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Calcium signalling in adult endothelial outgrowth cells

William G. Pierce [a], Christopher Zanette [a], Noel M. Caplice [b], John J. Mackrill [a,*]

[a] Department of Physiology, University College Cork, BioSciences Institute, College Road, Cork, Ireland
[b] Centre for Research in Vascular Biology, University College Cork, BioSciences Institute, College Road, Cork, Ireland

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ABSTRACT

Endothelial outgrowth cells (EOCs) derived from blood mononuclear cells can differentiate to an endothelial-like phenotype. There are deficits in understanding of the biology of these cells, particularly detailed characterisation of their Ca^{2+} signalling mechanisms. In the current study, it was found that human EOCs express two forms of ryanodine receptor (RyR1 and RyR2) Ca^{2+} release channel in their endoplasmic reticulum. Individual EOCs display heterogeneous Ca^{2+} responses to physiologically relevant regulators fibrinogen and collagen. Some EOCs showed distinctive, multiphasic Ca^{2+} responses to fibrinogen consisting of rapid decreases, transient increases then a gradual return to the resting levels. Transient elevations in Ca^{2+} required both L-type voltage gated calcium channels and RyRs. Decreases in Ca^{2+} stimulated by fibrinogen depended on plasma membrane Ca^{2+} ATPase pumps, but did not require thapsigargin-sensitive Ca^{2+} ATPases. These results indicate that EOCs possess sophisticated Ca^{2+} signalling mechanisms, capable of generating distinct Ca^{2+} waveforms in response to different physiologically relevant cues.

1. Introduction

Adult blood mononuclear cells can act as circulating vascular progenitors, capable of differentiating into phenotypes resembling vascular endothelial or smooth muscle cells [1–3]. Since their identification in 1997 [1], evidence has accumulated to indicate that endothelial progenitor cells (EPCs) are involved in repair of the vascular endothelium, or in maladaptive remodelling in pathologies such as atherosclerosis [2]. Populations of cells derived from peripheral blood mononuclear cells can differentiate toward endothelial outgrowth cell (EOC) [4,5] or smooth muscle outgrowth cell phenotypes [6]. These cells have clinical potential, for generating tissue engineered vessels, coating of surgical devices, and graft endothelialisation [3]. Inverse correlations exist between EPC numbers or mobility and coronary artery disease risk factors, including plasma fibrinogen (FB) [5,7]. Consequently, delineating signalling mechanisms regulating EPC proliferation, migration, differentiation and homing to sites of vascular injury is essential for understanding roles of these cells in health and disease [2,5].

EOCs interact with components of the extracellular matrix and plasma via heterodimeric cell-surface receptors called integrins. These receptors facilitate adhesion and also participate in signal transduction, coupling information about the extracellular environment to control of intracellular processes [8]. In addition to being a plasma clotting factor, FB is enriched at sites of vascular injury, acting as an integrin ligand, binding to receptors containing the β2 or β3 subunit. Elevated plasma FB is also a risk factor for cardiovascular disease [9]. Human EOCs express a range of integrins that regulate adhesion and homing, displaying high expression levels of αVβ3, αVβ5, α2β1, β1, and α1, but low expression of α4β1 [10]. In contrast to a differentiated endothelial cell-line, HUVEC, human EOCs express high levels of β2 integrins facilitating adhesion to FB *in vitro*, promoting neovascularisation [11]. In addition, FB enhances vascular endothelial growth factor-induced differentiation of EPCs, although to a lesser extent than fibronectin [12]. Other extracellular matrix components, including collagens, influence EPC growth and differentiation both *in vivo* and *in vitro* [7,12,13].

Extracellular cues, including integrin ligands, can regulate intracellular processes by altering cytoplasmic levels of the second messenger Ca^{2+}. Despite the fundamental importance of Ca^{2+} as a second messenger [14], little is known about Ca^{2+} signalling mechanisms in EOCs. Laboratory and clinical studies indicate beneficial effects of dihydropyridine calcium channel blockers on EOC functions. *In vitro*, benidipine enhances murine EPC endothelial differentiation [15]. In human subjects with essential hypertension the dihydropyridine nisoldipine, but not the angiotensin receptor antagonist telmisartan, increased circulating EPC numbers and enhanced their ability to form colonies *in vitro* [16]. In patients with type 1 hypertension, nifedipine significantly increased circulating EPC numbers, enhanced their differentiation and decreased cell-death [17]. However, direct demonstration of functional VGCCs in EPCs or EOCs has not been reported.

