Neurochemistry International 60 (2012) 631–639

Contents lists available at SciVerse ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

# Estrogen receptor alpha and beta differentially mediate C5aR agonist evoked $Ca^{2+}$-influx in neurons through L-type voltage-gated $Ca^{2+}$ channels

Imre Farkas [a,*], Miklós Sárvári [a], Máté Aller [b], Noriko Okada [c], Hidechika Okada [d], István Likó [e], Zsolt Liposits [a,f]

[a] Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary
[b] Laboratory of Cellular Pharmacology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary
[c] Department of Immunology, Nagoya City University, Nagoya, Japan
[d] Choju Medical Institute, Toyohashi, Japan
[e] Gedeon Richter Plc., Budapest, Hungary
[f] Department of Neuroscience, Faculty of Information Technology, Pázmány Péter Catholic University, Budapest, Hungary

ARTICLE INFO

*Article history:*
Available online 2 March 2012

*Keywords:*
GT1-7 neuron
Complement C5a receptor
Estrogen receptor alpha
Estrogen receptor beta
Voltage-gated calcium channel

ABSTRACT

Complement C5a is associated primarily with inflammation. The widespread expression of its receptors, C5aR and C5L2 in neuronal cells, however, suggests additional regulatory roles for C5a in the CNS. C5aR agonist (PL37-MAP) evokes $Ca^{2+}$-influx in GT1-7 neuronal cell line and the $Ca^{2+}$-influx is regulated by estradiol. In the present study, we examined further the mechanism of $Ca^{2+}$-influx and the contribution of the two estrogen receptor (ER) isotypes, ER$\alpha$ and ER$\beta$, to estrogenic modulation of intracellular $Ca^{2+}$-content. GT1-7 neurons were treated with isotype selective ER agonists for 24 h then C5aR agonist evoked $Ca^{2+}$-responses were measured by $Ca^{2+}$-imaging. Transcriptional changes were followed by real-time PCR. We found that not only estradiol (100 pM), but the ER$\alpha$ selective agonist PPT (100 pM) enhanced the PL37-MAP-evoked $Ca^{2+}$-influx (E2: 215%, PPT: 175%, compared to the PL37-MAP-evoked $Ca^{2+}$-influx). In contrast, the ER$\beta$ selective agonist DPN (100 pM) significantly reduced the $Ca^{2+}$-influx (32%). Attenuated $Ca^{2+}$-response (25%) was observed in Ca-free environment and depletion of the $Ca^{2+}$-pool by CPA eliminated the remaining elevation in the $Ca^{2+}$-content, demonstrating that the majority of $Ca^{2+}$ originated from the extracellular compartment. L-type voltage-gated $Ca^{2+}$-channel (L-VGCC) blocker nifedipine abolished the $Ca^{2+}$-influx, while R-type $Ca^{2+}$-channel blocker SNX-482 had no effect, exemplifying the predominant role of L-VGCC in this process. Acute pre-treatments (8 min) with ER agonists did not affect the evoked $Ca^{2+}$-influx, revealing that the observed effects of estrogens were genomic. Therefore, we checked estrogenic regulation of C5a receptors and L-VGCC subunits. ER agonists increased C5aR mRNA expression, whereas they differentially regulated C5L2. Estradiol decreased transcription of $Ca_v1.3$ L-VGCC subunit. Based on these results we propose that estradiol may differentially modulate C5a-induced $Ca^{2+}$-influx via L-VGCCs in neurons depending on the expression of the two ER isotypes.



## 1. Introduction

The complement (C) system is an ancient immune pathway comprised of numerous elements activated in a cascade leading to the elimination of pathogens (Speth et al., 2008). During C activation, the C5a anaphylatoxin, a 74 amino-acid long fragment of the fifth component of C is released when C5 is cleaved by the C5 convertase. C5a binds to two receptors, the "classical" G-protein coupled C5aR (CD88) and the non-G-protein coupled receptor C5L2 (GPR77). Both receptors are expressed on immune and non-immune cell types. C5a binding to C5aR leads to various events such as increased intracellular calcium level and activation of intracellular signaling cascades resulting in functional responses e.g. recruiting and activation of inflammatory cells, degranulation, delayed or enhanced apoptosis, phagocytosis, histamine release, and chemotaxis (Fujita et al., 2004; Guo and Ward, 2005). C5L2 may function as a decoy receptor regulating the inflammatory response resulted from the C5a/C5aR binding (Bamberg et al., 2010; Woodruff et al., 2011).

*Abbreviations:* C, complement system; C5aR, "classical" complement 5a receptor (G-protein coupled); C5L2, "second" complement 5a receptor (non-G-protein coupled); CPA, cyclopiazonic acid; DPN, diarylpropionitrile; E2, 17β-estradiol; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; GnRH, gonadotropin-releasing hormone; HBSS, Hanks' balanced salt solution; L-VGCC, L-type voltage-gated calcium channel; PPT, 4,4′,4″-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol; PVN, paraventricular nucleus; SON, supraoptic nucleus.

* Corresponding author. Address: Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Szigony. u. 43., H-1083 Budapest, Hungary. Tel.: +36 1 210 9400; fax: +36 1 210 9944.
*E-mail address:* farkas@koki.hu (I. Farkas).

0197-0186/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2012.02.024

Expression of C5aR has been demonstrated in astrocytes, microglia and neurons of the central nervous system (CNS) (Woodruff et al., 2010). The cellular expression pattern of C5L2 is similar to that seen for C5aR. Pyramidal neurons in the hippocampus and the cortex, Purkinje cells of the cerebellum and neuroblastoma cells express C5aR (Farkas et al., 1999, 1998a,b). Function of the C5aR in neurons remains elusive. A C5aR-related apoptotic pathway and the role of this receptor in neurodegenerative diseases such as Alzheimer's disease have been suggested (Farkas et al., 2003; Fonseca et al., 2009, 2011). In contrast, the neuroprotective role of C5a has also been demonstrated (Woodruff et al., 2010).

C5aR has recently been identified in hypothalamic neurons, including gonadotropin-releasing hormone (GnRH)-producing cells, immortalised GnRH-producing GT1-7 neurons and neurons of the paraventricular (PVN) and supraoptic (SON) nuclei (Farkas et al., 2008). GT1-7 neurons establish neuronal network with coordinated activity and produce GnRH in a pulsatile fashion (Liposits et al., 1991; Wetsel et al., 1992). Pulsatility and volume of the secretion is in strong correlation with synchronised firing (Moenter et al., 2003; Thiery and Pelletier, 1981; Wilson et al., 1984). Various factors released during inflammation can play role in the function of these cells (Karsch et al., 2002). Cannabinoids also affect GnRH neurons by utilising the retrograde endocannabinoid signaling mechanism (Farkas et al., 2010).

The estrogen receptor alpha and beta (ERα and ERβ) are expressed in numerous hypothalamic neurons, such as GnRH cells and the neurons of PVN and SON (Hrabovszky et al., 2004; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001), GT1-7 cells express both ER subtypes (Roy et al., 1999). 17β-estradiol (E2) can modulate the electric function of GT1-7 cells and exert both negative and positive feedback on the firing (Christian et al., 2005; Farkas et al., 2007).

Our previous experiments have shown that administration of a C5aR agonist results in robust calcium ($Ca^{2+}$) influx in GnRH neurons. In addition, E2 pre-treatment elevates this $Ca^{2+}$-response suggesting that the signal transduction pathways related to the C5aRs and the ERs, respectively, can modulate each other (Farkas et al., 2008). Change in the intracellular $Ca^{2+}$-milieu can heavily affect firing properties of the neurons. Firing can be fine-tuned for example by the opening and closing of the L-type voltage-gated $Ca^{2+}$-channels (L-VGCCs). $Ca_v1.2$ and $Ca_v1.3$ subunits of the L-VGCCs are strongly involved in spontaneous firing and pacemaking (Zuccotti et al., 2011). In the present study, therefore, we investigated further, how C5aR and ER subtypes interact using the immortalised GnRH-producing GT1-7 neurons as a neuronal model with $Ca^{2+}$-imaging and quantitative real-time PCR methods. Genomic and non-genomic actions of ER subtypes on the C5aR-mediated $Ca^{2+}$-influx were examined. Potential sources of the increased $Ca^{2+}$-content were also studied, including the role of various VGCCs and the intracellular $Ca^{2+}$-pool.

