J. Cell. Mol. Med. Vol 8, No 2, 2004 pp. 161-168

Invited Review

Intracellular calcium signals and control of cell proliferation: how many mechanisms?

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Received: April 1, 2004; Accepted: June 18, 2004

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Abstract

The progression through the cell cycle in non-transformed cells is under the strict control of extracellular signals called mitogens, that act by eliciting complex cascades of intracellular messengers. Among them, increases in cytosolic free calcium concentration have been long realized to play a crucial role; however, the mechanisms coupling membrane receptor activation to calcium signals are still only partially understood, as are the pathways of calcium entry in the cytosol. This article centers on the role of calcium influx from the extracellular medium in the control of proliferative processes, and reviews the current understanding of the pathways responsible for this influx and of the second messengers involved in their activation.

Keywords: calcium - cell proliferation - calcium channels - cell signaling

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Calcium and cell proliferation

Calcium ions are regulators of virtually all cellular processes; notably, these include the events that will decide the fate of a cell: survival, proliferation, motility, apoptosis, and differentiation [1]. In this review we will focus on the role of calcium signals in the control of cell proliferation, and on the pathways involved in their activation.

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 [2-7]. 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 [8, 9].

Calcium exerts its regulatory role by acting as an ubiquitous allosteric activator or inhibitor of several intracellular enzymes in the cytosol, in the organelles, and in the nucleus. Some proteins (calcium binding proteins, CBPs), other than enzymes, bind to calcium with different affinities and act as calcium buffers, thus limiting its diffusion. Other proteins do not have intrinsic enzymatic activity but, after their interaction with the ion, regulate calcium-dependent enzymes and ion channels. The best known example is calmodulin, 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 out to a direct involvement of CaMKII at several transition points during cell cycle progression [9]. Calcium-dependent enzymes also regulate the activation of several nuclear factors involved in the DNA division machinery, for example cdk and cyclins [10].

Since the early '80s, with the development of fluorescent and luminescent calcium indicators (fura, fluo, indo, recombinant aequorin and others), a new view of intracellular calcium homeostasis has been achieved, giving a more complex picture of the dynamic behavior of this ion [11].

Calcium concentrations inside the cytosol, $[Ca]_c$, are maintained very low (nearly 10^{-7} M in resting conditions) by active mechanisms mainly expressed in the plasma membrane (PM) and in the membrane 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 consumption. In the PM are also located secondary active calcium-extruding systems such as Na^+-Ca^{2+} exchanger [12], whose physiological relevance differs from tissue to tissue.

On the other hand, $[Ca]_c$ elevation is due to the activation of calcium channels that let the ion pass through the membranes in a passive way. The concentration of calcium in the extracellular medium and in ER lumen is much higher than $[Ca]_c$ (0.2-1 mM free calcium in ER) [1, 64]; thus calcium entry from outside and release from ER represent the main pathways to elevate $[Ca]_c$ in all cell types.

Release from intracellular calcium stores

Calcium channels in ER membranes are activated by intracellular messengers. The $InsP_3$ receptor ($InsP_3R$) is a multimeric calcium channel that opens after binding to inositoltrisphosphate (InsP3), released into the cytosol following phospholipase C (PLC) activation by several extracellular agonists [13]. The InsP3-induced calcium release generally gives rise to a very fast and short $[Ca]_c$ spike (see Fig. 1).

The ryanodine receptor (RyR) is another multimeric calcium channel modulated by calcium itself: in some models it can generate self-sustaining intracellular calcium oscillations due to a mechanism called calcium induced calcium release (CICR, [14]).

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

Extracellular calcium influx

Voltage operated calcium channels (VOCs) are activated by a depolarization and are involved in the electrical activity of excitable cells (neurons, muscle and secretory cells). Actually some members of the calcium VOC family are modulated by phosphorylation and/or interaction with intracellular messengers, released following stimulation with external agonists, including agonists involved in mitogenic effects, but the evidence of their involve-

ment in proliferative processes is scarce [15, 16]. Calcium VOCs are highly selective.

Receptor operated channels (ROCs) are activated by the interaction with extracellular agonists, and are typically involved in chemical synaptic processes, where they trigger the postsynaptic potential: ROCs are often non selective calcium channels, also permeable to Na^+ and K^+ ions.

Second messenger operated calcium channels (SMOCs) open following interaction with intracellular messengers, released as a consequence of external agonist binding to its specific membrane receptor. This is one of the mechanisms through which external agonists, including mitogens, stimulate $[Ca]_c$ elevation in non excitable cells [17]. Due to the complexity of intracellular signal transduction pathways induced by different agonists in different cell types, the identification and classification of agonist-activated calcium channels has proven to be a quite complex task.

Only few of these channels have been cloned (*e.g.* cyclic nucleotide gated channels, [18]) and their physiological roles still have to be identified.

