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*Published in:*

Journal of Vascular Research

*DOI:*

[10.1159/000354252](https://doi.org/10.1159/000354252)

2013

[Link to publication](#)

*Citation for published version (APA):*

Holm, A., Hellstrand, P., Olde, B., Svensson, D., Leeb-Lundberg, F., & Nilsson, B.-O. (2013). The G Protein-Coupled Estrogen Receptor 1 (GPER1/GPR30) Agonist G-1 Regulates Vascular Smooth Muscle Cell Ca Handling. *Journal of Vascular Research*, 50(5), 421-429. <https://doi.org/10.1159/000354252>

*Total number of authors:*

6

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# **The G protein-coupled estrogen receptor 1 (GPER1/GPR30) agonist G-1 regulates vascular smooth muscle cell Ca<sup>2+</sup> handling**

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**Running head:** GPER1 and Ca<sup>2+</sup> handling

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## Abstract

The G protein-coupled estrogen receptor GPER1/GPR30 is implicated in blood pressure regulation but the mechanisms are not identified. Here, we hypothesize that GPER1 controls blood pressure by regulating vascular smooth muscle cell  $\text{Ca}^{2+}$  handling. Treatment with the GPER1 agonist G-1, in the  $\mu\text{M}$  concentration range, acutely reduced spontaneous and synchronous  $\text{Ca}^{2+}$  spike activity in A7r5 vascular smooth muscle cells expressing mRNA for GPER1. Furthermore, G-1 (1  $\mu\text{M}$ ) attenuated thromboxane A2 analogue U46619-stimulated  $\text{Ca}^{2+}$  spike activity but had no effect on U46619-induced increase in basal level of  $\text{Ca}^{2+}$ . The voltage-sensitive L-type  $\text{Ca}^{2+}$  channel blocker nifedipine (100 nM) reduced  $\text{Ca}^{2+}$  spike activity similar to G-1. Pharmacological, but not physiological, concentration of the estrogen  $17\beta$ -estradiol reduced  $\text{Ca}^{2+}$  spike activity. The GPER1 antagonist G-15 blocked G-1-induced down-regulation of  $\text{Ca}^{2+}$  spike activity supporting a GPER1-dependent mechanism. G-1 (1  $\mu\text{M}$ ) and nifedipine (100 nM) attenuated the 30 mM KCl-evoked rise in intracellular  $\text{Ca}^{2+}$  concentration, suggesting that G-1 blocks inflow of  $\text{Ca}^{2+}$  via voltage-sensitive  $\text{Ca}^{2+}$  channels. In conclusion, we demonstrate that the GPER1 agonist G-1 regulates vascular smooth muscle cell  $\text{Ca}^{2+}$  handling by lowering  $\text{Ca}^{2+}$  spike activity, suggesting a role for this mechanism in GPER1-mediated control of blood pressure.

**Key words:** calcium; calcium spikes; estrogen; estrogen receptor; GPR30; nifedipine; vascular smooth muscle cells

## Introduction

Estrogen regulates cardiovascular function through the classical estrogen receptors (ERs) ER $\alpha$  and ER $\beta$ , but also through the newly discovered G protein-coupled estrogen receptor 1 (GPER1)/GPR30 [1-6]. Estrogen exerts cardiovascular protective and beneficial effects through stimulation of NO formation, attenuation of vascular smooth muscle cell proliferation, reduction of inflammation-promoter-induced recruitment of monocytes and by promoting vascular re-endothelialisation, all of which are reported to involve ER $\alpha$  [7-13]. Thus, many studies suggest that vascular ER $\alpha$  is important, whereas much less information is available regarding the importance of ER $\beta$  and GPER1 in the cardiovascular system.

Lack of a functional GPER1 gene causes elevated blood pressure in female mice, suggesting that GPER1 is involved in blood pressure regulation [14]. The increase in blood pressure develops over time and is associated with reduced circumference and increased media-to-lumen ratio of resistance arteries, indicating inward remodeling [14]. Probably, this change in arterial structure, due to lack of GPER1, takes time to develop explaining why high blood pressure is observed in old but not young animals. Acute intravenous infusion of the GPER1 agonist G-1 has been reported to cause a slight reduction in mean arterial pressure in normotensive male rats [15]. Furthermore, chronic infusion of G-1 via an osmotic minipump over a 2 weeks period lowers mean arterial pressure in hypertensive and ovariectomized female mRen2.Lewis rats [16], further strengthening the idea that GPER1 is involved in regulation of blood pressure. G-1, at 1-10  $\mu$ M, relaxes agonist-induced force in different vascular preparations such as rat mesenteric arteries, rat aorta, rat carotid arteries and human

internal mammary arteries [15-17], suggesting that GPER1 regulates vascular contractility. However, this receptor also seems to play a role in vascular smooth muscle and endothelial cell proliferation, which is inhibited by G-1, in the  $\mu\text{M}$  concentration range [15, 18]. Although activation of vascular GPER1 is supposed to regulate vascular contractility as well as vascular smooth muscle and endothelial cell proliferation, very little is known about the molecular mechanisms behind these effects [15, 18]. Here, we hypothesize that the mechanism behind reported GPER1 effects in the cardiovascular system involves regulation of vascular smooth muscle cell  $\text{Ca}^{2+}$  handling. Changes in intracellular  $\text{Ca}^{2+}$  levels may have a broad impact on vascular smooth muscle cell function, since  $\text{Ca}^{2+}$  plays an important role regulating contractility, apoptosis, transport as well as proliferation [19]. Oscillations in the intracellular  $\text{Ca}^{2+}$  concentration in vascular smooth muscle cells are thought to depend on both intracellular  $\text{Ca}^{2+}$  release and inflow of  $\text{Ca}^{2+}$  from the extracellular space [20, 21].  $\text{Ca}^{2+}$  oscillations may regulate vascular reactivity by inducing rhythmical contractions [20].

