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IN HUMAN ENDOTHELIAL PROGENITOR CELLS**

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**A FUNCTIONAL TRANSIENT RECEPTOR POTENTIAL
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9 **A FUNCTIONAL TRANSIENT RECEPTOR POTENTIAL VANILLOID 4 (TRPV4)**
10 **CHANNEL IS EXPRESSED IN HUMAN ENDOTHELIAL PROGENITOR CELLS**
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48 **ruthenium red, proliferation**

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ABSTRACT

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Endothelial progenitor cells (EPCs) are mobilized into circulation to replace damaged endothelial and recapitulate the vascular network of injured tissues. Intracellular Ca^{2+} signals are key to EPC activation, but it is yet to be elucidated whether they are endowed with the same blend of Ca^{2+} -permeable channels expressed by mature endothelial cells. For instance, endothelial colony forming cells (ECFCs), the only EPC subset truly committed to acquire a mature endothelial phenotype, lack canonical transient receptor potential channels 3, 5 and 6 (TRPC3, 5 and 6), which are widely distributed in vascular endothelium; on the other hand, they express a functional store-operated Ca^{2+} entry (SOCE). The present study was undertaken to assess whether human circulating EPCs possess TRP vanilloid channel 4 (TRPV4), which plays a master signalling role in mature endothelium, by controlling both vascular remodelling and arterial pressure. We found that EPCs express both TRPV4 mRNA and protein. Moreover, both GSK1016790A (GSK) and phorbol myristate acetate and, two widely employed TRPV4 agonists, induced intracellular Ca^{2+} signals uniquely in presence of extracellular Ca^{2+} . GSK- and PMA-induced Ca^{2+} elevations were inhibited by RN-1734 and ruthenium red, which selectively target TRPV4 in mature endothelium. However, TRPV4 stimulation with GSK did not cause EPC proliferation, while the pharmacological blockade of TRPV4 only modestly affected EPC growth in the presence of a growth factor-enriched culture medium. Conversely, SOCE inhibition with BTP-2, La^{3+} and Gd^{3+} dramatically decreased cell proliferation. These data indicate that human circulating EPCs possess a functional TRPV4 protein before their engraftment into nascent vessels.

INTRODUCTION

Vascular endothelial cells line the lumen of blood and lymphatic thereby forming a multifunctional signal transduction platform that maintains cardiovascular homeostasis (Mancardi et al., 2011; Moccia et al., 2013b). Endothelial injury is recognized as initial step in the onset of severe cardiovascular diseases, including myocardial infarction, brain stroke, atherosclerosis, and diabetes (Moccia et al., 2010; Moccia et al., 2012a). Endothelial progenitor cells (EPCs) are a sub-population of mononuclear cells (MNCs) which is recruited from either bone marrow (BM) or arterial wall to replace damaged/senescent endothelial cells and recapitulate the vascular network of lesioned organs (Yoder, 2012b). They act by either stimulating local angiogenesis via paracrine signalling or by physically engrafting within neovessels (Yoder, 2012b). It is, therefore, not surprising that circulating EPCs are currently regarded as one of the most promising tools for the cell-based therapy of CV pathologies (Alev et al., 2011; Murasawa and Asahara, 2005). Among the different subtypes of EPCs described in the literature (Yoder, 2012a), the so-called endothelial colony forming cells (ECFCs) are unique in their truly belonging to the endothelial lineage and in their ability of forming capillary like structures *in vitro* and patent vessels *in vivo* (Basile and Yoder, 2014; Yoder, 2012b). It is, however, largely unknown whether they already express the signal transduction pathways that enable mature endothelial cells to properly respond to external stimuli (Lodola et al., 2012; Moccia et al., 2012c; Moccia et al., 2014b).

An increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is the earliest response of vascular endothelium to extracellular stimulation (Moccia et al., 2012b; Pla et al., 2012a). The endothelial Ca^{2+} signals are mainly shaped by the interplay between inositol-1,4,5-trisphosphate (InsP_3)-gated Ca^{2+} release from the endoplasmic reticulum (ER) and Ca^{2+} influx across the plasma membrane (Moccia et al., 2012b; Moccia et al., 2013b). Store-operated Ca^{2+} entry (SOCE) sustains a myriad of endothelial

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9 functions, ranging from cell proliferation to the control of luminal permeability (Abdullaev et al., 2008;
10 Moccia et al., 2012b; Tirupathi et al., 2006). Recent work from our group has disclosed that a
11 functional SOCE is also present in human circulating EPCs, where it is mediated by the interaction
12 between Stim1, the ER Ca²⁺ sensor, and Orai1 and canonical transient receptor potential channel 1
13 (TRPC1), which contribute to the Ca²⁺-permeable pore-forming domain on the plasma membrane
14 (Lodola et al., 2012; Moccia et al., 2013a; Moccia et al., 2012d; Sánchez-Hernández et al., 2010).
15 Store-dependent Ca²⁺ inflow controls cell proliferation and *in vitro* tubulogenesis in EPCs isolated
16 from both peripheral (Dragoni et al., 2011; Dragoni et al., 2014; Lodola et al., 2012) and umbilical cord
17 blood (Dragoni et al., 2013). Conversely, circulating human EPCs lack the diacylglycerol-sensitive
18 canonical transient receptor potential (TRP) channels 3 (TRPC3) and TRPC6, and the polymodal
19 channel TRPC5 (Fiorio Pla and Munaron, 2014; Moccia et al., 2012b; Pla et al., 2012a). This feature is
20 rather surprising when considering the important roles played by these pathways in mature endothelium
21 (Fiorio Pla and Munaron, 2014; Moccia et al., 2012b; Moccia et al., 2014a; Pla et al., 2012a). For
22 instance, TRPC3 and TRPC6 may sustain the pro-angiogenic Ca²⁺ response to VEGF in microvascular
23 endothelial cells (Cheng et al., 2006; Hamdollah Zadeh et al., 2008). An additional store-independent
24 Ca²⁺ entry route in mature endothelium is provided by vanilloid TRP channel 4 (TRPV4) (Fiorio Pla
25 and Gkika, 2013; Hatano et al., 2013; Pla et al., 2012a; Schierling et al., 2011; Troidl et al., 2010;
26 Troidl et al., 2009). This is a polymodal TRP channel which acts as a molecular integrator of both
27 chemical and physical stimuli (Earley and Brayden, 2010; Moccia et al., 2012b). Endothelial TRPV4
28 may be activated by changes in osmotic pressure, fluid shear stress and temperature, by the phorbol
29 derivatives, 4 α -phorbol-12,13-didecanoate (4 α PDD) and phorbol myristate acetate (PMA), by
30 arachidonic acid (AA) and its metabolites, and by the specific small molecule agonist, N-((1S)-1-[[4-
31 ((2S)-2-[[[(2,4-dichlorophenyl)sulfonyl]amino]-3-hydroxypropanoyl]-1-piperazinyl]carbonyl]-3-
32 methylbutyl)-1-benzothiophene-2-carboxamide (GSK1016790A or GSK) (Baylie and Brayden, 2011;
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9 Sonkusare et al., 2012; Sullivan et al., 2012). A growing number of studies revealed that TRPV4-
10 mediated Ca^{2+} inflow contributes to endothelial cell proliferation and vascular remodelling (i.e.
11 arteriogenesis) which occurs as a consequence of arterial occlusion in a number of vascular districts
12 (Hatano et al., 2013; Pla et al., 2008; Pla et al., 2012b; Schierling et al., 2011; Troidl et al., 2010; Troidl
13 et al., 2009). Moreover, TRPV4 may regulate arterial pressure by activating a variety of vasodilatory
14 mechanisms, such as nitric oxide (NO), prostaglandin I_2 (PGI_2), and intermediate- and small-
15 conductance Ca^{2+} -activated K^+ channels ($\text{IK}_{\text{Ca}}/\text{SK}_{\text{Ca}}$)-mediated endothelial-dependent hyperpolarizing
16 factor (EDHF) (Baylie and Brayden, 2011; Earley and Brayden, 2010). In the light of the key signalling
17 function accomplished by TRPV4 in differentiated endothelial cells, we thus undertook the present
18 investigation to assess whether this Ca^{2+} -permeable route is also expressed in human circulating EPCs.
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MATERIALS AND METHODS

