UNIVERSITÀ DEGLI STUDI DI TORINO

Topics in anti-cancer research vol3 pp 179-201, **DOI:**
10.2174/9781608059089140301

CALCIUM SIGNALLING AND ANGIOGENESIS

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Running title: Angiogenesis and calcium signals

Keywords: calcium signalling, calcium channels, TRP, calcium channel blockers, calcium entry, angiogenic factors, VEGF, angiogenesis, neovascularization, antiangiogenic factors, endothelial cells, tumor-derived endothelial cells, signal transduction, tyrosine kinase receptors, arachidonic acid, PLA2, eicosanoids, nitric oxide, NOS, calcium-dependent transcription factors.

ABSTRACT

Proliferation and migration of vascular endothelial cells (ECs) are critical steps in angiogenesis and are strictly controlled by a number extracellular stimula. Proangiogenic peptides binding to tyrosine kinase receptors (i.e. VEGFs and FGFs) are released by several cell types, including ECs and tumor cells. Proangiogenic intracellular signalling cascades involve many messengers working in a sort of network. In particular, in this review we describe the properties and functions of the intracellular calcium signals (Ca_i), a universal, evolutionary conserved and highly versatile pathway involved in the regulation of EC proliferation and migration.

Angiogenic factors generate Ca_i rises *via* two mechanisms: entry from extracellular medium, through the opening of calcium-permeable channels in the plasmamembrane, or release from intracellular Ca^{2+} stores. Calcium entry, the main topic of this review, can be dependent on previously InsP3-activated emptying of calcium stores (store-operated Ca^{2+}

entry - SOCE), or independent on it (non store-operated Ca^{2+} entry - NSOCE). The intracellular pathways underlying endothelial Ca^{2+} entry involve, among the other pathways, arachidonic acid (AA) and nitric oxide (NO) metabolism. Even if some Ca^{2+} entry blockers are under clinical trial with encouraging results, a better knowledge about the molecular nature of proangiogenic Ca^{2+} channels and their intracellular regulation in healthy and pathological processes could lead to new and more powerful strategies in the therapeutical approaches aimed to interfere with altered tissue vascularization. Here, we discuss the state of the art in the field of calcium signaling and angiogenesis, the related recent literature and patents.

INTRODUCTION

Role of Ca2+ in the control of EC proliferation and migration

Intracellular calcium (Ca_i) signals are a highly conserved and ubiquitous mode for the control of cell survival, proliferation, motility, migration, apoptosis, and differentiation [1-4]. They are involved at different critical phases in the regulation of angiogenesis, both in healthy and in altered conditions [5-9].

Endothelial cells (EC) are the major actors of new blood vessels formation, and particular attention has been focused on them. During angiogenesis, ECs leave the preexisting vessel moving through the matrix, proliferate and finally stop their mitogenic activity and reorganize in a new tube [10-23]. Both migration and proliferation are strictly controlled by Ca_i dynamics, specifically modulated by extracellular proangiogenic agents such as vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGFs) [5-9, 24, 25].

Calcium entry from external medium is usually mediated by Ca^{2+}-permeable cationic channels in the plasmamembrane, which show varying degrees of selectivity and can support longer lasting signals (up to tens of minutes). While voltage-operated Ca^{2+}

channels open following a simple, direct stimulus such as a depolarization step, the activation of voltage-independent channels, largely prevalent in endothelium, needs the involvement of metabolic pathways stimulated by receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs), leading to the production of second messengers that modulate channel activity [26, 27].

We usually distinguish between two major types of calcium entry: store-operated Ca^{2+} entry - SOCE, secondary to- and dependent on a previously activated depletion of intracellular stores, and non store-operated Ca^{2+} entry - NSOCE, carried by channels sensitive to intracellular messengers released upon receptor activation [28-30]. SOCE and NSOCE often coexist in the same cell, in some cases depending on agonist concentration or on the level of expression of the same channel. In some cell types, SOCE and NSOCE could cross-regulate [31-34].