Calcium signals activated by mitogens

Mitogenic factors include a large variety 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 lipids including steroid compounds. Members of all these groups are able to increase $[Ca]_c$.

Most of the literature reports peptidic mitogens binding to membrane receptors and in this review we will focus on them: however, also agonists that act by binding to intracellular receptors, such as estrogens, have been shown to be able to induce $[Ca]_c$ elevations [19].

In particular, growth factors (such as 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 to cytosolic TKs, and other mitogens act *via* G-protein-coupled receptors (GPCRs), spanning seven times the plasma membrane (such as bradikinin, ATP, oxytocin, colecystokinin, many neuropeptides); 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 crosstalking (such as transactivations, see *e.g.* [20, 21]) 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 common (*e.g.* [22]), pointing to the fact that some signaling 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 the different factors can exert specific and unique effects on the same cellular model. We will deal with the latter issue in one of the following paragraphs.

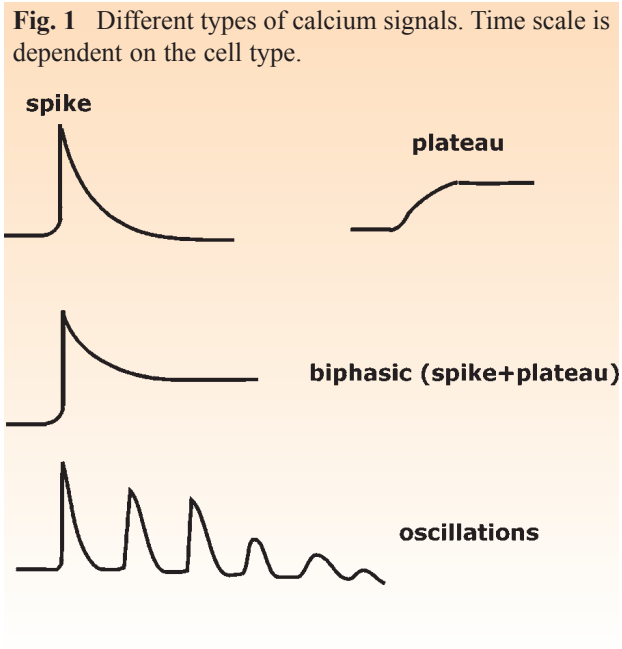
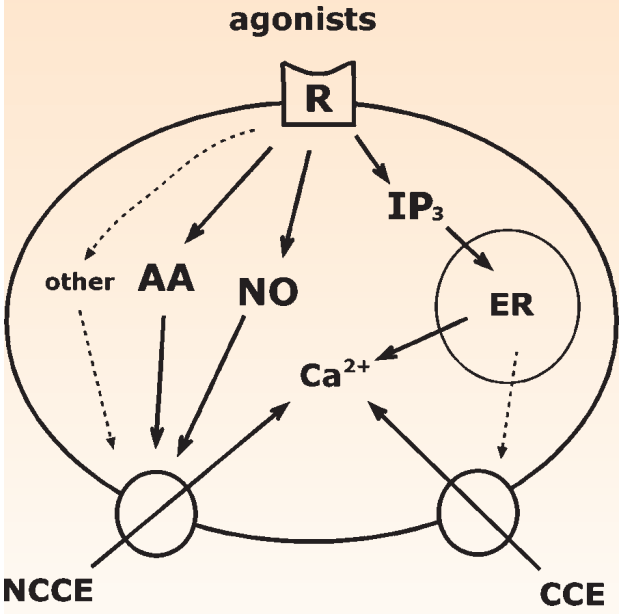


Fig. 1 Different types of calcium signals. Time scale is dependent on the cell type.

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 signals activated after membrane receptor recruitment have been detected in virtually every type of normal and transformed cell lines: calcium signaling emerges as one of the most conserved responses immediately triggered in the tar-

Fig. 2 Simplified picture showing the main pathways involved in the activation of mitogen-induced calcium entry.



CCE: capacitative calcium entry; NCCE: non capacitative calcium entry; R: receptor; ER: endoplasmic reticulum; AA: arachidonic acid; NO: nitric oxide.

get cells in which they exert a mitogenic activity. Activation of calcium increases has been observed, in some instances, also in response to the application of antibodies directed against extracellular epitopes of different receptors [16, 23]. In this context, the antibody has a stimulatory role; on the other hand, there are several examples of antibodies directed against either receptors or channels that can inhibit calcium influx (see *e.g.* [50]).

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 [24, 25], even if some mitogenic agonists can influence the expression or the functional properties of components of the signaling pathway [26, 27].

