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

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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, ERα and ERβ, to estrogenic modulation of intracellular Ca^{2+}-content. GT1-7 neurons were treated with isotype selective ER agonists for 24h 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 (100pM), but the ERα selective agonist PPT (100pM) enhanced the PL37-MAP-evoked Ca^{2+}-influx (E2: 215%, PPT: 175%, compared to the PL37-MAP-evoked Ca^{2+}-influx). In contrast, the ERβ selective agonist DPN (100pM) 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 (8min) 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.

Keywords: GT1-7 neuron, complement C5a receptor, estrogen receptor alpha, estrogen receptor beta; voltage-gated calcium channel

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

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).

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; Farkas et al., 1998a; Farkas et al., 1998b). 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; Fonseca et al., 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 co-ordinated 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 24hrs. 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 (100pM - 20nM) for 24h 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 8min 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 (1μM; Molecular Probes, Eugene, Oregon) in loading buffer Hanks' Balanced Salt Solution (HBSS) containing 0.1% DMSO and Pluronic-F127 (1μM, Molecular Probes) for 1.5h 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, 2.5μM). 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 1min 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 1min 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, 10μM, 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 30min before starting the measurements.

The VGCC blockers nifedipine (10μM, SIGMA) and SNX-482 (100nM) 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 250nM for the probe and 900nM 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 (ΔΔCt) were used for analysis of Applied Biosystems TaqMan gene expression assays. Two house-keeping genes (Gapdh, Hprt) were applied as internal controls. The geometric mean of Ct values of Gapdh and Hprt1 was used for subsequent ΔCt 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 ($n \geq 7$) 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 (2.5μM) triggered robust Ca^{2+}-influx in GT1-7 neurons (Fig. 1a). Onset of the elevation of intracellular Ca^{2+}-concentration was within 1-1.5min after application of the peptide. When the neurons were pre-treated with E2 (100pM, 24h), the evoked Ca^{2+}- influx started earlier (in less than 30 seconds) 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 (100pM, 24h) 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 (100pM, 24h), the elevation of the Ca^{2+}-content was much lower and started in 1.5-2min 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 (100pM, 24h) or PPT (100pM, 24h), revealing facilitation of the PL37-MAP-evoked Ca^{2+}-influx by both E2 and PPT. On the other hand, DPN (100pM, 24h) 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 (20nM, 24h) increased the PL37-MAP-evoked Ca^{2+}-influx whereas DPN (20nM, 24h) 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 (8min) 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 PL37-MAP was also examined when CPA (10μM, 30min) 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 (20nM, 24h) 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 (20nM, 24h) 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 (100nM), 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, 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) (Figs. 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 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; Farkas et al., 2008; Hrabovszky et al., 2004; Hrabovszky et al., 2000; Hrabovszky et al., 2001; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Stahel et al., 1997a; Stahel et al., 1997b; 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 5min. 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; Wu et al., 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 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.

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Figure legends

Figure 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 (100pM, 24h) 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 (100pM, 24h) resulted in an elevated Ca^{2+}-influx. d) DPN pre-treatment (100pM, 24h), 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 (8min) 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.

Figure 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.

Figure 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-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.

Figure 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 legend

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 "-".

