

Intracellular Calcium, Endothelial Cells and Angiogenesis

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Abstract: The proliferation and motility of vascular endothelial cells (ECs) are critical steps in angiogenesis and are strictly controlled by different extracellular signals. Among mitogens, peptides binding to tyrosine kinase receptors (i.e. VEGFs and FGFs) are well known and are released by several cell types, including ECs and tumor cells. The binding of mitogens to their specific receptors triggers intracellular signaling cascades, involving a number of messengers working in a sort of network. In particular, in this review we describe the increases of calcium levels in the cytosol, a universal, evolutionary conserved and highly versatile signal involved in the regulation of EC's proliferation and motility.

Most mitogens, including angiogenic factors, generate cytosolic calcium rises through two mechanisms: entry from extracellular medium, through the opening of calcium permeable channels in the plasma membrane, or release from intracellular organelles (mainly endoplasmic reticulum, ER).

Calcium entry, the main topic of this review, can be dependent on previously IP₃-activated emptying of calcium stores (store-dependent or capacitative calcium entry - CCE), or independent on it (non capacitative calcium entry, NCCE). The intracellular pathways underlying calcium entry are under investigation and recently arachidonic acid (AA) and nitric oxide (NO) metabolism have been suggested to play a key role, at least in some cell types. Even if some calcium entry blockers are under clinical trial with encouraging results, a better knowledge about the molecular nature of calcium channels and their intracellular regulation, together with a more detailed description of spatiotemporal dynamics of intracellular calcium events, could lead to new and more specific strategies in therapeutical approach to cancer progression and angiogenesis.

Keywords: Calcium, angiogenesis, endothelial cells, signal transduction, tyrosine kinase receptors, eicosanoids, nitric oxide.

INTRODUCTION

General Role of Calcium in the Control of Cell Proliferation

Intracellular calcium signals are a highly conserved and ubiquitous mode for the control of cell survival, proliferation, motility, apoptosis, and differentiation [1-3]. They are involved at different critical phases in the regulation of the complex and multisteped process of angiogenesis [4,5].

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; both motility and proliferation are strictly controlled by intracellular calcium dynamics, specifically modulated by extracellular agents such as vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), insulin like growth factors (IGFs), platelet derived growth factor (PDGF) and epidermal growth factor (EGF): in this review we will discuss in detail the mitogenic action of calcium for ECs and its role in the control of angiogenesis.

In the last decades, a relevant amount of evidence has accumulated pointing to a critical role of this ion in the control of proliferative events [6-11]. Increases in cytosolic free calcium concentration are associated with the progression through the cell cycle: the exit from quiescence in early G1 phase, the G1/S transition, and other checkpoints during S and M phases [12,13].

Calcium exerts its regulatory role by acting as a ubiquitous allosteric activator or inhibitor of several intracellular enzymes in the cytosol, organelles and nucleus. Some proteins (calcium binding proteins, CBPs), other than enzymes, interact with calcium showing different affinities and acting as calcium buffers: the effect is a limitation of free calcium diffusion in the intracellular environment; examples are parvalbumin, calbindin-D and calretinin [14]. Other proteins interact with the ion and regulate calcium-dependent enzymes and ion channels. The best known example is calmodulin (CaM), probably the most relevant calcium decoder for cell proliferation: it regulates, among others, the family of calcium-calmodulin dependent kinases type II (CaMKII) and several membrane channels. A great amount of data points to a direct involvement of CaMKII at several transition points during cell cycle progression [13]. Calcium-dependent enzymes also mediate the activation of several nuclear factors involved in the DNA division machinery, for example cdk and cyclins [15].

Since the early '80s, with the development of fluorescent and luminescent calcium indicators (fura, fluo, indo,

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recombinant aequorin and others), a new picture of intracellular calcium homeostasis has been achieved, giving a more complex view of the dynamic behavior of this ion [16].

GENERATION OF CALCIUM SIGNALING

Free calcium concentration inside the cytosol, $[Ca]_c$, is maintained very low (nearly 10^{-7} M in resting conditions) by active mechanisms mainly expressed in the plasma membrane (PM) and in the membranes of endoplasmic reticulum (ER). Calcium pumps are present both in PM and in ER membranes (respectively PMCA and SERCAs) extruding the ion from the cytosol by direct energy (ATP) consumption. A secondary active calcium-extruding system, the Na-Ca exchanger, is located in the PM: it contributes to the maintenance of the resting cytosolic calcium levels and its physiological relevance differs from tissue to tissue [17].

On the other hand, $[Ca]_c$ elevation is due to the opening of calcium channels that let the ion pass through the membranes in a passive way (following its electrochemical gradient). Calcium concentration in the extracellular medium and in ER lumen is much higher (respectively in the mM and μ M range) than $[Ca]_c$: thus calcium entry from outside and release from ER represent the main pathways to elevate $[Ca]_c$ in all cell types.

Compressively, the dynamic steady state of calcium in the cytosol is the result of the balance between active and passive fluxes through the cell membranes and it is strictly regulated at least for two functional reasons: to control intracellular transduction (low calcium levels allow even small calcium fluxes to be a significant intracellular chemical signal) and to avoid toxic effects due to prolonged and uncontrolled calcium elevations [14].

Release from Intracellular Calcium Stores

Calcium channels in ER membranes can be activated by intracellular messengers. The $InsP_3$ receptor ($InsP_3R$) is a multimeric calcium channel that opens in a calcium-dependent fashion after binding to inositoltrisphosphate ($InsP_3$), released into the cytosol following phospholipase C (PLC) activation by several extracellular agonists [18,19]. The $InsP_3$ -induced calcium release usually generates a very fast and short $[Ca]_c$ spike. The ryanodine receptor (RyR) is another multimeric calcium channel modulated by calcium itself: in some models it can trigger self-sustaining intracellular calcium oscillations due to a mechanism called Calcium-Induced Calcium Release (CICR) [20].

Since calcium content in ER lumen is limited, store depletion can occur rapidly and replenishment mechanisms (mainly *via* SERCAs) must be activated in order to restore initial conditions.

Calcium Entry from Extracellular Medium

Calcium entry from external medium is usually mediated by calcium-permeable cationic channels in the plasma membrane, which show varying degrees of selectivity and can support longer lasting signals, in the range of minutes or tens of minutes. It is necessary for the proliferation of several nonexcitable cell lines, including fibroblasts and ECs [9].