* Corresponding author. Address: Department of Physiology, University College Cork, Western Gateway Building, Western Road, Cork, Ireland. Fax: +353 (0) 214205370.
E-mail address: j.mackrill@ucc.ie (J.J. Mackrill).

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EPCs express multiple cell surface receptors that activate ER Ca^{2+} release via the PLC-IP_3-IP_3R pathway. Human EPCs possess a receptor for platelet activating factor, coupled to transient rises in cytoplasmic Ca^{2+} concentration and decreases in cell proliferation [18]. Stimulation of the insulin-like growth factor-2 receptor stimulates phospholipase C β2, Ca^{2+} transients, chemotaxis and expression of matrix metalloprotease-9 in human EPCs, implying a role for this pathway in homing to sites of neovascularisation [19].

Ca^{2+} responses of EPC-derived cells to other physiologically significant regulators including FB and collagen have not been reported. In differentiated vascular endothelial cells, interactions between fibronectin and αV integrins trigger increases in cytoplasmic Ca^{2+} [20]. In A7r5 rat aortic smooth muscle cells, FB elicits concentration-dependent Ca^{2+} influx via dihydropyridine-insensitive calcium channels. Intracellular Ca^{2+} buffering inhibited adhesion of A7r5 myocytes to FB, suggesting that this integrin ligand modulates cell function via this second messenger [21].

The current study aimed to examine Ca^{2+} responses of individual human EOCs to FB. This unveiled distinctive spatiotemporal patterns of Ca^{2+} signalling between different individual cells. Expression of RyRs, VGCCs and other calcium signalling components was explored and their contributions to FB triggered Ca^{2+} responses were evaluated.

2. Materials and methods

2.1. Materials

Endothelial cell growth medium-2 (EGM-2) was from Lonza Group Ltd. (Basel, Switzerland). Fura-2 acetoxymethyl (AM) ester, BODIPY-FLX-ryanodine and thapsigargin were purchased from Invitrogen Ltd. (Paisley, UK). Fibrinogen (FB) from human plasma was from Merck Biosciences Ltd. (Nottingham, UK). Type 1 collagen from rat tail and fibronectin from human plasma was from Sigma–Aldrich (Dublin, Ireland). Caloxin 3A1 was from Cambridge Biosciences (Cambridge, UK). Uncoated glass-bottom microscope dishes (35 mm diameter, 10 mm diameter coverslip) were from MatTek Corp. (Oregon, USA).

2.2. Cell culture

Ethical approval for this study was obtained from the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Human EOCs were isolated from the blood of healthy volunteers and were maintained as described previously [6,10].

2.3. Fluorescent microscopy

Cells were grown to approximately 50% confluency on glass-bottom tissue culture dishes. To assess cytoplasmic Ca^{2+} levels, cells were loaded with 2 μM fura-2 acetoxymethyl ester and were imaged using fluorescent videomicroscopy, essentially as described for differentiated U937 cells [22]. For detection of RyRs in live EOCs, dishes were washed twice (2 × 1 mL) with Krebs–Henseleit Buffer [22] and then stained for 30 min with 100 nM BODIPY-FLX-ryanodine [23] in the presence or absence of 100 μM unlabelled ryanodine. EOCs were washed twice with KHB, then images were captured using an epifluorescent microscope (100× oil-immersion objective, 1.3 NA, 480 nm excitation, EGFP emission filter).

2.4. Western blot analyses

Cells were lysed, extracted proteins resolved on 7.5% SDS–PAGE minigels and transferred onto nitrocellulose as described [24], except that transfer was performed using a BioRad mingel wet blotter at 70 V constant voltage for 2 h. Blots were immunostained with antisera recognising specific RyR subtypes [25] or a 1:5000 dilution of mouse anti-β-actin.

2.5. Statistics

Mean values (±SEM) were compared using an unpaired student's *t*-test, with *p*-values of <0.05 taken as significant. Where more than two mean values were compared, data were analysed using one way analysis of variance followed by Tukey's Multiple Comparison test using GraphPad software, taking *p*-values of <0.05 as significant.

