



LUND UNIVERSITY

The G Protein-Coupled Estrogen Receptor 1 (GPER1/GPR30) Agonist G-1 Regulates Vascular Smooth Muscle Cell Ca Handling.

Holm, Anders; Hellstrand, Per; Olde, Björn; Svensson, Daniel; Leeb-Lundberg, Fredrik; Nilsson, Bengt-Olof

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

General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

The G protein-coupled estrogen receptor 1 (GPER1/GPR30) agonist G-1 regulates vascular smooth muscle cell Ca²⁺ handling

Anders Holm, Per Hellstrand, Björn Olde, Daniel Svensson, L.M. Fredrik Leeb-Lundberg, Bengt-Olof Nilsson

Department of Experimental Medical Science, Lund University, Lund, Sweden

Running head: GPER1 and Ca²⁺ handling

Correspondence: Dr. Bengt-Olof Nilsson

Department of Experimental Medical Science

Lund University

BMC D12

SE-221 84 Lund

Sweden

Phone:+46-46-2227767

Fax:+46-46-2224546

E-mail: bengt-olof.nilsson@med.lu.se

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 Ca^{2+} handling. Treatment with the GPER1 agonist G-1, in the μM concentration range, acutely reduced spontaneous and synchronous Ca^{2+} spike activity in A7r5 vascular smooth muscle cells expressing mRNA for GPER1. Furthermore, G-1 (1 μM) attenuated thromboxane A2 analogue U46619-stimulated Ca^{2+} spike activity but had no effect on U46619-induced increase in basal level of Ca^{2+} . The voltage-sensitive L-type Ca^{2+} channel blocker nifedipine (100 nM) reduced Ca^{2+} spike activity similar to G-1. Pharmacological, but not physiological, concentration of the estrogen 17β -estradiol reduced Ca^{2+} spike activity. The GPER1 antagonist G-15 blocked G-1-induced down-regulation of Ca^{2+} spike activity supporting a GPER1-dependent mechanism. G-1 (1 μM) and nifedipine (100 nM) attenuated the 30 mM KCl-evoked rise in intracellular Ca^{2+} concentration, suggesting that G-1 blocks inflow of Ca^{2+} via voltage-sensitive Ca^{2+} channels. In conclusion, we demonstrate that the GPER1 agonist G-1 regulates vascular smooth muscle cell Ca^{2+} handling by lowering 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 α and ER β , 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 α [7-13]. Thus, many studies suggest that vascular ER α is important, whereas much less information is available regarding the importance of ER β 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 μ 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 μM 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 Ca^{2+} handling. Changes in intracellular Ca^{2+} levels may have a broad impact on vascular smooth muscle cell function, since Ca^{2+} plays an important role regulating contractility, apoptosis, transport as well as proliferation [19]. Oscillations in the intracellular Ca^{2+} concentration in vascular smooth muscle cells are thought to depend on both intracellular Ca^{2+} release and inflow of Ca^{2+} from the extracellular space [20, 21]. 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 Ca^{2+} homeostasis, we investigate here the effects of the GPER1-selective agonist G-1 on intracellular Ca^{2+} levels in rat aortic smooth muscle A7r5 cells. Interestingly, we show that G-1 acutely reduces Ca^{2+} spike activity in A7r5 cells expressing GPER1 in a similar manner as the conventional voltage-sensitive L-type 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 Ca^{2+} spikes and the cells respond to agonist-stimulation with rise in intracellular Ca^{2+} concentration and increased Ca^{2+} spike activity as demonstrated by Byron and Taylor [22]. The Ca^{2+} spikes depend on cell-cell communication and inflow of extracellular Ca^{2+} through voltage-sensitive L-type 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 μ 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 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 Ca^{2+} spike activity was performed. The frequency of the 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. 17β -estradiol (E_2) was purchased from Sigma Chemicals, St Louis, MO, USA. G-1, G-15 and E_2 were dissolved in DMSO. The thromboxane A₂ analogue U46619 (Tocris Bioscience, Bristol, United Kingdom) and the L-type 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 Ca²⁺ 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 ± 0.69 (n=18) compared to 12.73 ± 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 Ca²⁺ 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 Ca²⁺ spikes were included in the study. The baseline spontaneous Ca²⁺ spike frequency was 3.5 ± 0.2 per min (n=61). Treatment of A7r5 cells with the selective GPER1 agonist G-1 (1 μ M) acutely reduced both Ca²⁺ spike amplitude and frequency (Figs. 2B, C and D). Lower concentrations of G-1 (300 nM) reduced Ca²⁺ spike amplitude but had no effect on Ca²⁺ spike frequency (Figs. 2C and D). Vehicle-control (0.1% DMSO) had no effect on Ca²⁺ spike activity (Fig. 2A). The spontaneous Ca²⁺ spike frequency was 2.9 ± 0.4 per min (n=13) before inclusion of 0.1% DMSO compared to 3.1 ± 0.5 per min (n=13) in the presence of DMSO. In fact, 1 μ M G-1 made the Ca²⁺ 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 Ca²⁺ spike activity is reversible since the Ca²⁺ spikes re-appeared after wash-out of G-1 (data not shown). The voltage-sensitive L-type Ca²⁺ channel blocker nifedipine (100 nM) fully inhibited Ca²⁺ spike activity within 200-300 s (Fig. 3), showing that nifedipine acts similar to G-1. Stimulation with the thromboxane A2 analogue U46619 (1 μ M) increased the intracellular Ca²⁺ plateau level and the Ca²⁺ spike frequency (Figs. 4 A and B). Addition of G-