Available evidence refers mainly to the progression through G1 and the G1/S transition: experiments based on extracellular application of the calcium chelating agent EGTA, or of either pharmacological or inorganic blockers of mitogen-induced calcium entry, suggest that only calcium entry immediately triggered by mitogens (i.e. occurring during the first 2-4 hours of mitogen stimulation) is critical for cell cycle progression, while later calcium entry events during G1 phase are not effective [6-8,21,22].

Voltage Operated Channels (VOCs)

The first informations on the structure of Ca^{2+} permeable channels have been obtained twenty years ago concerning the voltage-dependent Ca^{2+} channels (Ca_v) of the skeletal muscle [23]. Additional work has shown that these channels possess the pentameric structure 1 2 [reviewed by 24]: the 1 subunit alone exhibits the essential functional properties of the channel (binding sites for agonists and antagonists, voltage-sensor, ion-conducting pore), while the additional smaller subunits play a modulatory role. The Ca_v 1 subunit has the same structure as the subunit of voltage-dependent Na channels and shows four homologous repeated domains (I-IV), each spanning six times the plasma membrane (transmembrane segments are indicated as S1-S6); both N- and C-terminal regions are intracellular; cytoplasmic loops between the four domains present consensus sequences for protein kinase A-mediated phosphorylation and the binding region for the Ca^{2+} /calmodulin-mediated inactivation. The pore region is formed by the alignment of the four aminoacidic loops between segments S5 and S6 of the four repeated domains. The S4 segment acts as a voltage sensor: its secondary structure is represented by an α -helix with a positively charged aminoacid every two hydrophobic residues; the position of this charged segment is influenced by the membrane potential and can be modulated by a depolarization, thus promoting a change in the global conformation of the channel and the opening of the pore region. Recently, this largely accepted model of VOC function has been questioned, in particular regarding the gating mechanism [25].

Even if all Ca_v channels display this common structure, their functional features are very different, since several isoforms of 1 and auxiliary subunits, with splice variants, can be translated from different homologous genes. Based on electrophysiological and pharmacological studies, Ca_v channels have been classified as L-, N, P/Q-, R- and T-types: they show functional differences in activation and inactivation, single channel conductance, kinetics of channel opening, block by divalent metal ions, sensitivity to dihydropyridine compounds [26].

Being the first Ca^{2+} channels to be described and characterized, VOCs were the first obvious target of the search for Ca^{2+} influx pathways accounting for normal and altered proliferation. However, since most of the models used to study these processes are non excitable cells expressing relatively low levels of these channels, the data supporting this hypothesis are far from abundant, as compared with either second messenger-activated or store-activated channels. One relevant exception may be represented by smooth muscle cells, in which Ca^{2+} VOCs are

expressed at relatively high densities, and that can actively proliferate in inflammation and hypertrophic growth of blood vessels and other organs; in this case, too, most of the evidence is indirect, based on the effects of VOCs blockers [see *e.g.* 4, 27-30]. Expression of L-type channels has been associated with long-term exposure to transforming growth factor (TGF) and ensuing transformation of hepatic stellate cells [31]. Moreover, some transformed models appear to express relatively high levels of T-type channels (as compared to their differentiated counterparts), and some recently developed VOCs antagonists, such as mibefradil [32] have an antiproliferative effect. On the other hand, expression of T-type channels in HEK 293 cells does not increase proliferative events, thus pointing to cell-specific effects [33]. Some observations are even more conflicting: suppression of T-type channels, in many non excitable cell types, has been associated with malignant transformation by H-ras or other oncogenes [6,34,35]. It must be considered, however, that the functional role of these channels in this context has still to be clarified.

Agonist-Activated Channels

While Ca^{2+} VOCs open following a simple, direct stimulus such as a depolarization step, activation of voltage-independent channels needs the involvement of metabolic pathways stimulated by receptor tyrosine kinases (RTKs) or G-protein-coupled receptors (GPCRs), leading to the production of various second messengers that modulate channel activity.

In spite of the variability of the mechanisms, two major pathways for the induction of calcium entry are known: capacitative or store-dependent calcium entry (CCE) is secondary to and dependent on a previously activated depletion of intracellular stores, while non capacitative entry (NCCE) is carried by store-independent calcium channels regulated by intracellular messengers released after receptor activation [36].

These two types of fluxes may coexist in the same cell, in some cases depending on agonist concentration or on the level of expression of the same channel [37]: usually low doses activate NCCE, while higher doses trigger CCE. Some Authors suggest a cross-inactivation mechanism, like a sort of switch operated by the same intracellular messenger, possibly arachidonic acid (AA) [see below; 38,39].

CCE is the mechanism that has been more extensively associated with Ca^{2+} influx related to cell proliferation and involves a heterogeneous class of channels, whose characterization is far from being exhaustive. The best known members are I_{crac} (Ca^{2+} release activated current) channels; even if their molecular nature is still unknown, their electrophysiological properties have been well described, mainly in blood cells. They are opened following store depletion induced by InsP_3 and show unique electrophysiological features, such as very low single channel conductance and high selectivity for Ca^{2+} ions over monovalent cations [40]. In leukemic T cells, block of I_{crac} caused an arrest in the G0/G1 phase [41]. Examples of CCE involvement in cell proliferation have been provided for several cell types, including basophilic leukaemia cells [42],

Ha-ras transformed fibroblasts [43], and bronchial smooth muscle cells [44].

Several other examples are related to proliferation dependent on NCCE mechanisms [see *e.g.* 11,21,45,46]; for others, finally, the mechanism is not specified [see *e.g.* 47]. Among the second messengers proposed to play a role in NCCE activation, arachidonic acid (AA) and other lipidic molecules are the most relevant (see below). In particular, in cultured bovine aortic ECs (BAE-1), AA directly opens calcium-permeable channels independently from store depletion and promotes calcium-entry-dependent cell proliferation [21,48,49; Fig. 1].

A calcium channel whose opening is not store-dependent but induced by low concentrations of arachidonic acid has been observed in HEK293 cells [50]: this channel, indicated as I_{ARC} (arachidonate-regulated Ca^{2+} current), has not been associated with cell cycle control so far.

Plasma membrane channels responsible for mitogen-activated calcium entry are a still elusive family of proteins: a great amount of data is available about their functional properties, but few and contradictory are the facts about their structure and physiological role. Electrophysiological measurements using patch clamp technique (in whole cell and single cell configuration) and fluorimetric evidences using calcium-sensitive fluorescent dyes suggest that they form a heterogeneous family, showing different biophysical properties (conductance, selective permeability, kinetic modulation). Many of them are non selective cationic channels, permeable to calcium, sodium, and potassium ions. Moreover the same intracellular messenger (such as AA or NO, see below) can modulate more than one channel in the same cell type [21,51,52].