3. Results and discussion

3.1. Human EOCs express functional RyRs and L-type VGCCs

BODIPY-FLX-ryanodine stained human EOCs with a reticular pattern, which was particularly intense around the nucleus, consistent with ER labelling (Fig. 1A) [23]. This staining is specific, being reduced by competition with excess unlabelled ryanodine. Expression of RyR2 has been demonstrated in guinea pig/rabbit vascular and endocardial endothelium [27]; RyR3 is expressed in human mesenteric artery endothelial cells [28]; and RyR3 is also present in rat spleen sinus endothelial cells [29]. Experimental evidence indicates that RyRs, in combination with SERCA and PMCA, mediate vectorial Ca^{2+} transport and extrusion by rat aortic endothelial cells [30]. To test which RyR subtypes are expressed in EOCs, western blots of cell lysates were probed with antibodies selective for each of the three mammalian RyR subtypes [25]. This indicated that RyR1 and RyR2 proteins were present in EOCs prepared from two different individuals (Fig. 1B), whereas RyR3 was undetectable. To test if RyRs expressed in human EOCs are functional, cells were challenged with 500 μM 4-chloro-*meta*-cresol (CmC) [26]. CmC rapidly increased intracellular Ca^{2+}, increasing fura-2 ratio from 0.486 ± 0.015 to a peak of 0.706 ± 0.069 ($n = 3$) (Fig. 1C). Pretreatment of EOCs with 10 μM ryanodine (locking RyRs into a long-lasting open state, depleting ER Ca^{2+} stores) increased the resting ratio to 0.678 ± 0.007 ($p < 0.01$), see (Fig. 3C), and inhibited the response to 500 μM CmC (0.219 ± 0.069 versus 0.029 ± 0.025, $n = 3$, $p < 0.05$) (Fig. 1C).

RyR channels can be activated by direct allosteric coupling to L-type calcium channels or by CICR triggered by Ca^{2+} influx via such VGCCs. Expression of L-type channels has been reported in a variety of differentiated endothelial cell types, including those from bovine adrenal medulla [31]. To test for presence of VGCCs in EOCs, cells were depolarised by extracellular application of 60 mM KCl. This rapidly increased cytoplasmic Ca^{2+}, with a peak elevation in ratio of 0.401 ± 0.003 above a resting value of 0.532 ± 0.015 ($n = 4$) (Fig. 1D). This rise was inhibited (to 0.031 ± 0.004 ($p < 0.01$)) by preincubation of cells with 1 μM nifedipine.

3.2. Individual human EOCs display heterogeneous Ca^{2+} responses to fibrinogen (FB)

FB was applied at a concentration of 0.5 mg/mL, a value representing the upper limits reported for human plasma [9]. Individual EOCs displayed distinct Ca^{2+} responses to FB. Most frequently (229/462 cells, 49.6%) there was no detectable change (Fig. 2A). Another subset of EOCs (18.6%) displayed an initial decrease in Ca^{2+} (ratio decrease of −0.153 ± 0.020), followed by a gradual rise lasting more than 5 min (Fig. 2B). The remaining EOCs (31.8%) displayed a distinctive multiphasic Ca^{2+} response to FB, consisting an initial decrease of −0.145 ± 0.023 lasting 16.7 ± 0.1 s of addition, followed