In order to assess the possible involvement of GPER1 in the regulation of vascular smooth muscle cell  $\text{Ca}^{2+}$  homeostasis, we investigate here the effects of the GPER1-selective agonist G-1 on intracellular  $\text{Ca}^{2+}$  levels in rat aortic smooth muscle A7r5 cells. Interestingly, we show that G-1 acutely reduces  $\text{Ca}^{2+}$  spike activity in A7r5 cells expressing GPER1 in a similar manner as the conventional voltage-sensitive L-type  $\text{Ca}^{2+}$  channel blocker nifedipine, suggesting a role for this mechanism in GPER1-mediated control of blood pressure.

## Material and Methods

### *Cells and cell culture*

The vascular smooth muscle cell line A7r5 was from American Type Tissue Culture Collection (ATCC, Manassas, VA, USA) and cultured in accordance with recommendations from ATCC. A7r5 is a well-characterized rat embryonic aortic smooth muscle cell line. The A7r5 cells develop spontaneous and synchronous  $\text{Ca}^{2+}$  spikes and the cells respond to agonist-stimulation with rise in intracellular  $\text{Ca}^{2+}$  concentration and increased  $\text{Ca}^{2+}$  spike activity as demonstrated by Byron and Taylor [22]. The  $\text{Ca}^{2+}$  spikes depend on cell-cell communication and inflow of extracellular  $\text{Ca}^{2+}$  through voltage-sensitive L-type  $\text{Ca}^{2+}$  channels [22]. Before experiments, normal culture medium was replaced with phenol red-free and fetal calf serum-free culture medium for 24 h to make the cells quiescent and to remove the estrogen-like activity of phenol red. Experiments were performed in confluent A7r5 cells.

### *RT-PCR*

Total RNA was extracted and purified using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Concentration and purity of RNA was measured at 260/280 nm in a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). cDNA was generated and mRNA for GPER1 determined as previously described by Mårtensson et al. [14]. Reverse transcriptase-negative controls were included to exclude the presence of contaminating genomic DNA. The sequence of GPER1 primers were: forward, GATCGTTAGATTAACAGAGCAG; reverse, CCTGGGAGCCTGTTAGTCTCAG. This

primer pair produces a GPER1 specific product [14]. Moreover, we have previously confirmed, using the same GPER1 primer pairs, that RAW264.7 cells, regarded as a positive control for GPER1, express a strong band at the correct predicted size [18]. For quantitative real-time PCR the RNA samples were subjected to one-step quantitative real-time PCR measurements using QuantiFast SYBR Green RT-PCR kit (Qiagen) and QuantiTect primer assays (Qiagen) on a Step One Plus real-time thermal cycler (Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in duplicate. Gene expression was calculated using the delta CT method as described by Pfaffl [23]. The RNA samples were used for quantitative RT-PCR measurements applying glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as house-keeping reference gene. The PCR primers (QuantiTect Primer Assays) for GPER-1 (Rn\_Gper\_1\_SG), CACNA 1c (Rn\_Cacna1c\_2\_SG) and GAPDH (Rn\_Gapd\_1\_SG) were purchased from Qiagen.

#### *Determination of intracellular $Ca^{2+}$ concentration*

For determination of intracellular  $Ca^{2+}$  concentration the cells were cultured on glass bottom cell culture Petri dishes (MatTek, Ashland, MA, USA). After reaching confluence, the cells were washed with HEPES buffered physiological salt solution of the following composition in mM: NaCl 135.5, KCl 5.9,  $CaCl_2$  2.5,  $MgCl_2$  1.2, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) 11.6 and glucose 11.5. The cells were incubated with the  $Ca^{2+}$  sensitive fluorescent dye Fluo 4-AM (3  $\mu$ M, Invitrogen, Carlsbad, CA, USA) for 40 min at room temperature and then washed carefully. During the  $Ca^{2+}$  measurements the cells were incubated in HEPES buffered physiological salt solution containing 2.5 mM  $Ca^{2+}$ . Fluorescence was recorded using a laser scanning confocal microscope (LSM 5 PASCAL, Carl Zeiss AG, Göttingen, Germany). The excitation wavelength was 488 nm and the

emission wavelength 505 nm. A summarized fluorescence signal was collected from all cells (about 500 cells) within the visual field at x100 magnification. The confocal pinhole setting was identical for all experiments. For each dish the  $\text{Ca}^{2+}$  spike activity in the presence of G-1 and G-15 was compared with that observed in the presence of vehicle prior to inclusion of the drugs, i.e. in each cell preparation a paired comparison of  $\text{Ca}^{2+}$  spike activity was performed. The frequency of the  $\text{Ca}^{2+}$  spikes and their mean amplitude was determined over a 10 min time-period directly upon addition of G-1 and G-15. Spikes were generally well resolved and their frequency and amplitude easy to determine unambiguously (please see original recordings in figures 2-4 and figure 6).

### *Drugs*

G-1 was purchased from Cayman Chemical, Ann Arbor, MI, USA. G-15 was kindly supplied by Prof. Roger Olsson, Lund University.  $17\beta$ -estradiol ( $\text{E}_2$ ) was purchased from Sigma Chemicals, St Louis, MO, USA. G-1, G-15 and  $\text{E}_2$  were dissolved in DMSO. The thromboxane A<sub>2</sub> analogue U46619 (Tocris Bioscience, Bristol, United Kingdom) and the L-type  $\text{Ca}^{2+}$  channel blocker nifedipine (Sigma Chemicals) were dissolved in DMSO. Controls received DMSO as vehicle. The final concentration of DMSO was 0.1-0.3%.

### *Statistics*

Values are presented as means  $\pm$  S.E.M. Statistical significance was calculated using ANOVA and Student's two-tailed t-test for paired comparisons with Bonferroni correction for post hoc analysis as appropriate. P values less than 0.05 were regarded to denote statistical significance.