Cell isolation

Blood samples (40 mL) were obtained from healthy human volunteers aged from 22 to 28 years old. The Institutional Review Board at “Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo Foundation” in Pavia approved all protocols and specifically approved the study. Informed consent was obtained according to the Declaration of Helsinki. Informed written consent was obtained according to the Declaration of Helsinki. We focussed on the so-called endothelial colony forming cells (ECFCs) (Ingram et al., 2004), a subgroup of EPCs which are found in the CD34⁺ CD45⁻ fraction of circulating mononuclear cells, exhibit robust proliferative potential and form capillary-like structures *in vitro* (Ingram et al., 2004; Moccia et al., 2012d). To isolate ECFCs, mononuclear cells (MNCs) were separated from peripheral blood (PB) by density gradient centrifugation on lymphocyte separation medium for 30 min at 400g and washed twice in EBM-2 with 2% FCS. A median of 36 x 10⁶ MNCs (range 18-66) were plated on collagen-coated culture dishes (BD Biosciences) in the presence of the endothelial cell growth medium EGM-2 MV Bullet Kit (Lonza) containing endothelial basal medium (EBM-2), 5% foetal bovine serum, recombinant human (rh) EGF, rhVEGF, rhFGF-B, rhIGF-1, ascorbic acid and heparin, and maintained at 37°C in 5% CO₂ and humidified atmosphere. Discard of non-adherent cells was performed after 2 days; thereafter medium was changed three times a week. The outgrowth of endothelial cells from adherent MNCs was characterized by the formation of a cluster of cobblestone-appearing cells (Sánchez-Hernández et al., 2010). That ECFC-derived colonies belonged to endothelial lineage was confirmed as described in (Sánchez-Hernández et al., 2010) and (Lodola et al., 2012). Adult human dermal microvascular endothelial cells (HMVEC-d) and adult human cardiac microvascular endothelial cells (HMVEC-c) were purchased from Lonza and grown in EGM2-MV medium. Cells were used at passages 3–10.

Solutions

Physiological salt solution (PSS) had the following composition (in mM): 150 NaCl, 6 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 Glucose, 10 Hepes. In Ca²⁺-free solution (0Ca²⁺), Ca²⁺ was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Solutions were titrated to pH 7.4 with NaOH. The osmolality of the extracellular solution, as measured with an osmometer (Wescor 5500, Logan, UT), was 300-310 mmol/kg.

[Ca²⁺]_i measurements and statistics

EPCs were loaded with 4 μM fura-2 acetoxymethyl ester (fura-2/AM; 1 mM stock in dimethyl sulfoxide) in PSS for 1 hour at room temperature. The Ca²⁺ measuring system has been extensively described in (Berra-Romani et al., 2013; Di Buduo et al., 2014). The Ca²⁺ fluorochrome has been excited at 340 and 380 nm and the light emitted detected at 510 nm. [Ca²⁺]_i was monitored by measuring, for each ROI, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly termed "ratio"). An increase in [Ca²⁺]_i causes an increase in the ratio (Berra-Romani et al., 2013; Di Buduo et al., 2014). Ratio measurements were performed and plotted on-line every 3 s. The experiments were performed at room temperature (22°C). All the data have been collected from ECFCs isolated from the peripheral blood of at least three healthy volunteers. The amplitude of the peak Ca²⁺ response was measured as the difference between the ratio at the peak and the mean ratio of 1 min baseline before the peak. Pooled data are given as mean ± SE and statistical significance ($P < 0.05$) was evaluated by the Student's *t*-test for unpaired observations.

RNA isolation and RT-PCR

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Total RNA was extracted from the ECFCs using the QIAzol Lysis Reagent (QIAGEN, Italy). Single cDNA was synthesized from RNA (1 µg) using random hexamers and M-MLV Reverse Transcriptase (Invitrogen S.R.L., Italy). Reverse transcription was always performed in the presence or absence (negative control) of the reverse transcriptase enzyme. PCR was performed as previously described by using specific primers (intron-spanning primers) designed for human TRPV4 (sense, 5'-TCCAGCTGCTCTACTTCATC-3'; antisense, 5'-CTGAAGCGTAGCCGATCAT -3') and TRPV1 (sense, 5'-AGCTACTACAAGGCCAGACA-3'; antisense, 5'-AACTTCACGATGCCAGCTGG-3') (Lodola et al., 2012; Sánchez-Hernández et al., 2010). The RT-PCR reactions were normalized using β -actin as housekeeping gene (primers used: Hs_ACTB_1_SG, QuantiTect Primer Assay QT00095431, Qiagen). First, the sequence of the TRPV4 band was checked by using the Big dye terminator cycle sequencing kit (Applied Biosystem, PE, USA). PCR products were separated with agarose gel electrophoresis, stained with ethidium bromide, and acquired with the Image Master VDS (Amersham Biosciences Europe, Italy). The molecular weight of the PCR products was compared to the DNA molecular weight marker VIII (Roche Molecular Biochemicals, Italy).

Protein extraction and Western blot analysis

Protein extraction and Western blot analysis were carried out as described previously (Lodola et al., 2012; Sánchez-Hernández et al., 2010). The anti-TRPV4 antibody was raised against the peptide C-DGHQQGYAPKWRAEDAPL in rabbits (Loftstrand Labs). The resulting rabbit serum was then affinity purified to obtain the anti-TRPV4 antibody. The specific controls were performed as previously described (Pla et al., 2012b).

Proliferation assay

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9 A total of 1×10^5 ECFC-derived cells (1st passage) were plated in 30-mm collagen-treated
10 dishes in endothelial basal medium (EBM-2; Lonza) supplemented with 5% foetal bovine serum (FBS)
11 in the presence of 20 nM GSK. As a control experiment, cells were seeded in the presence of in the
12 presence of the endothelial cell growth medium EGM-2 MV Bullet Kit (Lonza) containing EBM-2, 5%
13 FBS, recombinant human (rh) EGF, rhVEGF, rhFGF-B, rhIGF-1, ascorbic acid and heparin. Cultures
14 were incubated at 37 °C, 5% CO₂ and cell growth assessed every day until confluence was reached in
15 the control dishes. Cells were then recovered by trypsinization and their number assessed by counting
16 in a hemocytometer. The percentage of growth stimulation was calculated by dividing the total number
17 of cells obtained in presence of GSK by the number of cells in control experiments (i.e. EGM-2 MV)
18 and multiplying the ratio by 100. The effect of VEGF was evaluated by plating the cells with 10 ng/ml
19 VEGF and with or without BAPTA (30 μM), a membrane-permeable intracellular Ca²⁺ buffer (Dragoni
20 et al., 2011; Dragoni et al., 2013). Alternatively, 1×10^5 ECFC-derived cells were plated in 30-mm
21 collagen-treated dishes in EGM-2 MV Bullet Kit in the absence (control) and presence of either RN-
22 1734 (20 μM) or ruthenium red (10 μM), two well known TRPV4 inhibitors. The effect of TRPV4
23 blockade on EPC proliferation was evaluated as illustrated above.
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38 *Chemicals*