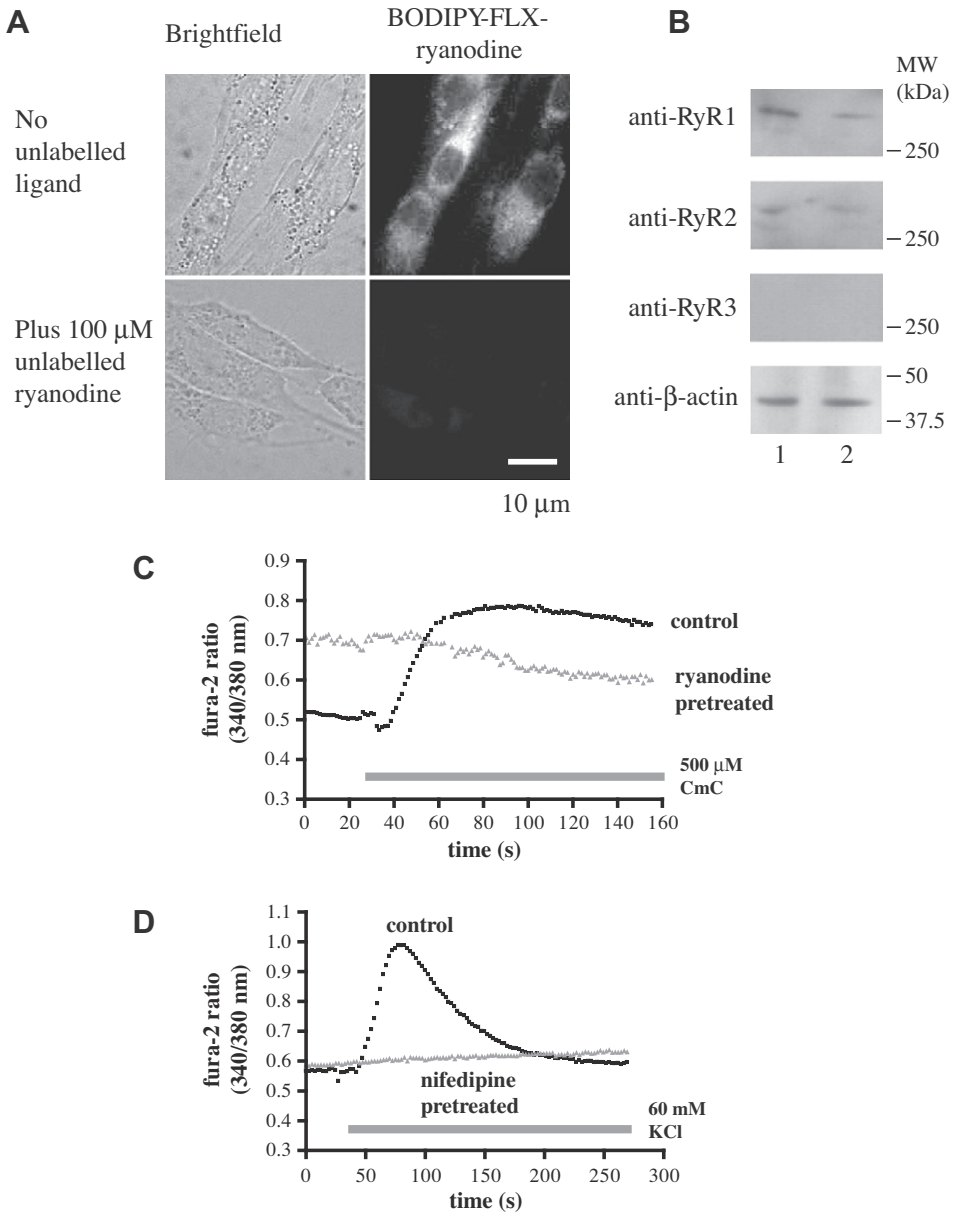

Fig. 1. Human EOCs express RyRs and VGCCs. (A) EOCs were stained with 100 nM BODIPY-FLX-ryanodine with or without 100 μM unlabelled ligand ($n = 3$). (B) Western blot analyses of protein (60 μg/lane) from two different EOC preparations (lanes 1 and 2) indicate they express detectable levels of RyR1 and RyR2, but not RyR3. Values of the right-hand side of the figure indicate the position and molecular weight of SDS–PAGE marker proteins. (C) CmC, an RyR agonist stimulates a rapid increase in cytoplasmic Ca^{2+} in EOCs (black trace). Pretreatment of cells with 10 μM ryanodine abolished this rise (grey trace, $n = 3$). (D) EOCs respond to depolarisation with an increase in Ca^{2+} (black trace). This effect was abolished by pretreatment with 1 μM nifedipine (grey trace, $n = 4$).

by a peak of 0.07 ± 0.01 lasting 10.1 ± 1.5 s, a decline of −0.14 ± 0.019 below the resting ratio, then a gradual rise lasting more than 5 min (Fig. 2C). EOCs did not show Ca^{2+} responses to KHB alone, fibronectin (5 μg/mL), or the synthetic peptide integrin ligand GRGDSP (*data not shown*). In contrast, 5 μg/mL type 1 collagen caused a rapid decline in Ca^{2+} in most EOCs (40/55 cells, 73%), represented by a decrease of −0.078 ± 0.007 from a resting ratio of 0.613 ± 0.003 ($n = 5$) (Fig. 2D), resembling the response of some EOCs to FB (Fig. 2B). In HUVECs, a differentiated endothelial cell type, most cells ($n = 7$, 34/56 cells, 60.7%) responded to FB with a gradual decrease in fura-2 ratio of −0.065 ± 0.023, lasting at least 5 min (*data not shown*), resembling that observed in some EOCs (Fig. 2B).