SOCE has been extensively associated with Ca^{2+} influx related to cell proliferation and involves the association of the endoplasmic Ca^{2+} sensor STIM-1 with channel-forming proteins including members of TRP and Orai1 [28, 35-41].

Several other examples are related to proliferation dependent on NSOCE; for others, finally, the mechanism is not specified. Among the second messengers proposed to play a role in NSOCE activation, arachidonic acid (AA) and other lipidic molecules are the most relevant (see below). In particular, in bovine and human ECs from normal and tumoral tissues, AA opens calcium-permeable channels independently from store depletion and promotes Ca^{2+}-entry-dependent cell proliferation and migration [42-52]. A store-independent Ca^{2+} channel, activated by low concentrations of AA, has been observed in HEK293 cells: this channel, responsible for I_{ARC} (arachidonate-regulated Ca^{2+} current), is not associated with cell migration and proliferation and its biophysical properties are significantly different from endothelial AA-activated calcium entry [31-34, 53, 54].

It is now well accepted that members of transient receptor potential (TRP) superfamily of channels contribute to agonist-activated Ca^{2+} entry in all tissues in a great number of physiological and pathological events [27, 55-63]. TRPs are cationic channels, some of which are directly activated by intracellular messengers, including diacylglicerol and AA [42, 50, 64].

Endothelial TRP channels (19 different TRP members) regulate angiogenesis, vascular tone and permeability, EC proliferation, motility and differentiation. Moreover they are involved in the progression of a number of human cardiovascular diseases [24, 41, 56, 58, 60, 61, 63, 65-82].

Beside TRPs, other channels play a role in proangiogenic signalling. Orai1 and Stim1, the components of the so-called calcium release activated currents (CRAC) channels, have been proposed to mediate VEGF-mediated SOCE in HUVECs and *in vivo* [39, 83]. In addition to their activity on mature ECs, Both TRPs and Orai1 are components of SOCE in endothelial progenitor cells (EPCs) that concur to neovascularization in tumors [83, 84].

CALCIUM CHANNELS BLOCKERS

Several Ca^{2+} channel blockers are available, with different chemical structure and variable selectivity and specificity [5, 6, 24, 25, 41, 72-82, 85-87]. Both voltage-dependent and independent Ca^{2+} channels are competitively blocked by inorganic ions, such as divalent transition ions (Ni^{2+}, Cd^{2+}, Co^{2+}) and lanthanides (La^{3+}, Gd^{3+}). The use of this ions in clinical protocols is severely limited by their toxicity, and, at least in some cases, by their aspecificity.

A number of organic compounds are known to interfere with endothelial Ca^{2+} channels and angiogenic process [88] (figure 1).

CAI. Carboxyamidotriazole (CAI, L651582, NSC 609974) is a synthetic Ca^{2+} influx blocker, extensively associated with inhibitory effects on cell proliferation [89-98]. The antiproliferative effects of CAI have been ascribed to its ability to inhibit intracellular pathways involving phospholipase-Cγ, $InsP_3$ and arachidonic acid release, as well as to block Ca^{2+} channels. CAI inhibits proliferation and invasive properties of several tumor cell lines *in vitro*, including prostate, glioblastoma, hepatoma, small cell lung and breast-derived cell lines. It also significantly reduces proliferation of several types of ECs (HUVECs, HAECs, BAECs) and affects angiogenesis induced *in vitro* by VEGF. The effect on angiogenesis is mediated by the block of calcium-mediated nitric oxide synthase-vascular endothelial growth factor pathway. In HUVECs, a reduction of NO release was observed, while no significant effect on VEGFR, PLCγ, ERK1/2, NFAT activities was detected, supporting the high specificity of the drug. In BAECs, CAI partially inhibits calcium entry activated by AA, critically involved in the control of proliferation. Thus, the use of calcium channel inhibitors, at least in some cell types including endothelial and smooth muscle cells, suggests a role for NO and AA in the control of cell proliferation and secondarily of physiopathological processes (see below): however, the insurgence of complex feedbacks of AA and NO on calcium homeostasis renders the experimental data difficult to be clearly interpreted.