Recently, a large amount of evidence suggests the involvement of transient receptor potential (TRP) superfamily of channels as a relevant route of agonist-induced calcium entry.

Structure and Function of TRP

The prototype of these channels was firstly described in mutant photoreceptors of *Drosophila melanogaster* [53] and then several mammalian TRP homologous channels have been cloned, and now classified into six families by sequence homology [54,55]. The TRP-Canonical (TRPC) family is composed by seven proteins (TRPC1-7) bearing highest homology to *Drosophila* TRP and is indicated also as short TRP since they are relatively short proteins (700-900 aminoacids); the TRPV family is named based on the first member described, the vanilloid receptor, involved in pain transduction; a third group is the TRPM family, from melastatin, a long TRP homologous protein.

Common architecture of TRP channels shows six transmembrane hydrophobic segments, with the loop between segments 5 and 6 forming the ion-conducting pore, intracellular N- and C- terminal domains, and ankyrin-repeats in the amino termini of TRPC and TRPV proteins; functional channels have been suggested to be homo- or heterotetramers with defined interactions between different subunits [56]. In particular, TRPC channels have been

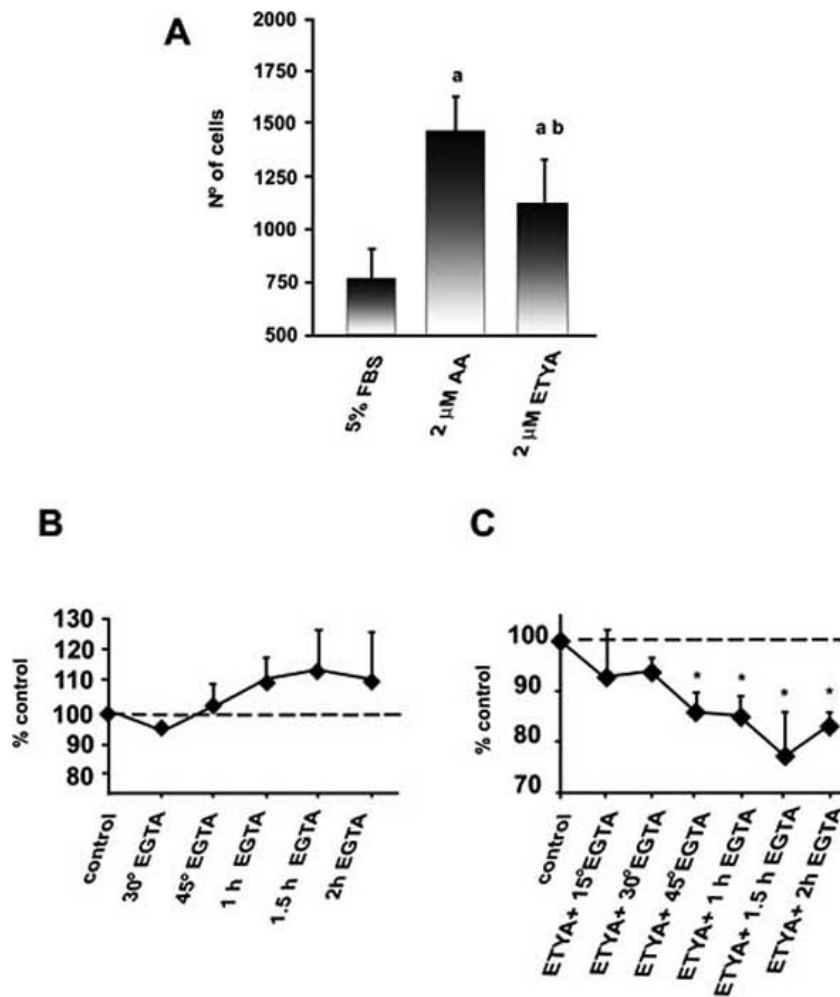


Fig. (1). Mitogenic effect of AA and ETYA and its dependence on calcium entry.

A. A representative experiment (of 5) showing the mitogenic effect of AA or ETYA in BAECs.

Cells were starved one day with a medium containing 1% FBS, then treated for one day with 2 μM AA or 2 μM ETYA added to medium containing 5% FBS. Each point is the mean ± S.D. of 6 measurements. (a) Significant increase of proliferation induced by AA or ETYA compared to control cultures not exposed to the fatty acids (5% FBS alone) ($p < 0.05$ estimated by Student-Newman-Keuls test); (b) significant increase of proliferation induced by ETYA compared to cultures exposed to AA ($p < 0.05$ estimated by Student-Newman-Keuls test).

B. Effect of 3 mM EGTA addition to the bath during the first 30', 45', 1, 1.5, and 2 h of 5% FBS application. Control stands for 5% FBS alone. Each point is the mean ± S.D. of 4 independent experiments. Protocol as in A. Data are expressed as percentage of the control.

C. Calcium sensitivity of cell proliferation induced by 2 μM ETYA stimulation using the same experimental protocol as in A.

Effect of 3 mM EGTA addition to the bath respectively during the first 15', 30', 45', 1, 1.5 h, and 2 h of fatty acid application. Each point is the mean ± S.D. of 5 independent experiments. Control stands for growth induced by ETYA in absence of EGTA. Values differing significantly ($p < 0.05$ estimated by Student-Newman-Keuls test) from the control are marked with an asterisk. Data are expressed as percentage of the control.

(From Fiorio Pla & Munaron, Cell Calcium 2001).

proposed to play a role in both capacitative and non capacitative Ca^{2+} influx [reviewed in 36].

TRP channels are a heterogeneous family of cationic channels with different calcium selectivity [36] and some of them are directly activated by intracellular messengers, including diacylglycerol (TRP and TRPL, 57) and fatty acids including AA (TRPC3, 4, 6) [56,58]. Moreover in an EC line endogenously expressing TRPC1, this channel seems to be involved in bFGF-induced calcium entry [59]. In pulmonary

artery smooth muscle cells, endogenous TRPC1 channels regulate a store-dependent calcium influx critical for proliferation: basal levels of TRPC1 protein expression are significantly higher in proliferating than in growth-arrested cells [60], and application of antisense oligonucleotides against TRPC1 mRNA reduces proliferation [47]. TRPC1 plays a role also in the proliferation control of airway smooth muscle cells [61]. On the contrary, down-regulation of TRPC6 has been observed in tumor mast cells during

progression to malignancy with increased proliferation rate due to autocrine production of IL-3 [62].