## 2. Materials and methods

### 2.1. Cell culture

GnRH-producing immortalised GT1-7 neurons were cultured in Dulbecco Modified Eagle Medium (DMEM) containing high-glucose and supplemented with 10% fetal calf serum (FCS) and 5% horse serum (HS). Prior to ER agonist treatment the culturing medium was replaced with a steroid/thyroid- and phenol red-free one and cells were cultured in this medium for 24 h. Subsequently, the cells were treated with 17β-estradiol (E2, SIGMA), the highly potent ERβ receptor agonist DPN (Diarylpropionitrile, Tocris) and the ERα receptor agonist PPT (4,4′,4″-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol, Tocris) at various concentrations (100 pM–20 nM) for 24 h and then used for calcium imaging and RT-PCR experiments. In the experiments examining effect of acute treatment, the E2, DPN, and PPT were added to the cells 8 min before starting the calcium imaging recording.

### 2.2. Calcium imaging

Cultured GT1-7 cells were loaded with the calcium-sensitive fluorescent dye Fura-2 AM (1 μM; Molecular Probes, Eugene, Oregon) in loading buffer Hanks' balanced salt solution (HBSS) containing 0.1% DMSO and Pluronic-F127 (1 μM, Molecular Probes) for 1.5 h at room temperature (RT). After washing with HBSS, the experiments were carried out at RT. The antisense homology box peptide fragment of the C5a (RAARISLGPRCIKAFTE) was synthesised in multiple antigenic peptide form (termed PL37-MAP, 2.5 μM). Sequence of the PL37 is a fragment sequence of the C5a, representing a "strong" antisense homology box region in the C5a (Baranyi et al., 1995). Our previous works applying both C5a and PL37, respectively, demonstrated that PL37 is a potent agonist of the C5aR preserving the biological activity of the C5a and triggering responses similar to that of the C5a (Baranyi et al., 1996; Fujita et al., 2004). The peptide was pipetted directly onto the cells in HBSS after a 1 min baseline recording and then the diluted peptide remained in the HBSS during recording. In the case of E2, DPN, PPT pre-treatment, the cells were pre-treated with them as described in the "Cell culture" section and all of the rinsing and extracellular solutions contained the same concentration of E2, DPN and PPT. After the 1 min baseline recording the PL37-MAP peptide was introduced into the bath fluid containing E2, DPN, and PPT and then the diluted peptide remained in the HBSS-E2 or HBSS-DPN or HBSS-PPT mixture during recording.

When PL37-MAP was applied in $Ca^{2+}$-free extracellular solution (phosphate buffered salt solution = PBS, pH 7.4), the HBSS was changed to PBS just before starting the recording, except when the cells were treated with cyclopiazonic acid (CPA, 10 μM, Tocris). CPA is a specific blocker of the $Ca^{2+}$-ATP-ase of the intracellular $Ca^{2+}$-store endoplasmic reticulum and depletes these $Ca^{2+}$-stores. CPA was applied to the GT1-7 neurons in PBS 30 min before starting the measurements.

The VGCC blockers nifedipine (10 μM, SIGMA) and SNX-482 (100 nM) were added to the HBSS just before starting the $Ca^{2+}$-imaging measurement and remained in the HBSS during recording.

The experiments were carried out with an ARGUS HiSCA $Ca^{2+}$-imaging system (Hamamatsu Photonics, Hamamatsu, Japan) or with an Olympus BX50WI microscope equipped with a Polychrome II monochromator (TILL Photonics), a cooled CCD camera (Photometrics Quantix, Tucson, AZ, USA), and controlled by the Axon Imaging Workbench 6.0 software (Axon Instruments, Union City, CA, USA). The ratio of the fluorescent signals obtained at excitation wavelengths of 340 and 380 nm was used to determine changes in the intracellular $Ca^{2+}$-concentration.

The surface density of the cultured cells was 500,000–750,000 cells/cm$^2$, the magnification of the objective lens used was 40x, and the area of the calcium imaging acquisition was 0.038 mm$^2$. Before starting the calcium imaging recordings, an area was chosen where at least 7 individual cells without overlaps could be clearly observed and measured (7–15 neurons depending on the surface density of the cells in the area measured).

### 2.3. Total RNA isolation from GT1-7 cells

Total RNA was isolated from GT1-7 cells using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA analytics included capillary electrophoresis using Agilent 2100 Bioanalyzer (Santa Clara, CA). All RNA samples displayed RNA integrity numbers (RIN) above 8.5.

### 2.4. Quantitative real-time PCR

Inventoried TaqMan assays were selected to study in depth the regulation of genes of our interest by quantitative real-time PCR. Each assay consisted of a FAM dye-labeled TaqMan MGB probe and two PCR primers. Every assay was optimised by the manufacturer to run under universal thermal cycling conditions with a final reaction concentration of 250 nM for the probe and 900 nM for each primer. Reverse transcription and real-time PCR were run as described earlier (Sarvari et al., 2010). RealTime StatMiner (Integromics, Granada, Spain) software and relative quantification against calibrator samples ($\Delta\Delta$Ct) were used for analysis of Applied Biosystems TaqMan gene expression assays. Two housekeeping genes (Gapdh, Hprt) were applied as internal controls. The geometric mean of Ct values of Gapdh and Hprt1 was used for subsequent $\Delta$Ct calculation (Vandesompele et al., 2002). Relative quantity (RQ) represents the change in the expression of a given gene in response to a treatment compared to basal (control) expression of the given gene. We considered changes with RQ > 1.5 as up-regulation or RQ < 0.67 as down-regulation (21).

### 2.5. Statistical analysis

$Ca^{2+}$-imaging recordings using the fluorescence ratio obtained at 340 and 380 nm wavelengths were baseline corrected, then the area-under-curve data of the records representing the net $Ca^{2+}$-influx were analyzed. Group data of the cells ($n \geqslant 7$) were expressed as mean ± standard error (SEM). Statistical significance was analyzed using ANOVA followed by Newman–Keuls (NK) test (GraphPad Software Inc., USA), and considered at $p < 0.05$.

## 3. Results

### 3.1. Estrogens differentially modulate the $Ca^{2+}$-influx evoked by the C5aR agonist

The C5aR agonist peptide PL37-MAP (2.5 μM) triggered robust $Ca^{2+}$-influx in GT1-7 neurons (Fig. 1a). Onset of the elevation of intracellular $Ca^{2+}$-concentration was within 1–1.5 min after application of the peptide. When the neurons were pre-treated with E2 (100 pM, 24 h), the evoked $Ca^{2+}$-influx started earlier (in less than