## Results

### *G-1 reduces Ca<sup>2+</sup> spike activity in A7r5 cells expressing GPER1*

The vascular smooth muscle A7r5 cells expressed mRNA for GPER1 as demonstrated by RT-PCR (Fig. 1). The delta CT value for GPER1 was  $27.35 \pm 0.69$  (n=18) compared to  $12.73 \pm 0.56$  (n=18) for the house-keeping gene GAPDH, as demonstrated by quantitative real-time RT-PCR, indicating a relatively high GPER1 mRNA expression in A7r5 cells. Spontaneous and synchronous Ca<sup>2+</sup> spikes, monitored by laser-scanning confocal microscopy of Fluo 4-AM labeled cells, were observed regularly (about 80 out of 100 Petri dishes) in confluent A7r5 vascular smooth muscle cells. Only dishes exhibiting spontaneous Ca<sup>2+</sup> spikes were included in the study. The baseline spontaneous Ca<sup>2+</sup> spike frequency was  $3.5 \pm 0.2$  per min (n=61). Treatment of A7r5 cells with the selective GPER1 agonist G-1 (1  $\mu$ M) acutely reduced both Ca<sup>2+</sup> spike amplitude and frequency (Figs. 2B, C and D). Lower concentrations of G-1 (300 nM) reduced Ca<sup>2+</sup> spike amplitude but had no effect on Ca<sup>2+</sup> spike frequency (Figs. 2C and D). Vehicle-control (0.1% DMSO) had no effect on Ca<sup>2+</sup> spike activity (Fig. 2A). The spontaneous Ca<sup>2+</sup> spike frequency was  $2.9 \pm 0.4$  per min (n=13) before inclusion of 0.1% DMSO compared to  $3.1 \pm 0.5$  per min (n=13) in the presence of DMSO. In fact, 1  $\mu$ M G-1 made the Ca<sup>2+</sup> spikes disappear totally within 200-300 s in 11 out of 17 experiments, i.e. in about 65% of the experiments (Figs. 2B, C and D). The G-1-induced attenuation of Ca<sup>2+</sup> spike activity is reversible since the Ca<sup>2+</sup> spikes re-appeared after wash-out of G-1 (data not shown). The voltage-sensitive L-type Ca<sup>2+</sup> channel blocker nifedipine (100 nM) fully inhibited Ca<sup>2+</sup> spike activity within 200-300 s (Fig. 3), showing that nifedipine acts similar to G-1. Stimulation with the thromboxane A2 analogue U46619 (1  $\mu$ M) increased the intracellular Ca<sup>2+</sup> plateau level and the Ca<sup>2+</sup> spike frequency (Figs. 4 A and B). Addition of G-

1 (1  $\mu\text{M}$ ) to U46619-stimulated cells markedly reduced the  $\text{Ca}^{2+}$  spike activity but had no effect on the plateau level of the U46619-evoked  $\text{Ca}^{2+}$  response (Figs. 4A and B). The U46619-evoked increase in  $\text{Ca}^{2+}$  spike frequency was completely inhibited by 1  $\mu\text{M}$  G-1 (Fig. 4B). Furthermore, 1  $\mu\text{M}$  G-1 reduced U46619-evoked spike activity also when G-1 was added to the cells before inclusion of U46619 (data not shown).

#### *Effects of estrogen on $\text{Ca}^{2+}$ spike activity in A7r5 cells*

Treatment with a high physiological concentration (100 nM) of 17 $\beta$ -estradiol ( $\text{E}_2$ ) had no effect on  $\text{Ca}^{2+}$  spike amplitude and frequency (Fig. 5). However,  $\text{E}_2$  reduced  $\text{Ca}^{2+}$  spike frequency, but not amplitude, at a 10 times higher concentration (1  $\mu\text{M}$ ) of  $\text{E}_2$  (Fig. 5). The plasma concentration of estrogen at ovulation is about 2 nM but increases severalfold in pregnancy [24]. Thus, 100 nM  $\text{E}_2$  is considered physiological, while 1  $\mu\text{M}$   $\text{E}_2$  represents a pharmacological estrogen concentration.

#### *Down-regulation of $\text{Ca}^{2+}$ spike activity by G-1 is reversed by the GPER1 antagonist G-15 in A7r5 cells*

In order to investigate the involvement of GPER1 in G-1-induced down-regulation of  $\text{Ca}^{2+}$  spike activity, the GPER1 antagonist G-15 was included at 10 min before G-1 inclusion and then present throughout the experiment [25, 26]. G-15 (1  $\mu\text{M}$ ) alone slightly, but not significantly, reduced  $\text{Ca}^{2+}$  spike amplitude, whereas G-1 (1  $\mu\text{M}$ ) alone reduced  $\text{Ca}^{2+}$  spike amplitude by about 70% (Figs. 6A, B and C). G-1 (1  $\mu\text{M}$ ) had no effect on  $\text{Ca}^{2+}$  spike amplitude in the presence of 1  $\mu\text{M}$  G-15 (Figs. 6A and C). In the combined presence of G-15 and G-1 (both at 1  $\mu\text{M}$ ) spiking persisted in 11 out of 14 experiments, and with a higher

concentration of G-15 (5  $\mu\text{M}$ ) spiking persisted in 5 out of 5 experiments. In contrast, treatment with 1  $\mu\text{M}$  G-1 alone fully inhibited  $\text{Ca}^{2+}$  spike activity in 11 out of 17 experiments, i.e. in the presence of 1  $\mu\text{M}$  G-1 alone spiking persisted only in 6 out of 17 experiments. Taken together, these data show that G-15 inhibits down-regulation of  $\text{Ca}^{2+}$  spike activity by G-1, suggesting that down-regulation of  $\text{Ca}^{2+}$  spike activity involves GPER1.