The involvement of other calcium channels, related or unrelated to TRP channels, in mitogen-induced signalling cannot be excluded at present: for example, a calcium-permeable cationic channel with homology to the TRPC family has been characterized in Balb/c 3T3 fibroblasts and named growth-factor channel (GCR): it is activated by insulin-like growth factor (IGF-I), that induces its translocation from the intracellular pools to the plasma membrane [63].

CALCIUM CHANNEL BLOCKERS

Several calcium channel blockers are available, with different chemical structure and variable selectivity and specificity [45, 46]. They can be divided into two major groups: inorganic ions and organic molecules. This classification does not reflect a specific action of these substances, since many of them interfere with more than one type of channel.

Inorganic (Ionic) Blockers

Both voltage-dependent and independent Ca^{2+} channels are competitively blocked by metal ions, such as divalent transition ions (Ni^{2+} , Cd^{2+} , Co^{2+}) and lanthanides (La^{3+} , Gd^{3+}) [23]. Given their structural simplicity, these cations have been among the first Ca^{2+} channels blockers used: they reduce Ca^{2+} influx and the related proliferation in several types of cells, both normal and transformed [6,38,64-66]. Estacion and Mordan (1993) have shown that, in normal mouse fibroblasts (C3H 10T1/2 cells) primed with insulin, PDGF induced a large long-lasting Ca^{2+} increase, completely blocked by addition of 10 μM La^{3+} to the extracellular solution; moreover, La^{3+} also inhibited progression to S phase. Similar effects could be obtained also by Ni^{2+} (20 μM) and Co^{2+} (200 μM), but not Cd^{2+} (200 μM). In Balb/c 3T3 fibroblasts, IGF-I induced Ca^{2+} oscillations, dependent on extracellular Ca^{2+} and reversibly abolished by 50 μM Co^{2+} ; this cation also attenuated proliferation [7,64]. It is worth noting that in the former case the effect was associated with a block of VOCs, in the latter to a block of voltage-independent channels. In serum-starved primary cultures of human pulmonary artery smooth muscle cells, store depletion increased capacitative Ca^{2+} influx, associated with proliferation and blocked by 0.5 mM Ni^{2+} [60]. In vascular smooth muscle cells (A7r5), vasopressin caused a biphasic rise in $[\text{Ca}]_i$, where the sustained phase was totally blocked by 1 mM La^{3+} or Gd^{3+} , with marked inhibitory effects on $[\text{H}^3]$ thymidine incorporation [49]. In the same cells, Ca^{2+} fluxes induced by vasopressin were mediated by voltage independent channels, activated by both capacitative and non capacitative mechanisms, with different sensitivity to Gd^{3+} block: pre-treatment of A7r5 cells with 1 μM Gd^{3+} completely abolished the capacitative pathway, without affecting the non capacitative component, which could be blocked only at higher concentrations (100 μM Gd^{3+}) [38]. In Muller glial cells, ATP provoked a Ca^{2+} influx, mediated by the P2Y metabotropic purinergic receptors, related to DNA synthesis and blocked by 40 μM Ni^{2+} ; nickel abolished both ATP-stimulated Ca^{2+} entry and proliferation, showing

the same effects as intracellular Ca^{2+} chelation by BAPTA/AM [65]. In human colon carcinoma cells (HRT-18), which require Ca^{2+} for proliferation, La^{3+} inhibited both SOCE and cell proliferation [66]. In another colon carcinoma-derived cell line (Caco-2), EGF induced Ca^{2+} influx through SOC channels, related to proliferation and reduced by 25 μM La^{3+} ; this ion also significantly attenuated EGF-stimulated mitogenesis [67].

Dihydropyridines

Dihydropyridines (nifedipine and related molecules) are the most widely used blockers of L-type voltage-dependent Ca^{2+} channels, being effective in the μM range. They are usually considered to be quite specific for this class of channels, and their effects can be associated with an identified Ca^{2+} influx pathway. However, some of them (nifedipine and nicardipine) can block a voltage-insensitive Ca^{2+} influx and suppress proliferation [68].

Evidence for the ability of such blockers to interfere with Ca^{2+} influx-dependent proliferation has been provided in several cell types, mainly vascular smooth muscle cells from normal and pathologic tissues [27-30, 69,70].

Other organic blockers of Ca^{2+} VOCs, associated with inhibition of proliferation, include verapamil [27], whose selectivity of action has been extensively questioned, and mibefradil, a drug considered to be selective for T-type channels expressed in some types of cancer cells [32].

Other Organic VOCs Blockers

Carboxyamidotriazole (CAI)

Carboxyamidotriazole (CAI, L651582, NSC 609974) is a synthetic Ca^{2+} influx blocker, extensively associated with inhibitory effects on cell proliferation [71-74; Fig. 2].

The antiproliferative effects of CAI have been ascribed to its ability to inhibit intracellular pathways involving phospholipase-C, InsP_3 and arachidonic acid release, as well as to block Ca^{2+} channels. Early studies on muscarinic acetylcholine receptor-transfected chinese hamster ovary cells showed that 10 μM CAI inhibited receptor mediated Ca^{2+} influx in response to the muscarinic agonist carbachol, in addition to an immediate effect on arachidonic acid release and a minimal effect on inositol phosphatase release.