39 EBM and EGM-2 were purchased from Clonetics (Cell System, St. Katharinen, Germany).
40 Fura-2/AM was obtained from Molecular Probes (Molecular Probes Europe BV, Leiden, The
41 Netherlands). GSK has been synthesized as described in (Zaninetti et al., 2011). All other chemicals
42 were obtained from Sigma Chemical Co. (St. Louis, MO, USA).
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RESULTS

TRPV4 is expressed in human endothelial colony forming cells

The expression of TRPV4 mRNA in human EPCs was assessed by RT-PCR. Figure 1A depicts the results of agarose gel electrophoresis of representative PCR reaction products. Single bands of the expected size of cDNA fragments were amplified (246 and 146 bp for TRPV4 and β -actin, respectively) in isolated cells. In the absence of RT, no PCR-amplified products were detected, thereby showing the specificity of the reaction (Fig. 1A). These data are consistent with the presence of TRPV4 mRNA in human EPCs. In order to determine whether TRPV4 is expressed at protein level, we performed a Western Blot analysis by using affinity-purified antibodies, which revealed a prominent band at 70 kDa (Fig. 1B, right). As a control, we utilized human dermal (HMVEC-d) and coronary (HMVEC-c) microvascular endothelial cells, which both display a protein of the same molecular size (Fig. 1B, left). Therefore, TRPV4 is present at both mRNA and protein level in circulating EPCs.

TRPV4 agonists elicit intracellular Ca^{2+} signals in endothelial colony forming cells

In order to assess whether these TRPV4 proteins conduct extracellular Ca^{2+} , EPCs were loaded with Fura-2 and exposed to GSK (20 nM) or PMA (10 μ M), two specific TRPV4 agonists (Everaerts et al., 2010). As a consequence, GSK has been extensively utilized to assess the biological outcomes of TRPV4 activation in vascular endothelium (Mergler et al., 2011; Sonkusare et al., 2012; Sullivan et al., 2012). Figure 2A shows that GSK evoked a transient increase in $[Ca^{2+}]_i$ in 121 out of 125 (96.8%) circulating EPCs: the magnitude of the Ca^{2+} signal was 0.075 ± 0.005 (n=121), whereas the average lag time between the exposition to the agonist and the onset of the response was 46.7 ± 1.2 sec (n=121). GSK-induced Ca^{2+} signals were reversibly prevented by removal of extracellular Ca^{2+} (Fig. 2B) and by RN-1734 (20 μ M; n=117) (Fig. 2C), a selective TRPV4 antagonist (Bagher et al., 2012; Zheng et al.,

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9 2013). Ruthenium red (RR) has also been used as a pharmacological tool to validate TRPV4 expression
10 in vascular endothelium (Hatano et al., 2013; Willette et al., 2008; Zheng et al., 2013). As expected, the
11 Ca^{2+} response to GSK was reversibly suppressed by pre-incubating the cells with this compound (10
12 μM ; n=104) (Fig. 2D).

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16 Subsequently, we probed the effect of PMA, a structurally distinct TRPV4 agonist (Everaerts et
17 al., 2010). Figure 3A shows that PMA (10 μM) elicited a rapid and monophasic increase in $[\text{Ca}^{2+}]_i$ in
18 251 out of 306 EPCs (82%). The amplitude of the Ca^{2+} peak was 0.125 ± 0.007 (n=251), while the
19 latency of the signal ranged between 10 sec and 430 sec, the average value being 107.8 ± 78.9 sec
20 (n=184). PMA-evoked increase in $[\text{Ca}^{2+}]_i$ was reversible (Fig. 3B) and disappeared in absence of
21 extracellular Ca^{2+} (Fig. 3C). Notably, readmission of Ca^{2+} to the bathing solution always restored the
22 $[\text{Ca}^{2+}]_i$ raise (Fig. 2C), as well as removal of extracellular Ca^{2+} caused the rapid decline of the $[\text{Ca}^{2+}]_i$
23 elevation to the baseline (Fig. 3D). Similar to GSK, both RN-1734 (20 μM ; n=102) (Fig. 3E) and RR
24 (10 μM ; n=170) (Fig. 3F) abrogated PMA-evoked Ca^{2+} signals in human EPCs. Consistent with these
25 results, the acute application of RR (10 μM) abrogated the Ca^{2+} response to PMA in 53 out of 53
26 (100%) cells (Fig. 3G). Phorbol esters might also stimulate TRPV1 channels (Baylie and Brayden,
27 2011), whose transcripts are expressed in circulating EPCs (see Fig. S1). However, PMA-induced Ca^{2+}
28 signals were unaffected by capsazepine (10 μM) (Fig. 3H), a specific TRPV1 inhibitor. This result
29 strongly hints at TRPV4 as the molecular target of PMA. Overall, the pharmacological profile (i.e.
30 activation by phorbol esters and GSK, and blockade by RN-1734 and RR) herein described concurs
31 with that reported in mature human endothelium, at both micro- and macrovascular level (Bubolz et al.,
32 2012a; Hatano et al., 2013; Pla et al., 2008; Pla et al., 2012b; Sonkusare et al., 2012; Sullivan et al.,
33 2012), and demonstrate that the TRPV4 protein detected by immunoblotting serves as a Ca^{2+} -
34 permeable membrane channel in human EPCs. Conversely, neither hypotonic stimulation (n=141) (Fig.
35 4A) or 1-2 μM arachidonic acid (AA) (n=102) (Fig. 4B), which have been associated to TRPV4-

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9 mediated Ca^{2+} inflow in mature endothelium (Moccia et al., 2012b; Pla et al., 2008), ignited any
10 detectable increase in $[\text{Ca}^{2+}]_i$ in EPCs responsive to 20 nM GSK.
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15 ***TRPV4-induced Ca^{2+} inflow is not amplified by intracellular Ca^{2+} release in EPCs***

16 Extracellular Ca^{2+} influx gated through TRPV4 channels may be further amplified by the
17 mechanism of Ca^{2+} -induced Ca^{2+} release (CICR), thereby resulting in a massive Ca^{2+} mobilization
18 from the intracellular Ca^{2+} pool. For instance, TRPV4-gated Ca^{2+} influx recruits InsP_3 -dependent Ca^{2+}
19 release and triggers propagating Ca^{2+} oscillations in mouse astrocyte endfeet (Dunn et al., 2013). We
20 have widely established that the ER Ca^{2+} content in human EPCs is mainly sensitive to InsP_3 -
21 dependent stimulation, while functional ryanodine receptors seem to be absent (Dragoni et al., 2011;
22 Dragoni et al., 2013; Lodola et al., 2012; Sánchez-Hernández et al., 2010). In order to assess the
23 contribution of the endogenous Ca^{2+} reservoir to TRPV4-induced Ca^{2+} signals, we depleted the InsP_3 -
24 regulated Ca^{2+} pool with cyclopiazonic acid (CPA). CPA is a selective inhibitor of Sarco-Endoplasmic
25 Reticulum Ca^{2+} -ATPase (SERCA) activity which impairs ER Ca^{2+} storage ability. The application of
26 CPA (10 μM) in the presence of extracellular Ca^{2+} led to an initial increase in $[\text{Ca}^{2+}]_i$ due to
27 interruption of the ER Ca^{2+} cycle between SERCA-mediated Ca^{2+} re-uptake and Ca^{2+} efflux through
28 ER leakage channels. The following decay of the intracellular Ca^{2+} peak to a plateau level was due to
29 SOCE activation following depletion of the intracellular Ca^{2+} reserve (Lodola et al., 2012; Sánchez-
30 Hernández et al., 2010). Thirty minutes of pre-treatment with 10 μM CPA have been shown to fully
31 empty the InsP_3 -sensitive Ca^{2+} store in human EPCs (Dragoni et al., 2011; Sánchez-Hernández et al.,
32 2010). However, the subsequent addition of GSK (20 nM) caused an increase in $[\text{Ca}^{2+}]_i$ which did not
33 significantly ($p < 0.05$) differ from that observed in untreated cells (Fig. 5A-C). In order to confirm this
34 data, we challenged with GSK (20 nM) EPCs pre-incubated with 2-APB (50 μM), a popular InsP_3 R
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9 inhibitor (Dragoni et al., 2011; Dragoni et al., 2013). In according with our previous results, 2-APB did
10 not significantly ($p < 0.05$) affect GSK-induced Ca^{2+} signals in EPCs (Fig. 5E-F).