*G-1 reduces the KCl-evoked rise in intracellular  $\text{Ca}^{2+}$  concentration in A7r5 cells*

Confluent A7r5 cells exhibited spontaneous  $\text{Ca}^{2+}$  spikes and showed a rise in intracellular  $\text{Ca}^{2+}$  concentration in response to stimulation with 30 mM KCl (Fig. 7), and, furthermore, the A7r5 cells expressed transcript for the *Cacna 1c* gene coding for the alpha-1c subunit of the voltage-sensitive L-type  $\text{Ca}^{2+}$  channel (data not shown), suggesting that confluent A7r5 cells express voltage-sensitive  $\text{Ca}^{2+}$  channels. Treatment with G-1 (1  $\mu\text{M}$ ) attenuated the  $\text{Ca}^{2+}$  response to 30 mM KCl by about 20%, suggesting that G-1 blocks inflow of  $\text{Ca}^{2+}$  through voltage-sensitive  $\text{Ca}^{2+}$  channels (Figs. 7B and D). The L-type  $\text{Ca}^{2+}$  channel blocker nifedipine (100 nM) completely inhibited the 30 mM KCl-evoked  $\text{Ca}^{2+}$  response (Fig. 7C). Addition of 0.1% DMSO as vehicle-control had no effect on the 30 mM KCl-induced rise in intracellular  $\text{Ca}^{2+}$  concentration (Fig. 7A).

## Discussion

Here, we investigate, using a pharmacological approach, the involvement of the putative estrogen receptor GPER1 in vascular smooth muscle cell  $\text{Ca}^{2+}$  handling. We show that A7r5 vascular smooth muscle cells express GPER1 mRNA. Furthermore, our results reveal that the selective GPER1 agonist G-1 acutely reduces  $\text{Ca}^{2+}$  spike activity, and that this effect is blocked by the GPER1 antagonist G-15. Vascular smooth muscle A7r5 cells exhibit spontaneous, synchronous and transient rises in intracellular  $\text{Ca}^{2+}$  concentration named  $\text{Ca}^{2+}$  spikes [22]. Vascular smooth muscle cell  $\text{Ca}^{2+}$  spike activity depends on both  $\text{Ca}^{2+}$  inflow and cell/cell contact [22, 27]. In the present study, we demonstrate that G-1 reduces  $\text{Ca}^{2+}$  spike activity similarly to the voltage-sensitive L-type  $\text{Ca}^{2+}$  channel blocker nifedipine, suggesting that GPER1 regulates  $\text{Ca}^{2+}$  handling by reducing L-type  $\text{Ca}^{2+}$  channel activity. G-1 attenuates also the thromboxane A2 U46619-stimulated A7r5 cell  $\text{Ca}^{2+}$  spike activity but has no effect on U46619-stimulated basal level of  $\text{Ca}^{2+}$ , suggesting that G-1 specifically blocks  $\text{Ca}^{2+}$  inflow associated with spikes but not mechanisms responsible for setting the basal intracellular  $\text{Ca}^{2+}$  concentration. Importantly, we also show that G-1 attenuates the 30 mM KCl-evoked  $\text{Ca}^{2+}$  response similar to nifedipine. The KCl-induced  $\text{Ca}^{2+}$  response is associated with membrane depolarization and inflow of  $\text{Ca}^{2+}$  via voltage-sensitive  $\text{Ca}^{2+}$  channels, and thus, the G-1-induced reduction of the KCl-evoked  $\text{Ca}^{2+}$  response provides further evidence that G-1 blocks voltage-sensitive  $\text{Ca}^{2+}$  channel activity. It should be noted that spikes in A7r5 cells are elicited by  $\text{Ca}^{2+}$ -dependent action potentials [27]. Addition of G-1 usually resulted in a gradual reduction of spike amplitude, which may reflect an increasing proportion of cells ceasing to fire action potentials. This could however not be analyzed due to limited spatial resolution in the present recordings on confluent A7r5 cells.

The involvement of GPER1 in the G-1-induced acute reduction of  $\text{Ca}^{2+}$  spike activity was investigated using the GPER1 antagonist G-15. G-1 reduced  $\text{Ca}^{2+}$  spike amplitude in the absence of G-15 but had no effect when administered in combination with G-15, suggesting that G-1-induced reduction of  $\text{Ca}^{2+}$  spike activity is mediated through GPER1. Importantly, treatment with 1  $\mu\text{M}$  G-1 alone inhibited completely  $\text{Ca}^{2+}$  spike activity in 11 out of 17 experiments (65%), while G-1 fully inhibited  $\text{Ca}^{2+}$  spike activity in only 3 out of 14 experiments (21%) in the presence of G-15, and in the presence of an excess of G-15 (5  $\mu\text{M}$ ), G-1 (1  $\mu\text{M}$ ) was unable to fully inhibit spike activity. Thus, G-1 seems to act through GPER1. Furthermore, we show that 17 $\beta$ -estradiol at 1  $\mu\text{M}$ , regarded as a pharmacological concentration of estrogen [24], also reduces vascular smooth muscle cell  $\text{Ca}^{2+}$  spike activity. This is in line with previous studies showing that 17  $\beta$ -estradiol, at  $\mu\text{M}$  concentrations, reduces  $\text{Ca}^{2+}$  currents in cultured vascular smooth muscle cells [28].

Previously, data have been presented suggesting that activation of GPER1 by estrogen causes a rise in intracellular  $\text{Ca}^{2+}$  concentration in GPER1-expressing COS7 cells [2]. Furthermore, intracellular injection of G-1 (3  $\mu\text{M}$ ) is reported to cause a fast and transient increase in intracellular  $\text{Ca}^{2+}$  concentration in human aortic smooth muscle cells [15]. These authors report also that pretreatment with G-1, by intracellular injection, blunts the subsequent 5-HT-induced  $\text{Ca}^{2+}$  response [15]. In the present study, we demonstrate that activation of vascular smooth muscle cell GPER1 may influence  $\text{Ca}^{2+}$  handling by reducing  $\text{Ca}^{2+}$  spike activity probably via attenuation of  $\text{Ca}^{2+}$  inflow through voltage-sensitive L-type  $\text{Ca}^{2+}$  channels. It has been shown that release of  $\text{Ca}^{2+}$  from intracellular stores does not influence  $\text{Ca}^{2+}$  spike activity in A7r5 cells, and thus our results do not exclude effects of G-1 on intracellular  $\text{Ca}^{2+}$  release

in other cell types. In summary, we propose that the GPER1 agonist G-1 regulates vascular smooth muscle cell  $\text{Ca}^{2+}$  handling by reducing  $\text{Ca}^{2+}$  spike activity, and that this may be a mechanism by which GPER1 controls vascular reactivity and blood pressure.