CAI inhibited proliferation and invasive properties of several tumor cell lines *in vitro*, including prostate, glioblastoma, hepatoma, small cell lung and breast-derived cell lines [72,75-77]. It also significantly reduced proliferation of several types of ECs (HUVECs, HAECs, BAECs) and inhibited 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 [78]. In HUVECs, a reduction of NO release was observed, while no significant effect on VEGFR, PLC, ERK1/2, NFAT activities was detected, supporting the high specificity of the drug [79]. In BAECs, CAI partially inhibits calcium entry activated by AA, critically involved in the control of proliferation [22]. Thus, the use of calcium channel inhibitors, at least in some cell types including endothelial and smooth muscle cells,

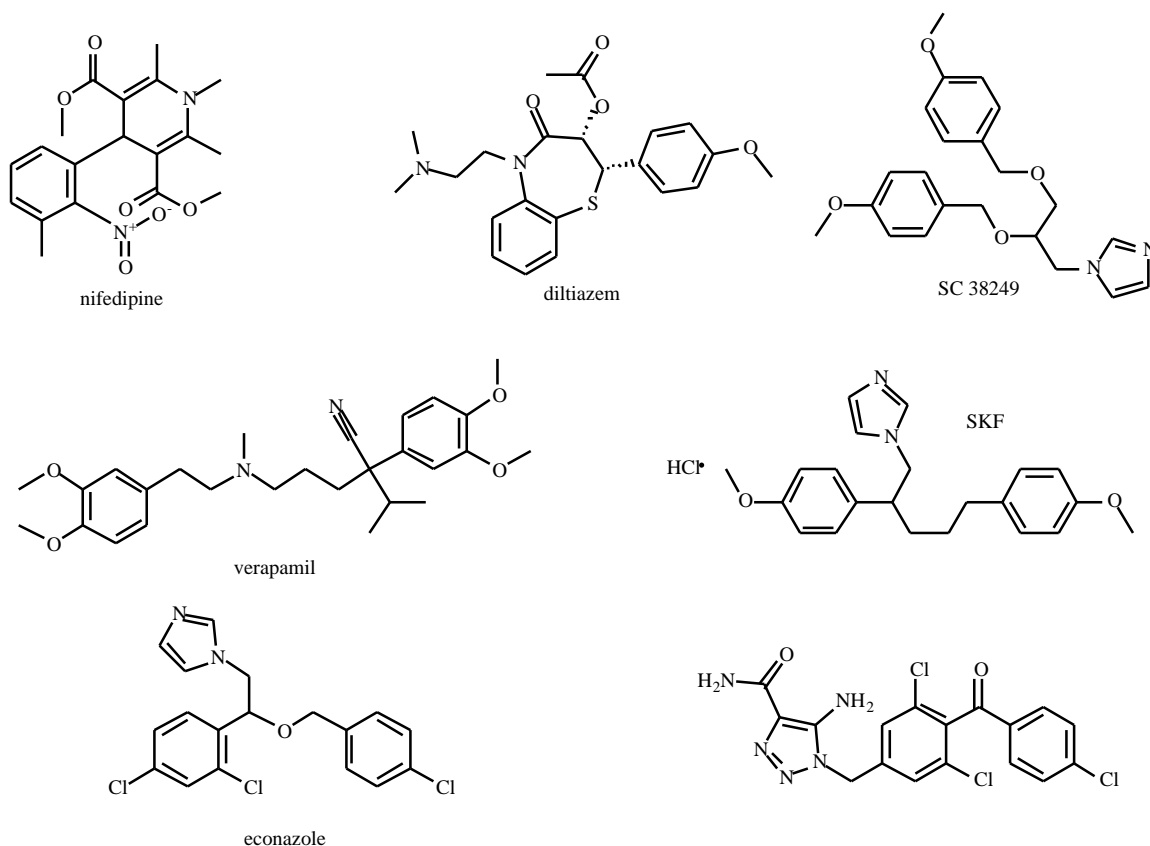


Fig. (2). Structure of some widely used blockers of mitogen-activated calcium entry.

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 [78-82].

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 [83], and the expression of metalloproteinase-2 is modulated by Ca^{2+} influx and down regulated by CAI [84]. Inhibition of angiogenesis and metastasis has been detected in *in vivo* studies on several types of solid tumors [75,85]. Administration of CAI, also in combination with other drugs, in preclinical and clinical investigations stabilized solid tumors including carcinomas and melanomas [86-88].

Imidazole Derivatives

Imidazole derivatives, such as clotrimazole, econazole, miconazole, ketoconazole, are antimycotic drugs widely used for the treatment of yeast infections. 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 [see e.g. 89-93]. 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 [94-96]; clotrimazole inhibits the activity of the sarcoplasmic reticulum Ca^{2+} pump in rabbit cardiomyocytes [97].

Notably, some imidazole derivatives have been shown to affect the activity of kinases strongly involved in mitogen-induced intracellular signalling [98,99]. However, since it cannot be excluded that these compounds also act directly on Ca^{2+} permeable channels (as for other drugs, such as SK&F 96365 and CAI) and since they (clotrimazole in particular) are also under trial as chemotherapeutic drugs, we report here some relevant data about their effects on Ca^{2+} influx.

The compound 1-(*o*-chloro-, -diphenylbenzyl)imidazole (clotrimazole) inhibits bradykinin-induced proliferation of smooth muscle cells and the related rise of $[\text{Ca}]_i$ [100].

The compounds 1-(2-[(4-chlorophenyl)methoxy]-2-[2,4-dichlorophenyl]ethyl)-1*H*-imidazole (econazole) and (\pm)-*cis*-1-acetyl-4-(4-[(2-[2,4-dichlorophenyl]-2-[1*H*-imidazol-1-yl-methyl]-1,3-dioxolan-4-yl)-methoxy]phenyl)piperazine (ketoconazole) are potential purging agents for residual leukaemia cells present in bone marrow, since proliferation of human leukaemia cells *in vitro* is more sensitive to these blockers than proliferation of normal hemopoietic progenitor cells; inhibition of tumoral cell proliferation by clotrimazole is ascribed to block of store operated Ca^{2+} influx [101]. The

use of clotrimazole in the treatment of proliferative disorders is being clinically tested [102].

Below we will review some imidazole derivatives whose role in blocking mitogen-activated Ca^{2+} influx has been more extensively studied.

SC38249

The compound ((±)-1,-2,3-bis-[(4-methoxyphenyl)methoxy]propyl)-1H-imidazole (SC38249) displays multiple actions on Ca^{2+} homeostasis, blocking both voltage-dependent and independent channels and also interfering with the activity of Ca^{2+} ATPases [103]. In NIH3T3 fibroblasts overexpressing the EGF receptors (EGFR-T17 cells) and lacking voltage-dependent Ca^{2+} channels, the mitogen EGF induced a biphasic rise of [Ca]_c, with long-lasting rhythmic fluctuations dependent on Ca^{2+} influx. SC38249 (10 μM) abolished Ca^{2+} influx and inhibited by 60% the proliferation induced by EGF. Interestingly, Ca^{2+} oscillations were paralleled by synchronous plasma membrane hyperpolarizations due to opening of Ca^{2+} -dependent K^+ channels, blocked by charybdotoxin. Charybdotoxin induced also a minor but significant reduction (-20%) of the EGF-stimulated mitogenic effect [104]. This observation is an example of how Ca^{2+} influx can be modulated not only by blocking calcium-permeable channels, but also by reducing the electrochemical gradient, thereby depolarizing the membrane potential.