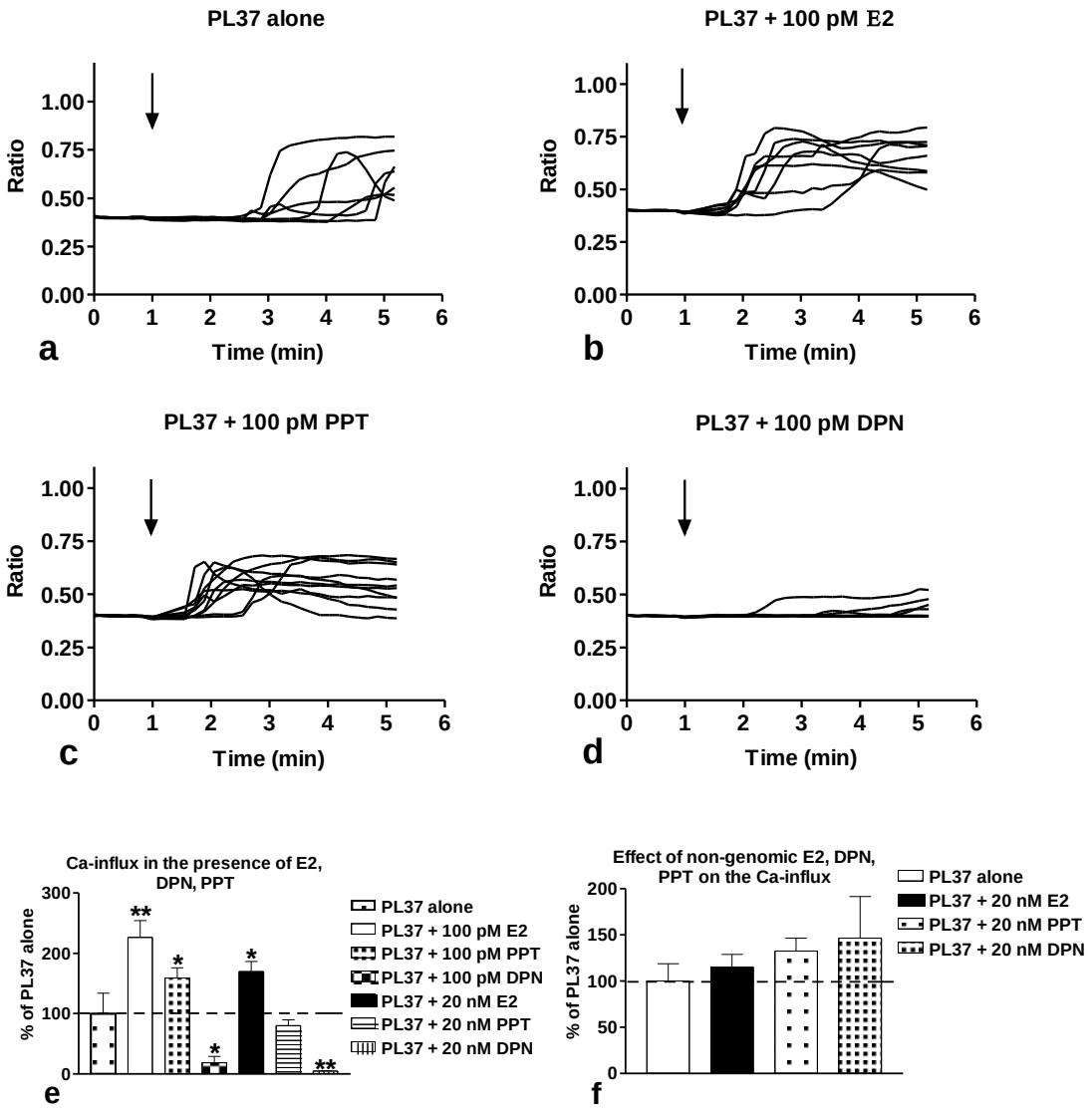
PL37 alone
PL37 + 100 pM E2
PL37 + 100 pM PPT
PL37 + 100 pM DPN
Ratio
Time (min)
a
b
c
d
Ca-influx in the presence of E2, DPN, PPT
% of PL37 alone
PL37 alone
PL37 + 100 pM E2
PL37 + 100 pM PPT
PL37 + 100 pM DPN
PL37 + 20 nM E2
PL37 + 20 nM PPT
PL37 + 20 nM DPN
e
Effect of non-genomic E2, DPN, PPT on the Ca-influx
PL37 alone
PL37 + 20 nM E2
PL37 + 20 nM PPT
PL37 + 20 nM DPN
f

Fig. 1.