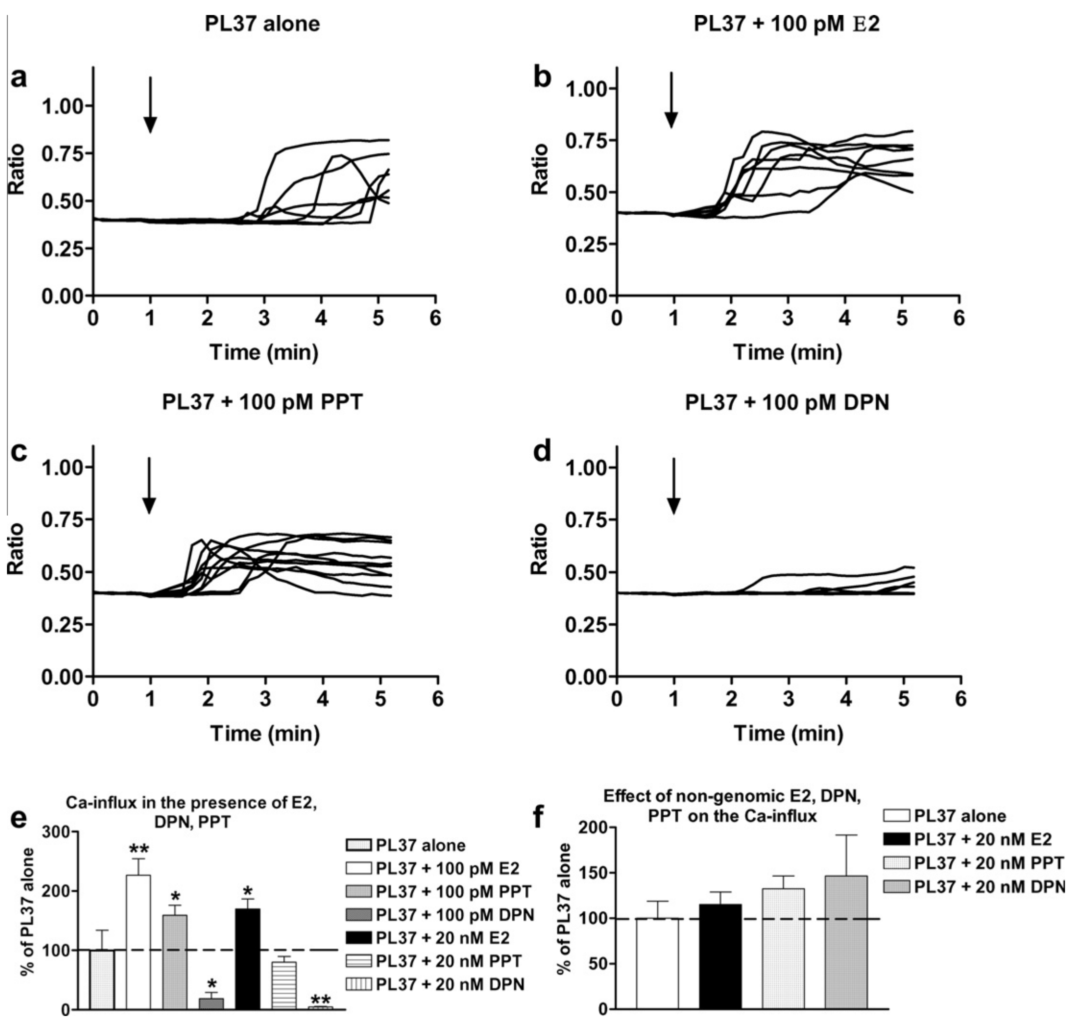


**Fig. 1.** $Ca^{2+}$-influx evoked by the C5aR agonist PL37-MAP (PL37) in the presence of E2, DPN, or PPT in the GT1-7 neurons. (a) The PL37-MAP induced robust $Ca^{2+}$-influx in the cells. (b) Pre-treatment of the GT1-7 cells with E2 (100 pM, 24 h) potentiated the $Ca^{2+}$-influx significantly. Onset of the response started earlier and the amplitude of it was higher than without pre-treatment. (c) Pre-treatment with the PPT (100 pM, 24 h) resulted in an elevated $Ca^{2+}$-influx. (d) DPN pre-treatment (100 pM, 24 h), however, attenuated the $Ca^{2+}$-influx evoked by the PL37-MAP, significantly. (e) The histogram shows the area-under-curve values, representing the net $Ca^{2+}$-influx in the neurons, in the percentage of the $Ca^{2+}$-influx measured with the PL37-MAP alone (PL37 + 100 pM E2: 226.6 ± 27.52%; PL37 + 100 pM PPT: 159.1 ± 16.74%; PL37 + 100 pM DPN: 18.6 ± 10.47%; PL37 + 20 nM E2: 169.7 ± 16.67%; PL37 + 20 nM PPT: 80.2 ± 9.73%; PL37 + 20 nM DPN: 10.9 ± 3.65%). (f) Acute (8 min) pre-treatments with the E2, DPN, or PPT showed no significant effect on the PL37-MAP-evoked $Ca^{2+}$-influx, demonstrating that the observed effect of E2, DPN, or PPT was genomic (PL37 + E2: 115.1 ± 13.78%; PL37 + PPT: 132.1 ± 13.97%; PL37 + DPN: 146.9 ± 45.22%). $^{*}p < 0.05$, $^{**}p < 0.01$. Arrow shows the onset of the administration of the PL37-MAP.

30 s) and the amplitude of the records was higher than that of the recordings evoked by the PL37-MAP alone (Fig. 1b). Pre-treatment with the selective ERα agonist PPT (100 pM, 24 h) elevated the PL37-MAP-triggered $Ca^{2+}$-influx. The elevation was similar to the increased $Ca^{2+}$-influx measured with E2 (Fig. 1c). In contrast, when the cells were pre-treated with the selective ERβ agonist DPN

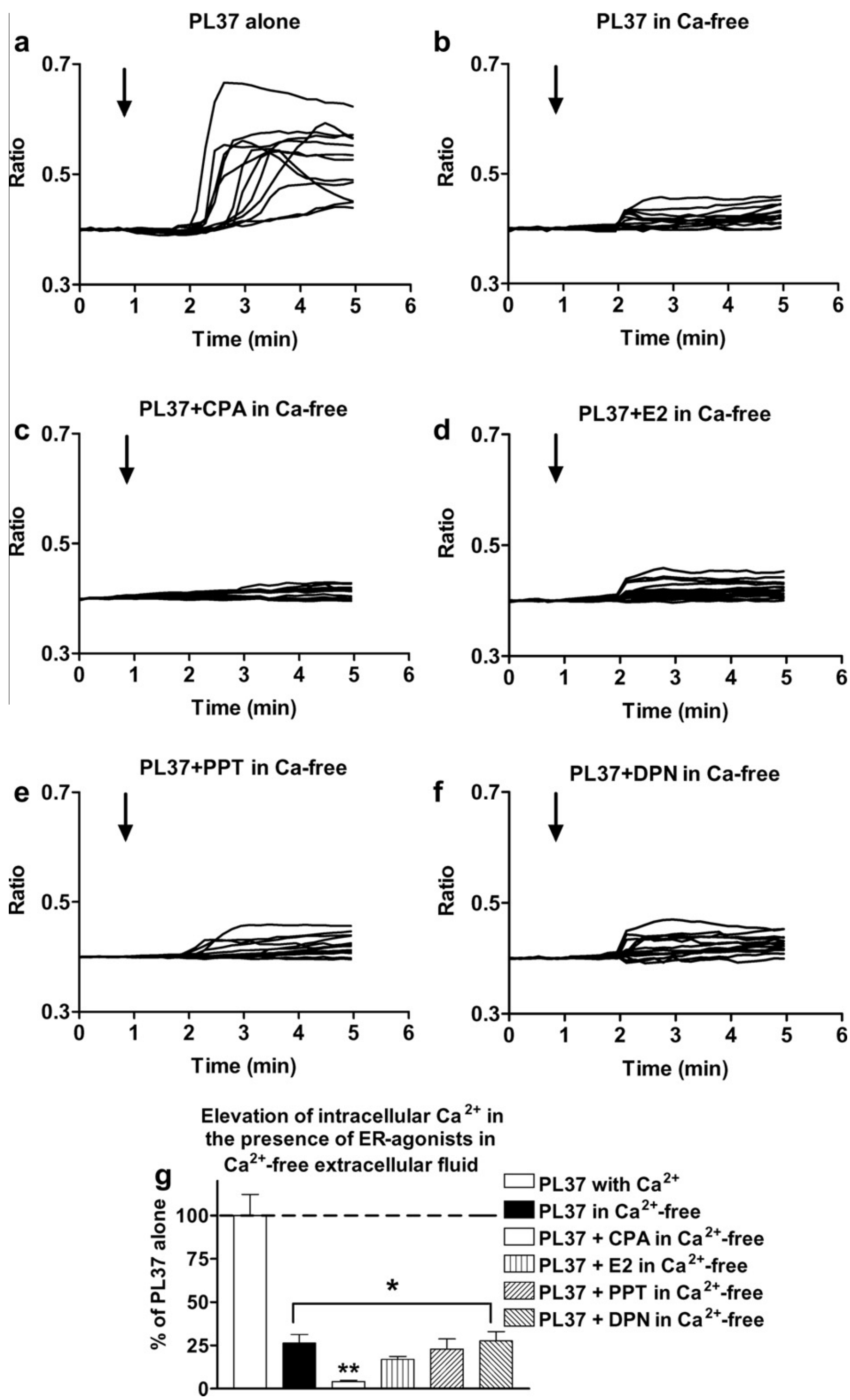


**Fig. 2.** $Ca^{2+}$-influx evoked by the PL37-MAP (PL37) in the presence of CPA, E2, DPN, or PPT in the GT1-7 neurons in $Ca^{2+}$-free extracellular solution. (a)–(f) Calcium imaging recordings show that extracellular calcium sources play important role in the $Ca^{2+}$-influx. (g) The histogram reveals that the $Ca^{2+}$-free extracellular environment decreased the $Ca^{2+}$-response significantly, demonstrating that majority of the elevation in the $Ca^{2+}$-concentration was from the extracellular source. This change was independent from the pre-treatment with E2, DPN or PPT. CPA, a depletor of the $Ca^{2+}$-pool in the endoplasmic reticulum, however, eliminated the remaining $Ca^{2+}$-response in the neurons, showing that the endoplasmic reticulum was the intracellular source of the remaining $Ca^{2+}$-response (PL37 in $Ca^{2+}$-free: 25.3 ± 4.91%; PL37 + CPA in $Ca^{2+}$-free: 3.2 ± 0.66%; PL37 + E2 in $Ca^{2+}$-free: 16.9 ± 1.63%; PL37 + PPT in $Ca^{2+}$-free: 22.9 ± 5.82%; PL37 + DPN in $Ca^{2+}$-free: 27.5 ± 5.45%). $^{*}p < 0.05$, $^{**}p < 0.01$. Arrow shows the onset of the administration of the PL37-MAP.

(100 pM, 24 h), the elevation of the $Ca^{2+}$-content was much lower and started in 1.5–2 min after introducing the PL37-MAP peptide into the bath fluid (Fig. 1d).