1 (1 μM) to U46619-stimulated cells markedly reduced the Ca^{2+} spike activity but had no effect on the plateau level of the U46619-evoked Ca^{2+} response (Figs. 4A and B). The U46619-evoked increase in Ca^{2+} spike frequency was completely inhibited by 1 μM G-1 (Fig. 4B). Furthermore, 1 μM 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 Ca^{2+} spike activity in A7r5 cells

Treatment with a high physiological concentration (100 nM) of 17 β -estradiol (E_2) had no effect on Ca^{2+} spike amplitude and frequency (Fig. 5). However, E_2 reduced Ca^{2+} spike frequency, but not amplitude, at a 10 times higher concentration (1 μM) of E_2 (Fig. 5). The plasma concentration of estrogen at ovulation is about 2 nM but increases severalfold in pregnancy [24]. Thus, 100 nM E_2 is considered physiological, while 1 μM E_2 represents a pharmacological estrogen concentration.

Down-regulation of 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 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 μM) alone slightly, but not significantly, reduced Ca^{2+} spike amplitude, whereas G-1 (1 μM) alone reduced Ca^{2+} spike amplitude by about 70% (Figs. 6A, B and C). G-1 (1 μM) had no effect on Ca^{2+} spike amplitude in the presence of 1 μM G-15 (Figs. 6A and C). In the combined presence of G-15 and G-1 (both at 1 μM) spiking persisted in 11 out of 14 experiments, and with a higher

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

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

Confluent A7r5 cells exhibited spontaneous Ca^{2+} spikes and showed a rise in intracellular 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 Ca^{2+} channel (data not shown), suggesting that confluent A7r5 cells express voltage-sensitive Ca^{2+} channels. Treatment with G-1 (1 μM) attenuated the Ca^{2+} response to 30 mM KCl by about 20%, suggesting that G-1 blocks inflow of Ca^{2+} through voltage-sensitive Ca^{2+} channels (Figs. 7B and D). The L-type Ca^{2+} channel blocker nifedipine (100 nM) completely inhibited the 30 mM KCl-evoked 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 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 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 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 Ca^{2+} concentration named Ca^{2+} spikes [22]. Vascular smooth muscle cell Ca^{2+} spike activity depends on both Ca^{2+} inflow and cell/cell contact [22, 27]. In the present study, we demonstrate that G-1 reduces Ca^{2+} spike activity similarly to the voltage-sensitive L-type Ca^{2+} channel blocker nifedipine, suggesting that GPER1 regulates Ca^{2+} handling by reducing L-type Ca^{2+} channel activity. G-1 attenuates also the thromboxane A2 U46619-stimulated A7r5 cell Ca^{2+} spike activity but has no effect on U46619-stimulated basal level of Ca^{2+} , suggesting that G-1 specifically blocks Ca^{2+} inflow associated with spikes but not mechanisms responsible for setting the basal intracellular Ca^{2+} concentration. Importantly, we also show that G-1 attenuates the 30 mM KCl-evoked Ca^{2+} response similar to nifedipine. The KCl-induced Ca^{2+} response is associated with membrane depolarization and inflow of Ca^{2+} via voltage-sensitive Ca^{2+} channels, and thus, the G-1-induced reduction of the KCl-evoked Ca^{2+} response provides further evidence that G-1 blocks voltage-sensitive Ca^{2+} channel activity. It should be noted that spikes in A7r5 cells are elicited by 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 Ca^{2+} spike activity was investigated using the GPER1 antagonist G-15. G-1 reduced 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 Ca^{2+} spike activity is mediated through GPER1. Importantly, treatment with 1 μM G-1 alone inhibited completely Ca^{2+} spike activity in 11 out of 17 experiments (65%), while G-1 fully inhibited 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 μM), G-1 (1 μM) was unable to fully inhibit spike activity. Thus, G-1 seems to act through GPER1. Furthermore, we show that 17 β -estradiol at 1 μM , regarded as a pharmacological concentration of estrogen [24], also reduces vascular smooth muscle cell Ca^{2+} spike activity. This is in line with previous studies showing that 17 β -estradiol, at μM concentrations, reduces 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 Ca^{2+} concentration in GPER1-expressing COS7 cells [2]. Furthermore, intracellular injection of G-1 (3 μM) is reported to cause a fast and transient increase in intracellular 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 Ca^{2+} response [15]. In the present study, we demonstrate that activation of vascular smooth muscle cell GPER1 may influence Ca^{2+} handling by reducing Ca^{2+} spike activity probably via attenuation of Ca^{2+} inflow through voltage-sensitive L-type Ca^{2+} channels. It has been shown that release of Ca^{2+} from intracellular stores does not influence Ca^{2+} spike activity in A7r5 cells, and thus our results do not exclude effects of G-1 on intracellular Ca^{2+} release