On ECs, CAI-sensitive Ca^{2+} influx has been correlated to cell adhesion, spreading, proteolysis and migration, all processes involved in tumor invasion. Endothelial cell spreading on type IV collagen, but not on type I, is specifically regulated by CAI-sensitive Ca^{2+} influx, and the expression of metalloproteinase-2 is modulated by Ca^{2+} influx and down regulated by CAI. Inhibition of angiogenesis and metastasis has been detected in *in vivo* studies on several types of solid tumors. Administration of CAI, also in combination

with other drugs, in preclinical and clinical investigations stabilized solid tumors including carcinomas and melanomas.

Imidazole derivatives. Imidazole derivatives, such as clotrimazole, econazole, miconazole, ketoconazole, are antimycotic drugs widely used for the treatment of yeast infections [5, 99]. Imidazole antimycotics are potent inhibitors of many mammalian cytochrome P450-dependent reactions. Secondary to their effects on cytochrome P450 activity, they have also been extensively used as non-specific blockers of Ca^{2+} influx. Moreover, other effects on Ca^{2+} homeostasis have been described: miconazole, clotrimazole and econazole, in the μM range, promote Ca^{2+} entry in canine kidney cells; clotrimazole inhibits the activity of the sarcoplasmic reticulum Ca^{2+} pump in rabbit cardiomyocytes. Notably, some imidazole derivatives have been shown to affect the activity of kinases strongly involved in mitogen-induced intracellular signalling. The compound SK&F 96365 is widely used, and it is commonly suggested to act on TRPC channels [100]. However, it has been in turn classified as a specific blocker of SOCs, NSOCs, or as a non-specific blocker, acting also on VOCs. Moreover, other aspecific effects have been reported, both of activation and block of other types of channels. The isoquinoline derivative LOE-908 is an inhibitor of SOCE and NSOCE that interferes with endothelial permeability. It has been used as TRPC inhibitor [88, 100].

Calcium Trifluoroacetate. A recent report described the effects of Calcium Trifluoroacetate, $Ca(TFA)_2$, a complex, poorly dissociated salt with low toxicity [101]. $Ca(TFA)_2$ inhibits VEGF-induced ECs proliferation *in vitro* and angiogenesis *in vivo*. Interestingly, it triggers [Ca]i signals in HMECs preventing [Ca]i signaling induced by VEGF [102]. The detailed mechanism of through which $Ca(TFA)_2$ reduces the efficiency of signal transduction triggered by the growth factor has not been investigated. It could be able to activate calcium-permeable channels, as well as to regulate calcium extrusion systems and potassium channels.

CALCIUM SIGNALS RELATED TO ANGIOGENESIS

Proangiogenic factors. Most angiogenic factors (FGFs, EGF, PDGF, VEGFs, IGF-I), exert their effect by the interaction with intrinsic tyrosine kinase receptors (RTKs); cytokines (such as interleukins, ILs) bind to receptors associated with cytosolic TKs, while other growth factors (such as bradikinin, ATP, oxytocin, colecystokinin, many neuropeptides) act *via* G-protein-coupled receptors (GPCRs) [6, 10, 103-111]. While each of these classes has its peculiarities, and involves specific cascades of intracellular events, the distinction is actually not so sharp, since in many instances crosstalks between different pathways have been described and may represent a general and physiological process. Moreover, a high degree of convergence on the same effector (a channel or an enzyme) is quite usual, suggesting that some signalling modules (among them calcium signals) are well conserved and are employed by different agonists in different contexts; this finding prompts another question, i.e. how such an interwoven web of signals can be reconciled with the evidence that different factors can exert specific and unique effects on the same cells (figure 2).

Calcium rises activated upon membrane receptor recruitment have been detected in virtually every type of normal and tumor-derived cell lines. Ca_i signals are very early events, starting typically after a delay of seconds from the agonist exposure. On the basis of the time course, we can distinguish four types of Ca_i increases: a single spike usually due to release from intracellular stores; a slower and more persistent Ca^{2+} signal dependent on Ca^{2+} entry from the extracellular medium; a biphasic Ca_i elevation resulting from the combination of the two mechanisms; finally, Ca_i oscillations.