Histogram of the normalised area-under-curve data representing the net changes in the intracellular free $Ca^{2+}$ concentration showed significant increase when PL37-MAP was applied to GT1-7 neurons in the presence of either E2 (100 pM, 24 h) or PPT (100 pM, 24 h), revealing facilitation of the PL37-MAP-evoked $Ca^{2+}$-influx by both E2 and PPT. On the other hand, DPN (100 pM, 24 h) significantly decreased (Fig. 1e) the $Ca^{2+}$-influx evoked by PL37-MAP (E2: 226.6 ± 27.52%; PPT: 159.1 ± 16.74%; DPN 18.6 ± 10.47% of the $Ca^{2+}$-influx evoked by PL37-MAP alone; $p < 0.001$). Application of higher concentrations of ER agonists resulted in similar effect except in the case of PPT (Fig. 1e). E2 (20 nM, 24 h) increased the PL37-MAP-evoked $Ca^{2+}$-influx whereas DPN (20 nM, 24 h) attenuated it significantly (E2: 169.7 ± 16.67%; DPN 10.9 ± 3.65% of the $Ca^{2+}$-influx evoked by PL37-MAP alone; $p < 0.001$).

### *3.2. The effects of ER agonists on the $Ca^{2+}$-influx are genomic*

In order to determine whether the observed effect of ER agonists on the PL37-MAP-evoked $Ca^{2+}$-influx was genomic, PL37-MAP was applied after an acute (8 min) application of E2, PPT, and DPN. $Ca^{2+}$-imaging experiments demonstrated that short administration of ER agonists failed to influence the change in the intracellular free $Ca^{2+}$-concentration triggered by PL37-MAP (Fig. 1f). Examination of the area-under-curve data showed no significant differences (E2: 115.1 ± 13.78%; PPT: 132.2 ± 13.97%; DPN 146.9 ± 45.22% of the $Ca^{2+}$-influx evoked by PL37-MAP alone). The results revealed that genomic effects of the E2, DPN, and PPT were necessary to modulate the $Ca^{2+}$-response.

### *3.3. The major $Ca^{2+}$-source is extracellular*

Potential sources of the increase in the intracellular $Ca^{2+}$-content were also investigated with $Ca^{2+}$-imaging on the GT1-7 neurons (Fig. 2a–g). Application of PL37-MAP in $Ca^{2+}$-free extracellular fluid (PBS) resulted in a significantly lower $Ca^{2+}$-response than in HBSS, which was independent from the ER agonist pre-treatment ($p < 0.001$). Administration of PL37-MAP resulted in a significantly attenuated elevation in $Ca^{2+}$-concentration (25.3 ± 4.91% of the $Ca^{2+}$-increase evoked by PL37-MAP in $Ca^{2+}$-containing extracellular fluid). Nevertheless, the effect of the PL37-MAP was not entirely eliminated in PBS, suggesting, that intracellular $Ca^{2+}$-sources were also activated during the process. Therefore, the effect of

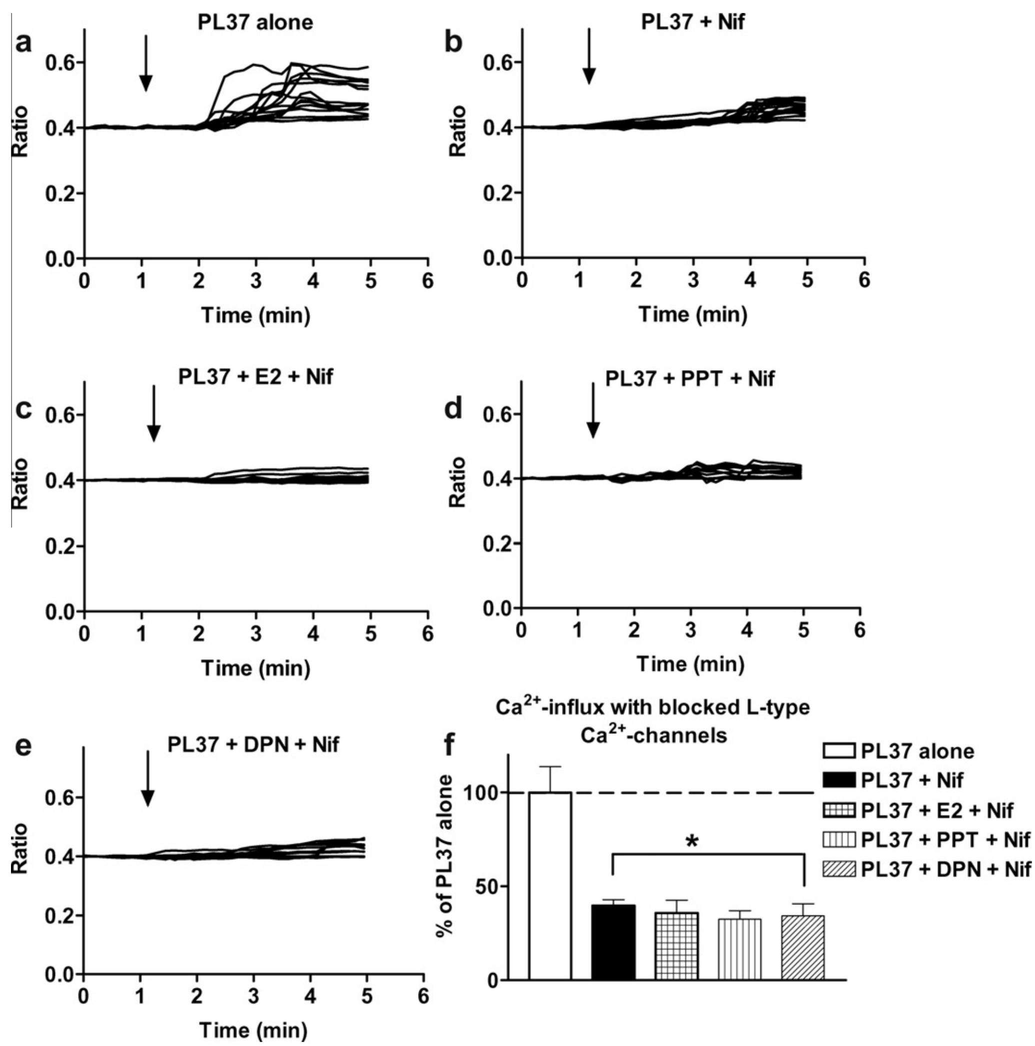


**Fig. 3.** $Ca^{2+}$-influx evoked by the PL37-MAP in the presence of nifedipine (blocker of L-type $Ca^{2+}$-channels), E2, DPN, or PPT in the GT1-7 neurons. (a) and (b) Calcium imaging recordings show that comparing to the control, nifedipine (Nif) eliminated the $Ca^{2+}$-influx significantly. (c)–(e) This decrease was independent from the presence of the E2, DPN, or PPT, demonstrating role of the L-type $Ca^{2+}$-channels. (f) The histogram of the area-under-curve data shows the values expressed in the percentage of the $Ca^{2+}$-influx measured with the PL37-MAP alone (PL37 + Nif: 39.7 ± 3.29%; PL37 + E2 + Nif: 35.8 ± 6.94%; PL37 + PPT + Nif: 32.4 ± 4.66%; PL37 + DPN + Nif: 32.4 ± 4.66%). $^{*}p < 0.05$. Arrow shows the onset of the administration of the PL37-MAP.

PL37-MAP was also examined when CPA (10 μM, 30 min) was present in the PBS. Depletion of the $Ca^{2+}$-store in the endoplasmic reticulum by CPA resulted in ablation of the increase in the intracellular free $Ca^{2+}$-content (3.2 ± 0.66% of the $Ca^{2+}$-increase evoked by PL37-MAP in $Ca^{2+}$-containing extracellular fluid). Application of PL37-MAP in PBS in the presence of E2, DPN, or PPT (20 nM, 24 h) demonstrated significant decrease of the $Ca^{2+}$-response (E2: 16.9 ± 1.63%; PPT: 22.9 ± 5.82%; DPN: 27.5 ± 5.45% of the $Ca^{2+}$-increase evoked by PL37-MAP in $Ca^{2+}$-containing extracellular fluid), however, these data did not differ from the $Ca^{2+}$-increase evoked by PL37-MAP in $Ca^{2+}$-free solution.

