Abstract. Most peptidic growth factors (GFs) that act as mitogens for different cell types bind to tyrosine kinase receptors (RTKs) and trigger complex intracellular signal transduction pathways finally leading to cell proliferation. Among the early events induced by GFs, cytosolic calcium increase plays a key role, and in particular calcium entry from extracellular medium appears to be a widespread signal. Even if a great amount of data has been provided during the last decades, several issues remain to be fully clarified: the nature of the calcium-permeable channels involved, their regulation by intracellular messengers, and the mechanisms underlying calcium-dependent cell proliferation. This review focuses on the relationship between calcium signals and angiogenesis, a process in which endothelial cell proliferation, mainly triggered by basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), is a critical step.

Mitogens are a set of diffusible factors, including hormones and peptidic growth factors, lipidic compounds, and other molecules, that control progression through the different phases of the cell cycle. They act via autocrine and/or paracrine mechanisms and exert their effects interacting with high affinity receptors located in the plasma-membrane, triggering a cascade of intracellular reactions, finally leading to DNA synthesis and cell duplication.

One of the early events immediately induced by mitogens is the increase of free calcium concentration in the cell cytosol. Calcium signalling regulates several cellular events, among them early gene transcription by the recruitment of cytosolic and nuclear transcription factors (NF-AT, NF-κB, and CREB): the specificity of the effect depends on the spatiotemporal features of intracellular calcium waves.

The free cytosolic calcium ([Ca]c) increases stimulated by external agonists are due either to the release from intracellular stores, mainly represented by endoplasmic reticulum (ER), or to the influx from the extracellular medium through the opening of calcium-permeable channels: these two pathways are not exclusive and can coexist in response to the stimulation with the same agonist (Fig. 1).

The release from intracellular calcium stores was the first mechanism described: it is usually due to the production of inositol 1,4,5 trisphosphate (InsP3), a diffusible cytosolic messenger binding to specific calcium channels located in the ER membranes (InsP3Rs), and causing their opening. Usually the signal is of great amplitude and fast, showing a duration of a few minutes: it is a spike whose return to the basal level is dependent on the activity of calcium pumps (PMCA in the plasma-membrane and SERCA in ER membranes), Na-Ca exchangers, and calcium binding proteins in the cytosol.

The second pathway, calcium entry from external solution, is mediated by the opening of calcium-permeable voltage-independent ion channels in the plasmamembrane. The signal is often smaller than the one triggered by release from the stores, but shows a long lasting duration, in the order of minutes, tens of minutes, and hours.

The modes and roles of mitogen-induced calcium entry in pathophysiological processes will be discussed in particular detail.

2. Calcium and proliferation

Several peptidic growth factors (GFs) bind to receptors with intrinsic tyrosine kinase activity (RTKs) and exert their action
locally via autocrine and/or paracrine modes. The majority of GFs interact with receptors expressed by a wide range of cell types, while some exert more restricted effects: an example is the family of VEGFs that are mitogens for endothelial cells (2).

Calcium measurements using fluorescent probes (fura-2, indo-1, fluo-3, recombinant aequorin, and others) (7), developed and improved in the last decades, have revealed that most GFs are able to trigger an increase of calcium in the cytosol that can be associated to their mitogenic activity.

Platelet derived growth factor (PDGF) (8-10), epidermal growth factor (EGF) (11,12), basic fibroblast growth factor (bFGF) (13-15), insulin-like growth factor-I (IGF-I) (15,16), and vascular endothelial growth factors (VEGFs) (17-20) trigger significant increases of \([\text{Ca}]_{\text{c}}\) in different cell types. The typical time course of calcium elevation induced by external agonists, including mitogens, its long lasting duration, provides an interesting mode for activation of specific pattern of genes, different from those regulated by brief spikes, generally due to release from intracellular stores (6,21).