In particular, a striking feature of proangiogenic Ca_i signalling is its heterogeneity. Both amplitude and time course of the response is highly variable from cell to cell. This observation can be ascribed to the differential expression of the receptors, the Ca^{2+}

channels, or the intracellular machinery leading to the response. Vascular endothelium is a heterogeneous tissue: macro- and microvascular EC lines, as well as ECs obtained from different tissues, display biochemical and functional differences (see also concluding remarks).

Angiogenic inhibitors: Angiostatin and Endostatin. A number of endogenous inhibitors of angiogenesis are associated with tumors: the best known are angiostatin and endostatin [112-114]. Angiostatins are constituted by the first three, four, or five kringle domains of the plasminogen molecule: they are released by matrix metalloproteases secreted by tumor-infiltrating macrophages . Endostatin is a 20 kDa C-terminal fragment of collagen XVIII, probably generated by proteases and elastates activity [113, 115-117]. Endostatin and angiostatin inhibit EC migration and proliferation, and both induce EC apoptosis. Interestingly the antiproliferative effects of angiostatin seem specific for ECs while other cell types are not affected. Even if some studies have been performed by the analysis of acute intracellular effects of these peptides, several aspects of their mechanisms of action (including the putative membrane receptors) are unknown [112, 114, 115]. Ca_i signalling related to angiostatin and endostatin stimulation has been described in BAECs, HMECs, and CPAECs [117]. The response is dependent on INSP3 release following PLC activation and is composed by an initial release from intracellular stores followed by a prolonged Ca2+ entry. Another interesting observation is that prolonged exposure to endostatin attenuates acute calcium signalling in response to subsequent treatment with VEGF and FGF. Kringle domains of urokinase, another antiangiogenic endogenous factor, are also able to promote calcium increases in HUVECs but not in other cell types.

ARACHIDONIC ACID, NITRIC OXIDE AND Ca^{2+}

Arachidonic acid metabolism and Ca^{2+}

In resting cells, AA is stored within the cell membrane, esterified to glycerol in phospholipids. Three enzymes (the phospholipases A2, C and D), with different sites of attack on the phospholipid backbone, mediate the deacylation reaction that releases the fatty acid [5, 51, 99]. While PLA2 releases arachidonate in a single-step reaction, PLC and phospholipase D (PLD) do not produce AA directly; rather, they generate lipid products containing arachidonate, respectively DAG and phosphatidic acid; the latter can be metabolized to DAG by phosphatidic acid phosphatase (PA-PH). From DAG, AA can be subsequently released by diacylglycerol lipase (DAG lipase) .

In mammalian cells, PLA2 is present in several isoforms, classified in 11 groups differing in structure, intracellular localization, regulation, calcium dependence and pharmacological inhibition [118-124]. One of them is the secretory PLA2 (sPLA2), a low molecular weight enzyme (14 kDa), whose activity is dependent on high calcium concentration (in the millimolar range); the cytosolic PLA2 (cPLA2, 85 kDa) is the form that has been investigated more extensively and is the enzyme stimulated mainly by growth factors through a MAPK-dependent phosphorylation on Ser-505. Its activation requires also a translocation to the membrane, in order to interact with its substrate, in a calcium-dependent fashion, at the physiological Ca^{2+} levels reached after agonist stimulation. Another form of PLA2 (iPLA2) is Ca^{2+}-independent and its functional roles are not well characterized; recently, in a colon carcinoma cell line (Caco-2 cells), evidence has been provided for its involvement in serum-induced AA release and cell proliferation.

In vascular ECs, bFGF receptor activation leads to the recruitment of several adapter proteins (FRS2, Grb2) followed by the indirect activation of ras and the mitogen activated protein kinase (MAPK) cascade. MAPK triggers a series of downstream events, including the activation of phospholipase A2 (PLA2): this enzyme in turn catalyzes the hydrolysis of phospholipids at the sn-2 position, where arachidonic acid (AA) is acetylated.

