phosphoinositides, thereby making them inaccessible to PLC [20]. Pretreatment of C6 glioma cells with neomycin (100 μM) also inhibited accumulation of free arachidonate and docosahexaenoate by DOI (Table 2).

Table 1
DOI-stimulated release of DG and FFA from [^{3}H]AA- and [^{14}C]DHA-prelabeled C_6 glioma cells

	[^{3}H]	[^{14}C]
DG		
Control	0.27 [b] ± 0.06	0.02 ± 0.01
DOI [a] (100 nM)	0.23 ± 0.01	0.03 ± 0.01
FA		
Control	0.26 ± 0.01	0.49 ± 0.06
DOI (100 nM)	0.42 ± 0.01 **	0.71 ± 0.01 **

C6 glioma cells were prelabeled with 0.5 μCi of both [^{3}H]AA and [^{14}C]DHA for 24 h and were stimulated as indicated in Section 2. The radioactivity released to the medium was analyzed by thin layer chromatography.

[a] DOI, (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride, $5HT_{2A/2C}$ receptor agonist.

[b] Values represent mean ± S.D. of percentage calculated against the total cellular radioactivity. Total cell radioactivity was 167,297 dpm for [^{3}H]AA and 408,700 dpm [^{14}C]DHA. The experiment has been repeated three times with similar results.

** $P < 0.01$, statistical significance of stimulation by DOI tested using Student's *t*-test.

Table 2
Effects of PLA_2 inhibitors on DOI-stimulated release of [^{3}H]AA- and [^{14}C]DHA-labeled C_6 glioma cells

Treatment	% of basal	
	[^{3}H]AA	[^{14}C]DHA
DOI (100 nM)	185 ± 17 ***	193 ± 16 ***
DOI + $AACOCF_3$ (50 μM)	90 ± 13	88 ± 14
DOI + mepacrine (100 μM)	94 ± 10	84 ± 3
DOI + neomycin (100 μM)	97 ± 2	95 ± 9

C_6 glioma cells were prelabeled for 24 h with both [^{3}H]AA and [^{14}C]DHA. Cells were then washed and preincubated in the presence or absence of inhibitors before the addition of DOI. The results are the mean ± S.D. of triplicate wells from a representative experiment which has been repeated three times with similar results.

*** $P < 0.001$, data compared to the basal (unstimulated) values of each treatment using Student's *t*-test.

Table 3
Effect of $5\text{-}HT_{2A/2C}$ receptor agonists and antagonists on the release of [^{3}H]AA and [^{14}C]DHA from C6 glioma cells

	% of basal	
	[^{3}H]AA	[^{14}C]DHA
Basal	100	100
DOI (100 nM)	190 ± 17 [a] ***	152 ± 12 [a] **
Ketanserin (1 μM)	112 ± 23	107 ± 11
Ketanserin + DOI (1 μM + 100 nM)	128 ± 10 [b] **	105 ± 6 [b] ***
MDL 100,907 (1 μM)	88 ± 12	102 ± 10
MDL 100,907 + DOI (1 μM + 100 nM)	87 ± 11 [b] ***	82 ± 12 [b] ***

C6 glioma cells were prelabeled with 0.5 μCi each of both [^{3}H]AA and [^{14}C]DHA for 24 h, washed and then incubated at 37°C in the absence or presence of the agonist DOI, for 10 min. The antagonists ketanserin or MDL 100,907 were added 15 min before the addition of DOI.

The radioactivity released to the medium was counted by liquid scintillation. The results are the mean ± S.D. of triplicate wells from a representative experiment which has been repeated three times with similar results. Basal release was 5506 ± 682 dpm for [^{3}H]AA and 17,776 ± 1749 dpm for [^{14}C]DHA.

[a] Data were compared to the basal values using Student's *t*-test: ** $P < 0.01$, *** $P < 0.001$.

[b] Data were compared to the DOI-stimulated release using Student's *t*-test: ** $P < 0.01$, *** $P < 0.001$.

The confirmation that the mobilization of polyunsaturated fatty acids by DOI is mediated by the type-2 receptors was obtained using 5-$HT_{2A/2C}$ receptor antagonists. Table 3 shows the effect of antagonists on the release of [^{3}H]AA and [^{14}C]DHA stimulated by DOI. Ketanserin (1 μM), which preferentially blocks 5-HT_{2A} receptors but also shows affinity to 5-HT_{2C} receptors [24], significantly inhibited the DOI-stimulated released of [^{3}H]AA and [^{14}C]DHA. MDL 100,907, a specific 5-HT_{2A} receptor antagonist, abolished the DOI-stimulated release of both fatty acids. These results further support the involvement of the 5-HT_{2A} receptor subtype in this mobilization process.

