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Research paper

Participation of membrane calcium channels in erythropoietin-induced endothelial cell migration

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ABSTRACT

Calcium (Ca^{2+}) plays an important role in angiogenesis, as it activates the cell migration machinery. Different proangiogenic factors have been demonstrated to induce transient Ca^{2+} increases in endothelial cells. This has raised interest in the contribution of Ca^{2+} channels to cell migration, and in a possible use of channel-blocking compounds in angiogenesis-related pathologies. We have investigated the ability of erythropoietin (Epo), a cytokine recently involved in angiogenesis, to induce Ca^{2+} influx through different types of membrane channels in EA.hy926 endothelial cells. The voltage-dependent Ca^{2+} channel antagonists amlodipine and diltiazem inhibited an Epo-triggered transient rise in intracellular Ca^{2+}, similarly to a specific inhibitor (Pyr3) and a blocking antibody against the transient potential calcium channel 3 (TRPC3). Unlike diltiazem, amlodipine and the TRPC3 inhibitors prevented the stimulating action of Epo in cell migration and *in vitro* angiogenesis assays. Amlodipine was also able to inhibit an increase in endothelial cell migration induced by Epo in an inflammatory environment generated with TNF-α. These results support the participation of Ca^{2+} entry through voltage-dependent and transient potential channels in Epo-driven endothelial cell migration, highlighting the anti-angiogenic activity of amlodipine.

1. Introduction

Among the different mechanisms underlying the process of cell migration, the involvement of calcium (Ca^{2+}) has previously been reported (Giannone et al., 2004). A migrating cell extends a protrusion, called lamellipodium, toward the source of the stimulus, and retracts its rear end in order to move one "step" forward. Such morphological changes are accompanied by dynamic modifications in cytoskeleton, cell adhesions and localization of membrane and cytosolic proteins which constitute the migration machinery. The distribution of cytosolic Ca^{2+} in migrating cells is also polarized, being higher at the rear end. A lower basal Ca^{2+} concentration at the front allows for the occurrence of Ca^{2+} "flickers" in response to promigratory stimuli (Tsai et al., 2015). These transient elevations of the cation activate the detachment of the cell rear through actin-myosin contractile force and the disassembly of cell adhesions by calcium-dependent phosphatases and proteases (Lee et al., 1999).

The ability to migrate is a key feature of endothelial cells and, not surprisingly, angiogenic factors have been reported to trigger Ca^{2+} rises in this cell type. Human umbilical vein endothelial cells (HUVEC) exhibited an increase in cytosolic Ca^{2+} levels within 5–10 min of exposure to vascular endothelial growth factor (VEGF) (Faehling et al., 2002). Erythropoietin (Epo), the most important erythropoietic growth factor, is also recognized as a proangiogenic cytokine, as demonstrated in different *in vitro* and *in vivo* studies (Ribatti et al., 1999; Jaquet et al., 2002; Kertesz et al., 2004), and it has been shown to induce calcium transient increases in endothelial cells (Vogel et al., 1997; Maltaneri et al., 2017). A previous work by our group revealed the participation of extracellular calcium, as well as that of nitric oxide and reactive oxygen species, as a mediator of the effect exerted by Epo on endothelial cell migration (Maltaneri et al., 2017).

The involvement of calcium in cell motility has prompted

Abbreviations: Aml, amlodipine; Ca^{2+}, free calcium; DHP, dihydropyridine; Dil, diltiazem; Epo, erythropoietin; EpoR, erythropoietin receptor; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBS, phosphate-buffered saline; Pyr3, TRPC3 inhibitor; Qui, quinine; ROS, reactive oxygen species; SEM, standard error of the mean; Thap, thapsigargin; TNF-α, tumor necrosis factor-alpha; TNFR1, TNF-α receptor 1; TRPC3, transient potential calcium channel 3; VDCC, voltage-dependent calcium channel; VEGF, vascular endothelial growth factor

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researchers to investigate the contribution of Ca^{2+} channels to migration-related pathologies, such as metastasis and tumor vessel proliferation in cancer. In this context, the study of Ca^{2+} channels and their inhibitors may lead to interesting therapeutical applications.

Calcium channels represent an important fraction of ion channels in endothelial cells, and also constitute a varied group. Non-selective cation channels, which may be operated by different agonists, have been demonstrated to mediate Ca^{2+} entry in endothelial cells (Nilius et al., 1993). Store-operated calcium channels (SOCs) are, in contrast, highly selective for this cation, and become activated upon depletion of Ca^{2+} stores in the endoplasmic reticulum (Nilius and Droogmans, 2001). Transient receptor potential channels (TRP) are abundant in the endothelium, though their expression levels are frequently subjected to regulation by environmental stimuli (Cheng et al., 2016). It has been demonstrated that TRP channels of the canonical type (TRPC) mediate the proangiogenic effect of cytokines such as VEGF, and that their suppression leads to deficient cell migration (Hamdollah Zadeh et al., 2008). In addition, the contribution of TRP channels of the vanilloid type (TRPV) has recently been reported in erythropoietin-induced angiogenesis (Yu et al., 2017).

On the other hand, the presence of voltage-dependent calcium channels (VDCCs) in endothelial cells is still a matter of debate, since this is a non-excitable cell type. This family comprises L-type (high voltage activated) and T-type (low voltage activated) channels, which are essential for cardiac and vascular smooth muscle contractility, as they increase cytosolic Ca^{2+} levels through augmented influx and release from intracellular stores (Catterall, 2011).