SK&F 96365

The compound 1- β -[3-(4-methoxy-phenyl)propoxy]-4-methoxyphenethyl-1H-imidazole hydrochloride (SK&F 96365) [105], the best known of this class, has been widely used, and it is commonly suggested to act directly on Ca^{2+} permeable channels. However, it has been in turn classified as a specific blocker of store-operated channels [47,106], of non-store-operated ones [105, 106], or as a non-specific blocker, acting also on VOCs [8,105]. Moreover, other aspecific effects have been reported, both of activation and block of other types of channels [89,108]. Independently from its specificity, it acts as an antiproliferative agent on some cell types, among them Balb-c 3T3 fibroblasts [8] and mammary epithelial cells [109].

LOE 908

The isoquinoline derivative (*R,S*)-(3,4-dihydro-6,7-dimethoxy-quinoline-1-yl)-2-phenyl-*N,N*-di-[2-(2,3,4-trimethoxyphenyl)ethyl]acetamide (LOE 908) is an inhibitor of receptor- and store-operated Ca^{2+} influx [110,111], largely employed by Kawanabe *et al.* [see *e.g.* 112-115]. In rabbit vascular smooth muscle cells, the mitogen endothelin-1 activates Ca^{2+} influx through different pathways: two types of non-selective cation channels (NSCC-1 and NSCC-2), store-operated channels (SOC) and voltage-dependent channels. Contribution of these permeation pathways to Ca^{2+} influx relevant for endothelin-induced mitogenesis can be resolved using different blockers: application of nifedipine, blocking VOCs, has a minor role on proliferation stimulated by endothelin-1, that, conversely, is completely abolished by the combination of SK&F96365 and LOE908. In these cells SK&F96365 blocks NSCC-2 and SOC channels, while LOE908 affects both types of non-selective cation channels

[115]. By preventing Ca^{2+} influx, LOE908 inhibits proliferation in other different experimental models, such as C6 glioma cells [112] or A7r5 vascular smooth muscle cells stimulated with endothelin-1 [114], and Chinese hamster ovary (CHO) cells overexpressing the (1A)-adrenergic receptors treated with noradrenalin [113].

CALCIUM SIGNALS ACTIVATED BY MITOGENS AND ANGIOGENIC FACTORS

Growth Factors

Mitogenic factors are a highly heterogeneous family of agonists, most of which act in a pleiotropic way regulating other cellular processes (survival, differentiation, motility, secretion and apoptosis). Some are peptides, such as classical growth factors, cytokines and many hormones; others are lipidic and steroid compounds: members of all these groups are able to increase [Ca]_c [11,116-119]. Most of the literature is related to peptidic mitogens binding to membrane receptors and in this review we focus on them, even if agonists that act by binding to intracellular receptors, such as estrogens, have been shown to be able to induce [Ca]_c elevations [120].

In particular, growth factors, including some potent angiogenic factors (FGFs, EGF, PDGF, VEGFs, IGF-I), exert their effect by the interaction with intrinsic tyrosine kinase receptors (RTKs) [121]; cytokines (such as interleukins, ILs) bind to receptors associated with cytosolic TKs, and other mitogens (such as bradikinin, ATP, oxytocin, colecystokinin, many neuropeptides) act *via* G-protein-coupled receptors (GPCRs), spanning seven times the plasma membrane [see *e.g.* 117]. 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 [122], pointing to the fact 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 cellular model.

The fact that the same effector (*e.g.* a calcium permeable channel) can be recruited in response to a wide repertory of extracellular signals is one of the reasons why the mechanisms involved in mitogen-induced calcium signaling are so controversial, and a sharp picture is still lacking.

Calcium rises activated after membrane receptor recruitment have been detected in virtually every type of normal and transformed cell lines, emerging as one of the most conserved responses immediately triggered in the target cells in which they exert a mitogenic activity. These signals have been observed, in many instances, also in response to the application of antibodies directed against extracellular epitopes of different receptors [123].

A striking feature of mitogen-activated calcium increase is its heterogeneity: both amplitude and time course of the

response vary from cell to cell even in the same cell culture. This non-homogeneity can be ascribed to the differential expression of the receptors, the calcium channels, or the intracellular signalling machinery leading to the response. Variability can still be observed in some cell lines after serum deprivation: in these conditions most of the cells are quiescent (i.e. in G0/G1 phase), excluding a cell cycle-phase dependent effect as a major route for generating heterogeneity [48, 124-125] even if some mitogenic agonists can influence the expression or the functional properties of components of the signaling pathway [60,63]. Moreover ECs are a high heterogeneous tissue: biochemical and functional differences have been described between macro and microvascular EC lines and among ECs obtained from different tissues [80,126].

Increases in [Ca]_c start typically after a delay of seconds (from a few to several tens). On the basis of the time course, at least four types of [Ca]_c increase are detectable: a single spike due to release from intracellular stores (mainly ER), a slower and more persistent calcium signal dependent on calcium entry from the extracellular medium, a biphasic [Ca]_c elevation due to the combination of the two mechanisms, and calcium oscillations.

Angiostatin, Endostatin and Calcium

A number of endogenous angiogenesis inhibitors have been found to be particularly associated with the presence of tumors: the best known are angiostatin and endostatin [127, 128].

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 [129].

Endostatin is a 20 kDa C-terminal fragment of collagen XVIII, probably generated by proteases and elastases activity [130].

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.

Recently, calcium signaling related to angiostatin and endostatin stimulation has been described in BAECs, HMECs, and CPAECs. The response is dependent on IP₃ release following PLC activation and is composed by an initial release from intracellular stores followed by a prolonged calcium entry [131]. Another interesting observation is that prolonged exposure to endostatin attenuates acute calcium signalling in response to subsequent treatment with VEGF and FGF [131]. Kringle domains of urokinase, another antiangiogenic endogenous factor, are also able to promote calcium increases in HUVECs but not in other cell types [132].

COMPLEX RELATIONSHIP AMONG ARACHIDONIC ACID, NITRIC OXIDE AND CALCIUM

Arachidonic Acid Metabolism and Calcium

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 (Fig. 3). 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; 133,134).

