ORIGINAL ARTICLE

Effect of fluoxetine and adenosine receptor NECA agonist on G alpha q/11 protein of C6 glioma cells

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Abstract

OBJECTIVES: Trimeric G-proteins play a crucial role in the transmembrane signalling to intracellular pathways via effector phospholipase C (1,4,5 IP3) or adenylylcyclase (cAMP). G-protein modulation is considered to participate in the antidepressant mode of action by neurotransmitter G-protein coupled receptors (GPCR). Adenosine is naturally occured nucleoside and adenosine receptor belongs to GPCR family. Properties and functions of ubiquitous adenosine receptor were described with number of agonists and antagonists.

METHODS: In C6 glioma cells, we studied acute administration of SSRI antidepressants – fluoxetine, sertraline and citalopram. We used immunochemical estimation (ELISA) of the main types of G-protein alpha subunits from isolated membranes of C6 glioma cells. We also estimated effect of NECA agonist on fluoxetine induced signalling via 1,4,5 IP3 and its levels.

RESULTS: Results show involvement of the antidepressant drugs in the C6 glioma signal transduction cascades and their modulation in dependence on the antidepressant of SSRI type. We measured main G alpha protein profiles after fluoxetine, sertraline and citalopram administration. We found significant changes as following: decreased G alpha Gq/11 for fluoxetine, low G alpha s for sertraline and both high G alpha q/11 and high G alpha s for citalopram. Furthermore the NECA (5´-N-ethylcarboxamido- adenosine) agonist of adenosine receptor alone evoked high decrease of G alpha q/11 levels. Whereas fluoxetine influenced G alpha q/11 decline was abolished by NECA in concentration manner, especially at 10^{-8} and 10^{-9} M concentrations. These results support abolishion NECA effect on fluoxetin influenced 1,4,5 IP3 signalling via PLC.

CONCLUSION: Main G alpha profiles are dependent on SSRI type antidepressant. Abolishing both fluoxetine evoked G alpha q/11 and and 1,4,5 IP3 signalling can indicate parallel interference between G-protein coupled receptors (GPCR) and the cell response. Presented data are first findings about adenosine receptor interaction with fluoxetine signalling. Thus in vitro studies contribute to the clarification of the molecular basis of antidepressant action.

Abbreviations:

AR	- adenosine receptor
cAMP	- cyclic adenosine monophoshate
G-protein	- trimeric GTP binding protein
GPCR	- G-protein coupled receptor
GTP	- guanosine triphosphate
IP3	- 1,4,5 inositol triphosphate
NECA	- 5´-(N-ethylcarboxamido) adenosine
5-HT	- 5-hydroxytryptamine, serotonin
PLC	- phospholipase C
SSRI	- selective serotonin reuptake inhibitor

INTRODUCTION

The selective serotonin reuptake inhibitor (SSRI) fluoxetine is one of the most widely used antidepressant. The other commercially available SSRIs include sertraline, citalopram, escitalopram, fluvoxamine and paroxetine (Páv *et al.* 2008; Sghendo & Mifsud 2012).

The SSRI antidepressants are used to augment the actions of serotonin (5-hydroxytryptamine, 5-HT) in the nervous system. Serotonin plays a pivotal role in the regulation of mood. Although it is generally accepted that 5-HT-mediated processes are fundamental in depressive aetiology, a consensus regarding the specific 5-HT transporter and 5-HT receptor subtype alterations has not been reached (Millan 2006; Tardito *et al.* 2006, Páv *et al.* 2008; Sghendo & Mifsud 2012).

Seven serotonin receptors, members of trimeric G protein-coupled receptor (GPCR) family have been discovered to date. All serotonin receptors, except 5-HT3, are trimeric G-protein coupled receptors that activate an intracellular second messenger cascade. The 5-HT3 receptor is a ligand-gated ion channel members of G protein-coupled receptor (GPCR) family (Wettschureck & Offermanns 2005; Avissar and Schreiber 2006; Sghendo & Mifsud 2012). Furthermore, 5-HT1A receptors are known as autoreceptors since their stimulation inhibits the release serotonin in nerve terminals. Transmembrane signalling systems consist of heptahelical GPCR receptors that are coupled with limited repertoire of G proteins and interact with few effector molecules, including adenylyl cyclase (AC), phospholipase C (PLC), phospholipase A2, cyclic-GMP phosphodiesterase and others (Wettschureck Offermanns 2005; Hubbard & Hepler 2006).