### *3.4. L-type but not the R-type voltage-gated $Ca^{2+}$-channels are involved in the PL37-MAP-evoked $Ca^{2+}$-influx*

In order to investigate which $Ca^{2+}$-channel was involved in the $Ca^{2+}$-influx triggered by C5aR activation, PL37-MAP was applied to GT1-7 neurons in the presence of blockers of various VGCCs. The L- and the R-type $Ca^{2+}$-channels have been reported as the most abundant ones in the GT1-7 cells (Watanabe et al., 2004), therefore these two channels were examined. Application of nifedipine (10 μM), the inhibitor of the L-VGCC, resulted in a significantly reduced $Ca^{2+}$-influx evoked by PL37-MAP (Fig. 3a–f). Pre-treatment the cells with E2, PPT or DPN (20 nM, 24 h) did not modify effect of nifedipine (nifedipine alone: 39.7 ± 3.29%; E2: 35.8 ± 6.94%; PPT: 32.4 ± 4.66%; DPN: 34.2 ± 6.48% of the data measured with PL37-MAP alone; $p < 0.001$).

In contrast to nifedipine, SNX-482 (100 nM), the inhibitor of the R-type $Ca^{2+}$-channels, had no effect on the changes in the intracellular $Ca^{2+}$-concentration evoked by PL37-MAP (Fig. 4a, b and f). In the presence of SNX-482, the triggered $Ca^{2+}$-influx did not differ from the one measured with PL37-MAP alone (SNX: 81.6 ± 13.95%). In addition, block of the R-type $Ca^{2+}$-channels did not influence effect of the pre-treatment with E2, PPT or DPN (20 nM, 24 h) (Fig. 4c–f). The $Ca^{2+}$-influx increased upon E2 whereas decreased upon DPN pre-treatment significantly (E2: 139.5 ± 14.63%, PPT: 86.6 ± 5.33%, DPN: 43.3 ± 6.15%, $p < 0.001$).

### *3.5. Estrogenic modulation of the transcription of the C5a receptors and the $Ca_v1.3$ subunit of the L-type $Ca^{2+}$-channel in GT1-7 cells*

We examined the effects of E2 and isotype selective ER agonists on the transcription of genes encoding C5a receptors C5aR and C5L2, and L-VGCC subunits $Ca_v1.2$, and $Ca_v1.3$, by real-time PCR. We demonstrated estrogenic regulation of the C5a receptor genes (Table 1). C5ar1 (the classical C5aR) was up-regulated by the three

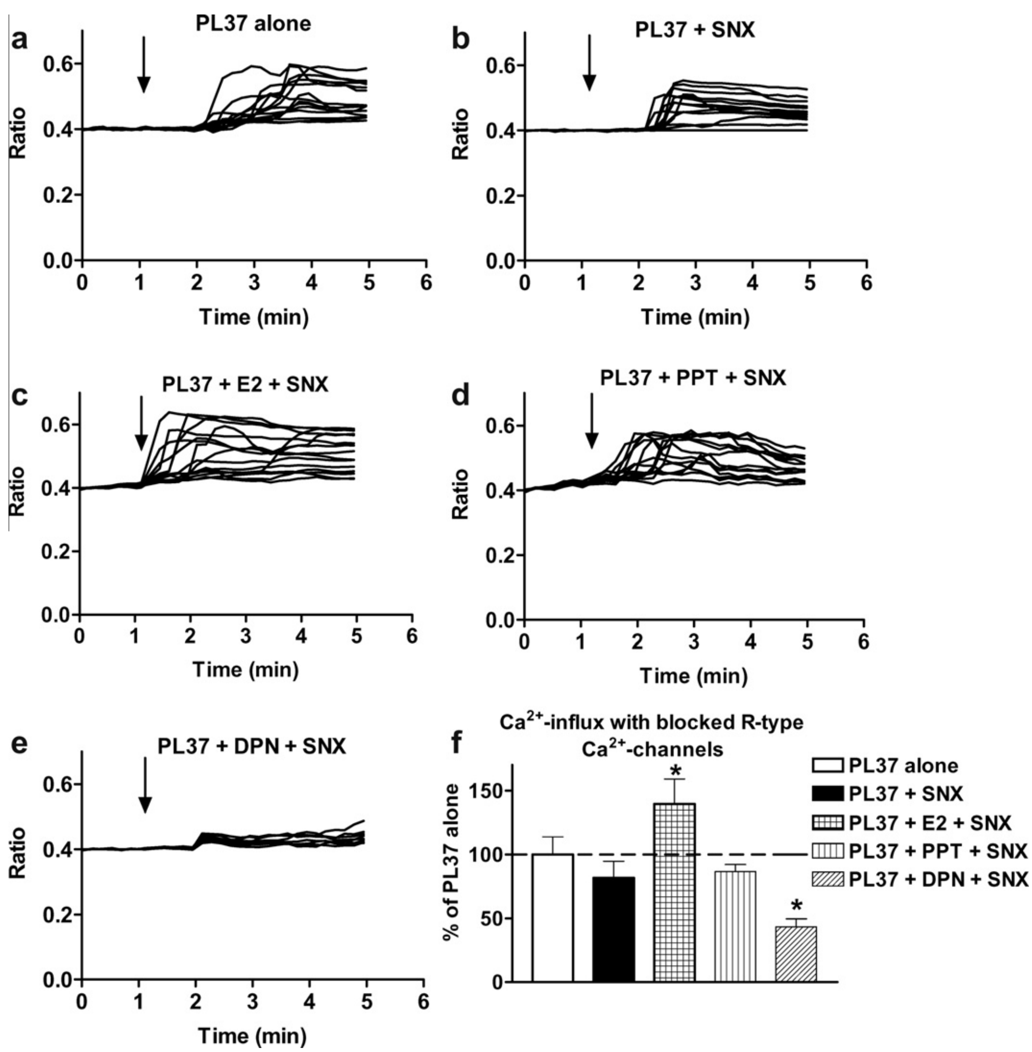


**Fig. 4.** $Ca^{2+}$-influx evoked by the PL37-MAP in the presence of SNX-482 (blocker of R-type $Ca^{2+}$-channels), E2, DPN, or PPT in the GT1-7 neurons. (a)–(e) The ratiometric graphs revealed that SNX-482 (SNX) showed no effect on the $Ca^{2+}$-influx evoked by the PL37-MAP, and it exerted no influence on the effect of the ER agonists, demonstrating that the R-type $Ca^{2+}$-channel plays no role in these processes. (f) The histogram of the area-under-curve data shows the values expressed in the percentage of the $Ca^{2+}$-influx measured with the PL37-MAP alone (PL37 + SNX: 81.6 ± 13.95%; PL37 + E2 + SNX: 139.5 ± 14.63%; PL37 + PPT + SNX: 86.6 ± 5.33%; PL37 + DPN + SNX: 43.3 ± 6.15%). $^{*}p < 0.05$. Arrow shows the onset of the administration of the PL37-MAP.

**Table 1**
Transcriptional modulation of L-type $Ca^{2+}$-channel subunits and C5a receptors. Transcription of L-type $Ca^{2+}$-channel $Ca_v1.2$ (Cacna1c) and $Ca_v1.3$ (Cacna1d) subunits, and C5a receptors CD88 (C5ar1) and C5L2 (Gpr77) was followed by real-time PCR. Table shows the arithmetic mean and standard deviation of relative quantities (RQ) from two independent experiments. Arrows show direction of regulation of the respective transcript where change is significant. The lack of evidence for regulation is denoted with "–".

| Gene symbol and physiological name | E2 | | PPT | | DPN | |
|---|---|---|---|---|---|---|
| Cacna1c (L-type $Ca^{2+}$-channel $Ca_v1.2$ subunit) | 0.867 ± 0.164 | – | 0.905 ± 0.073 | – | 0.748 ± 0.052 | – |
| Cacna1d (L-type $Ca^{2+}$-channel $Ca_v1.3$ subunit) | 0.413 ± 0.326 | ↓ | 0.960 ± 0.023 | – | 0.795 ± 0.117 | – |
| C5ar1 (C5aR) | 2.476 ± 0.715 | ↑ | 2.039 ± 0.241 | ↑ | 1.938 ± 0.743 | ↑ |
| Gpr77 (C5L2) | 1.634 ± 0.336 | ↑ | 0.562 ± 0.101 | ↓ | 0.955 ± 0.577 | – |

ER agonists. C5L2 was regulated differentially, E2 increased while PPT decreased its transcription. Cacna1d (gene for $Ca_v1.3$) was regulated only by E2 whereas Cacna1c ($Ca_v1.2$) showed no estrogenic regulation.

## 4. Discussion

In the present study, we examined further estrogenic modulation of the C5aR agonist-evoked $Ca^{2+}$-response using the GnRH-producing GT1-7 cell line as a neuronal model and applying isotype selective ER agonists. We demonstrated that (i) ERα and ERβ agonists differentially modulated the C5aR agonist-evoked $Ca^{2+}$-influx, (ii) estrogenic modulation was dependent on genomic effects, (iii) $Ca^{2+}$-influx was mediated primarily through L-VGCC, (iv) estrogens up-regulated C5aR mRNA expression while differentially regulated C5L2.