In mammalian cells, PLA₂ is known to be present in several isoforms, recently classified in 11 groups differing in structure, intracellular localization, regulation, calcium dependence and pharmacological inhibition [135]. 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 [136]. Its activation requires also a translocation to the membrane, in order to interact with its substrate, in a calcium-dependent fashion, at the physiological Ca²⁺ levels reached after agonist stimulation [137]. Another form of PLA₂ (iPLA₂) is Ca²⁺-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 [138].

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 [139]. 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 [133]. Metabolism of AA is carried out by three enzyme families: cyclooxygenases (COX), which generate prostaglandins, prostacyclins and thromboxanes, lipoxygenases (LOX), that produce leukotrienes, and cytochrome P450 monooxygenases, yielding a variety of epoxyeicosatrienoic and hydroperoxyeicosatetraenoic acids (see Fig. 3). All these compounds are collectively called eicosanoids, and act as autocrine or paracrine regulators of a variety of functions, particularly in inflammatory processes [140]. Notably, several lines of evidence suggest their involvement in the control of EC proliferation and angiogenesis progression [141]. In ECs

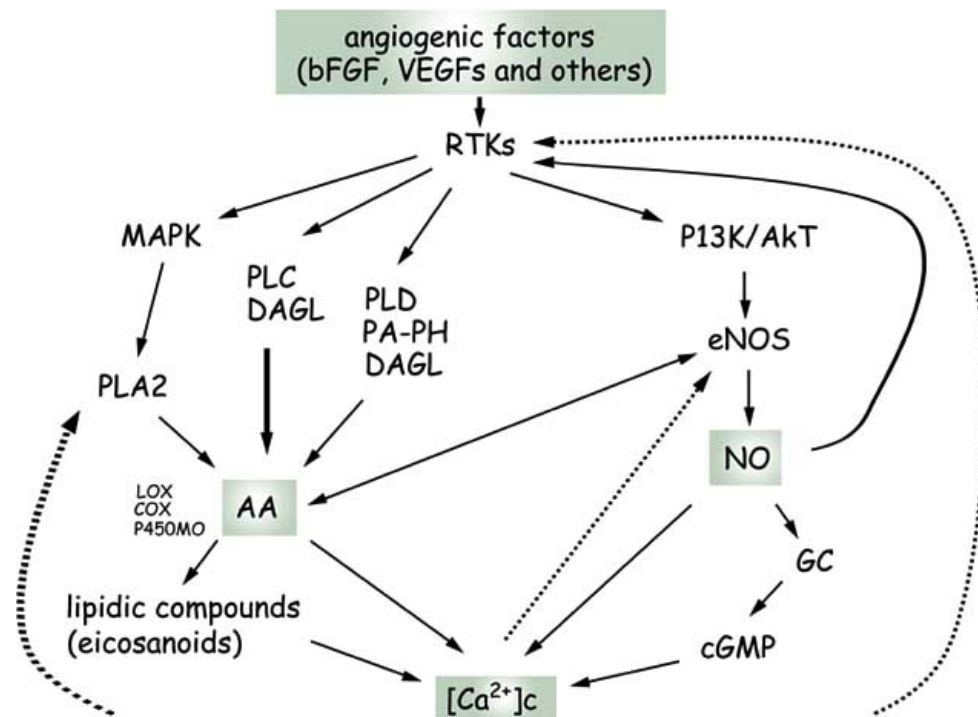


Fig. (3). Interplay among AA, NO and calcium in endothelial cells.

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), cytochrome P450 monooxygenases (P450-MO), Guanylyl Cyclase (GC), endothelial Nitric Oxide Synthase (eNOS), cytosolic free calcium concentration ([Ca]_c).

Dashed lines show the feedback effects of calcium on intracellular signaling.

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 [142]. In ECs isolated from bovine aorta, cPLA2 is activated by bFGF through p42 MAPK-dependent phosphorylation, triggering the release of AA [143]; moreover, in the same cell type, bFGF is a potent stimulator of proliferation through the LOX pathway of AA metabolism [144]. Therefore, in general, LOX metabolites display mitogenic activity on ECs [145], while eicosanoids produced by the cyclooxygenase pathway are considered to be predominantly involved in the stimulation of migration [143,146]. However, recent evidence for a role of COX metabolism in EC proliferation has been provided in BAECs [22]. Some of the pathways cited above can lead to other second messengers, potentially involved in the activation of calcium-permeable channels in the plasma membrane, notably diacylglycerol, DAG [84], and InsP3 itself [147,148]: for these two messengers, up to now no clear evidence is available that the related calcium fluxes may have a mitogenic role. Moreover, for InsP3-dependent calcium channels the mechanism of activation is still debated [36]: the case is particularly sensitive, since both store-dependent and independent mechanisms have been proposed [149,150].

AA and Calcium

AA itself and some of its metabolites are able to induce mitogenically-related calcium increases in fibroblasts and ECs [21,22,125]. 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 calcium entry [125]. In bovine aortic ECs (BAECs), AA induces a sustained NCCE involved in the control of proliferation: it is probably due to more than one type of calcium channels, whose molecular nature is not known [21]. In the same cells, NCCE is also activated by mitogenic growth factors, including bFGF, IGF-I and VEGF [48].

Nitric Oxide Metabolism and Calcium

NO and Angiogenesis

Several lines of evidence point to a relationship between NO release and angiogenesis progression [151-153]. However, the selective role of NO in the different steps of endothelial biochemical modifications during angiogenesis (mainly proliferation) 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 [154,155]. Similarly, transforming growth factor beta (TGFβ) or bFGF trigger NO release in ECs [156-158].

A number of different approaches show the global involvement of NO on angiogenesis. 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 NW-nitro-L-arginine methylester (L-NAME) [159,160]. The same effect of L-NAME is observed in the rabbit cornea, another model of angiogenesis [161].

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 [162,163]; it increases proliferation on some EC types and reduces it in others [161,164,165]. NO also promotes endothelial migration [166,167], possibly via the activation of podokinesis [168]. In addition, NO enhances matrix-endothelial cell interaction by inducing the expression of α_3 [167] (the ligand for the extracellular matrix protein Del-1, which is itself known to induce angiogenesis) and by increasing disruption of the extracellular matrix via the bFGF-induced up regulation of urokinase-type plasminogen activator [161]. 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 [169] 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 [170; see below]. 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 [171].