Different spatiotemporal patterns of Ca^{2+} signals result in distinct cellular responses [14]. For example, oscillating Ca^{2+} signals are more effective at activating Ca^{2+}/calmodulin-dependent protein kinase II, a key transducer and activator of gene expression, than are sustained Ca^{2+} signals [32]. Heterogeneity of Ca^{2+} responses within EOC populations does not necessarily imply variations in cell types present. Early fura-2 imaging studies reported heterogeneity of Ca^{2+} responses triggered by several hormones in individual BC3H1 smooth muscle cells, an apparently clonal cell-line [33]. Such heterogeneity in Ca^{2+} signalling was observed between daughter cells whilst undergoing division, implying underlying epigenetic mechanisms.

3.3. In EOCs, increases in Ca^{2+} in response to FB are dependent on VGCCs and RyRs

To examine mechanisms underlying increases in Ca^{2+} caused by FB in EOCs, cells were preincubated with dihydropyridines to block VGCCs (Fig. 3A), or with ryanodine or dantrolene to inhibit RyRs

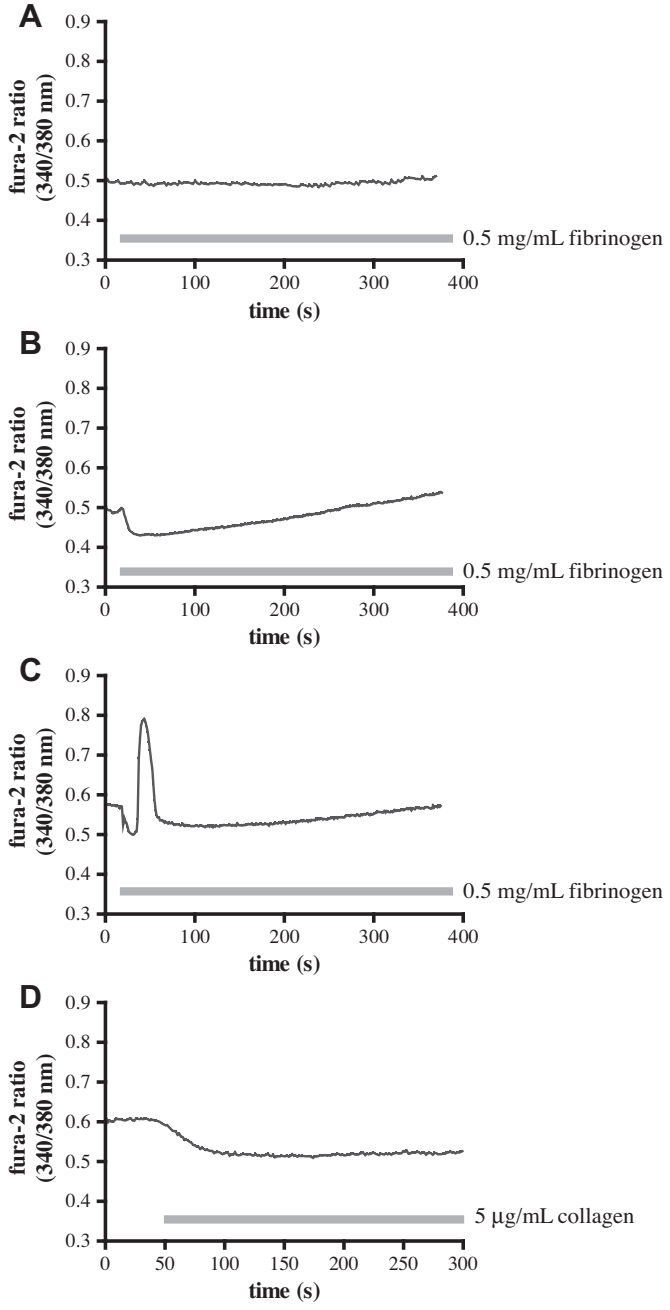

Fig. 2. Heterogeneous Ca^{2+} responses to FB or collagen in EOCs. Most cells (A), showed no response to FB, whereas others (B) displayed in rapid decline in fura-2 ratio followed by a gradual return to the resting value. In the remaining EOCs, (C) FB caused a distinctive multiphasic Ca^{2+} signal, consisting of a rapid decline, followed by a transient increase and then a gradual return to resting levels ($n = 61$; 462 individual cells). (D) In most EOCs, type 1 collagen stimulated a rapid decline in Ca^{2+} levels that was either sustained or gradually returned to resting levels ($n = 5$).