Among the GPCR of serotonin type, 5-HT2C receptors still raise particular attention in view of their implication in many physiological functions and a spectrum of psychiatric disorders (Millan 2006). Serotonin 5-HT2C receptor is a Gq-coupled receptor exhibiting a high degree of constitutive activity toward phospholipase C effector pathway, a process regulated by receptor mRNA editing (Labasque *et al.* 2010).

In the family of GPCR receptors, adenosine receptors, AR (P1 purinoceptors) are involved and are divided to A1, A2A, A2B, and A3 AR. Adenosine is a naturally occurring nucleoside, which exerts its physiological/biological effects by interacting with a family of adenosine receptors known as A1, A2A, A2B, and A3, (Cohen *et al.* 2010). The A(2B) subtype and A2A receptor couple to stimulation of adenylyl cyclase and phospholipase C The A(2B) receptors show a ubiquitous distributions. NECA is one of the most potent A(2B) adenosine receptor agonist (Baraldi *et al.* 2009). Furthermore, high-affinity A1 and A2a AR subtypes are known in contrast to low-affinity A2B and A3 AR subtypes (Baraldi *et al.* 2009). But the sensitivity of A2B adenosine receptors can be greatly increased by interaction with protein kinase C (Baraldi *et al.* 2009, Cohen *et al.* 2010).

Rat C6 glioma cells are an established and useful model for long time. C6 glioma cells are a suitable model to study the mechanism of antidepressant action because of no synaptic structures. The C6 glioma cells are also used in analyzes of antidepressant effects on Galpha subunit levels or production of growth factors, cytokines and gene expression (Hisaoka *et al.* 2007, Tsuchioka *et al.* 2008, Choi *et al.* 2011; Kovářů *et al.* 2000; 2011). Furthermore, C6 glioma cells have been shown to respond to antidepressants in a manner similar to primary astroglial cells (Hisaoka *et al.* 2007). In C6 glioma cells, role of 5HT2 receptors was examined in relationship ERK phosphorylation and the mechanism of 5-HT2 receptor-induced glia cell line-derived neurotrophic factor (GDNF) mRNA expression and a participation G alpha q/11 (Tsuchioka *et al.* 2008). Also adenosine receptors A1,A2A, A2B, and A3 were detected in C6 glioma cells (Castillo *et al.* 2007).

Here, we examined the acute effect of fluoxetine, sertraline and citalopram of SSRI group on G alpha subunit profiles of main G-protein types in C6 glioma cells. In other experiments, a possible role of adenosine receptor NECA agonist (5´-N-ethylcarboxamido- adenosine) on fluoxetine influenced G alpha q/11-protein signalling via PLC effector and second messenger 1,4,5 IP_3 formation by the enzyme was also analysed.

MATERIAL AND METHODS

C6 glioma cells

Cells were cultured in MEM medium, pH 7.3 with 5% fetal calf serum under standard conditions. Confluent cultures were exposed to antidepressant (final 1 μM) for up to 24 h (acute model). Then cells were washed with PBS, harvested by scraping with rubber policeman in PBS, containing proteolytic inhibitors, and cholate membrane extracts were prepared as described previously (Kovařů *et al.* 2011).

G alpha subunit estimation

G alpha chains ganges were analysed by ELISA technique with our rabbit antibodies against synthetic C-terminal dekapeptides of alpha chains of Gs, Gi1,2 and Gq/11. ELISA method of competitive inhibiton was performed and modified by use of Maxisorp microtitration plates (NUNC) for noncovalent peptide binding.

ELISA method assay estimation was compared with Western immunoblotting (Kovařů *et al.* 1998; 2011). Used monospecific antibodies against individual G alpha dekapeptides were without cross reactivity. Synthetic dekapeptides were prepared in the Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague, Czech Republic, and primary amino acid sequence of dekapeptides was checked by sequence analysis (Kovařů *et al.* 1998). For othe details see Kovářů et al (2010).

The 1,4,5 IP_3 was estimated according to protocol of TRK1000 assay system (Amersham Biosciences, UK).

Statistical analysis

All results are expressed as the arithmetical mean ± S.E.M. The differences between experimental samples were evaluated by Student´s t test for unpaired values.