in other cell types. In summary, we propose that the GPER1 agonist G-1 regulates vascular smooth muscle cell Ca^{2+} handling by reducing 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.

References

1. Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA: Mechanism of estrogen action. *Physiol Rev* 2001;81:1535-1565.
2. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER: A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 2005;307:1625-1630.
3. Thomas P, Pang Y, Filardo EJ, Gong J: Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology* 2005;146:624-632.
4. Olde B, Leeb-Lundberg LM: GPR30/GPER1: searching for a role in estrogen physiology. *Trends Endocrinol Metab* 2009;20:409-416.
5. Meyer MR, Prossnitz ER, Barton M: The G protein-coupled estrogen receptor GPER/GPR30 as regulator of cardiovascular function. *Vascul Pharmacol* 2011;55:17-25.
6. Nilsson BO, Olde B, Leeb-Lundberg LM: G protein-coupled oestrogen receptor 1 (GPER1)/GPR30: a new player in cardiovascular and metabolic oestrogenic signalling. *Br J Pharmacol* 2011;163:1131-1139.

7. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW: Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest* 1999;103:401-406.
8. Darblade B, Pendaries C, Krust A, Dupont S, Fouque MJ, Rami J, Chambon P, Bayard F, Arnal JF: Estradiol alters nitric oxide production in the mouse aorta through the alpha-, but not beta-, estrogen receptor. *Circ Res* 2002;90:413-419.
9. Nilsson BO: Modulation of the inflammatory response by estrogens with focus on the endothelium and its interactions with leukocytes. *Inflamm Res* 2007;56:269-273.
10. Pare G, Krust A, Karas RH, Dupont S, Aronovitz M, Chambon P, Mendelsohn ME: Estrogen receptor-alpha mediates the protective effects of estrogen against vascular injury. *Circ Res* 2002;90:1087-1092.
11. Pendaries C, Darblade B, Rochaix P, Krust A, Chambon P, Korach KS, Bayard F, Arnal JF: The AF-1 activation-function of ERalpha may be dispensable to mediate the effect of estradiol on endothelial NO production in mice. *Proc Natl Acad Sci USA* 2002;99:2205-2210.
12. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK: Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 2000;407:538-541.

13. Toutain CE, Filipe C, Billon A, Fontaine C, Brouchet L, Guery JC, Gourdy P, Arnal JF, Lenfant F: Estrogen receptor alpha expression in both endothelium and hematopoietic cells is required for the accelerative effect of estradiol on reendothelialization. *Arterioscler Thromb Vasc Biol* 2009;29:1543-1550.
14. Mårtensson UE, Salehi SA, Windahl S, Gomez MF, Swärd K, Daszkiewicz-Nilsson J, Wendt A, Andersson N, Hellstrand P, Grände PO, Owman C, Rosen CJ, Adamo ML, Lundquist I, Rorsman P, Nilsson BO, Ohlsson C, Olde B, Leeb-Lundberg LM: Deletion of the G protein-coupled receptor 30 impairs glucose tolerance, reduces bone growth, increases blood pressure, and eliminates estradiol-stimulated insulin release in female mice. *Endocrinology* 2009;150:687-698.
15. Haas E, Bhattacharya I, Brailoiu E, Damjanovic M, Brailoiu GC, Gao X, Mueller-Guerre L, Marjon NA, Gut A, Minotti R, Meyer MR, Amann K, Ammann E, Perez-Dominguez A, Genoni M, Clegg DJ, Dun NJ, Resta TC, Prossnitz ER, Barton M: Regulatory role of G protein-coupled estrogen receptor for vascular function and obesity. *Circ Res* 2009;104: 288-291.
16. Lindsey SH, Cohen JA, Brosnihan KB, Gallagher PE, Chappell MC: Chronic treatment with the Gpr30 agonist G-1 decreases blood pressure in ovariectomized mRen2.Lewis rats. *Endocrinology* 2009;150:3753-3758.
17. Broughton BR, Miller AA, Sobey CG: Endothelium-dependent relaxation by G protein-coupled receptor 30 agonists in rat carotid arteries. *Am J Physiol Heart Circ Physiol* 2010;298:H1055-H1061.