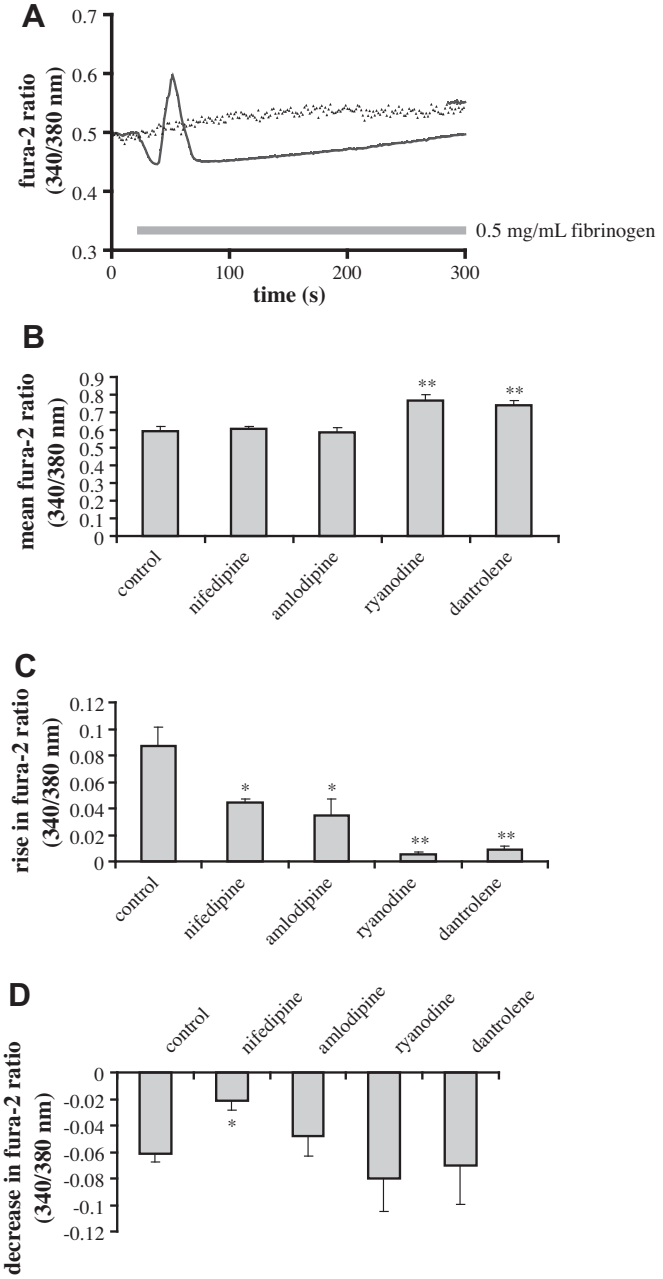

Fig. 3. Increases in Ca^{2+} triggered by FB depend on L-type VGCCs and RyRs. (A) Representative traces showing EOC Ca^{2+} responses to FB in the presence (broken trace) or absence (solid trace) of 1 µM nifedipine. (B) Whereas 1 µM nifedipine or amlodipine ($n = 4$) had no detectable effect, 10 µM ryanodine ($n = 15$) or 50 µM dantrolene ($n = 26$) elevated resting Ca^{2+} levels in EOCs. (C) Both L-type VGCC antagonists and RyR inhibitors significantly ablated FB stimulated Ca^{2+} elevations. (D) Only nifedipine caused a significant reduction in the Ca^{2+} decrease caused by FB ($^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$).

[26]. Although dihydropyridines had no significant effect on resting ratio, both ryanodine and dantrolene increased this parameter, from 0.593 ± 0.024 ($n = 7$) to 0.770 ± 0.031 ($p < 0.001$, $n = 15$) and 0.743 ± 0.029 ($p < 0.001$, $n = 26$), respectively (Fig. 3B). Inhibition of either VGCCs or RyRs decreased FB stimulated Ca^{2+} rises, from a value of 0.086 ± 0.014 in untreated EOCs to values of 0.044 ± 0.002 for nifedipine- ($p < 0.05$), 0.034 ± 0.013 for amlodipine- ($p < 0.05$), 0.005 ± 0.002 for ryanodine- ($p < 0.001$) and 0.009 ± 0.002 for dantrolene- ($p < 0.001$) pretreated cells (Fig 3C). Only nifedipine significantly inhibited the declining phase of the FB Ca^{2+} response, from −0.061 ± 0.006 in untreated cells to −0.021 ± 0.007 in pretreated EOCs ($p < 0.05$) (Fig. 3D).