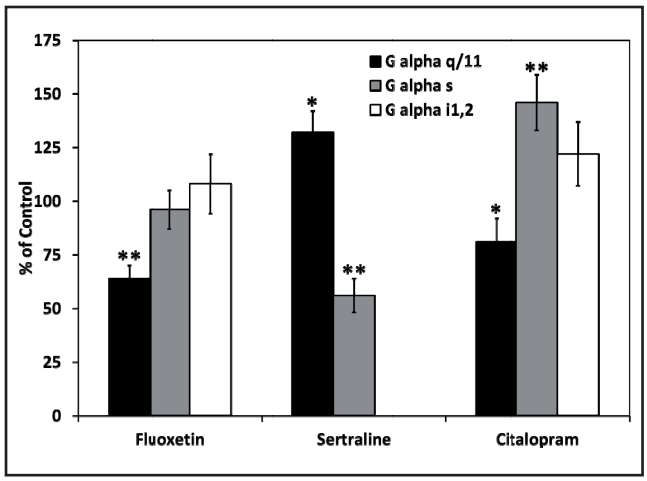

Fig. 1. Acute effect of fluoxetine, sertraline and citalopram on G alpha subunit profile of C6 glioma cells. Results are arithmetical mean of 3–4 experiments. Results are statistically significant $^{**}p<0.01$ and $^{*}p<0.05$.

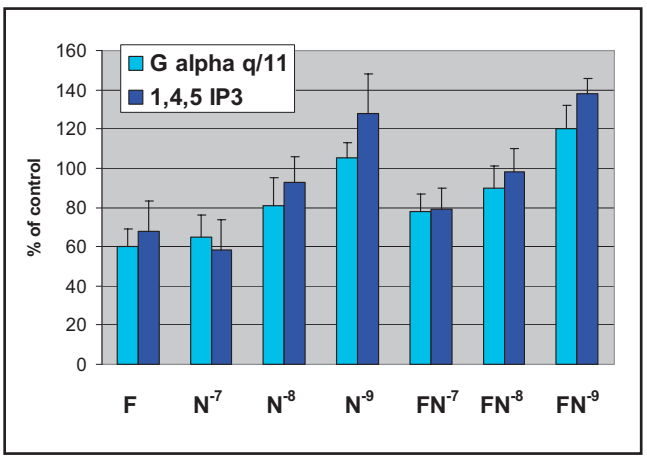

Fig. 2. Effect of fluoxetine (F,10-6 M) and receptor NECA agonist (N, 10^{-7},10^{-8}, 10^{-9} M) on G alpha q/11 and 1,4,5 IP_3 levels of C6 glioma cells. Results are arithmetical mean of three experiments. Each value was measured in tetraplet. All results are statistically significant $p<0.01$, except $p=0.05$ for FN^{-7} concentration.

RESULTS AND DISCUSSION

Figure 1 summarizes the acute effect of fluoxetine, sertraline and citalopram of SSRI type on C6 glioma cells. Results indicate that SSRI antidepressants induce different G alpha subunit profiles as followed: significantly decrease of G alpha q/11 for fluoxetine, low G alpha s for sertraline and and both significantly low G alpha q/11 and high G alpha s for citalopram. Results show that each SSRI antidepressant evoked various regulation of one or more G alpha subunits via both signalling effectors 1,4,5 IP_3 or cAMP, 2nd messengers. On the other hand, general knowledge is traditional in relationship to a chronic mode of administration that despite the very different molecular structures of SSRIs, they all have similar mechanisms of action, albeit with slightly different pharmacokinetic properties (Sghendo and Mifsud 2012).

Results show highly significant reduction of membrane G alpha q/11 level confirmed by highly decreased 1,4,5 IP_3 concentration (Figure 2), i.e. reduced signalling via G alpha q/11 to effector PLC. Beside this we determined in another study fluoxetine induced translocation of membrane G alpha q/11 subunit into cytosol of C6 glioma cells as a part of desensitization mechanisms (Kovařů *et al.* in preparation). Furthermore, in C6 glioma (astrocytoma) cells, fluoxetine effects can involve probably 5-HT2B receptor linked to G alpha q/11 pathway (Tsuchioka *et al.* 2008, Zhang *et al.* 2010, Choi *et al.* 2011). Possible role of 5-HT2B in various SSRI antidepressant-induced can be in accordance with participation of HT2B in multipathway cascades of transmembrane signalling (Baraldi *et al.* 2010, Cohen *et al.* 2010). However A3R role is not excluded because of G alpha q/11 signalling (Baraldi *et al.* 2009,Cohen *et al.* 2010, Labasque *et al.* 2010).