AA metabolites and angiogenesis

Once released, free AA has different potential fates: direct regulation of several target proteins (ion channels, enzymes), diffusion outside the cell, reincorporation into phospholipids and metabolism [122, 125]. Metabolism of AA is carried out by three enzyme families: cyclooxygenases (COX), which generate prostaglandins, prostacyclins and thromboxanes, lipoxygenases (LOX), that produce leukotrienes, and cytocrome P450 monooxygenases, yielding a variety of epoxyeicosatrienoic and hydroperoxyeicosatetraenoic acids. All these compounds are collectively called eicosanoids, and act as autocrine or paracrine regulators of a variety of functions, particularly in inflammatory processes. Notably, several lines of evidence suggest their involvement in the control of EC proliferation and angiogenesis progression. In ECs from bovine adrenal cortex capillaries, AA release and the LOX pathway play a critical role in vascular cell proliferation induced by bFGF, PDGF and serum. In ECs isolated from bovine aorta, cPLA2 is activated by bFGF through p42 MAPK-dependent phosphorylation, triggering the release of AA. Moreover, in the same cell type, bFGF is a potent stimulator of proliferation through the LOX pathway of AA metabolism. Therefore, in general, LOX metabolites display mitogenic activity on ECs, while eicosanoids produced by the cyclooxygenase pathway are considered to be predominantly involved in the stimulation of migration. Some of the pathways cited above lead to other well known regulators of calcium-permeable channels, such as diacylglicerol (DAG) and InsP3.

AA and Ca^{2+}

AA itself and some of its metabolites are able to induce Ca_i increases in ECs. The direct effect of AA, independent on its metabolites, has been revealed via two major strategies: the pharmacological inhibition of COX, LOX and P450 MO (using indomethacin, NDGA, econazole derivatives and other compounds, some of which highly aspecific) or the use of ETYA, an AA analogue not metabolized by the enzymes cited above, able to

mimic at least partially AA-dependent Ca^{2+} entry. In bovine aortic ECs (BAECs), human microvascular ECs, and tumor-derived human ECs (TECs, see below), AA induces a sustained NSOCE. Calcium entry is due to different Ca^{2+} channels, including TRPs, and regulates EC proliferation and migration. In the same cells, NSOCE is also activated by angiogenic factors (bFGF and VEGF) [42, 44, 45, 47-49, 51, 52, 126-128].

Nitric oxide metabolism and calcium

NO and angiogenesis

Several lines of evidence point to a relationship between NO release and angiogenesis progression [129, 130]. However, the selective role of NO in the different steps of endothelial biochemical modifications during angiogenesis is debated. Many angiogenic factors increase the expression of endothelial NO synthase (eNOS) and stimulate the release of endothelium-derived NO. VEGF augments the endothelial expression of NOS, and stimulates the biosynthesis of NO from cultured human umbilical venous ECs and vascular segments of rabbit thoracic aorta. Similarly, transforming growth factor beta (TGFß) or bFGF trigger NO release in ECs.

A number of different approaches show the global involvement of NO on angiogenesis [129-136]. Stimulation of human umbilical venous ECs in a three-dimensional gel with bFGF or VEGF triggers NO production and let them to form capillary-like structures. This process is abolished by the NOS antagonist *N*w-nitro-l-arginine methylester (l-NAME). The same effect of L-NAME is observed in the rabbit cornea, another model of angiogenesis. NO appears to affect more than one step of angiogenesis process, acting as a freely diffusible pleiotropic factor on different cell types: in particular, this ability is clearly exerted on endothelial and smooth muscle cells. NO is an endothelial survival factor, inhibiting apoptosis. It increases proliferation on some EC types and reduces it in others. NO also promotes endothelial migration, possibly via the activation of podokinesis. In addition, NO

enhances matrix–endothelial cell interaction by inducing the expression of $\alpha v\beta 3$ and by increasing disruption of the extracellular matrix *via* the bFGF-induced up regulation of urokinase-type plasminogen activator. Intriguing evidences point to the well known ability of NO to act as vasodilator: increased flow in the skeletal microcirculation has been observed to trigger endothelial cell proliferation and it is therefore possible that in addition to its direct effects on endothelial cell proliferation, NO may influence endothelial growth indirectly by increasing blood flow locally. Detailed mechanisms underlying this process are not known, but the effect of NO on endothelial mechanically gated calcium-permeable channels activated by shear stress could be a critical route. Finally, it should be noted that NO can induce the synthesis and release of VEGF from vascular cells, giving rise to a positive feedback mechanism.