18. Holm A, Baldetorp B, Olde B, Leeb-Lundberg LM, Nilsson BO: The GPER1 agonist G-1 attenuates endothelial cell proliferation by inhibiting DNA synthesis and accumulating cells in the S and G2 phases of the cell cycle. *J Vasc Res* 2011;48:327-335.
19. Berridge MJ, Lipp P, Bootman MD: The versatility and universality of calcium signaling. *Nat Rev Cell Biol* 2000;1:11-21.
20. Haddock RE, Hill CE: Rhythmicity in arterial smooth muscle. *J Physiol* 2005;566:645-656.
21. Bolton TB: Calcium events in smooth muscles and their interstitial cells; physiological roles of sparks. *J Physiol* 2006;570:5-11.
22. Byron KL, Taylor CW: Spontaneous Ca^{2+} spiking in vascular smooth muscle cell line is independent of the release of intracellular Ca^{2+} stores. *J Biol Chem* 1993;268:6945-6952.
23. Pfaffl MW: A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45.
24. Cornil CA, Ball GF, Balthazart J: Functional significance of rapid regulation of brain estrogen action: where do the estrogens come from? *Brain Res* 2006;1126:2-26.

25. Bologna CG, Revankar CM, Young SM, Edwards BS, Arterburn JB, Kiselyov AS, Parker MA, Tkachenko SE, Savchuck NP, Sklar LA, Oprea TI, Prossnitz ER: Virtual and biomolecular screening converge on a selective agonist for GPR30. *Nat Chem Biol* 2006;2:207-212.
26. Dennis MK, Burai R, Ramesh C, Petrie WK, Alcon SN, Nayak TK, Bologna CG, Leilao A, Brailoiu E, Deliu E, Dun NJ, Sklar LA, Hathaway HJ, Arterburn JB, Oprea TI, Prossnitz ER: In vivo effects of a GPR30 antagonist. *Nat Chem Biol* 2009;5:421-427.
27. Mani BK, Brueggemann LI, Cribbs LL, Byron KL: Opposite regulation of KCNQ5 and TRPC6 channels contributes to vasopressin-stimulated calcium spiking responses in A7r5 vascular smooth muscle cells. *Cell Calcium* 2009;45:400-411.
28. Zhang F, Ram JL, Standley PR, Sowers JR: 17 β -oestradiol attenuates voltage-dependent Ca^{2+} currents in A7r5 vascular smooth muscle cell line. *Am J Physiol* 1994;266:C975-C980.

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 μ M) attenuates spontaneous Ca^{2+} spike activity in A7r5 cells. Panels A and B show representative 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 Ca^{2+} spike amplitude and frequency, respectively. The intracellular 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 Ca^{2+} channel blocker nifedipine (100 nM) inhibits Ca^{2+} spike activity in A7r5 cells. Nifedipine was included at the arrow and present throughout the experiment. The intracellular 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 μ M) to U46619-stimulated (1 μ M) cells reduces Ca^{2+} spike activity but has no effect on the basal level of the U46619 evoked 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 Ca^{2+} spike frequency show that 1 μ M U46619 increases spike frequency almost 2-times and that 1 μ M G-1 completely blocks the U46619-induced increase in spike frequency (B). The intracellular 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 μ M) of estrogen (17 β -estradiol, E2) to A7r5 cells reduces Ca^{2+} spike frequency (B) but not amplitude (A). A high physiological concentration (100 nM) of estrogen has no effect on 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 μ M) blocks the G-1-induced (1 μ M) attenuation of 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 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 μ M) attenuates the Ca^{2+} response to 30 mM KCl in A7r5 cells (B and D). The L-type Ca^{2+} channel blocker nifedipine (100 nM) completely inhibits the 30 mM KCl-evoked Ca^{2+} response (C). Addition of 0.1% DMSO as vehicle-control has no effect on the 30 mM KCl-induced rise in intracellular Ca^{2+} concentration (A). Summarized data (D), assessing the maximal amplitude of the KCl-stimulated Ca^{2+} response, show that G-1 reduces the 30 mM KCl-induced Ca^{2+} response by about 20%. The Ca^{2+} response to 30 mM KCl was evaluated by measuring the maximal amplitude of the Ca^{2+} signal in the presence of G-1 or DMSO (Control) normalized to that of its respective reference KCl-induced 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 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.