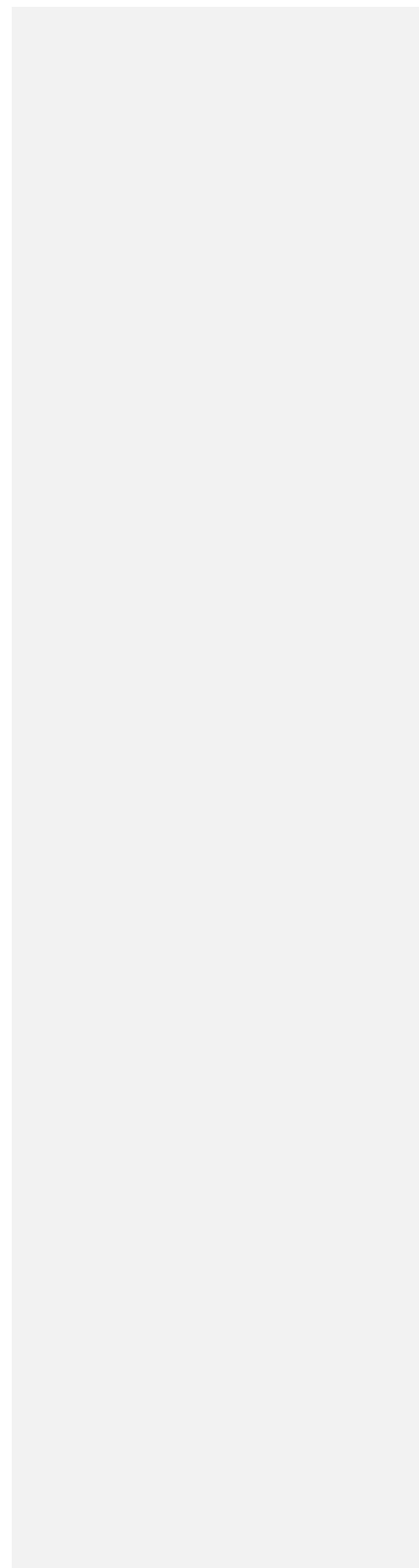
11 12 13 14 ***Effect of TRPV4-mediated Ca^{2+} entry on EPC proliferation***

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16 TRPV4-mediated Ca^{2+} entry promotes cell replication in a variety of mature endothelial cells
17 (Hatano et al., 2013; Pla et al., 2008; Schierling et al., 2011; Troidl et al., 2010; Troidl et al., 2009).
18 Therefore, we focussed on EPC proliferation to evaluate the physiological outcome of TRPV4
19 activation. Surprisingly, TRPV4 stimulation with GSK did not cause a statistically relevant ($p < 0.05$)
20 increase in cell number, when EPCs were grown in a basal medium devoid of growth factors and
21 supplemented with 5% foetal bovine serum (Figure 6A). Our control experiments demonstrated that
22 EPC proliferation occurs in a Ca^{2+} -dependent manner when the cells are stimulated with VEGF (10
23 ng/ml), as previously reported by our group (Fig. 6B) (Dragoni et al., 2011; Dragoni et al., 2013;
24 Lodola et al., 2012; Sánchez-Hernández et al., 2010). Therefore, TRPV4 activation by GSK does not
25 lead to cell replication in endothelial committed progenitors. We then sought to assess whether TRPV4
26 sustains EPC proliferation during EPC expansion with the EGM-2 MV Bullet Kit. Figure 6C shows
27 that RR (10 μ M) and RN-1734 (20 μ M) cause a modest inhibition in cell growth when compared to the
28 dramatic effect exerted by BTP-2 (20 μ M), a selective inhibitor of SOCE in EPCs (Lodola et al., 2012;
29 Moccia et al., 2012d; Moccia et al., 2014a; Sánchez-Hernández et al., 2010). However, BTP-2 has been
30 shown to affects conductances other than Orai1 and TRPC1 in other cellular settings (Moccia et al.,
31 2012d; Moccia et al., 2014a). Therefore, we confirmed this result by showing that low micromolar
32 doses of La^{3+} and Gd^{3+} , which selectively interfere with SOCE at this concentration (Moccia et al.,
33 2012d; Moccia et al., 2014a), dramatically reduced cell proliferation (Fig. 6C), as previously shown by
34 our group in healthy EPCs (Lodola et al., 2012). These data are consistent with those we have
35 previously described in both normal cells and EPCs isolated from RCC patients (Lodola et al., 2012;
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Sánchez-Hernández et al., 2010): overall, they strongly suggest that SOCE is more powerful in delivering pro-angiogenic Ca²⁺ signals to human EPCs than TRPV4.

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DISCUSSION

TRPV4 has recently been shown to promote mature EC proliferation both *in vitro* and *in vivo* due to the activation of a host of Ca^{2+} -dependent transcription factors, such as nuclear factor of activated T-cells, calcineurin-dependent 1 (NFATc1), Kv channel interacting protein 3, calsenilin (KCNIP3/CSEN/DREAM), and myocyte enhancer factor 2C (MEF2C). Moreover, TRPV4-mediated Ca^{2+} inflow drives TECs to both migrate and replicate, a feature which hints at this channel as a suitable target for alternative anti-angiogenic strategies. Apart from the classic process of sprouting angiogenesis, tumour vascularization may require the contribution of bone marrow-derived EPCs (Gao et al., 2009; Moccia et al., 2014a). We have recently suggested that the pharmacological blockade of extracellular Ca^{2+} inflow might be successfully employed in anti-cancer strategies when the target channel is present both in mature ECs and in more immature committed progenitors (Moccia et al., 2014a). Therefore, we endeavoured the present investigation to assess whether TRPV4 is expressed and controls proliferation in peripheral EPC, the only EPC subset truly belonging to the endothelial lineage and to physically engraft within neovessels *in vivo*.

A functional TRPV4 is expressed in human EPCs

We provided the evidence that TRPV4 is present in circulating human EPCs at both mRNA and protein level. Immunoblotting detected a single band at 70kDa, which is in the same range as that reported in human microvascular endothelium (Pla et al., 2012b). Conversely, human coronary ECs were recently found to express two TRPV4 bands at 110 and 98 kDa, respectively (Bubolz et al., 2012b). In this regard, immunoblotting conducted in both kidney extracts (Liedtke and Friedman, 2003) and rat cholangiocytes (Gradilone et al., 2007) revealed a double band pattern: the 107 kDa protein and a shorter 75 kDa isoform. The lower molecular band observed in both EPCs and