## **Acknowledgements**

This study was supported by grants from the Swedish Research Council, the Swedish Dental Society, the Greta and Johan Kocks Foundation, the Sven and Lilly Thuréus Foundation and the Vascular Wall Programme at Lund University. The authors thank Joanna Daszkiewicz-Nilsson and Ina Nordström for excellent technical assistance.

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## Figure legends

**Fig. 1.** The A7r5 vascular smooth muscle cells express a strong GPER1 mRNA band as demonstrated by RT-PCR at the correct predicted size (302 bp). The leftmost panel shows DNA ladder. No band was observed in reverse transcriptase-negative controls (RT-). This experiment was repeated twice.

**Fig. 2.** The GPER1 agonist G-1 (1  $\mu$ M) attenuates spontaneous  $\text{Ca}^{2+}$  spike activity in A7r5 cells. Panels A and B show representative  $\text{Ca}^{2+}$  traces in vehicle-control (DMSO, 0.1%) and G-1 stimulated cells, 1 out of 6 experiments for DMSO and 1 out of 17 experiments for G-1, respectively. G-1 or DMSO-vehicle was included at the arrow and present throughout the experiment. Panels C and D show summarized data on  $\text{Ca}^{2+}$  spike amplitude and frequency, respectively. The intracellular  $\text{Ca}^{2+}$  concentration was assessed by laser-scanning confocal microscopy of Fluo 4-AM labeled cells. Summarized data are presented as mean  $\pm$  SEM of 4-25 observations in each group. \* $P < 0.05$  and \*\*\* $P < 0.001$ , when compared to control.

**Fig. 3.** The voltage sensitive L-type  $\text{Ca}^{2+}$  channel blocker nifedipine (100 nM) inhibits  $\text{Ca}^{2+}$  spike activity in A7r5 cells. Nifedipine was included at the arrow and present throughout the experiment. The intracellular  $\text{Ca}^{2+}$  concentration was assessed by laser-scanning confocal microscopy of Fluo 4-AM labeled cells. This trace shows one representative experiment out of three.

**Fig. 4.** Addition of G-1 (1  $\mu$ M) to U46619-stimulated (1  $\mu$ M) cells reduces  $\text{Ca}^{2+}$  spike activity but has no effect on the basal level of the U46619 evoked  $\text{Ca}^{2+}$  response in A7r5 cells (A). U46619 and G-1 were included at the arrows and then present throughout the experiment (A). This trace shows one representative experiment out of five. Summarized data on  $\text{Ca}^{2+}$  spike frequency show that 1  $\mu$ M U46619 increases spike frequency almost 2-times and that 1  $\mu$ M G-1 completely blocks the U46619-induced increase in spike frequency (B). The intracellular  $\text{Ca}^{2+}$  concentration was assessed by laser-scanning confocal microscopy of Fluo 4-AM labeled cells. Summarized data are presented as mean  $\pm$  SEM of 5 observations in each group. \*P<0.05.

**Fig. 5.** Addition of a pharmacological concentration (1  $\mu$ M) of estrogen (17 $\beta$ -estradiol, E2) to A7r5 cells reduces  $\text{Ca}^{2+}$  spike frequency (B) but not amplitude (A). A high physiological concentration (100 nM) of estrogen has no effect on  $\text{Ca}^{2+}$  spike frequency and amplitude. Values are means  $\pm$  SEM of 9-12 observations in each group. \*P<0.05 when compared to control.

**Fig. 6.** The GPER1 antagonist G-15 (1  $\mu$ M) blocks the G-1-induced (1  $\mu$ M) attenuation of  $\text{Ca}^{2+}$  spike activity in A7r5 cells (A, B and C). Panels A and B show one representative recording out of 14 for each type of experiment. G-15 and G-1 were included at the arrows and present throughout the experiment. The intracellular  $\text{Ca}^{2+}$  concentration was assessed by laser-scanning confocal microscopy of Fluo 4-AM labeled cells. Summarized data on mean spike amplitude are presented as means  $\pm$  SEM of 14 observations in each group (C). \*P<0.05. N.S. = not significant.

**Fig. 7.** Treatment with G-1 (1  $\mu$ M) attenuates the  $\text{Ca}^{2+}$  response to 30 mM KCl in A7r5 cells (B and D). The L-type  $\text{Ca}^{2+}$  channel blocker nifedipine (100 nM) completely inhibits the 30 mM KCl-evoked  $\text{Ca}^{2+}$  response (C). Addition of 0.1% DMSO as vehicle-control has no effect on the 30 mM KCl-induced rise in intracellular  $\text{Ca}^{2+}$  concentration (A). Summarized data (D), assessing the maximal amplitude of the KCl-stimulated  $\text{Ca}^{2+}$  response, show that G-1 reduces the 30 mM KCl-induced  $\text{Ca}^{2+}$  response by about 20%. The  $\text{Ca}^{2+}$  response to 30 mM KCl was evaluated by measuring the maximal amplitude of the  $\text{Ca}^{2+}$  signal in the presence of G-1 or DMSO (Control) normalized to that of its respective reference KCl-induced  $\text{Ca}^{2+}$  response evoked prior to inclusion of G-1 or DMSO. Analysis of the area under the curve show similar results as the analysis of maximal amplitude. The intracellular  $\text{Ca}^{2+}$  concentration was assessed by laser-scanning confocal microscopy of Fluo 4-AM labeled cells. G-1, DMSO or nifedipine were included at the arrows and present throughout the respective experiment. The original traces show one representative recording out of 5-11 for each type of experiment. Summarized data are presented as mean  $\pm$  SEM of 5-11 observations in each group. \*\*\* $P < 0.001$  when compared to control.