In particular mitogen-induced calcium influx plays a key role in the control of proliferation of non-excitable cell. Serum-activated calcium entry in fibroblasts is required for the mitogenic effect and its inhibition causes a block in G2/M phases of the cell cycle, through mechanisms not clearly known (22); sustained calcium influx by PDGF is a requirement (even if not sufficient) for proliferation of mouse fibroblasts (9). Furthermore, bFGF-induced calcium entry, mediated by arachidonic acid, is needed for proliferation in endothelial cells (15,23). In most cases, the effect is specific for a calcium increase triggered in the early period of mitogen stimulation (up to 4 h), being not detectable in later periods (9,22).

The initiation of proliferation is dependent on long lasting intracellular calcium signalling, as has been clearly shown in several tissues, such as lymphocytes, fibroblasts, CHO cells, and endothelial cells (3). Calcium entry from extracellular medium can support \([\text{Ca}]_{\text{c}}\) elevations lasting for hours, and thus it appears more suitable than release from calcium stores, more limited sources, generally supporting only brief calcium signals.

### 3. Modes of calcium entry

Agonist-activated calcium entry (CE) is mediated by the opening of calcium-permeable channels in the plasma-membrane, whose properties appear to be quite heterogeneous, and whose molecular nature has not been unequivocally clarified (24,25).

Two modes of CE activation are described (Fig. 1). One, called capacitative (CCE) or store-dependent calcium influx (SDCI), is secondary and consequent to the depletion of...
intracellular calcium stores (ER). Even if CCE is a widespread mechanism that can be activated in several cell types, its physiological role is not clear (3,25). Partially unknown is the pathway linking ER depletion with plasmamembrane channel (SOCs, store operated channels) opening: more than one mechanism is probably involved, depending on the cell type considered (26).

The other mode for CE triggering is the non-capacitative (NCCE, or store-independent) one (Fig. 1B) it is not functionally related to the release from ER, even if it can co-exist with it. The channels involved are often cationic non-selective and they likely belong to a heterogeneous family of pore-forming proteins, directly opened by intracellular messengers, such as arachidonic acid (AA) and its metabolites, InsP3, diacylglycerol (DAG), and others (24,25,27-29) (Fig. 1).

4. Intracellular pathways and mechanisms involved in the induction of calcium entry

In recent years, our group and others have focused their attention on the signal transduction pathways leading to tyrosine kinase-dependent calcium entry induced by GFs in different cell types from mammalian tissues. The main question is: what is (are) the intracellular mediator(s) of RTK-activated calcium influx?

As a general mechanism, GFs, by binding to their surface receptors, induce RTKs dimerization and activation of their tyrosine kinase activity. A second step is the crossphosphorylation of RTKs, with the subsequent recruitment and the activation of intracellular proteins, that transmit the signal to a plethora of targets, including nuclear transcription factors. Among these signalling molecules, phospholipase C (PLCγ), phosphatidylinositol-3-kinase (PI-3K), ras activating protein (GAP), ras, mitogen-activated protein kinase (MAPK), and phospholipase A2 (PLA2) are the most commonly involved (2).

AA metabolism. In fibroblasts and endothelial cells (ECs), one of the major signalling pathways activated in response to bFGF and other GFs is the release of arachidonic acid (AA) via the recruitment of phospholipase A2 enzymes (30).

Arachidonic acid is a polyunsaturated fatty acid present in the mammalian membrane phospholipids, where it is esterified usually in the sn-2 position to form phospholipids. While other pathways may contribute to the release of the fatty acid, the activity of phospholipase A2 (PLA2) is thought to be the primary rate-limiting step controlling the supply of free arachidonate for further metabolism in most cell types, including ECs. PLA2s form a large family of enzymes, differently regulated: cytosolic PLA2 (cPLA2) is the form mainly activated by GFs, via PKC or MAP kinase recruitment, as shown in endothelial cells and other cell types (31-34).