Inhibition of RyRs and L-type VGCCs indicated their involvement in generating Ca^{2+} increases in response to FB. This does not strongly support allosteric- or CICR-coupling mechanisms between VGCCs and RyRs in EOCs, since effects of ryanodine or dantrolene were greater than those of the dihydropyridines. In bovine aortic endothelial cells, activation of αV integrin triggered oscillations in cytoplasmic Ca^{2+} that were inhibited with thapsigargin, or La^{3+}, a non-selective cation channel blocker [34]. This implies that integrins are coupled to both Ca^{2+} influx and release

in differentiated endothelial cells. Integrin activation in pulmonary artery vascular smooth muscle increased Ca^{2+} in a ryanodine-sensitive manner [35]. Mechanisms by which integrins activate RyRs in EOCs and other vascular cell types await investigation.

3.4. In EOCs, decreases in Ca^{2+} in response to FB are dependent on PMCA but not on SERCA pumps

Key mechanisms for removal of Ca^{2+} from the cytoplasm of cells are Ca^{2+}-ATPase transporter proteins located in the PM (PMCA) and in the SR/ER (SERCA pumps). To test the involvement of SERCA pumps in the declining phase of the EOC Ca^{2+} transients triggered by FB, cells were treated with thapsigargin, a selective, high-affinity inhibitor of these transporters [36]. Thapsigargin stimulated a rapid increase in cytoplasmic Ca^{2+} levels in EOCs, corresponding to an increase in ratio of 0.14 ± 0.03 (n = 5), reaching a sustained plateau within 2 min, demonstrating the presence of a major SERCA-dependent Ca^{2+} pool. Subsequent addition of FB induced a decrease in fura-2 ratio of −0.085 ± 0.034, not significantly different from that in untreated cells (−0.074 ± 0.020) (Fig. 4A).

To examine involvement of PMCA Ca^{2+} pumps in this declining phase, EOCs were pretreated for 5 min with either 20 μM caloxin 3A1, an 'engineered' peptide inhibitor of these transporters [37], or with 100 μM sodium orthovanadate ('vanadate'), a general P-type ATPase inhibitor [38]. Both reagents increased the resting ratio from 0.594 ± 0.066 (n = 7) in untreated cells, to 0.663 ± 0.026 (n = 3) and 0.744 ± 0.106 (n = 7) respectively, but this difference was only significant ($p < 0.01$) for vanadate (Fig. 4B). Neither caloxin 3A1 nor vandate significantly altered the increase in Ca^{2+} in response to FB (Fig. 4C). However, both PMCA blockers inhibited the declining phase of the FB triggered Ca^{2+} signal, from a maximal decrease of −0.061 ± 0.006 in control cells, to −0.014 ± 0.009 ($p < 0.01$) in caloxin 3A1 pretreated EOCs and −0.025 ± 0.006 ($p < 0.001$) in vanadate pretreated cells (Fig. 4D).

There are few reports of endogenous ligands causing decreases in cytoplasmic Ca^{2+} over a timescale of seconds, as observed here for FB and collagen. Soluble vitronectin, fibronectin or anti-β3 integrin antibodies inhibit divalent cation currents through L-type VGCCs in rat arteriolar smooth muscle cells [39]. This suggests that β3 integrin is coupled to inhibition of VGCCs in some cell types, but the lack of effect of fibronectin on EOCs indicates that this mechanism does not operate in these cells. Since type 1 collagen and FB both decrease cytoplasmic Ca^{2+} levels in EOCs, these ligands might operate by binding to a common integrin. A candidate receptor for this role is integrin αXβ2, also known as CD11c/CD18, which is a monocyte/macrophage marker expressed by EPCs [40]. However, activation of αXβ2 in human leukocytes triggers elevations, not decreases, in cytoplasmic Ca^{2+} [41].