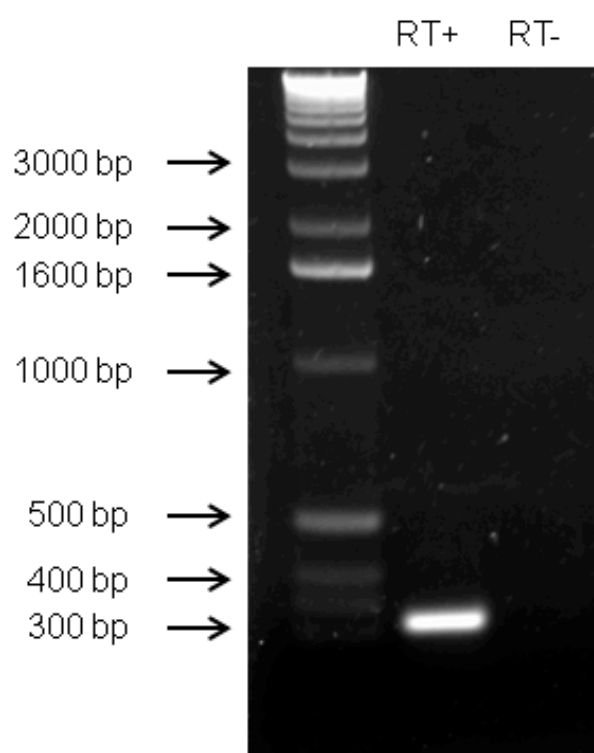
Fig. 1

Fig. 2

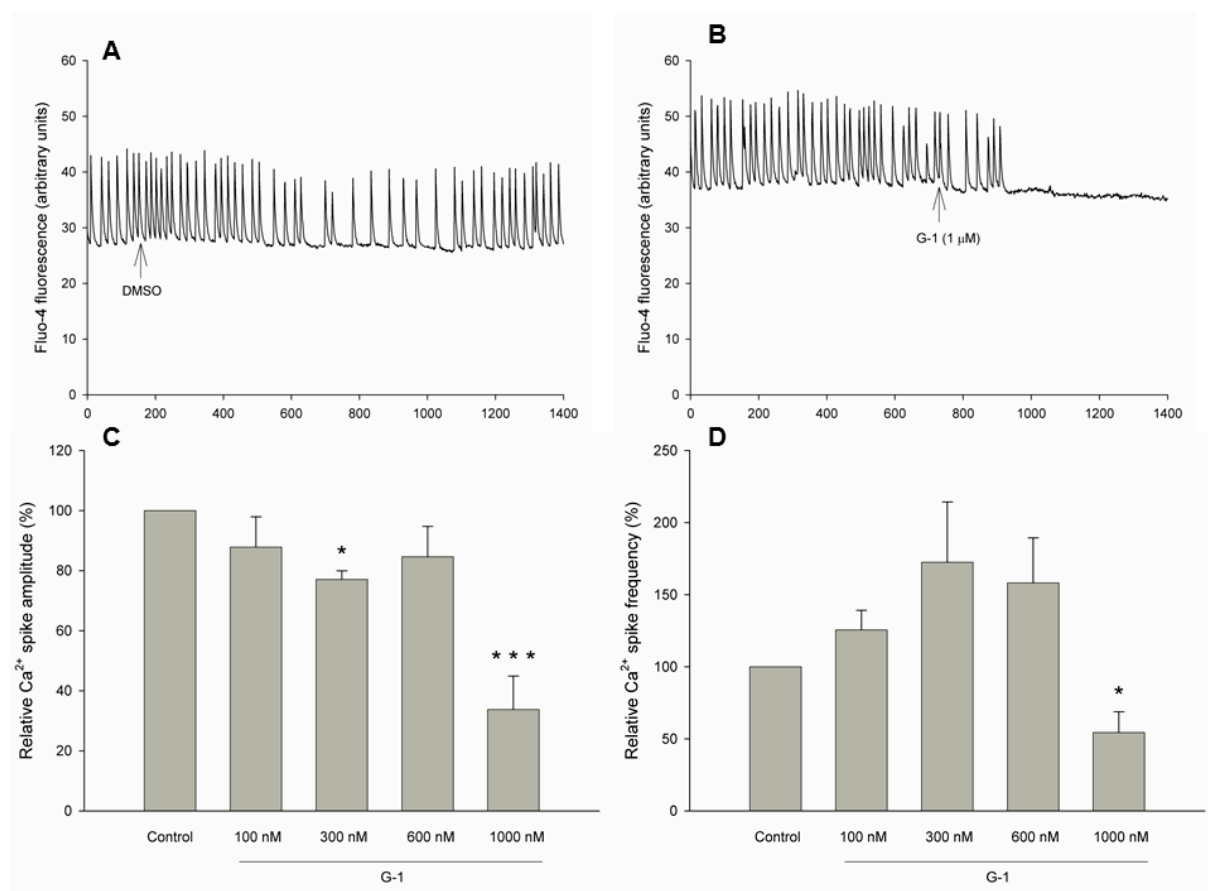