NO and Calcium

NO release is controlled by calcium elevation, due to eNOS calcium-sensitivity [172,173]. Accordingly to its plasma membrane association, eNOS has been proposed to be preferentially recruited by calcium entry more than by calcium release from intracellular stores in bovine aortic ECs [174]. Moreover, prolonged capacitative calcium entry strongly activates eNOS [175]. On the other hand, eNOS can be activated by calcium-independent additional mechanisms, such as PI3K-Akt signalling [160,176; see Fig. 3].

Nitric oxide has been shown to affect calcium homeostasis in ECs in different ways. Flow induced calcium entry, mediated by mechanically-gated calcium-permeable channels, is sensitive to a protein kinase G-activated conductance in rat aortic ECs [see above; 170,177]. In porcine pulmonary artery ECs (PAECs) NO upregulates the expression of cyclic nucleotide gated channels and activate a cGMP-independent calcium entry [178,179]. Moreover, in calf pulmonary artery ECs (CPAECs), NO inhibits CCE and enhances endoplasmic reticulum uptake of calcium [52]. 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 [51,81].

Notably the pathways leading to mitogen-induced intracellular calcium increase are calcium-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 [180,181].

SPECIFICITY OF CALCIUM SIGNALLING

Technical Advancements in Calcium Measurements

The synthesis of fluorescent calcium-sensitive dyes, in the seventies, allowed for the first time to measure intracellular calcium signals in living cells [182]. Now several calcium probes are available with different spectral properties. Some of them are excited by UV light (fura-2, indo-1); a special property is their ability to allow absolute calcium measurements through the production of a calibration curve and applying a ratiometric algorithm that describe the shift of excitation or emission spectra. Ratiometric measurements avoid the problems due to the variability of the optical pathway and the biological preparation over the time. However, UV excitation generates acute and chronic toxic effects and is filtered by low quality lens in the optical pathways [182]. Probes excited by visible light (fluo-3, calcium greens and others) give relative calcium estimation, but show a higher emission and are not biased by UV drawbacks.

These calcium-sensitive fluorescent probes are usually introduced into the cells as inactive acetoxymethyl esters, freely permeable through the plasma membrane and rapidly hydrolysed by cytosolic esterases: the final effect is that cytosolic acidic form of the active probe is compartmentalized in the cytosol. Another approach, usually combined with patch clamp, is the introduction of the active probe directly via the patch pipette.

Improvement of calcium measurements has been recently obtained by the use of two approaches: recombinant aequorin and confocal microscopy. Aequorin is a calcium-sensitive luminescent protein extracted from jellyfish *Aequorea* [183]. Aequorin fused with peptidic domains involved in the protein targeting allows calcium measurements in different intracellular organelles (nucleus, mitochondria, endoplasmic reticulum, Golgi membranes): in particular, this approach revealed the physiological role of mitochondria as intracellular calcium buffers associated with local microdomains in which the levels of calcium can reach high values [183,184].

A relevant change in our view of spatiotemporal intracellular calcium dynamics has been carried by the introduction of confocal laser scanning microscopy (CLSM): calcium microdomains have been revealed in excitable and nonexcitable cells, and particular attention has been focused on the basis of the specificity of the calcium-mediated cellular response (see below).

Spatial Dynamics of Calcium Signals: From Elementary to Global Events

The improvement of confocal laser scanning microscopy (CLSM) using calcium-sensitive fluorescent dyes allowed to increase spatial resolution and revealed the existence of localized intracellular calcium signals (microdomains called blips, puffs, quarks) in different cell types, including ECs [185-188]: when diffusional and regenerative mechanisms are triggered, these elementary events evolve to global calcium waves involving all the cell volume, including the nucleus. Calcium microdomains have been detected in several regions: near the plasma membrane calcium 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 release and capacitative calcium entry [187,188]. Mitochondria have been shown to play a critical role in the local regulation of capacitative calcium entry and store refilling in HUVECs [189, 190].

Caveolae, special membrane microdomains, may play a relevant role in controlling the spatial and temporal pattern of intracellular Ca signalling [191]. Endothelial caveolae include several components of intracellular signaling such as calcium pumps, IP₃ receptor-like proteins, eNOS, PLC, PKC, and both GPCR and RTKs [192].

Temporal Dynamics of Calcium Signals and Gene Expression

Increased concentrations of free Ca²⁺ in the cytosol 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 qualitative differential expression of receptors, signaling molecules, calcium channels and other elements of intracellular signalling [12,119].

The detailed mechanisms underlying the complex relationship between calcium signals with different time courses and gene activation are not known: however, some recent evidences will enable us to begin to answer this question [193,194; for a review see 195]. In T and B lymphocytes maturation and activation, the induction of a small transient spike due to calcium release from internal stores is sufficient to activate a specific pattern of signaling molecules and transcription factors such as NF- κ B and JNK. 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 calcium-dependent phosphatase calcineurin and translocates in the nucleus. As a result, only a long lasting calcium increase, mediated by calcium entry, is able to sustain NFAT activation [193]. DNA microarray analysis on T lymphocytes confirms these evidence providing further informations: calcium-dependent signalling mediates both gene induction and gene repression by integration of inputs from calcium store depletion, calcium entry, calcineurin activation and other downstream pathways [196]. Some authors suggest an opposite role of CREB and NFAT

transcription factors in the control of cell growth and proliferation [195]. Even if these observations have been provided on a particular and highly specialized cell type, they may be useful as a working hypothesis to be verified in other cell types, including ECs. A relevant goal could be to identify the role of calcium 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 a critical role for calcium in the control of angiogenesis and tumoral progression: in particular calcium entry from extracellular space, mediated by the opening of plasma membrane calcium-permeable channels, is involved in the control of proliferation in virtually all cell types.

For these reasons, this event could be in principle a potential target for the development of anti-cancer drugs: however, despite the discovery of several natural or synthetic calcium channels blockers, some relevant concerns limit their therapeutical applications. Firstly, a great amount of calcium channel types, voltage-dependent and agonist-activated, have been detected, differentially activated or modulated by mitogens: notably, at least in some cell types, more than one calcium channel type can be activated by the same intracellular messenger. Moreover, the molecular nature of several mitogen-activated calcium channels, whose biophysical properties have been described in many cell types, is still elusive, making very difficult the development of specific blockers. Finally, the expression pattern of calcium channels by different cell types, and even by cells within the same population, is highly heterogeneous. Proteomic approach will help us to overcome some of these difficulties, providing more detailed informations concerning the identity of calcium channels involved in the control of cell proliferation in normal and tumoral tissues, and their relationship with specific gene expression patterns.

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