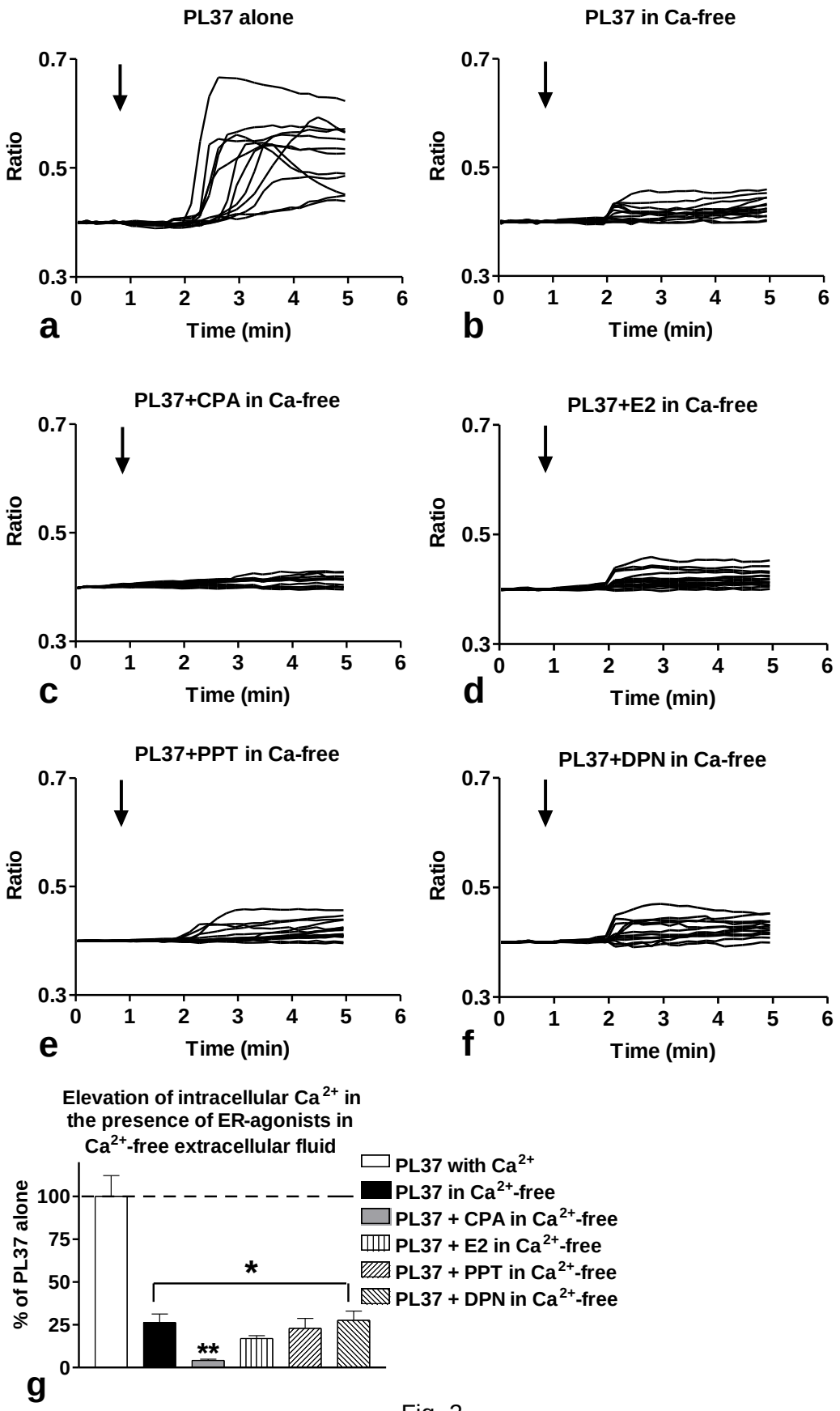
PL37 alone
PL37 in Ca-free
PL37+CPA in Ca-free
PL37+E2 in Ca-free
PL37+PPT in Ca-free
PL37+DPN in Ca-free
Ratio
Time (min)
a
b
c
d
e
f
Elevation of intracellular Ca2+ in the presence of ER-agonists in Ca2+-free extracellular fluid
% of PL37 alone
PL37 with Ca2+
PL37 in Ca2+-free
PL37 + CPA in Ca2+-free
PL37 + E2 in Ca2+-free
PL37 + PPT in Ca2+-free
PL37 + DPN in Ca2+-free
*
**
g

Fig. 2.