Fig. 3

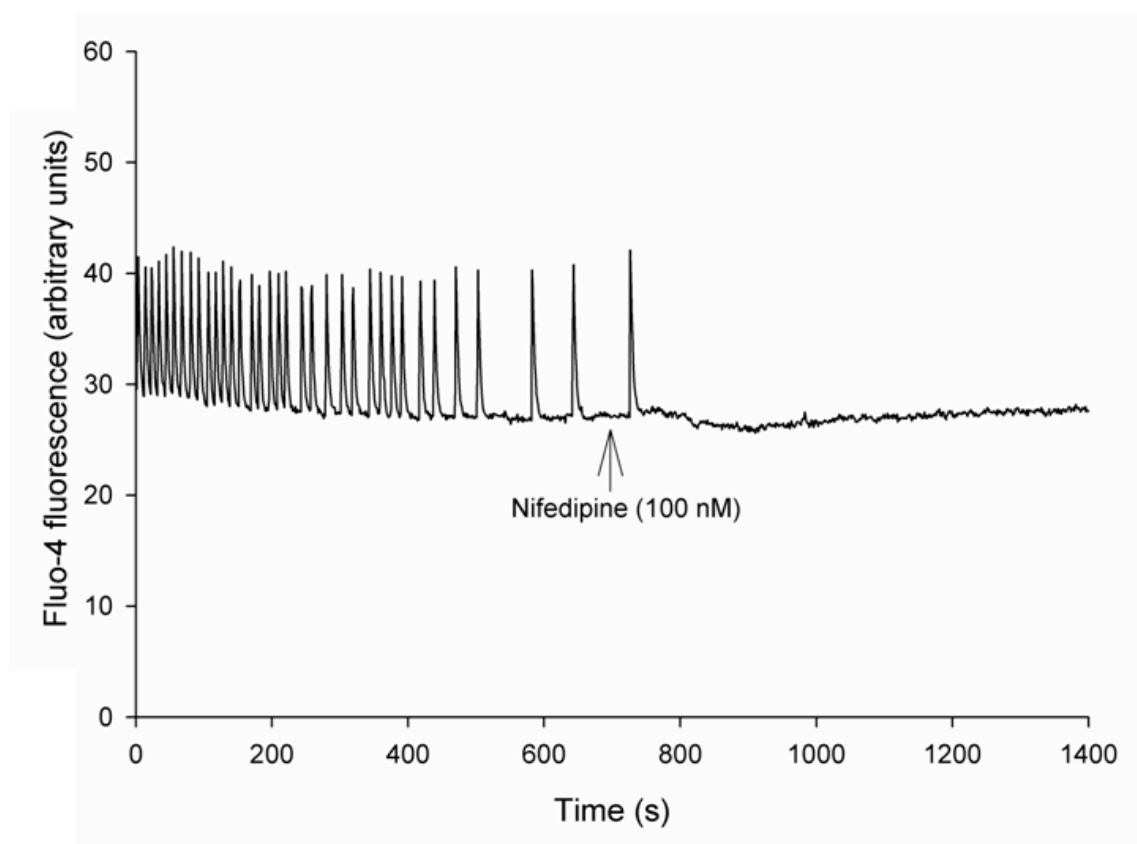


Fig. 4