### *4.1. Estrogens differentially modulate the PL37-MAP-evoked $Ca^{2+}$-influx*

Our present results showed that the C5aR agonist-evoked $Ca^{2+}$-influx was differentially mediated by various ER agonists in GT1-7 neurons. Expression of C5aR, and ERα and ERβ has long been reported in various types of neurons (Farkas et al., 2003, 2008; Hrabovszky et al., 2004, 2000, 2001; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Stahel et al., 1997a,b; Wilson et al., 2002; Woodruff et al., 2010). Differential modulation by ERα and ERβ could be important, because $Ca^{2+}$-influx evoked by the activation of C5aR can differentially affect functions of a neuronal cell, such as firing pattern, shape of after-hyperpolarisation and depolarising after-potentials, neurotransmitter release, plasticity, gene transcription, and vulnerability (Berridge, 1998; Zuccotti et al., 2011).

In the present experiments DPN decreased the $Ca^{2+}$-influx evoked by PL37-MAP. Nevertheless, the amplitude of the inward ion current in the ERβ-expressing GnRH neurons from slices obtained from E2 substituted mice was higher than those from ovariectomized mice (Farkas et al., 2008). The reasons of the discrepancy may lie in the differences between the two models.

### *4.2. Estrogenic modulation of the evoked $Ca^{2+}$-influx is dependent on genomic effects*

Estrogenic modulation of the $Ca^{2+}$-signal evoked by the activated C5aR was genomic rather than rapid in our experiments. Numerous estradiol-regulated genes have already been identified in GT1-7 neurons by expression profiling (Varju et al., 2009). Majority of the responding genes were up-regulated in these cells, including potassium channel subunits and transporters, transcription factors, molecules related to cell death, immune response, neurotransmitter, hormone and neuropeptide receptors, regulators of G-protein signaling. Those results support our present data showing up-regulation of C5aR.

In our present experiments, we found no acute effect of E2 on the $Ca^{2+}$-influx evoked in the GT1-7 cells. In another model, published from another laboratory (Sun et al., 2010), both genomic and rapid changes resulted from the E2 administration were reported by potentiating the $Ca^{2+}$-current in GnRH neurons in the acute brain slice in 5 min. This report described, however, that percentage of the responding cells depended upon the concentration of E2 and only doses of E2 much higher than used in our experiments could evoke response with high rate of success. In, addition, the GnRH neurons presented an "all or none" ability to respond to acutely administered E2 (Sun et al., 2010). The observed discrepancies may reflect differences in the basic physiology and regulation of GnRH neurons integrated within the preoptic brain slice preparation versus the immortalised GT1-7 neurons cultured *in vitro*.

### *4.3. L-type but not R-type $Ca^{2+}$-channels are involved in the PL37-MAP-evoked $Ca^{2+}$-influx*

In GT1-7 cells, the two major VGCCs are the L- and R-type channels (Watanabe et al., 2004). Both of them play a critical role in the regulation of $Ca^{2+}$-dependent GnRH-release. In addition, R-type $Ca^{2+}$-channels have been reported to be involved in the release of neurotransmitters in calyx-type synapses of the medial nucleus of the trapezoid body, oxytocin neurons, and adrenal chromaffin cells (Albillos et al., 2000; Wang et al., 1999; Wu et al., 1998, 1999). R-type channels are responsible for the dendritic $Ca^{2+}$-influx induced by action potentials in CA1 pyramidal neurons of the hippocampus (Magee and Johnston, 1995; Sabatini and Svoboda, 2000). L-VGCCs contribute to $Ca^{2+}$-dependent gene transcription and can modulate firing properties of neurons (Gomez-Ospina et al., 2006; Zuccotti et al., 2011). Our results have now revealed that the $Ca^{2+}$-ion current resulted from the activation of the C5aR in GT1-7 neurons passed through the L-VGCCs but not via R-type channels. In addition, differential modulation of the $Ca^{2+}$-influx by ER agonists affected the function of the L-type channel. These data suggest that physiological functions of the L-VGCC such as regulation of the GnRH release, parameters of the firing, and various gene transcriptional events are affected by C5aR activation. Modulation of the L-VGCC by E2 and DPN has recently been reported in GnRH neurons of the acute brain slice of the mice demonstrating changes in the ion current via these channels under physiological conditions (Sun et al., 2010). Similarly, modulation of the T-type voltage-gated $Ca^{2+}$-channels by estradiol was also demonstrated (Bosch et al., 2009; Qiu et al., 2006; Zhang et al., 2009).

It is an intriguing question, how the activation of C5aR can regulate opening of a VGCC. One possibility is a change in the threshold level by phosphorylation of the L-VGCC, occurring as a downstream event of the C5aR activation. G-protein-coupled receptor activation can result in activation of diverse pathways involving enzymes such as protein kinase A or protein kinase C yielding phosphorylation of the L-VGCC, in particular its $Ca_v1.2$ or $Ca_v1.3$ subunits (Dai et al., 2009; Dolphin, 2009). This phosphorylation could eventually modify various electric parameters of the neurons such as open probability of the channel or the threshold level. Nevertheless, the existence of a C5aR-related phosphorylation of L-VGCC requires further examination. Other G-protein mediated mechanisms can also be involved such as direct G-protein related modulation of the L-VGCCs (Currie, 2010; Tedford and Zamponi, 2006). In this paradigm, the L-VGCC molecule possesses residues interacting directly with the βγ subunits of the G-protein. The interaction is membrane-delimited, i.e. involves a second messenger molecule that remains associated with the plasma membrane, rather than diffusing to the channel via a cytoplasmic pathway (Hille, 1994).

Our $Ca^{2+}$-imaging measurements showed that in addition to the extracellular sources, intracellular $Ca^{2+}$-stores were also involved in the elevation of the $Ca^{2+}$-content evoked by the activation of the C5aR. The intracellular $Ca^{2+}$-stores could be triggered to release $Ca^{2+}$ directly via the C5aR-related signal transduction pathway (Nishiura et al., 2010). Another possible pathway for this action is that C5aR-activation opens the L-VGCCs first and then the L-VGCCs activate the intracellular $Ca^{2+}$-stores via a putative coupling (Kim et al., 2007; Kolarow et al., 2007), however, these opportunities require further elaboration.

### *4.4. C5aR agonist and estrogens modulate function of L-VGCC crucial in firing*

The characteristic firing pattern is a crucial feature of the hormone secreting neurons. Pulsatile release of the GnRH, for example, is indispensable for the proper function of the reproductive system (Moenter et al., 2003). In addition, the synchronous firing of the GnRH-producing neurons correlates with this pulsatility (Moenter et al., 2003). The $Ca^{2+}$-channels mediates how the neurons fire, therefore, effects disturbing the intracellular $Ca^{2+}$-milieu could have an effect on the firing properties of the GnRH cells and consequently, the pulsatile secretion of GnRH. The L-VGCC is considered as one of the key mediators of the firing pattern (Zuccotti et al., 2011). Its $Ca_v1.2$ and $Ca_v1.3$ subunits are expressed in neurons and endocrine cells, such as pancreatic beta, adrenal chromaffin cells (Catterall et al., 2005) and contribute to the spontaneous firing and pacemaking of the neurons (Zuccotti et al., 2011). However, literature data show activity-dependent differences between them. $Ca_v1.3$ is more effective at low levels of activity such as during interburst intervals, and $Ca_v1.2$ is more efficient at high levels of activity such as during interspike intervals in the bursts (Zhang et al., 2006). It has recently been reported that E2 decreases mRNA expression of the $Ca_v1.2$, but has no effect on the $Ca_v1.3$ subunit in the hippocampus of aged female rats (Brewer et al., 2009). Our real-time PCR measurements revealed that the $Ca_v1.3$ subunit was down-regulated by E2 in GT1-7 cells. This discrepancy might originate from the cell-type differences in the two experimental models.

The point of convergence of the signals coming from C5aR and ERs, was not the expression of the $Ca^{2+}$-channel, because the $Ca_v1.2$ and $Ca_v1.3$ subunits were not regulated by DPN and PPT. Therefore, our present data suggest that any differential transcriptional regulation of elements of C5a/C5aR signaling which might be involved in the explored differential effects of the ER agonists on the evoked $Ca^{2+}$-influx should be upstream of L-VGCC. Possible candidates could be the regulator molecules of G-protein signaling (RGS2, RGS9 and RGS 10) which were earlier shown to be regulated by E2 in the GT1-7 cells (Varju et al., 2009).