NO and Ca^{2+}

NO release is controlled by calcium elevation, due to eNOS calcium-sensitivity Accordingly to its plasmamembrane association, eNOS has been proposed to be preferentially recruited by calcium entry more than by calcium release from intracellular stores in bovine aortic ECs. Moreover, prolonged capacitative calcium entry strongly activates eNOS. On the other hand, eNOS can be activated by calcium-independent additional mechanisms, such as PI3K-Akt signalling.

Nitric oxide affects endothelial calcium homeostasis in different ways [48, 49, 132]. Flow induced calcium entry, mediated by mechanically-gated calcium-permeable channels, is sensitive to a protein kinase G-activated conductance in rat aortic ECs. In porcine pulmonary artery ECs (PAECs) NO upregulates the expression of cyclic nucleotide gated channels and activate a cGMP-independent calcium entry. Moreover, in calf pulmonary artery ECs (CPAECs), NO inhibits CCE and enhances endoplasmic reticulum uptake of calcium. In these reports, NO is not explicitly associated with cell proliferation: nevertheless, due to the ability of several mitogens to release this messenger, it could play

a critical role in the control of calcium signals related to proliferative processes. Interestingly, NO has been recently suggested as a mediator of AA-induced calcium entry in smooth muscle cells and isolated mouse parotid cells.

Notably the pathways leading to proangiogenic Ca_i increase are Ca^{2+}-dependent: some members of the phospholipase A2 (PLA2) (that release AA), PLC (releasing DAG and InsP3), NOS and TK families are calcium-regulated. This may establish a non-linear positive loop contributing to the complexity of the signal.

SPECIFICITY OF CALCIUM SIGNALLING

Spatial dynamics of calcium signals: from elementary to global events

Technical improvements during the last decades revealed the existence of localized Ca_i signals (microdomains called blips, puffs, quarks) in different cell types, including ECs [48, 137-146]. When diffusional and regenerative mechanisms are triggered, these elementary events evolve to global Ca_i waves involving all the cell volume, including the nucleus. Calcium microdomains have been detected in several regions: near the plasmamembrane Ca^{2+} channels and close to ER releasing sites. Such signals can remain localized and activate and/or recruit effectors in the vicinity. In bovine vascular ECs, focal ATP stimulation results in spatially restricted Ca^{2+} release and SOCE. Mitochondria have been shown to play a critical role in the local regulation of capacitative Ca^{2+} entry and store refilling in HUVECs. Caveolae, special membrane microdomains, may play a relevant role in controlling the spatial and temporal pattern of Ca_i signalling. Endothelial caveolae include several components of intracellular signalling such as Ca^{2+} pumps, InsP3 receptor-like proteins, eNOS, PLC, PKC, and both GPCR and RTKs.

Temporal dynamics of calcium signals and gene expression

Intracellular calcium signals trigger arrays of both rapid and sustained events, respectively ranging in seconds/minutes and hours/days. These responses are highly variable from a cell type to another. Moreover, single cell analysis points to the existence of a variability also in the same cell population, depending on quali-quantitative differential expression of receptors, signalling molecules, Ca^{2+} channels and other elements of intracellular signalling.