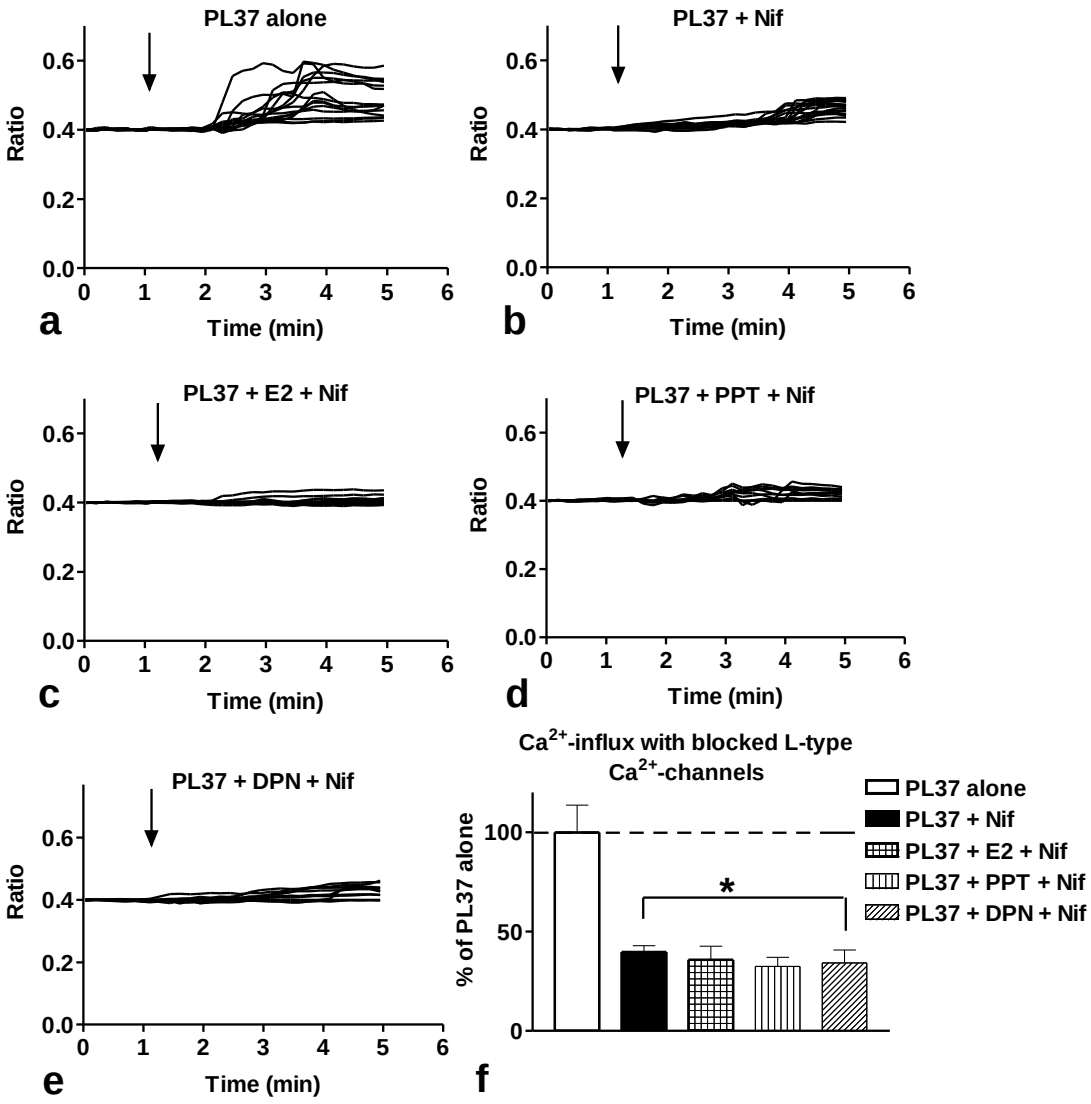
PL37 alone
PL37 + Nif
PL37 + E2 + Nif
PL37 + PPT + Nif
PL37 + DPN + Nif
Ratio
Time (min)
a
b
c
d
e
f
Ca2+-influx with blocked L-type Ca2+-channels
% of PL37 alone
PL37 alone
PL37 + Nif
PL37 + E2 + Nif
PL37 + PPT + Nif
PL37 + DPN + Nif
*

Fig. 3.