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9 microvascular ECs might, therefore, be attributed to an alternative splice variant or to post-translational
10 modifications of TRPV4. For instance, the anti-TRPV4 immunoblot showed only the 75 kDa band
11 when rat cholangiocyte homogenates were treated with N-glycosidase (Gradilone et al., 2007). These
12 results indicate that, unlike TRPC3, TRPC5 and TRPC6, TRPV4 is expressed by EPCs before they
13 engraft within the foci of neovascularisation. It is, however, likely that specific environmental cues
14 instruct EPCs to promote TRPV4 glycosylation when they acquire a macrovascular endothelial
15 phenotype (Aird, 2012). The expression of a functional TRPV4 protein in EPCs has then been
16 demonstrated by the Ca^{2+} signals occurring in response to two structurally distinct TRPV4 agonists,
17 namely PMA and GSK. Accordingly, both PMA- and GSK-evoked elevations in $[\text{Ca}^{2+}]_i$ have been
18 observed in the presence, but not in the absence, of extracellular Ca^{2+} and are inhibited by both RR,
19 which is an unselective TRPV inhibitor, and RN 1734, which specifically blocks TRPV4. The kinetics
20 of the Ca^{2+} response to PMA and GSK are, however, different. Both compounds have widely been
21 employed to assess the expression of a conductive TRPV4 channel in mature ECs both *in vitro* and *in*
22 *vivo* (Hatano et al., 2013; Schierling et al., 2011; Troidl et al., 2010; Troidl et al., 2009). Phorbol esters
23 activate TRPV4 independently on protein kinase C, i.e. by physically interacting with a binding pocket
24 located within the transmembrane III-IV region of the channel protein (Everaerts et al., 2010).
25 Conversely, the activating mechanism of GSK is yet to be elucidated. Unlike PMA, the Ca^{2+} response
26 to GSK is transient and rapidly decays to the baseline. The rapid recovery of intracellular Ca^{2+} in the
27 continuous presence of the agonist might be due to GSK-induced channel desensitization, as recently
28 described in (Jin et al., 2011). These authors demonstrated that GSK specifically activates TRPV4-
29 gated Ca^{2+} inflow, thereafter causing a rapid down-regulation of the plasmalemmal protein that is
30 associated to the shut-down of channel activity (Jin et al., 2011). This feature, however, does not apply
31 to each vascular district, as human corneal endothelial cells display a sustained increase in $[\text{Ca}^{2+}]_i$ even
32 upon agonist washout (Mergler et al., 2011). As compared to PMA, however, the onset of GSK-

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9 induced Ca^{2+} signals is relatively fast. The long delay before the development of the Ca^{2+} response to
10 phorbol esters is a well documented hallmark of TRPV4 signalling (Nilius et al., 2004), and has been
11 reported both in micro- and macrovascular endothelial cells (Bubolz et al., 2012a; Pla et al., 2012b).
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13 This feature is related to the dynamics of actin cytoskeleton: arachidonic acid (AA)-induced actin
14 polymerization dramatically reduces the latency distribution of the Ca^{2+} response to $4\alpha\text{PDD}$ in breast
15 tumour-derived endothelial cells (Pla et al., 2012b). Future experiments will have to assess this
16 mechanism also in EPCs.
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24 ***Hypotonic cell swelling and arachidonic acid do not stimulate TRPV4-mediated Ca^{2+} entry in***
25 ***EPCs***

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27 TRPV4 is a polymodal channel that may be also activated by mechanical stimulation and
28 endogenous second messengers, such as AA and its metabolites, including epoxyeicosatrienoic acids
29 (EETs) (Everaerts et al., 2010; Moccia et al., 2012b). For instance, a hypotonic challenge has been
30 shown to produce TRPV4-mediated Ca^{2+} signals in human corneal endothelial cells and carotid artery
31 endothelial cells (Hartmannsgruber et al., 2007). Similarly, low micromolar doses of AA recruit
32 TRPV4 in mouse aortic endothelial cells (Vriens et al., 2004), human coronary ECs (Zheng et al.,
33 2013) and in B-TECs (Pla et al., 2012b). However, both hypotonic stimulation and low μM doses of
34 AA failed to induce intracellular Ca^{2+} signals in GSK-sensitive EPCs. These results imply a difference
35 in the gating mechanism between mature endothelial cells and their committed progenitors. It has been
36 suggested that AA and its metabolites bind to a LSRKFKD domain, that is homolog to the archidonate
37 recognition sequence (ARS) of melastatin TRPM2 (TRPM2) (ISXXTKE) (Everaerts et al., 2010).
38 Alternatively, TRPV4 stimulation by either cell swelling or low μM concentrations of AA may activate
39 Ca^{2+} entry at a lower extent as compared to GSK and PMA, thereby caused a sub-membranal Ca^{2+}
40 elevation that is missed by our Ca^{2+} imaging system. Whatever its molecular underpinnings, the lack of
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9 a Ca^{2+} response to AA explains EPC insensitivity to hypotonic stress; accordingly, cell swelling elicits
10 TRPV4-mediated Ca^{2+} influx by stimulating phospholipase A2 (PLA2) to produce AA, which is in turn
11 metabolized by P450 epoxyoxidases to epoxyeicosatrienoic acids (Everaerts et al., 2010). Thus, if AA
12 fails to evoke a detectable increase in $[\text{Ca}^{2+}]_i$, neither hypotonic challenge is expected to augment
13 intracellular Ca^{2+} levels.
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Intracellular Ca^{2+} mobilization does not sustain TRPV4-mediated Ca^{2+} inflow

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20 We have recently shown that EPCs may utilize extracellular Ca^{2+} influx to stimulate Ca^{2+}
21 mobilization from the InsP_3 -sensitive intracellular Ca^{2+} pool. For instance, in umbilical cord derived-
22 EPCs, TRPC3 gates an influx of Ca^{2+} which subsequently promotes InsP_3 -dependent Ca^{2+} release
23 (Dragoni et al., 2013). However, the depletion of ER Ca^{2+} pool does not affect the Ca^{2+} response to
24 GSK in their peripheral counterparts. Similarly, the pharmacological inhibition of InsP_3 Rs does not
25 impair TRPV4-evoked Ca^{2+} signalling in circulating EPCs. This result concurs with the findings
26 reported in endothelial cells of mouse mesenteric arteries (Sonkusare et al., 2012), where TRPV4-
27 induced Ca^{2+} inflow is an event temporally and spatially distinct from InsP_3 -dependent Ca^{2+} release,
28 but not with those described in mouse astrocytes (Dunn et al., 2013). Perivascular astrocytic endfeet
29 generate intracellular Ca^{2+} waves by impinging on the dynamic synergy between TRPV4-mediated
30 Ca^{2+} entry and InsP_3 -evoked Ca^{2+} release: these propagated Ca^{2+} elevations are abrogated by
31 superfusing the cells with either CPA or xestospongin C, a well known InsP_3 R inhibitor. Another
32 observation suggests that TRPV4 channels on the plasma membrane are uncoupled from the
33 intracellular Ca^{2+} releasing machinery sitting on the underlying ER membranes. TRPV4 activity is
34 potentiated by a modest increase Ca^{2+} levels in close proximity of the inner mouth of the channel pore,
35 while it is inhibited by higher Ca^{2+} elevations (Everaerts et al., 2010). Conversely, the global elevation
36 in $[\text{Ca}^{2+}]_i$ elicited by SERCA inhibition does not interfere with TRPV4-dependent Ca^{2+} inflow in EPCs.
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9 This feature suggests that this massive $[Ca^{2+}]_i$ raise is not translated into a change in Ca^{2+} concentration
10 in vicinity of the COOH-terminal calmodulin-binding site of TRPV4 protein, which senses the changes
11 in environmental Ca^{2+} .
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15 16 ***TRPV4 plays a minor role in controlling EPC proliferation***