AA is rapidly metabolized within the cell by lipoxygenase (LOX), cyclooxygenase (COX) and P450 monoxygenase (MO) enzymes, giving rise to the release of a large family of compounds, called eicosanoids (35). The effects of AA metabolites on blood vessels are well known, including the control of EC motility and proliferation and smooth muscle contractility: eicosanoids regulate inflammatory processes and the induction of angiogenesis. In bovine capillary endothelial cells from adrenal cortex, bFGF and PDGF regulate vascular cell growth through the LOX pathway of AA metabolism (36); in bovine aortic endothelial cells (BAECs), bFGF is a potent stimulator of proliferation and movement, and triggers AA metabolism (37,38). cPLA2 activity and the subsequent AA release are correlated with the proliferating state of several types of ECs (32).

AA and calcium entry. Limited literature is available on the relationship between AA in calcium, however, some eicosanoids have been shown to activate calcium influx in different cell types: leukotriene C4 (LTC4) in EGFR-T17 cells (39); epoxyeicosatrienoic acids (EETs), cytochrome P450 MO metabolites, in endothelial and smooth muscle cells (40,41).

Recent evidence provided by our group and others points to a direct contribution of AA to calcium-regulated biological processes. In some cell types, AA plays a direct role in the control of calcium homeostasis, and in particular in the induction of calcium entry from the extra-cellular medium: AA-activated calcium influx has been described in exocrine cells from the avian nasal gland, HEK cells, Balb-C 3T3 fibroblasts, smooth muscle cells, and in endothelium (23,42-46). The mechanisms vary regarding some features, but at least three properties are shared: calcium entry is: a) directly triggered by AA and not by its metabolites; b) independent from intracellular calcium store depletion (i.e. non-capacitative); c) activated by low AA concentrations (1-8 µM), below the critical mycellar concentration. Recently our group has shown that store-independent calcium influx induced by AA in BAECs is involved in the control of proliferation of this cell type (23) (Fig. 2).

5. Mitogen-activated calcium channels

Details about functional properties of mitogen-activated calcium mainly come from single channel patch clamp experiments (47). The use of cell-attached and inside-out

![Figure 2. Putative mechanism responsible for bFGF-induced non-capacitative calcium entry involved in the control of endothelial cell proliferation. Arachidonic acid (AA) plays a key and direct role in the activation of calcium-permeable ion channels in the plasma-membrane. AA release is mainly dependent on MAPK-cPLA2 signalling, even if other pathways are probably involved.](image-url)
configurations allow to identify different calcium conductances activated by external and internal agonists (growth factors and related intracellular messengers). Using this approach, biophysical properties of agonist-activated calcium channels (conductance, ion selectivity, modulation, kinetic behaviour) can be analysed in detail in highly controlled experimental conditions.

Measurements performed by our group on fibroblasts and ECs suggest the existence of a family of calcium conductances with different reversal potentials (15,23,43). Ionic selectivity may vary, but usually these channels are non-selective cationic pores, permeable to sodium, calcium, and potassium ions. Some inhibitors have been described in different models: among inorganic ions, lanthanum is a widely used and effective blocker (24); SK&F 96365 and related compounds (imidazole derivatives) inhibit calcium entry in several cell types: however, relevant non-specific effects have been detected (22,48-50).

The molecular structure of calcium channels involved in calcium entry triggered by mitogens is not known, even if recently some putative candidates have been proposed (51).

The family of transient receptor potential channels (TRPCs), structurally related to those expressed in Drosophila photoreceptors, has been suggested to be responsible for agonist-activated calcium entry in several tissue types (52).

In a recent report we proposed the putative involvement of TRPC1 channel in bFGF-triggered calcium entry in BAECs (51).

6. Calcium entry and angiogenesis: discussion and perspectives

The knowledge of the early events that regulate cell proliferation, among them calcium signals, is the basis for the understanding of some physiological and pathological processes. From this point of view, angiogenesis, that requires the controlled proliferation of endothelial cells, can be an useful paradigm.

Factors such as bFGF and VEGF are involved in angiogenesis at different steps: after hypoxia they stimulate EC activation, enzymatic degradation of basal lamina, migration, proliferation, and organization in new blood vessels (53).