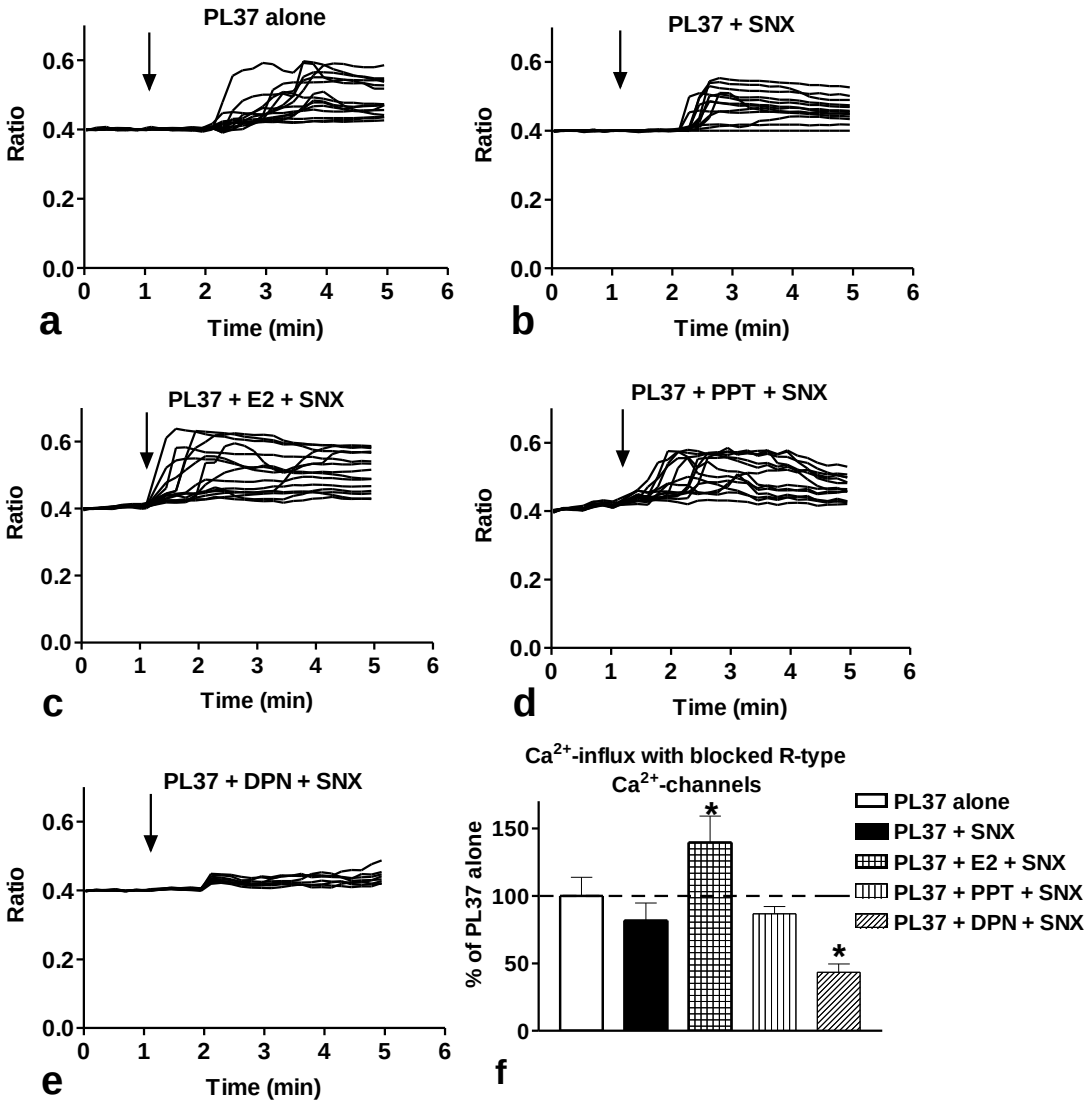
PL37 alone
PL37 + SNX
PL37 + E2 + SNX
PL37 + PPT + SNX
PL37 + DPN + SNX
Ratio
Time (min)
a
b
c
d
e
f
Ca2+-influx with blocked R-type Ca2+-channels
% of PL37 alone
PL37 alone
PL37 + SNX
PL37 + E2 + SNX
PL37 + PPT + SNX
PL37 + DPN + SNX

Fig. 4.

Gene symbol and physiologic 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	–

Table 1.