Inhibition of these voltage-dependent channels in vascular smooth muscle cells as well as in cardiomyocytes not only results in coronary and peripheral artery vasodilation, but also in decreased cardiac contractility. Stemming from this, the clinical use of calcium antagonists has been demonstrated to successfully prevent stroke, ischemic heart disease and mortality in hypertensive patients (Grossman and Messerli, 2004).

According to their chemical structure, calcium antagonists may be classified as phenilalkylamine, benzothiazepine and dihydropyridine drugs. Verapamil and diltiazem, which belong to the first two classes, respectively, are antihypertensive drugs with marked effects on cardiac conduction, for which they are employed as treatment for supraventricular arrhythmias. Dihydropyridines (DHPs), on the other hand, are more potent vasodilators with minimal effect on heart contractility. From the first generation of drugs released in the '60s, such as nifedipine, advances have been made to give rise to more pharmacokinetically-stable, and less cardio-selective compounds. Among the latter group, amlodipine has become a drug of choice to treat hypertension (Fares et al., 2016).

Considering the involvement of calcium in the migration of different cell types, the aim of this work was to investigate the participation of transient potential and voltage-dependent channels in the Ca^{2+} influx triggered by Epo in endothelial cells. In addition to this, and given that the administration of Epo as a proangiogenic compound represents a potential benefit in coronary ischemia (Buemi et al., 2002), we were also interested in investigating its performance in the presence of the calcium antagonists amlodipine and diltiazem —which are used to treat hypertensive patients— both in normal conditions and in a proinflammatory environment, characteristic of cardiovascular disease.

2. Materials and methods

2.1. Materials and reagents

All the reagents used in this work were of analytical grade. Human recombinant erythropoietin (specific activity: 125 IU/μg) was kindly provided by Zelltek (Santa Fe, Argentina). The culture media IMDM and M199, the cell dissociation reagent TrypLE Select, the Geltrex growth factor-reduced basal membrane matrix, the Low Serum Growth Kit (LSGS) for culture of primary endothelial cells, the penicillin–streptomycin antibiotic mixture and human recombinant TNF-α (specific activity: $5 \times 10^7 - 2 \times 10^7$ U/mg) were from Gibco – Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Natocor (Córdoba, Argentina) and Gibco – Thermo Fisher Scientific. The TRIzol reagent, the BAPTA-AM calcium chelator, the Alexa-Fluor 488-conjugated secondary antibodies, the fluorescent probe Fluo 4-AM (calcium) and the recombinant Taq DNA polymerase, as well as the primers against GAPDH, EpoR and TNFR1, were from Thermo Fisher Scientific (Waltham, MA, USA). The primary antibodies used were: anti-EpoR (sc-697) from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and anti-TRPC3 (ACC-016) from Alomone Labs (Jerusalem, Israel). The fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA, ROS) and the channel blockers amlodipine besylate, diltiazem and quinine were from Sigma Aldrich (Saint Louis, MO, USA). Cytofix/Cytoperm and PermWash buffer were from BD Biosciences (San José, CA, USA). Human recombinant VEGF-165 was purchased from BioLegend (San Diego, CA, USA). The reagents used for reverse transcription, comprising Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, RNAsin RNAse inhibitor and oligodTs were from Promega Corporation (Madison, WI, USA).The SERCA-pump inhibitor thapsigargin was from Sigma Aldrich, while the TRPC3 inhibitor Pyr3 was from Cayman Chemicals.

2.2. Cell cultures

The endothelial cell line EA.hy926, obtained by fusion of HUVEC cells with the adenocarcinoma cell line A549 (Edgell et al., 1983), was kindly provided by Dr. Fernanda Parborell (IBYME-CONICET, Buenos Aires) with permission from Dr. Gareth Owen (Pontificia Universidad Católica, Chile). Previous experiments by our group have demonstrated the ability of Epo to stimulate migration in EA.hy926 cells (Maltaneri et al., 2017), as observed in HUVEC cells. Cultures were maintained on Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Natocor), 100 U/mL penicillin and 100 μg/mL streptomycin (growth medium), and were used no further than passage 18, due to cell senescence.

Human Umbilical Vein Endothelial Cells (HUVEC; C-003-5C; Life Technologies) were cultured in M199 medium supplemented with Low Serum Growth Kit (Gibco), to the following final concentrations: 1 μg/mL hydrocortisone; 10 ng/mL EGF (Epidermal Growth Factor); 3 ng/mL bFGF (basic Fibroblast Growth Factor); 10 μg/mL heparin, 0.2 μg/mL bovine serum albumin; 1X gentamicin / amphotericin and 2% (v/v) FBS. Cells were used between passages 2 and 5 due to senescence.

Cultures were grown at 37 °C and 5% CO_2, with replacement of culture media every two days. Cells were divided into separate flasks upon reaching 80–90% confluence. In order to reproduce the physiological quiescent state of the endothelium, cells were FBS-deprived before treatment, and experiments were performed in FBS-free medium.

2.3. Cell migration (wound healing) assay

Cells were seeded on 24-well plates (65,000 viable cells/well) and cultured overnight on growth medium. After washing with PBS, cells were FBS-deprived for 8 h before scratching with a pipette tip and adding the corresponding treatments. FBS (10% for EA.hy926 cells, 2% for HUVEC cultures) was used as a positive control of cell migration. Images were acquired at the beginning (t = 0) and at the end of the experiment (t = 15 h for EA.hy926 cells and t = 24 h for HUVEC cells) using an inverted microscope Axiovert 135 (Carl Zeiss) and a Nikon Coolpix 5000 camera, and digitalized with the Axiovision software. For each independent experiment, cell migration quantifications of 4 different fields *per* treatment were averaged. Inhibitors were added at least 10 min before the assays.