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18 TRPV4-mediated Ca^{2+} inflow has long been known to control endothelial proliferation and
19 vascular remodelling due to the activation of several Ca^{2+} -dependent transcription factors, such as
20 nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1), Kv channel
21 interacting protein 3, calsenilin (KCNIP3/CSEN/DREAM), and myocyte enhancer factor 2C (MEF2C)
22 (Hatano et al., 2013; Pla et al., 2008; Pla et al., 2012b; Schierling et al., 2011; Thodeti et al., 2009;
23 Troidl et al., 2010; Troidl et al., 2009). For instance, TRPV4-dependent Ca^{2+} signals drive the shear
24 stress- and 4 α PDD-induced growth of collateral vessels (arteriogenesis) which occurs as a consequence
25 of arterial occlusion in a number of vascular districts (Schierling et al., 2011; Troidl et al., 2010; Troidl
26 et al., 2009). In addition, TRPV4-induced Ca^{2+} entry promotes proliferation of human brain capillary
27 endothelial cells (Hatano et al., 2013) and of breast tumour-derived endothelial cells (B-TECs) (Pla et
28 al., 2008; Pla et al., 2012b). However, selective stimulation of TRPV4 with GSK did not promote EPC
29 growth when the cells were plated in the absence of any further growth factor. These results are
30 different from those reported in human brain capillary endothelial cells and in B-TECs, which are
31 induced to replicate when TRPV4 is stimulated with 4 α PDD and AA, respectively (Hatano et al., 2013;
32 Pla et al., 2008). Likewise, endothelial cells are prompted to undergo mitosis upon *in vivo* application
33 of 4 α PDD in rat carotid artery (Schierling et al., 2011), rabbit femoral artery (Troidl et al., 2009), and
34 pig limb arteries (Troidl et al., 2010). Therefore, TRPV4-gated Ca^{2+} inflow *per se* does not entail EPC
35 proliferation and is likely to require the concomitant activation of additional Ca^{2+} entry pathways or
36 signal transduction cascades. Consistent with this hypothesis, when the cells were expanded in a culture
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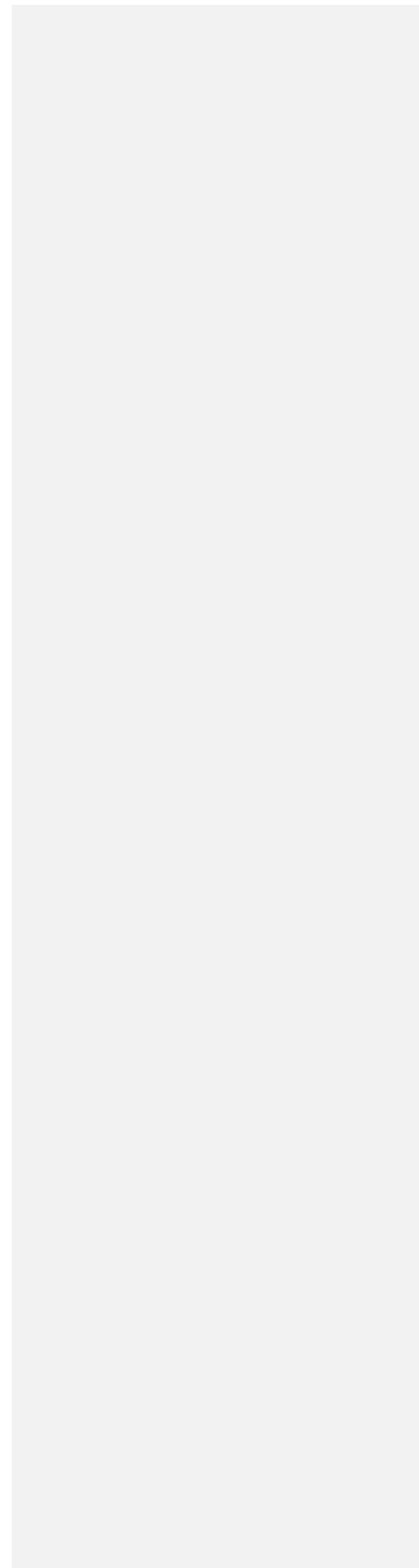
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9 medium supplemented with several growth factors and serum, the pharmacological inhibition of
10 TRPV4 with either RR or RN-1734 only partially reduced EPC proliferation. Interestingly, RR exerted
11 a stronger effect as compared to RN-1374. However, while the latter is a drug designed to selectively
12 impair TRPV4-mediated Ca^{2+} inflow, the former may affect other TRPV channels. In particular, RR
13 may block TRPV1-gated Ca^{2+} entry in human corneal endothelial cells (Mergler et al., 2011). It is,
14 therefore, conceivable that the higher extent of inhibition caused by this compound is due to the
15 blockade of a TRPV1-dependent Ca^{2+} influx. Unlike RR and RN-1734, however, the blockade of
16 SOCE with three structurally distinct inhibitors, i.e. BTP-2, La^{3+} and Gd^{3+} , fully abrogated cell
17 replication. Therefore, Orai1 and TRPC1 deliver a more powerful pro-angiogenic Ca^{2+} stimulus to
18 endothelial committed progenitors as compared to TRPV4. It is tempting to speculate that the Ca^{2+} -
19 sensitive machinery that translates TRPV4-mediated Ca^{2+} inflow into a mitogenic signal relocates in
20 vicinity to the inner mouth of the channel pore only after EPC engraftment into the vasculature and
21 subsequent acquisition of a mature endothelial phenotype. Alternatively, we might invoke the recent
22 finding that TRPV4 activity is confined to a few sites on the endothelial membrane, even though the
23 channel is evenly distributed along the whole cell surface (Sullivan et al., 2012). In this view, the
24 agonist-dependent recruitment of TRPV4 channels selectively coupled to the decoders of the
25 proliferative stimulus might occur only after full EPC differentiation to endothelial lineage. Yet, future
26 studies will have to unveil whether TRPV4 is already capable of triggering NO and/or PGI_2 synthesis
27 and/or EDHF activation in EPCs as it does in mature endothelium.
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Competing interests

None declared.

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13
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16 who passed by on August 30th, 2011, at the age of 23 yo.
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FIGURE LEGENDS

Figure 1. Expression of TRPV4 transcript and protein in ECFCs. A, gel electrophoresis of PCR products. Representative semiquantitative RT-PCR of TRPV4. Reverse transcription was performed in the presence (+) or absence (-) of reverse transcriptase enzyme. TRPV4 mRNA expression was normalized to β -actin. The 246 and 146 bp bands correspond to the TRPV4 and β -actin-specific PCR products, respectively. MW: molecular weight marker. B, representative western blots showing TRPV4 and β -actin expression in normal human microvascular ECs (HMEVC-d, HMEVC-c) and EPCs.

Figure 2. GSK stimulates Ca^{2+} inflow in EPCs. A, GSK (20 nM) induces a transient elevation in $[Ca^{2+}]_i$ in EPCs. B, the Ca^{2+} response to GSK arises in the presence, but not in the absence ($0Ca^{2+}$), of extracellular Ca^{2+} . C, 30 min pre-treatment with RN-1734 (20 μ M) inhibits GSK-induced Ca^{2+} entry, which promptly resumes upon drug washout. D, 30 min pre-incubation with ruthenium red (RR; 10 μ M) reversibly prevents GSK-evoked Ca^{2+} entry.