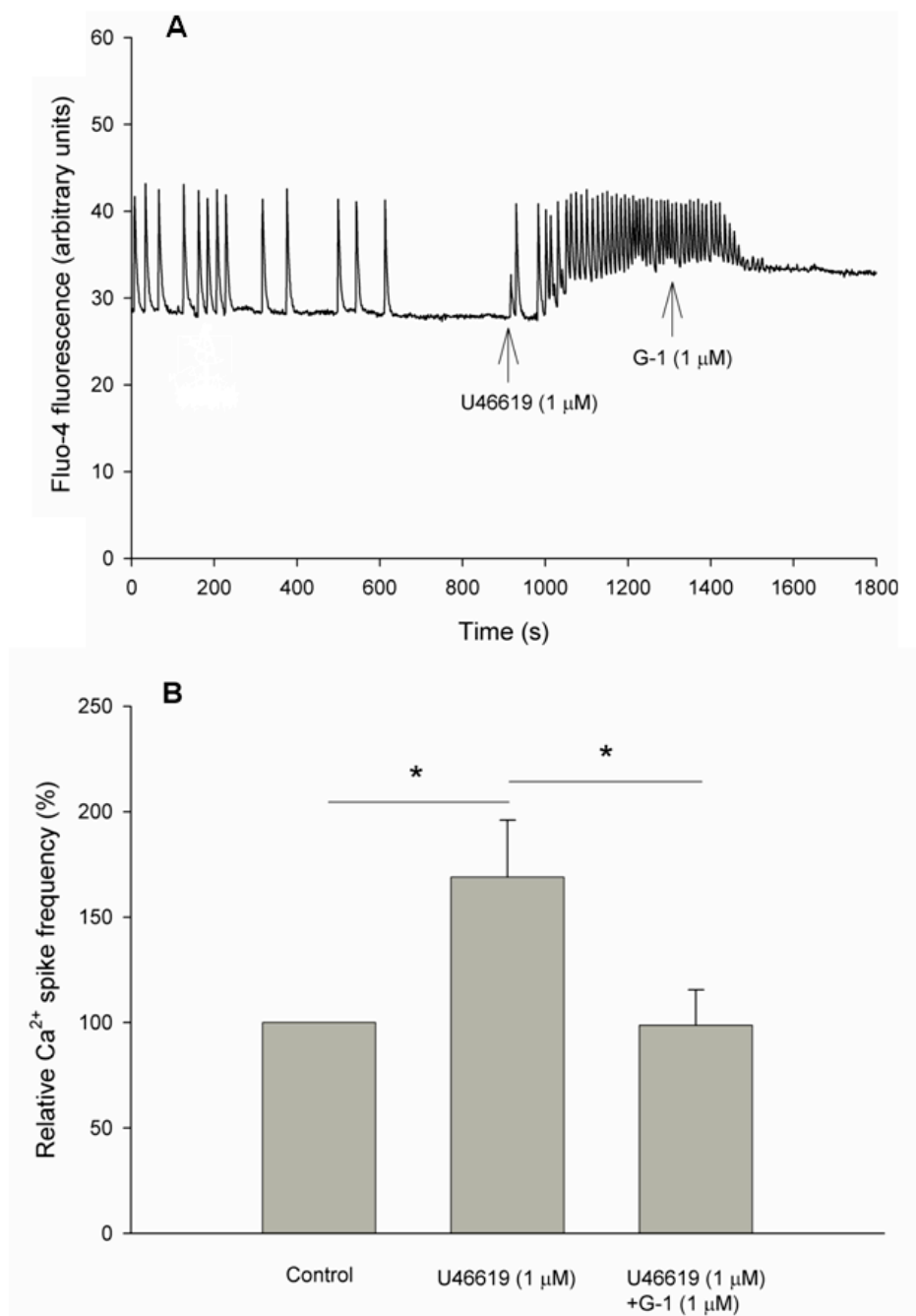


Fig. 5

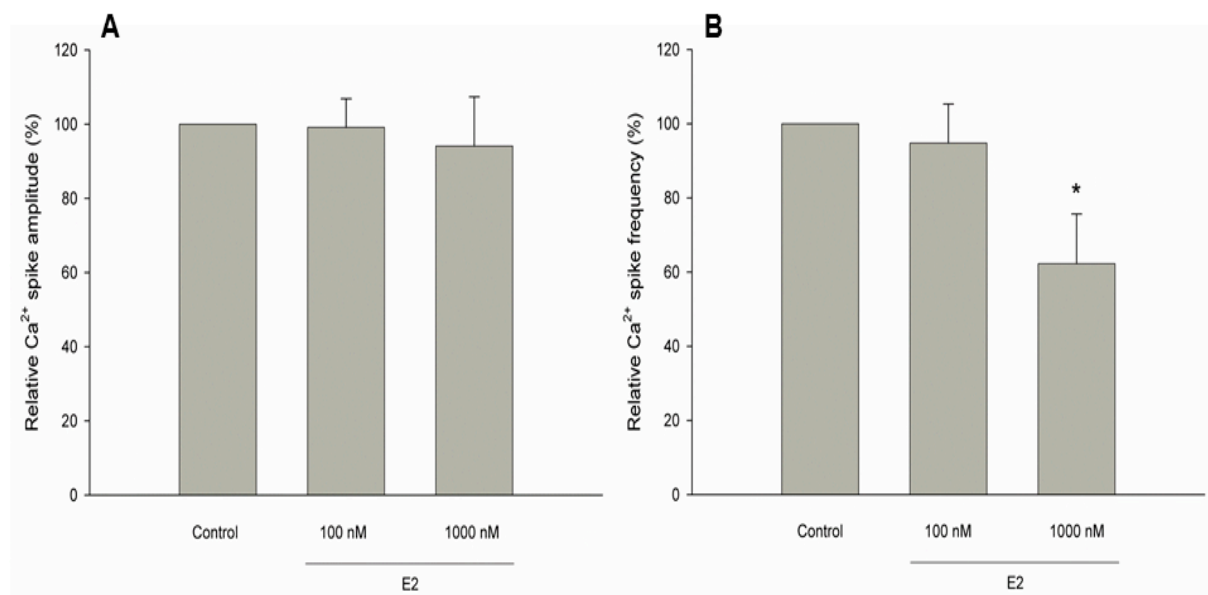