4. Discussion

The expression of serotonin receptors has been demonstrated in C6 glioma cells [18,31] and in astrocytes [14,33], but their role in the release of arachidonic acid has not been well characterized. In many cell types, the release of arachidonic acid occurs secondary to receptor-mediated activation of PLC leading to the formation of inositol phosphates that raise intracellular Ca^{2+}. Receptors for 5-HT have been reported to affect intracellular Ca^{2+} mobilization [28] and phosphoinositide hydrolysis [9,11]. In C6 glioma cells, both responses have been shown to be coupled to the 5-HT_{2A}-receptors [1,12,18]. Nevertheless, it has not been demonstrated that polyunsaturated fatty acids can be released upon stimulation of astroglial 5-HT receptors. This report is the first documenting that serotonin, particularly 5-HT_{2A} receptors, can mediate the release of both arachidonic and docosahexaenoic acid in cells of astroglial origin.

5-HT_2 receptors on astroglial cells are more abundant in the brain stem and cortex than in the hippocampus and striatum [14]. It has been previously reported that 5-HT_2 receptor agonists stimulate the release of arachidonate from hippocampal neurons, but not from hippocampal glial cells [13]. In a separate report, DOI-induced activation of PLA_2 was absent in rat brain cerebellar granule cells [6]. Our results were obtained from a cell line presenting homogeneous populations of astroglia which express abundant 5-HT_{2A} receptors [12,31]. As we have demonstrated here, lower concentrations of the 5-$HT_{2A/2C}$ receptor agonist, DOI, can mobilize both arachidonate and docosahexaenoate from C6 glioma cells.

In several cellular models, receptor–agonist interactions results in activation of PLA_2 directly [20] or by the elevation of the cytoplasmic Ca^{2+} concentration after phosphoinositides metabolism by PLC [7]. The fact that the radioactivity released resides mostly in fatty acids as well as the complete inhibition by PLA_2 and PI-PLC inhibitors strongly suggests that mobilization of AA and DHA by serotonergic agonist DOI is most probably due to activation of PI-PLC and PLA_2 pathways. In fact, we have obtained preliminary results based on immunodetection which indicate the presence of cytosolic PLA_2 in C6 glioma cells (data not shown).

Free arachidonic acid (AA) and other polyunsaturated fatty acids are toxic at high concentrations which may be attainable by stimulation of 5-HT_{2A} receptors. However, we have observed during the course of enrichment with polyunsaturates that up to 50 μM free fatty acids were not toxic for C6 glioma cells in the presence of Vitamin E (40 μM). Under normal physiological conditions, the level of free fatty acids are tightly controlled by the deacylation–reacylation cycle and/or further metabolism. In C6 glioma cells, conversion of arachidonic acid to oxygenated metabolites has been observed [15]. These metabolites may be involved in the further signaling pathways upon stimulation of serotonin receptors, although in some cases the observed toxicity of free arachidonic acid may be attributed to this conversion.

Docosahexaenoic acid, an essential fatty acid for normal development of the brain, is also released upon stimulation of serotonin type 2A receptors. Interestingly, it has been suggested that 5-HT may be involved in the process of differentiation of astroglia [17]. Serotonin receptors in astroglia have also been linked to the production of a factor which regulates the development of serotonergic neurons [32]. In this regard, the released DHA may be important for the neuronal development.

DOI is an agonist primarily for 5-HT_{2A} receptors, but it also shows affinity for the 5-HT_{2C} receptors [24]. Thus, DOI-induced release of PUFA may be mediated by stimulation of 5-HT_{2A} and/or 5-HT_{2C} receptors. Both receptor subtypes have similar molecular structures, and their activation is coupled to guanine nucleotide regulatory proteins and increases phosphatidylinositol turnover [19]. Our results indicated that the preferential 5-HT_{2A} receptor antagonist ketanserin completely blocked the release of AA and DHA. Furthermore, pretreatment with the novel phenylpiperidine antagonist MDL 100,907 abolished the DOI-induced release of PUFA. MDL has been reported to have higher affinity for 5-HT_{2A} ($>$ 200-fold) [24,26] than for 5-HT_{2C} receptors. In addition, in C6 glioma cells 5-HT_{2C} receptors are poorly expressed. Therefore, 5-HT_{2A} rather than 5-HT_{2C} receptors seem to play a predominant role in mediating DOI-induced mobilization of AA and DHA in C6 glioma cells. However, we cannot exclude the possibility that 5-HT_{2C} receptors can also mediate the release of polyunsaturated fatty acids in other systems.

The 5-HT_{2A} receptor is ubiquitous and mediates contraction of vascular and nonvascular smooth muscles, platelet aggregation and neuroexcitation [23]. In the central nervous system it has been shown that activation of 5-HT_{2A} receptors evokes affective disorders while 5-HT_{2A} receptor antagonists are used in the treatment of psychosis and sleep disorders [16,23]. Abnormal signal transduction via altered free fatty acid production may be one of underlying mechanisms involved in these psychiatric disorders.

In summary, 5-HT induced the release of polyunsaturated fatty acids and 5-HT_{2A} receptor activation is involved in this process. Our results provide a strong evidence for the mobilization of polyunsaturated fatty acids as another cellular mechanism responding to serotonin. The regulation of both free arachidonate and docosahexaenoate by serotonin from astroglial cells may play an important role in some of the physiological responses to serotonin.

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