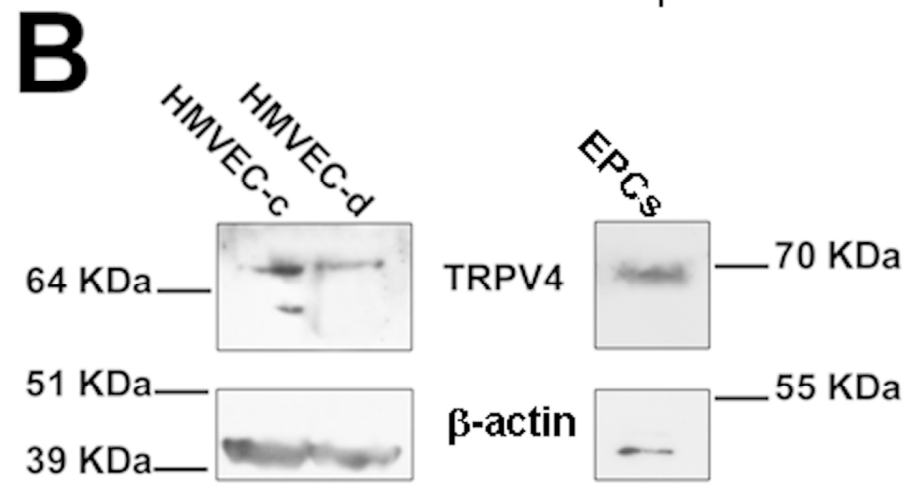
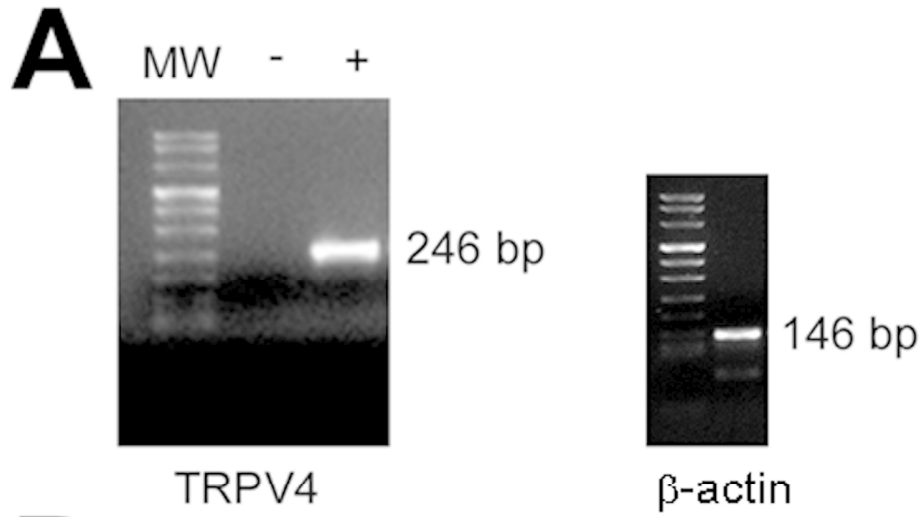
Figure 3. PMA triggers Ca^{2+} entry in EPCs. A, PMA (10 μ M) evokes a sustained increase in $[Ca^{2+}]_i$ in circulating EPCs. B, PMA-evoked Ca^{2+} signals rapidly return to the baseline upon removal of the agonist from the bath. C, PMA does not elicit any detectable increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} ($0Ca^{2+}$); restitution of Ca^{2+} to the external solution quickly resumes the Ca^{2+} response. D, removal of extracellular Ca^{2+} ($0Ca^{2+}$) reversibly abolishes PMA-induced elevation in $[Ca^{2+}]_i$. E, 10 min pre-incubation with ruthenium red (RR; 10 μ M) prevents PMA-evoked Ca^{2+} signalling until drug removal from the perfusate. F, the acute addition of ruthenium red (RR; 10 μ M) abrogates the Ca^{2+} response to PMA. G, 10 min pre-incubation with capsazepine (10 μ M) does not

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9 alter PMA-induced increase in $[Ca^{2+}]_i$. H, mean \pm SE of the amplitude of PMA-evoked Ca^{2+} signals in
10 the presence and absence of capsazepine.
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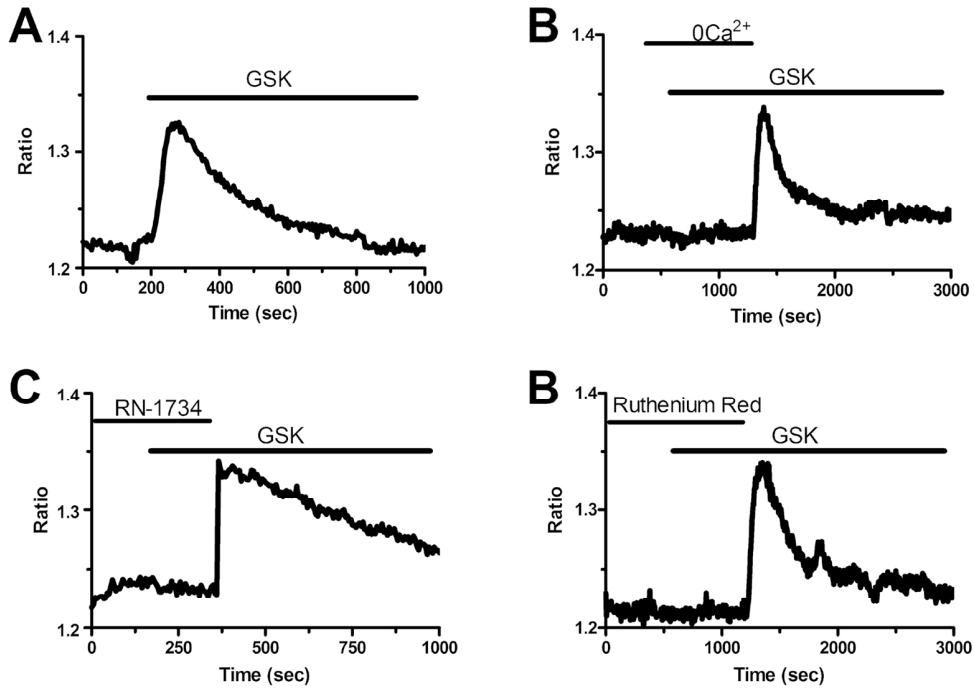
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15 **Figure 5. Intracellular Ca^{2+} mobilization does not support GSK-induced Ca^{2+} inflow in**
16 **EPCs.** Ca^{2+} response to GSK (20 nM) in the absence (A) and presence of (B) of cyclopiazonic acid
17 (CPA; 10 μ M), which blocks SERCA activity thereby depleting the endogenous Ca^{2+} reservoir. C,
18 mean \pm SE of the amplitude of GSK-induced Ca^{2+} signals under the designated treatments. Ca^{2+}
19 response to GSK (20 nM) in the absence (D) and presence of (E) of 2-APB (50 μ M), which blocks
20 InsP3Rs and prevents intracellular Ca^{2+} discharge. F, mean \pm SE of the amplitude of GSK-induced Ca^{2+}
21 signals under the designated treatments.
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30 **Figure 6. The effect GSK, ruthenium red and RN-1734 on EPC proliferation.** A, GSK (20
31 nM) does not stimulate ECFC proliferation after three days in culture. As a positive control the cells
32 have been grown in the presence of the growth factors-enriched medium EGM-2. B, VEGF (10 ng/ml)
33 induces ECFC proliferation in the absence, but not in the presence, of BAPTA (30 μ M). BAPTA was
34 added 30 min before challenging the cells with VEGF to buffer intracellular Ca^{2+} levels, as shown in
35 (Dragoni et al., 2011). C, mean \pm SE of the percentage of EPC growth in the presence of EGM-2,
36 ruthenium red (RR; 10 μ M), RN-1734 (20 μ M; RN), BTP-2 (20 μ M), La^{3+} (10 μ M) and Gd^{3+} (10 μ M).
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43 *, compared with control and after Bonferroni's correction (Student's t test for unpaired samples).
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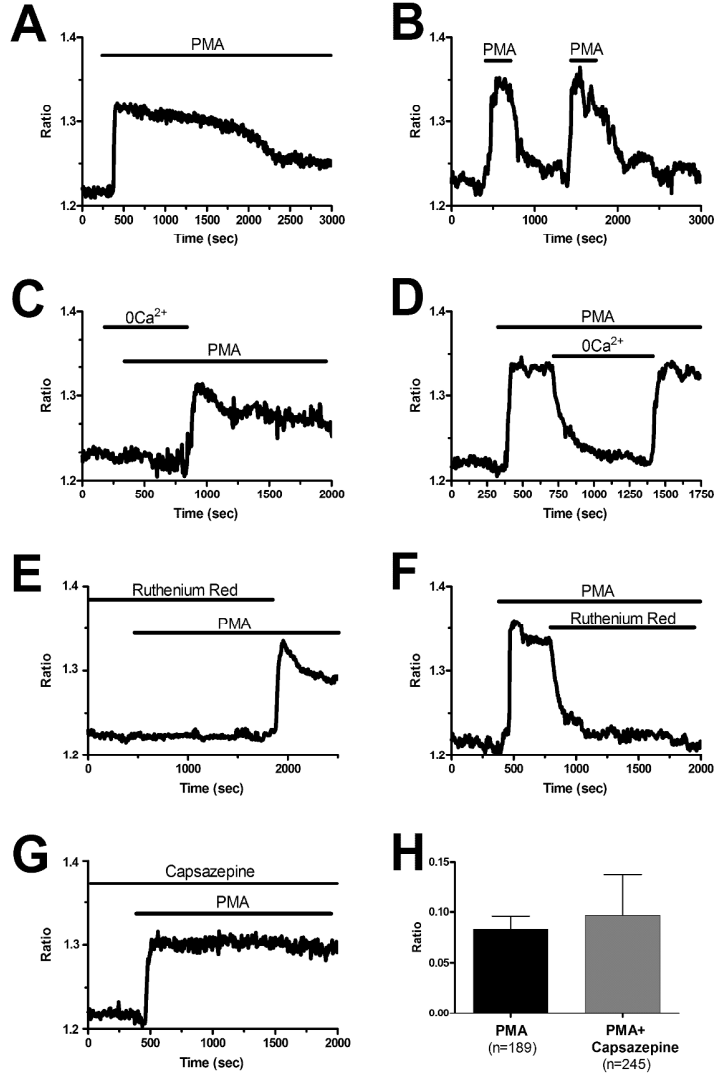