Fig. 6

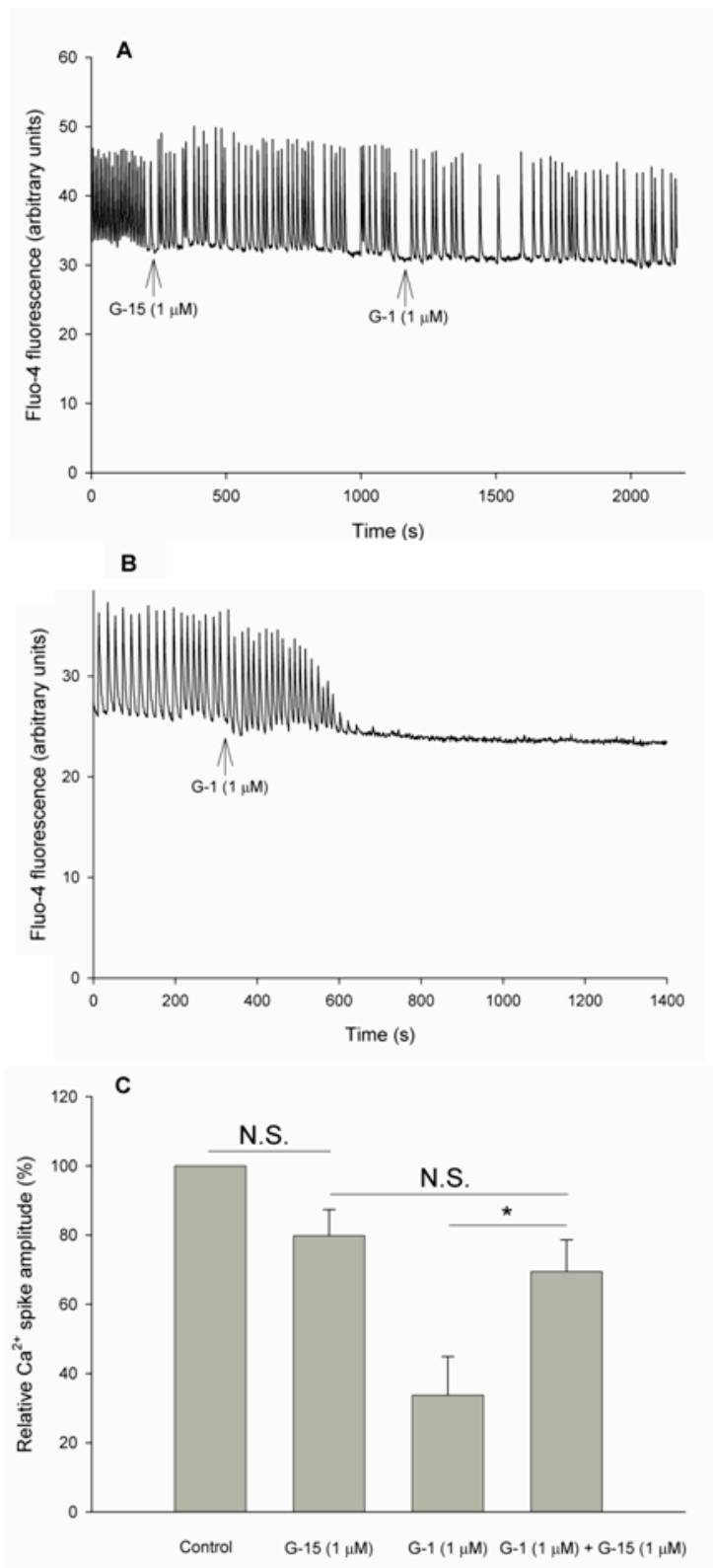


Fig. 7