### *4.5. C5aR is up-regulated by ER agonists*

In our studies, the expression of classical C5aR was up-regulated by E2 and the used isotype selective ER agonists, suggesting that neurons could respond more effectively to the inflammatory mediator C5a in the presence of estrogens. In contrast, the expression of C5L2 was differentially regulated by ER agonists, displaying the up-regulation of this receptor by E2 and the attenuation of its expression by PPT. As the decoy receptor modulates the performance of the classical C5aR, the elucidation of this inverse regulatory trend warrants further investigation.

The results of the present *in vitro* study raise the questions of how activation of C5aR could occur by its ligand, the C5a in hormone secreting neurons *in vivo*. The hypophysiotrophic axonal projections of numerous hormone secreting neurons terminate outside the blood brain barrier, suggesting that these neurons are capable of monitoring C5a released either in the hypothalamus or the blood. Since several rodent hypothalamic neurons have been shown to express functional C5a receptors (Farkas et al., 2008), it is reasonable to assume that the inflammatory mediator C5a can alter the physiological properties and cellular functions of these neurons. In these mechanisms, the $Ca^{2+}$-influx occurring via L-VGCCs might have a pivotal role.

## 5. Conclusions

Summing up, this study provided evidence that C5aR-mediated $Ca^{2+}$-signaling can be differentially modulated via ERα and ERβ. In addition, estrogens potentiate the sensitivity of GT1-7 neurons for C5a by up-regulation of C5aR through ERα and ERβ. C5aR activation leads to $Ca^{2+}$-influx through L-VGCCs. Although the transcription of the $Ca_v1.3$ L-VGCC subunit is regulated by E2, the isotype specific ligands had no effect, indicating that these subunits are not the primary targets of ERα and ERβ agonist actions upon C5a/C5aR signaling. The significance of the present findings relates to the better understanding of the differential impact of estrogens on the C5a-evoked response of neurons which express ERα and/or ERβ.

## Disclosure summary

The authors have nothing to disclose.

## Acknowledgement

We thank Drs. Tibor Zelles and Lajos Baranyi for their invaluable advices. We are grateful to Dr. Pamela Mellon for providing us the GT1-7 neuronal cell line.

This research was supported by Grants from the Hungarian Scientific Research Fund (OTKA T73002) and the Hungarian Medical Research Foundation (403-03). The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under Grant agreement No. 245009.

## References

Albillos, A., Neher, E., Moser, T., 2000. R-Type $Ca^{2+}$ channels are coupled to the rapid component of secretion in mouse adrenal slice chromaffin cells. J. Neurosci. 20, 8323–8330.

Bamberg, C.E., Mackay, C.R., Lee, H., Zahra, D., Jackson, J., Lim, Y.S., Whitfeld, P.L., Craig, S., Corsini, E., Lu, B., Gerard, C., Gerard, N.P., 2010. The C5a receptor (C5aR) C5L2 is a modulator of C5aR-mediated signal transduction. J. Biol. Chem. 285, 7633–7644.

Baranyi, L., Campbell, W., Ohshima, K., Fujimoto, S., Boros, M., Okada, H., 1995. The antisense homology box: a new motif within proteins that encodes biologically active peptides. Nat. Med. 1, 894–901.

Baranyi, L., Campbell, W., Okada, H., 1996. Antisense homology boxes in C5a receptor and C5a anaphylatoxin: a new method for identification of potentially active peptides. J. Immunol. 157, 4591–4601.

Berridge, M.J., 1998. Neuronal calcium signaling. Neuron 21, 13–26.

Bosch, M.A., Hou, J., Fang, Y., Kelly, M.J., Ronnekleiv, O.K., 2009. 17Beta-estradiol regulation of the mRNA expression of T-type calcium channel subunits: role of estrogen receptor alpha and estrogen receptor beta. J. Comp. Neurol. 512, 347–358.

Brewer, L.D., Dowling, A.L., Curran-Rauhut, M.A., Landfield, P.W., Porter, N.M., Blalock, E.M., 2009. Estradiol reverses a calcium-related biomarker of brain aging in female rats. J. Neurosci. 29, 6058–6067.

Catterall, W.A., Perez-Reyes, E., Snutch, T.P., Striessnig, J., 2005. International Union of Pharmacology. XLVIII. Nomenclature and structure–function relationships of voltage-gated calcium channels. Pharmacol. Rev. 57, 411–425.

Christian, C.A., Mobley, J.L., Moenter, S.M., 2005. Diurnal and estradiol-dependent changes in gonadotropin-releasing hormone neuron firing activity. Proc. Natl. Acad. Sci. USA 102, 15682–15687.

Currie, K.P., 2010. G protein modulation of $Ca_V2$ voltage-gated calcium channels. Channels (Austin) 4, 497–509.

Dai, S., Hall, D.D., Hell, J.W., 2009. Supramolecular assemblies and localized regulation of voltage-gated ion channels. Physiol. Rev. 89, 411–452.

Dolphin, A.C., 2009. Calcium channel diversity: multiple roles of calcium channel subunits. Curr. Opin. Neurobiol. 19, 237–244.

Farkas, I., Baranyi, L., Kaneko, Y., Liposits, Z., Yamamoto, T., Okada, H., 1999. C5a receptor expression by TGW neuroblastoma cells. Neuroreport 10, 3021–3025.

Farkas, I., Baranyi, L., Liposits, Z.S., Yamamoto, T., Okada, H., 1998a. Complement C5a anaphylatoxin fragment causes apoptosis in TGW neuroblastoma cells. Neuroscience 86, 903–911.

Farkas, I., Baranyi, L., Takahashi, M., Fukuda, A., Liposits, Z., Yamamoto, T., Okada, H., 1998b. A neuronal C5a receptor and an associated apoptotic signal transduction pathway. J. Physiol. 507 (Pt 3), 679–687.

Farkas, I., Kallo, I., Deli, L., Vida, B., Hrabovszky, E., Fekete, C., Moenter, S.M., Watanabe, M., Liposits, Z., 2010. Retrograde endocannabinoid signaling reduces GABAergic synaptic transmission to gonadotropin-releasing hormone neurons. Endocrinology 151, 5818–5829.

Farkas, I., Takahashi, M., Fukuda, A., Yamamoto, N., Akatsu, H., Baranyi, L., Tateyama, H., Yamamoto, T., Okada, N., Okada, H., 2003. Complement C5a receptor-mediated signaling may be involved in neurodegeneration in Alzheimer's disease. J. Immunol. 170, 5764–5771.

Farkas, I., Varju, P., Liposits, Z., 2007. Estrogen modulates potassium currents and expression of the Kv4.2 subunit in GT1-7 cells. Neurochem. Int. 50, 619–627.

Farkas, I., Varju, P., Szabo, E., Hrabovszky, E., Okada, N., Okada, H., Liposits, Z., 2008. Estrogen enhances expression of the complement C5a receptor and the C5a-agonist evoked calcium influx in hormone secreting neurons of the hypothalamus. Neurochem. Int. 52, 846–856.

Fonseca, M.I., Ager, R.R., Chu, S.H., Yazan, O., Sanderson, S.D., LaFerla, F.M., Taylor, S.M., Woodruff, T.M., Tenner, A.J., 2009. Treatment with a C5aR antagonist decreases pathology and enhances behavioral performance in murine models of Alzheimer's disease. J. Immunol. 183, 1375–1383.

Fonseca, M.I., Chu, S.H., Berci, A.M., Benoit, M.E., Peters, D.G., Kimura, Y., Tenner, A.J., 2011. Contribution of complement activation pathways to neuropathology differs among mouse models of Alzheimer's disease. J. Neuroinflammation 8, 4.

Fujita, E., Farkas, I., Campbell, W., Baranyi, L., Okada, H., Okada, N., 2004. Inactivation of C5a anaphylatoxin by a peptide that is complementary to a region of C5a. J. Immunol. 172, 6382–6387.

Gomez-Ospina, N., Tsuruta, F., Barreto-Chang, O., Hu, L., Dolmetsch, R., 2006. The C terminus of the L-type voltage-gated calcium channel Ca(V)1.2 encodes a transcription factor. Cell 127, 591–606.

Guo, R.F., Ward, P.A., 2005. Role of C5a in inflammatory responses. Annu. Rev. Immunol. 23, 821–852.

Hille, B., 1994. Modulation of ion-channel function by G-protein-coupled receptors. Trends Neurosci. 17, 531–536.

Hrabovszky, E., Kallo, I., Steinhauser, A., Merchenthaler, I., Coen, C.W., Petersen, S.L., Liposits, Z., 2004. Estrogen receptor-beta in oxytocin and vasopressin neurons of the rat and human hypothalamus: Immunocytochemical and in situ hybridization studies. J. Comp. Neurol. 473, 315–333.