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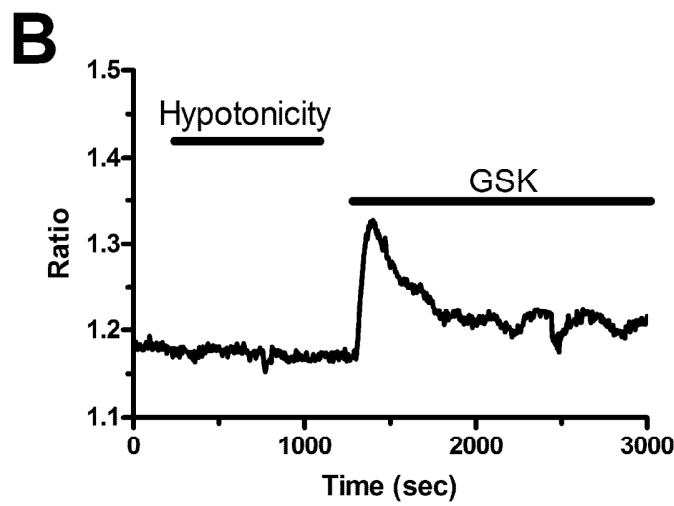
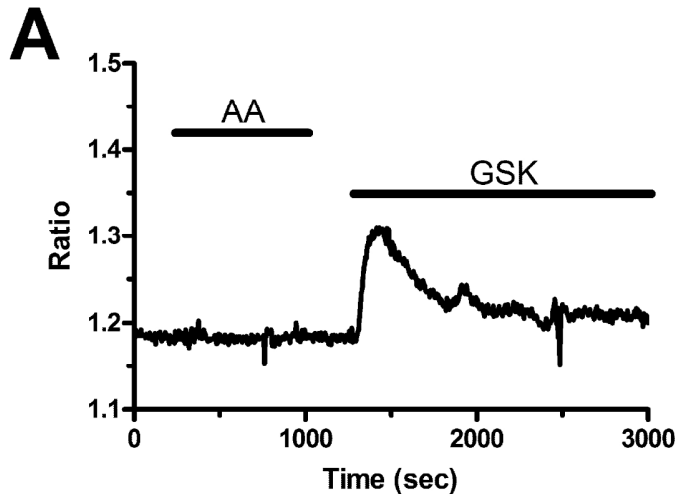
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Dragoni et al. - Figure 3

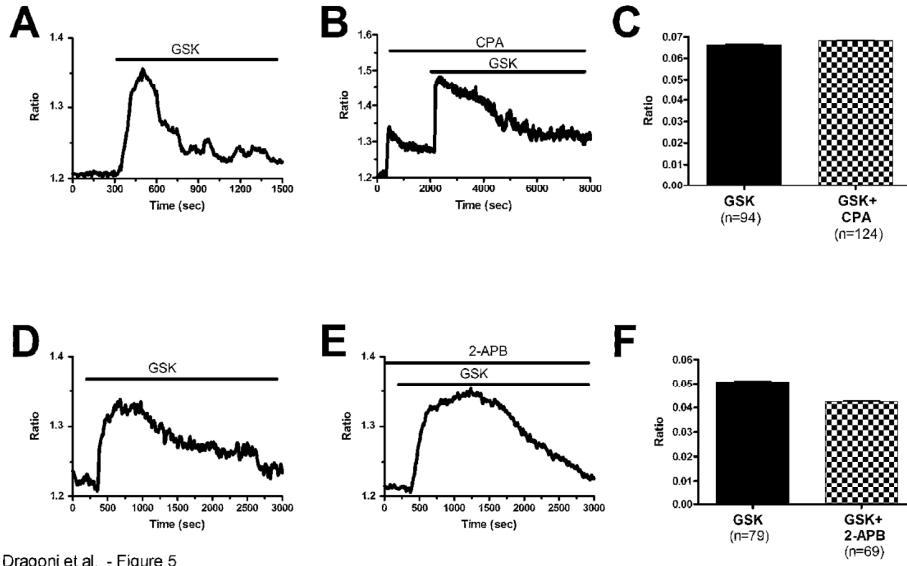
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Dragoni et al. - Figure 4

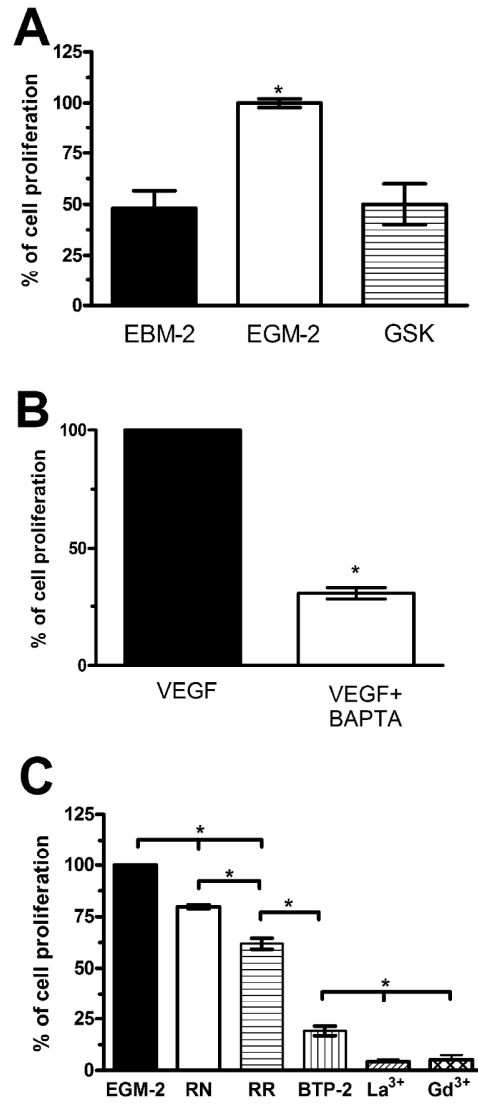
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Dragoni et al. - Figure 5

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Dragoni et al. - Figure 6

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