In EOCs, Ca^{2+} decreases in response to FB result from PMCA pump rather SERCA transporter activity. The mechanism by which this PMCA-dependent decrease in Ca^{2+} occurs is unresolved at present. The only interaction between integrins and PMCA reported is an inhibition of PMCA4b due to tyrosine phosphorylation by focal adhesion kinase, a downstream effector of integrins [42].

3.5. Summary

The current study demonstrates that individual EOCs display heterogeneous Ca^{2+} responses to FB and collagen. Increases in Ca^{2+} in response to FB involve activation of both L-type VGCCs and RyRs. Decreases in Ca^{2+} require PMCA though not SERCA pump activity. HUVECs differ from EOCs in that they do not display Ca^{2+} increases in response to FB and in that they express distinct RyR subtypes. It is likely that these differences will have consequences on proliferation, differentiation, migration and homing of EOCs, particularly given the clinical effects of dihydropyridine calcium channel blockers on these parameters.

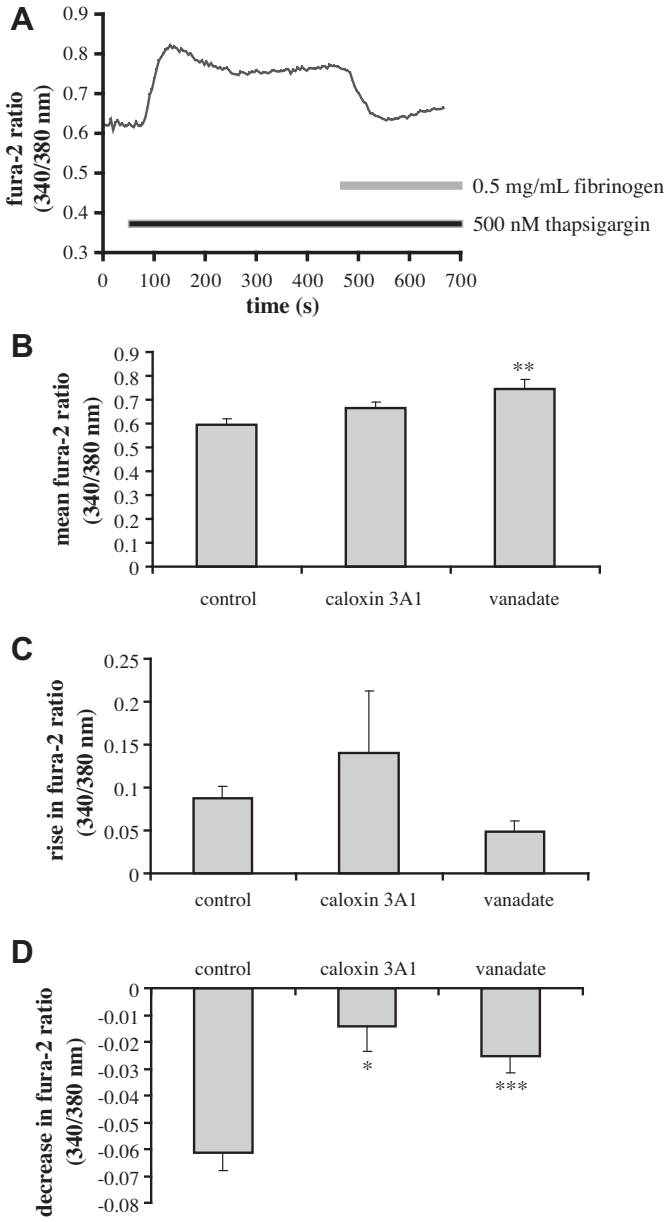

Fig. 4. Ca^{2+} decreases elicited by FB depend on PMCA but not on SERCA pumps. (A) Thapsigargin caused a rapid increase in Ca^{2+}, followed by a sustained plateau. Subsequent addition of FB stimulated decreases in fura-2 ratio in most cells (15/24 EOCs, n = 5). (B) PMCA inhibitors caloxin 3A1 (20 μM, n = 3) and sodium orthovanadate ('vanadate', 100 μM, n = 7) both elevated resting Ca^{2+} levels in EOCs, but this was only significant for vanadate. (C) Neither reagent had a significant effect on the FB induced Ca^{2+} rise. (D) However, both PMCA inhibitors significantly inhibited the decrease in Ca^{2+} triggered by FB ($^{*}p < 0.05$; $^{**}p < 0.01$).

Acknowledgments

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