On the other hand, fluoxetine was able to induce via G-protein coupled receptors pro-apoptic event leading to C6 glioma cell apoptosis, indicating distinct pathways in the cell response (Španova *et al.* 1997; Levkovitz *et al.* 2005, Kovařů *et al.* – in preparation). In contrast to G protein signalling (Figure 2) NECA agonist exhibited potentiation effect on fluoxetine induced apoptosis in C6 glioma cells (Kovářů *et al.* 2000). Beside this sertraline alone (signalling via G alpha s, Figure 1) or sertraline with NECA were not able to induce the apoptic response (Kovářů *et al.* 2000). Relationship could be linked to various signalling pathways for fluoxetine via major Gq/11 and sertraline via major Gs under these conditions..

Figure 2 show the acute effect of fluoxetine on C6 glioma cells measured by G alpha q/11 signalling and 1,4,5 IP_3 levels. Results indicate highly significant reduction of membrane G alpha q/11 level confirmed by highly decreased 1,4,5 IP_3 concentration i.e. reduced signalling via G alpha q/11 to effector PLC. The 5-HT2 receptors are present in C6 glioma cells and they are coupled via G alpha q/11 protein to the signalling via

PLC and stimulation of PI hydrolysis (Tsuchioka *et al.* 2008, Zhang *et al.* 2010). But A3R role is not excluded because of a role in G alpha q/11 signalling (Baraldi *et al.* 2009, Cohen *et al.* 2010).

The NECA AR agonist effect on G protein signalling (Figure 2). We estimated NECA agonist evoked marked decrease of G alpha q/11 level in C6 glioma cells. The G alpha q/11 pathway could indicate a role of A2B subtype of AR receptor (Tsuchioka *et al.* Cohen *et al.* 2010). NECA is a potent A2B adenosine receptor agonist and can be involved in the response (Baraldi *et al.* 2009).

Combined effect of fluoxetine and NECA AR agonist (Figure 2). Our results show that fluoxetine or NECA agents act via Gq/11 on effector PLC system, indicated by 2nd messenger 1,4,5 IP_3 formation. Furthermore, fluoxetine induced decrease of signalling by NECA is regulated in NECA dose dependent manner. Abolished G alpha q/11 signalling by fluoxetine was dependent on increased NECA concentration and was confirmed by declined levels of 1,4,5 IP_3 formation by PLC of C6 glioma cells. Data should suggest a posibility of a cooperation between fluoxetine and NECA induced GPCR receptors. It seems that NECA agonist of adenosine receptor (A2B) influenced fluoxetine transmembrane signalling.It was evident that both G alpha q/11 levels and 1,4,5 IP_3 signalling were very sensitive to low NECA concentration at 10^{-8}M and 10^{-9}M to its strong abolishing effect.

To date there are very limited data in literature about posibility of adenosine receptor interference with 5-HT receptor or 5-HT transporter signalling. Recently, evidence was presented that A3 adenosine receptor AR was colocalized with 5-HT transporter (5-HTT) in mouse midbrain serotonergic neurons (Zhang *et al.* 2011). The A3AR can exist within 5HT transporter complexes and the abundance of A3AR/ 5-HTT complexes was enhaced in a protein kinase G of I type (PKGI) dependent manner. Mechanisms by which A3 adenosine receptor activity control activation of 5-HTT proteins require an understanding whether regulation is indirect or is mediated by more confined, physical interactions or pathways coordinating A3AR signaling to 5-HT transporter can be spatially restricted and regulated. In presence, compartmentalizing mechanisms by which GPCRs can target one or more of these modulators to regulate 5-HT transporter without influencing other cytosolic and membrane effectors are unknown (Zhang *et al.* 2011).

Finally, our experiments are first finding about adenosine receptor effect on fluoxetine signalling. Undoubtedly, more detailed knowledge remains to be elucidated in an area cooperation of GPCR receptors in transmembrane signalling. However, in vitro studies contribute to the clarification of the molecular basis of antidepressant action and its regulation.