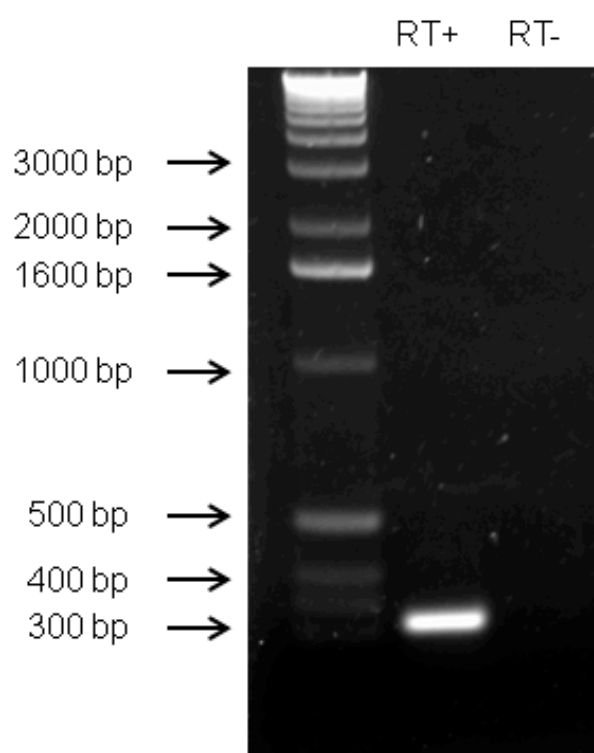
**Fig. 1**



Fig. 2

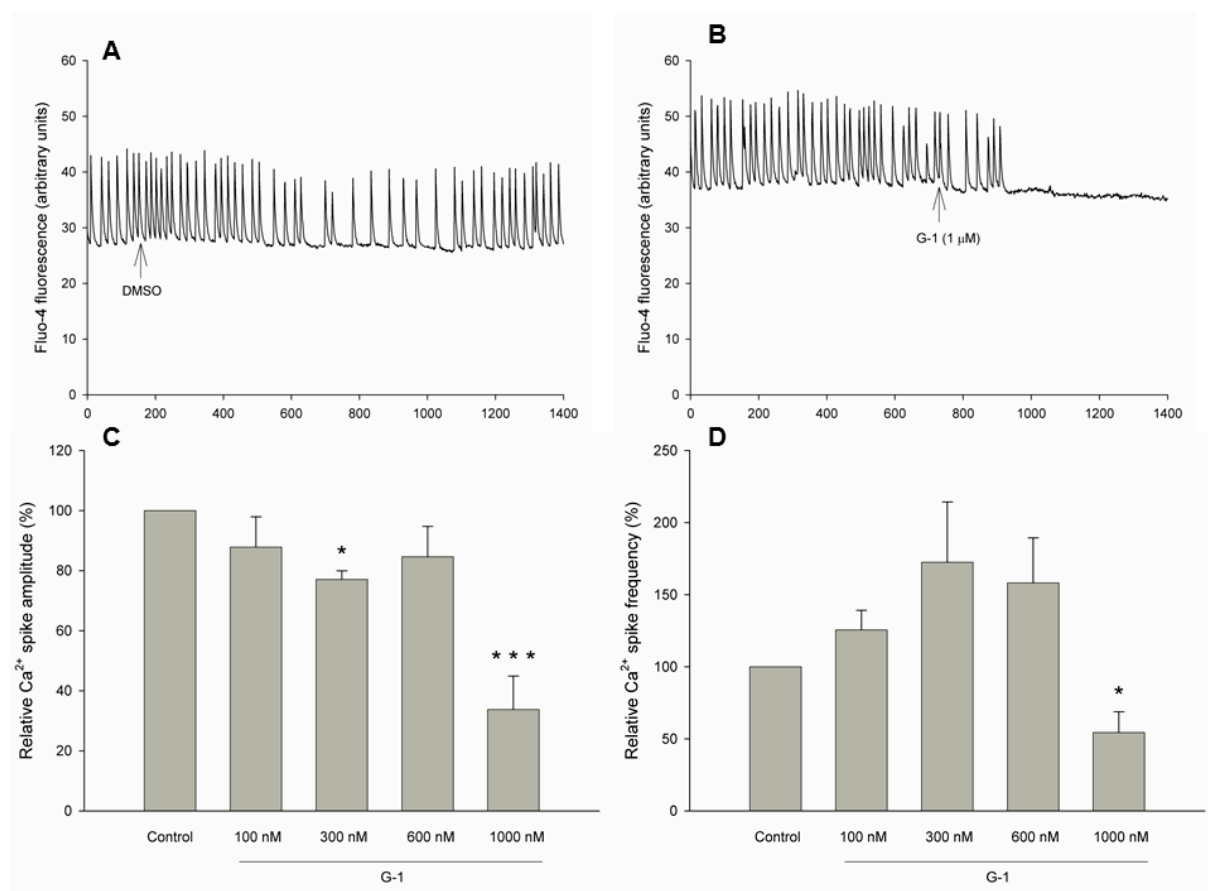