The detailed mechanisms underlying the complex relationship between Ca^{2+} signals with different time courses and gene activation are only partially known [147]. In T and B lymphocytes maturation and activation, the induction of a small transient spike due to Ca^{2+} release from internal stores is sufficient to activate a specific pattern of signalling molecules and transcription factors such as NF-kB and JNK [148-153]. However this brief calcium event fails to activate other transcription factors, notably NFAT. In resting cells the phosphorylated form of this protein is located in the cytosol: after stimulation, it is dephosphorylated by the Ca^{2+}-dependent phosphatase calcineurin and translocates in the nucleus. As a result, only a long lasting Ca_i increase, mediated by Ca^{2+} entry, is able to sustain NFAT activation. DNA microarray analysis on T lymphocytes confirms these evidence providing further informations: Ca^{2+}-dependent signalling mediates both gene induction and gene repression by integration of inputs from calcium store depletion, Ca^{2+} entry, calcineurin activation and other downstream pathways. Some authors suggest an opposite role of CREB and NFAT transcription factors in the control of cell growth and proliferation. Even if these observations have been provided on a particular and highly specialized cell type, they may be useful as a working hypothesis on ECs. A relevant goal could be to identify the role of Ca_i signals in the switch between the different events involving ECs during angiogenesis: proliferation, migration and reorganization in a new vessel.

CURRENT AND FUTURE DEVELOPMENTS

Several independent lines of evidences suggest that endothelial calcium signals play a role in physiological and pathological vascularization. In particular, Ca^{2+} entry mediated by proangiogenic Ca^{2+}-permeable channels regulates endothelial migration and proliferation. For these reasons, this event could be a target for the development of anti-cancer approaches aimed to inhibit tumor vascularization. However, despite the discovery of several natural or synthetic Ca^{2+} channels blockers, some relevant concerns limit their therapeutical applications. Firstly, a great amount of Ca^{2+} channel types are involved, activated or modulated by proangiogenic factors. In addition, while the same intracellular messenger can modulate different Ca^{2+} channels, some proangiogenic Ca^{2+} channels (i.e. TRPs) are co-regulated by a variety of intracellular pathways. Finally, the pattern of endothelial Ca^{2+} channels and their functional roles are strictly dependent on the tissue-specific microenvironment. This great variability has been well established for normal tissues, and particularly evident between macro- and microvasculature. More recently, this observation was extended to ECs obtained from human tumoral tissues.

Tumor neovascularization can be achieved by the recruitment of cell types other than ECs, including tumor cells, inflammatory cells and endothelial precursors, giving rise to very peculiar blood vessels [11, 104]. Tumor-derived human ECs (TECs) differ from normal ones for their functional behaviour and intracellular signalling [154, 155]. In particular, breast carcinoma- derived human ECs are more responsive to proangiogenic stimula in terms of calcium signals. The expression and functional role of some TRPs is altered [6, 42, 44, 45, 47, 128, 156-162].

A broad proteomic approach, combined to high-throughput functional screening assays, would be required to provide a more detailed information on the expression and regulation of Ca^{2+} channels involved in vascularization in health and disease.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

Disclosure

The chapter submitted for Patent Series eBook entitled "Topics in Anti-Cancer Research", Volume **2** is an update of our article "**Intracellular Calcium, Endothelial Cells and Angiogenesis**", published in the journal 'Recent Patents on Anti-Cancer Drug Discovery', Volume 1, Number 1, January, 2006, Page 105 to 119 with modified title, additional text and references.

Figure Legends

Figure 1
Structure of some widely used blockers of proangiogenic Ca^{2+} entry.
Carboxyamidotriazole (CAI), Calciumtrifluoroacetate ($Ca(TFA)_2$), SKF (SK&F 96365).

Figure 2
Interplay among AA, NO and Ca^{2+} in endothelial cells.
Tyrosine kinase receptors (RTKs), phospholipase D (PLD), phospholipase C (PLC), phospholipase A2 (PLA2), phosphatidic acid phosphatase (PA-PH), diacylglycerol lipase (DAG lipase), mitogen activated protein kinase (MAPK), cyclooxygenases (COX), lipoxygenases (LOX), cytocrome P450 monooxygenases (P450-MO), Guanylyl Cyclase (GC), endothelial Nitric Oxide Synthase (eNOS), proteine kinase A (PKA), cytosolic free calcium concentration ((Ca)i).
Dashed lines show the feedback effects of Ca^{2+} on intracellular signalling.

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