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REFERENCES

1 Avissar S, Schreiber G (2006). The involvement of G proteins and regulators of receptor-G protein coupling in the pathophysiology, diagnosis and treatment of mood disorders. Clin Chim Acta. **366**: 37–47.
2 Baraldi PG, Tabrizi MA, Fruttarolo F, Romagnoli R Preti D(2009). &Recent improvements in the development of A2B adenosine receptor agonists. Purinergic Signalling **5**: 3–19.
3 Castillo CA, Albasanz JL, Fernández M, Martín M. (2007). Endogenous expression of adenosine A1, A2 and A3 receptors in rat C6 glioma cells. Neurochem Res. **32**: 1056–1070.
4 Choi MR, Oh DH, Kim SH, Yang BH, Lee JS, Choi J, Jeon HS Chai YG, Park YC (2011). Fluoxetine Up-Regulates Bcl-xL Expression in Rat C6 Glioma Cells.Psychiat Invest. **8**: 161–168.
5 Cohen MV, Yang X, Downey JM (2010). A2b adenosine receptors can change their spots. Brit J Pharmacol. **159**: 1595–1597.
6 Hisaoka K, Takebayashi M, Tsuchioka M, Maeda TN, Nakata Y, Yamawaki S (2007). Antidepressants increase glial cell line derived neurotrophic factor production through monoamine in dependent activation of protein tyrosine kinase and extracellular signal-regulated kinase in glial cells. J Pharmacol Exp Ther. **121**: 148–157.
7 Hubbard KB, Hepler JR (2006). Cell signalling diversity of theGq alpha family of heterotrimeric G proteins. Cell Signal. **18**: 135–150.
8 Kovařů H, Fišerova A, Kovařů F, Paclt I, Lisa V (2000). Antidepressant or immunomodulator induced regulation of cell signalling in brain and immune system. Eur Neuropsychopharmacol. **Suppl. 3**: 269–270.
9 Kovařů H, Kovařů F, Žižkovsky V, Velek J (1998). Developmental changes of trimeric GTP-binding proteins in porcine brain and immune system. Acta Vet. Brno. **67**: 15–20.
10 Kovářů H, Kovaru F, Pav M, Dvorak R, Palikova M, Raboch J, Zidková J (2010). Effect of mitogen lectin on lymphocyte or brain cortex cell activation. Neuro Endocrinol Lett. **31**: 325–329.
11 Kovářů H, Kovářů F, Ondráčková P, Lisá V, Anna Fišerová (2011). Effect of fluoxetine or adenosine receptor NECA agonist on G-proteins of C6 glioma cells or NK immunocytes.Neuro Endocrinol Lett. **32**: 274–278.
12 Labasque M, Metre J, Carrat G, Besamel C, Bockaert J, Marin P (2010). Constitutive activity of serotonin 2C receptors at G protein-independent signaling: modulation by RNA editing and antidepressants. Mol Pharmacol. **78**: 818–826.
13 Levkovitz Y, Gil-Ad I, Zeldich E, Dayag M, Weizman A. (2005). Differential induction of apoptosis by antidepressants in glioma and neuroblastoma cell lines: evidence for p-c-Jun, cytochrome c, and caspase-3 involvement. JMol Sci. **27**: 29–42.
14 Millan MJ (2006). Multi-target strategies for the improved treatment of depressive states: conceptual foundations and neuronal substrates, drug discovery and therapeutic application. Pharmacol Ther. **110**: 135–370.
15 Páv M, Kovařů H, Fišerova A, Havrdova E, Lisa V (2008). Neurobiological aspects of depressive disorder and antidepressant treatment: role of glia. Physiol Res. **57**: 151–64
16 Španova A, Kovářů H, Lisa V, Lukašova E, Rittich B (1997). Estimation of apoptosis in C6 glioma cells treated with antidepressants. Physiol Res. **46**: 161–164.
17 Tardito D, Perez J, Tiraboshi E, Musazzi L, Racagni G, Popoli M (2006). Signaling pathways regulating gene expression, neuroplasticity, and neurotrophic mechanisms in the action of antidepressants: A Critical Overview. Pharmacol Rev. **58**: 115–134.

18 Tsuchioka M, Takebayashi M, Hisaoka K, Maeda N, Nakata Y(2008). Serotonin (5-HT) induces glial cell line-derived neurotrophic factor (GDNF) mRNA expression via the induces glial cell line-derived neurotrophic factor (GDNF) mRNA expression via the transactivation of fibroblast growth factor receptor 2 (FGFR2) in rat C6 glioma cells. J.Neurochem. **106**: 254–257.
19 Wettschureck N, Offermanns S (2005). Mammalian G proteins and their cell type specific functions. Physiol Rev. **85**: 1159–120.
20 Zhang S Lovatt D Song G. Goldmann S. Nedergaard M. Hertz L.Peng L (2010). 5HT2B receptors are expressed on astrocytes from brain and in culture are a chronic target for all five conventional serotonin-specific reuptake inhibitors'. Neuron Glia Biol. **2**: 113–125.
21 Zhu CB, Lindler KM, Campbell NG, Sutcliffe JS,. Hewlett WA, Blakely RD (2011). Colocalization and regulated physical association of presynaptic serotonin transporters with A3 adenosine receptors. Mol Pharmacol. **80**: 458–465.