Fig. 3

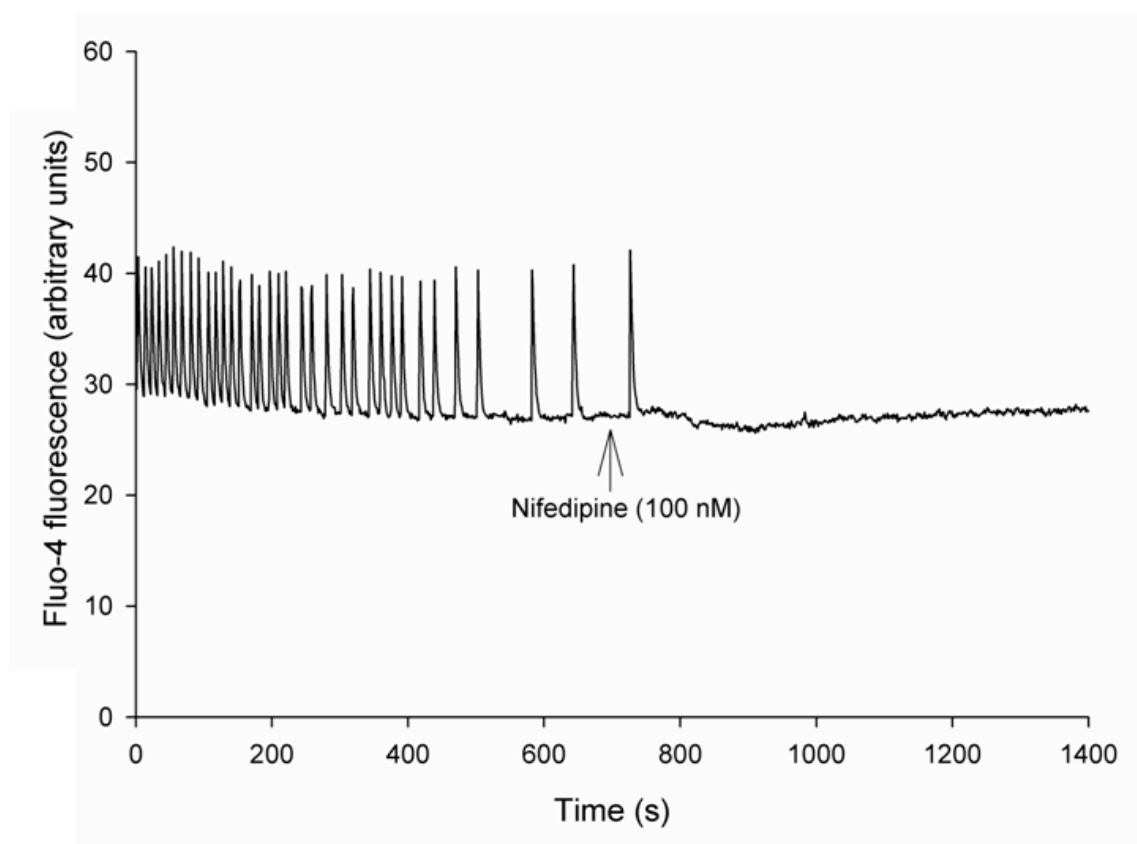
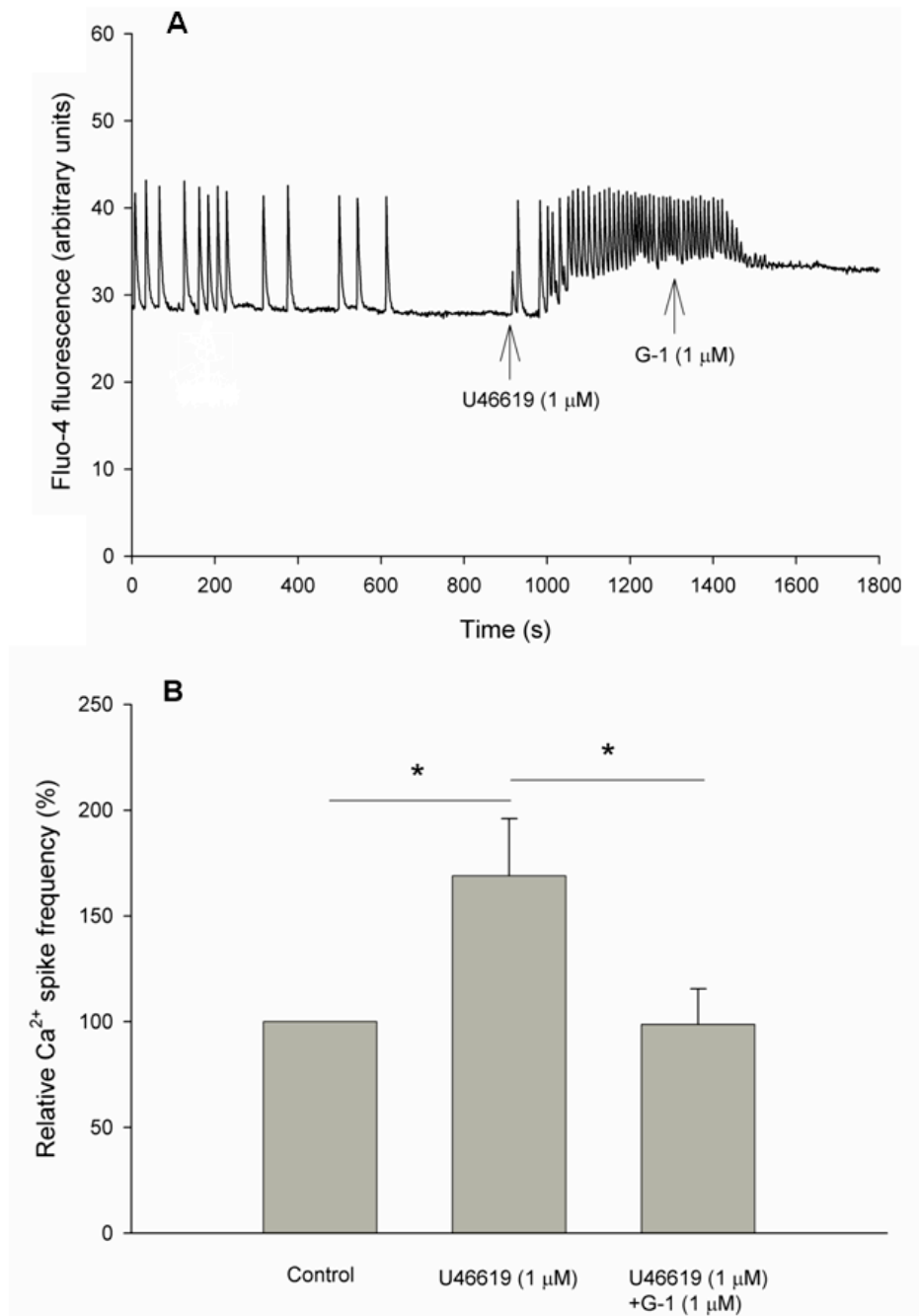