These two factors, the most potent endogenous angiogenic agents, bind to different and specific families of RTKs that trigger an increase of [Ca]c in ECs. There are some similarities in the intracellular substrates recruited by the two factors (54); a common requirement for VEGF and bFGF-induced angiogenesis is the activation of PLCγ (via MAPK-dependent pathway), the release of arachidonic acid, and the production of 12-LOX metabolites, involved in EC proliferation, migration, and tube differentiation (35).

Conversely, other intracellular pathways seem not to be triggered by either VEGF or bFGF, or play different physiological roles.

Even if in some EC types both GFs are able to activate PLCγ and subsequent Ca2+ release from intracellular stores, at least in choroidal ECs a direct role of PLCγ and calcium in VEGF-regulated cell growth has been suggested, whereas the same signalling pathway would not be linked to FGF-mediated effects (55).

Release of NO by eNOS (via Akt-dependent phosphorylation) has been clearly described after VEGF stimulation and is needed for angiogenesis dependent on this factor, while not involved in FGF-dependent one (17,18,56-58).

Although a great amount of evidence has been provided on intracellular signal transduction pathways, several problems have to be solved in order to obtain a more satisfactory view of the link between mitogens, calcium elevation, and angiogenesis. Here we discuss the major critical points.

The biological models used to study in further detail this issue are a critical discussion topic. A first issue is related to the heterogeneity of ECs. Indeed several reports focus on the existence of molecular, cellular and macroscopic divergences among ECs taken from different anatomical sites (59,60); macrovascular and microvascular, arterial and venous sites confer specialized functions to ECs. In particular, qualitative and quantitative expression of some ion channels is not the same, even if no specific data about agonist-activated voltage-independent calcium channels are available to support this hypothesis (60).

A second issue is related to the use of cultured cell lines (HUVEC, BAEC, CPAEC), that are largely employed to obtain cellular and molecular information on intracellular calcium signalling. It should be taken into consideration that cell cultures change their biochemical properties (expression of channels and critical intracellular enzymes) during culture passages, conferring different physiological features to the same cell type (61,62).

Another very interesting observation comes from the results of experiments at the single cell level: several groups reported that the responses to mitogens, detected using patch clamp or fluorimetric calcium measurements, vary from cell to cell in the same population of a cell line (same culture conditions and passage) (15,63). This heterogeneity may be related to several factors. Cells not fully synchronized are in different phases of the cell cycle, thus potentially expressing different amounts and types of receptors, intracellular signalling molecules, and channels. This is an intriguing topic, particularly regarding ECs: they may provide diverging calcium signals to the same factors during different phases of angiogenesis (confluent cells when they are in a vessel, moving and proliferating cells during intermediate phases and again confluent in the new vessel).

Beyond all the limits described above, the key role of calcium in the control of cell proliferation is strengthened by the fact that clinical applications of drugs affecting calcium influx are in progress in cancer therapy with promising results. An example is the use of CAI (carboxyamido-triazole), an inhibitor of voltage-independent calcium influx (64). CAI inhibits the proliferation and invasion of several tumor cell lines in vitro (breast, prostate, and glioblastoma): it also exerts antiangiogenic activity and inhibits the proliferation of human vein endothelial cells (HUVECs) and BAECs (Munaron, unpublished data). However, its additional effects (subsequent to the block of calcium entry?) on intracellular NO and AA release have to be considered to correctly evaluate eventual side effects in antiangiogenic therapy (65).

Endostatin and angioatin, two well known angiogenesis inhibitors (66,67), have been shown to induce calcium transients in endothelial cells derived both from small and
from large vessels: prolonged exposure to endostatin attenuates acute calcium signalling in response to subsequent treatment with bFGF or VEGF (68).

In the near future, a crucial task will be to identify the genes encoding the calcium channels involved in mitogen-induced calcium entry: this will allow their expression in heterologous systems, in order to analyse in detail their biophysical properties, to clarify the intracellular signalling pathways that regulate their activation, and to develop new and more specific drugs to be potentially employed in the approach to the clinical treatment of solid tumors.

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References


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