Hrabovszky, E., Shughrue, P.J., Merchenthaler, I., Hajszan, T., Carpenter, C.D., Liposits, Z., Petersen, S.L., 2000. Detection of estrogen receptor-beta messenger ribonucleic acid and 125I-estrogen binding sites in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology 141, 3506–3509.

Hrabovszky, E., Steinhauser, A., Barabas, K., Shughrue, P.J., Petersen, S.L., Merchenthaler, I., Liposits, Z., 2001. Estrogen receptor-beta immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. Endocrinology 142, 3261–3264.

Karsch, F.J., Battaglia, D.F., Breen, K.M., Debus, N., Harris, T.G., 2002. Mechanisms for ovarian cycle disruption by immune/inflammatory stress. Stress 5, 101–112.

Kim, S., Yun, H.M., Baik, J.H., Chung, K.C., Nah, S.Y., Rhim, H., 2007. Functional interaction of neuronal $Ca_v1.3$ L-type calcium channel with ryanodine receptor type 2 in the rat hippocampus. J. Biol. Chem. 282, 32877–32889.

Kolarow, R., Brigadski, T., Lessmann, V., 2007. Postsynaptic secretion of BDNF and NT-3 from hippocampal neurons depends on calcium calmodulin kinase II signaling and proceeds via delayed fusion pore opening. J. Neurosci. 27, 10350–10364.

Liposits, Z., Merchenthaler, I., Wetsel, W.C., Reid, J.J., Mellon, P.L., Weiner, R.I., Negro-Vilar, A., 1991. Morphological characterization of immortalized hypothalamic neurons synthesizing luteinizing hormone-releasing hormone. Endocrinology 129, 1575–1583.

Magee, J.C., Johnston, D., 1995. Characterization of single voltage-gated $Na^+$ and $Ca^{2+}$ channels in apical dendrites of rat CA1 pyramidal neurons. J. Physiol. 487 (Pt. 1), 67–90.

Moenter, S.M., DeFazio, A.R., Pitts, G.R., Nunemaker, C.S., 2003. Mechanisms underlying episodic gonadotropin-releasing hormone secretion. Front Neuroendocrinol. 24, 79–93.

Nishiura, H., Tokita, K., Li, Y., Harada, K., Woodruff, T.M., Taylor, S.M., Nsiama, T.K., Nishino, N., Yamamoto, T., 2010. The role of the ribosomal protein S19 C-terminus in Gi protein-dependent alternative activation of p38 MAP kinase via the C5a receptor in HMC-1 cells. Apoptosis 15, 966–981.

Qiu, J., Bosch, M.A., Jamali, K., Xue, C., Kelly, M.J., Ronnekleiv, O.K., 2006. Estrogen upregulates T-type calcium channels in the hypothalamus and pituitary. J. Neurosci. 26, 11072–11082.

Roy, D., Angelini, N.L., Belsham, D.D., 1999. Estrogen directly respresses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor-alpha (ERalpha)- and ERbeta-expressing GT1-7 GnRH neurons. Endocrinology 140, 5045–5053.

Sabatini, B.L., Svoboda, K., 2000. Analysis of calcium channels in single spines using optical fluctuation analysis. Nature 408, 589–593.

Sarvari, M., Kallo, I., Hrabovszky, E., Solymosi, N., Toth, K., Liko, I., Molnar, B., Tihanyi, K., Liposits, Z., 2010. Estradiol replacement alters expression of genes related to neurotransmission and immune surveillance in the frontal cortex of middle-aged, ovariectomized rats. Endocrinology 151, 3847–3862.

Shughrue, P.J., Lane, M.V., Merchenthaler, I., 1997. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. J. Comp. Neurol. 388, 507–525.

Shughrue, P.J., Merchenthaler, I., 2001. Distribution of estrogen receptor beta immunoreactivity in the rat central nervous system. J. Comp. Neurol. 436, 64–81.

Speth, C., Prodinger, W.M., Wurzner, R., Stoiber, H., Dierich, M.P., 2008. Complement. In: Paul, W.E. (Ed.), Fundamental Immunology, 6th ed. Lippincott Williams and Wilkins, Philadelphia, pp. 1047–1078.

Stahel, P.F., Frei, K., Eugster, H.P., Fontana, A., Hummel, K.M., Wetsel, R.A., Ames, R.S., Barnum, S.R., 1997a. TNF-alpha-mediated expression of the receptor for anaphylatoxin C5a on neurons in experimental Listeria meningoencephalitis. J. Immunol. 159, 861–869.

Stahel, P.F., Kossmann, T., Morganti-Kossmann, M.C., Hans, V.H., Barnum, S.R., 1997b. Experimental diffuse axonal injury induces enhanced neuronal C5a receptor mRNA expression in rats. Brain Res. Mol. Brain Res. 50, 205–212.

Sun, J., Chu, Z., Moenter, S.M., 2010. Diurnal in vivo and rapid in vitro effects of estradiol on voltage-gated calcium channels in gonadotropin-releasing hormone neurons. J. Neurosci. 30, 3912–3923.

Tedford, H.W., Zamponi, G.W., 2006. Direct G protein modulation of $Ca_v2$ calcium channels. Pharmacol. Rev. 58, 837–862.

Thiery, J.C., Pelletier, J., 1981. Multiunit activity in the anterior median eminence and adjacent areas of the hypothalamus of the ewe in relation to LH secretion. Neuroendocrinology 32, 217–224.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome. Biol. 3, RESEARCH0034.

Varju, P., Chang, K.C., Hrabovszky, E., Merchenthaler, I., Liposits, Z., 2009. Temporal profile of estrogen-dependent gene expression in LHRH-producing GT1-7 cells. Neurochem. Int. 54, 119–134.

Wang, G., Dayanithi, G., Newcomb, R., Lemos, J.R., 1999. An R-type Ca(2+) current in neurohypophysial terminals preferentially regulates oxytocin secretion. J. Neurosci. 19, 9235–9241.

Watanabe, M., Sakuma, Y., Kato, M., 2004. High expression of the R-type voltage-gated $Ca^{2+}$ channel and its involvement in $Ca^{2+}$-dependent gonadotropin-releasing hormone release in GT1-7 cells. Endocrinology 145, 2375–2383.

Wetsel, W.C., Valenca, M.M., Merchenthaler, I., Liposits, Z., Lopez, F.J., Weiner, R.I., Mellon, P.L., Negro-Vilar, A., 1992. Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. Proc. Natl. Acad. Sci. USA 89, 4149–4153.

Wilson, M.E., Rosewell, K.L., Kashon, M.L., Shughrue, P.J., Merchenthaler, I., Wise, P.M., 2002. Age differentially influences estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) gene expression in specific regions of the rat brain. Mech. Ageing Dev. 123, 593–601.

Wilson, R.C., Kesner, J.S., Kaufman, J.M., Uemura, T., Akema, T., Knobil, E., 1984. Central electrophysiologic correlates of pulsatile luteinizing hormone secretion in the rhesus monkey. Neuroendocrinology 39, 256–260.

Woodruff, T.M., Ager, R.R., Tenner, A.J., Noakes, P.G., Taylor, S.M., 2010. The role of the complement system and the activation fragment C5a in the central nervous system. Neuromolecular Med. 12, 179–192.

Woodruff, T.M., Nandakumar, K.S., Tedesco, F., 2011. Inhibiting the C5–C5a receptor axis. Mol. Immunol.

Wu, L.G., Borst, J.G., Sakmann, B., 1998. R-type $Ca^{2+}$ currents evoke transmitter release at a rat central synapse. Proc. Natl. Acad. Sci. USA 95, 4720–4725.

Wu, L.G., Westenbroek, R.E., Borst, J.G., Catterall, W.A., Sakmann, B., 1999. Calcium channel types with distinct presynaptic localization couple differentially to transmitter release in single calyx-type synapses. J. Neurosci. 19, 726–736.

Zhang, C., Bosch, M.A., Rick, E.A., Kelly, M.J., Ronnekleiv, O.K., 2009. 17Beta-estradiol regulation of T-type calcium channels in gonadotropin-releasing hormone neurons. J. Neurosci. 29, 10552–10562.

Zhang, H., Fu, Y., Altier, C., Platzer, J., Surmeier, D.J., Bezprozvanny, I., 2006. Ca1.2 and $Ca_V1.3$ neuronal L-type calcium channels: differential targeting and signaling to pCREB. Eur. J. Neurosci. 23, 2297–2310.

Zuccotti, A., Clementi, S., Reinbothe, T., Torrente, A., Vandael, D.H., Pirone, A., 2011. Structural and functional differences between L-type calcium channels: crucial issues for future selective targeting. Trends Pharmacol. Sci. 32, 366–375.