Fig. 4



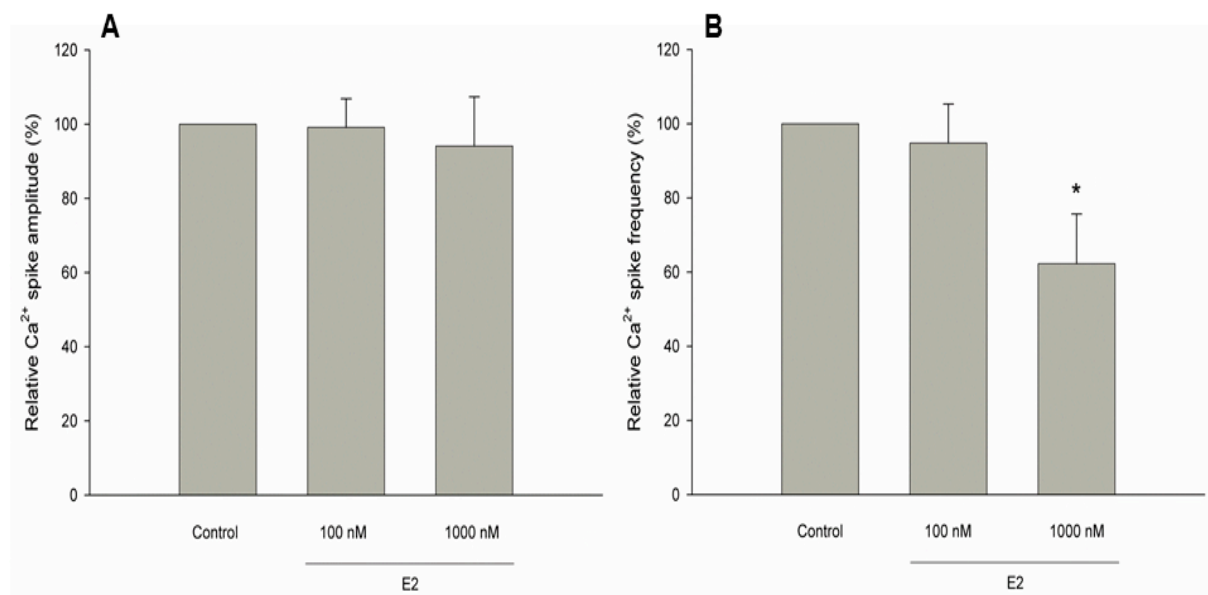
**Fig. 5**

Fig. 6

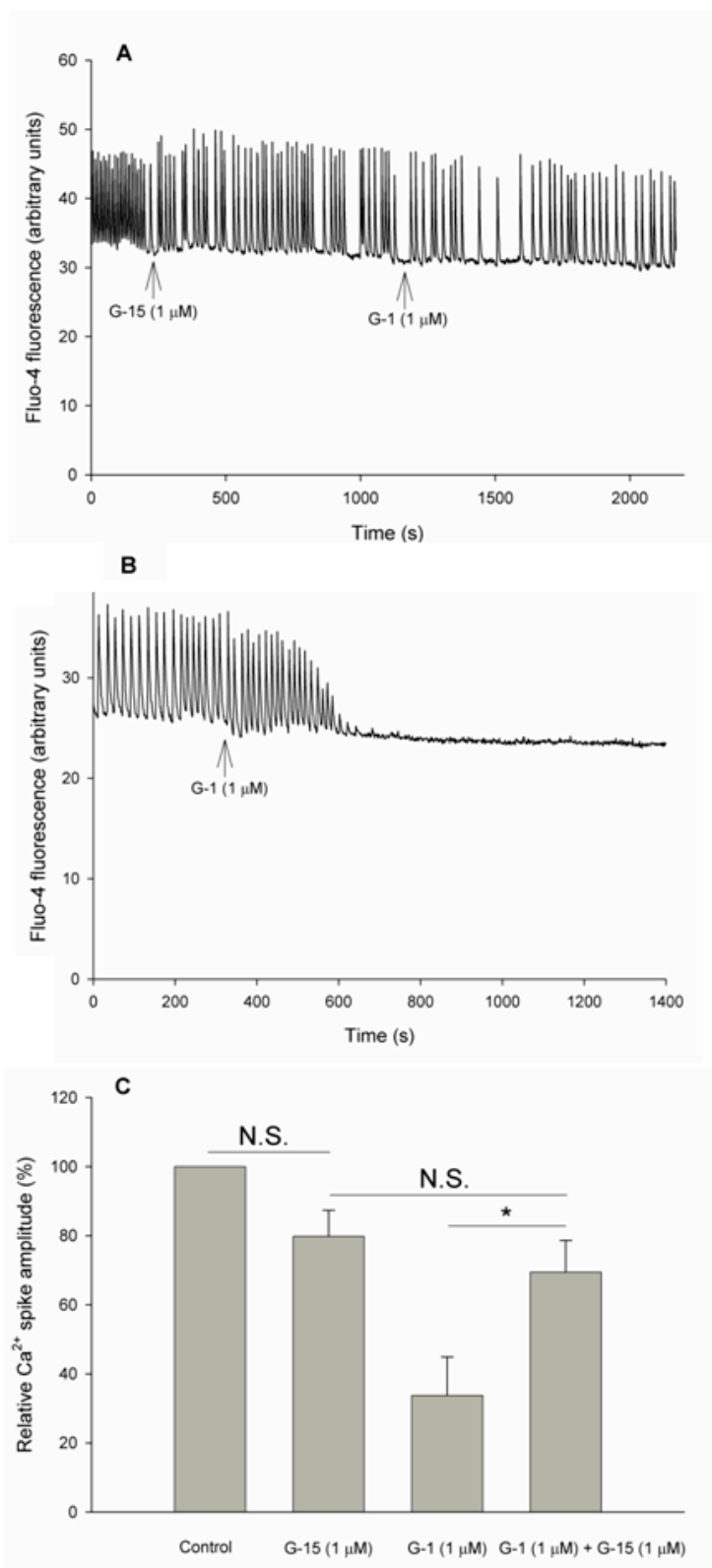


Fig. 7