Increases in $[Ca]_c$ start tipically 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: 1). a single spike due to release from intracellular stores (mainly ER); 2). a slower and more persistent calcium signal dependent on calcium entry from the extracellular medium; 3). a biphasic $[Ca]_c$ elevation due to the combination of the two mechanisms, and 4). calcium oscillations (Fig. 1).

Modes of calcium release and influx

Calcium release from intracellular stores is usually triggered by the production of InsP3 that can be stimulated by both GPCRs (*via* PLCβ) and RTKs (*via* PLCγ). The consequent $[Ca]_c$ spike is rapidly down regulated because of the limited volume of the stores and of the activity of SERCA and PMCA calcium pumps (Fig. 1).

On the other hand, 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 spite of the variability of the mechanisms, two major pathways for the induction of calcium entry are known: capacitative calcium entry (CCE) is secondary to and dependent on a previous depletion of intracellular stores, while non capacitative entry (NCCE) is carried by store-independent calcium channels regulated by intracellular messengers released after receptor activation [17].

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 [28]. Usually low concentrations of agonist activate NCCE, while higher doses activate CCE [29]. Some authors suggested a cross-inactivation mechanism, like a sort of switch operated by the same intracellular messenger, such as arachidonic acid (AA) [30].

Fig. 2 shows some of the proposed mechanisms.

As for NCCE, the first level of variability is represented by the second messengers involved in the activation of calcium permeable channels. The relatively scarce data related to mitogen-activated pathways have to be considered in the more gener-

al context of agonist-dependent calcium influx, so that in most cases the proposed mechanisms are somehow speculative.

Several groups, including ours, have focused their attention on the intracellular pathways leading to arachidonic acid. AA release can be obtained *via* at least three distinct multistep pathways (depending respectively on PLA2, PLC and PLD - the last two involving also DAG lipase); AA itself is rapidly modified, giving rise to many intracellular cascades involving short-lived lipid compounds (eicosanoids), some of which are biologically active. They include prostaglandins, leucotrienes, epoxyeicosatrienoic acids: both AA and some of its metabolites are able to induce calcium increases in different cell types, such as fibroblasts, HEK293, endothelial and secretory cells [31-35].

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 DAG [36], and InsP3 itself [37, 38]: until now there is no clear evidence that the calcium fluxes induced by these two messengers may have a mitogenic role.

Moreover, for InsP3-dependent calcium channels the mechanism of activation is still debated [17] : the case is particularly sensitive, since both store-dependent and independent mechanisms have been proposed [39, 40].

Recent evidence points to a role of nitric oxide, NO (released by NOS, nitric oxide synthase) as either a positive or a negative modulator of calcium entry in smooth muscle and endothelial cells [30, 41-44]. In these reports, NO is not explicitly associated to 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. Additionally, some authors have suggested that NO may act as a mediator of AA-induced calcium influx [30, 45].

It should be noted that in many cases the pathways leading to intracellular calcium increase are calcium-dependent too: some members of the phospholipase A2 (PLA2) (that release AA) and PLC (releasing DAG and InsP3) and NOS families are calcium-regulated. This may establish a non linear positive loop contributing to the complexity of the signal. Conversely, it has been shown that receptor and cytosolic TKs are inhibited by calcium elevation, thus generating a negative feeback [20, 46].

How many channels?

Another level of variability is related to the molecular identity of the channels involved. Plasma membrane channels responsible for mitogen-activated calcium entry are a still elusive family of proteins: a great amount of data are available about their functional properties, but few and contradictory are the facts about their structure and physiological role.

Electrophysiological measurements (in whole cell and single cell configuration) and fluorimetric evidences suggest that they form a heterogeneous family, showing different biophysical properties (conductance, selective permeability, kinetic behaviour). Many of them are non selective cationic channels, permeable to calcium, sodium, and potassium ions. Moreover the same intracellular messenger (such as AA and NO) can stimulate more than one channel in the same cell type [34].

Recently cloned transient receptors potential (TRP) channels (so called because of their involvement in the response of *Drosophila* photoreceptors) are a heterogeneous family of cationic channels, with different calcium selectivity [17]: some TRPC channels have been shown to be activated directly by intracellular messengers, such as diacylglicerol (TRP and TRPL, [47]) and fatty acids including AA (TRPC3, 4, 6; [48, 49]), Moreover in an endothelial cell line endogeneously expressing TRPC1, this channel seems to be involved in bFGF-induced calcium entry [50]. 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 [27], and application of antisense oligonucleotides against TRPC1 mRNA reduces proliferation [51] ; in these cells, the mitogenic factor PDGF stimulates proliferation and enhances expression levels of TRPC6 mRNA [52]. TRPC1 plays a role also in the proliferation control of airway smooth muscle cells [53]. On the contrary, down-regulation of TRPC6 has been observed in tumor mast cells during progres-

sion to malignancy with increased proliferation rate due to autocrine production of IL-3 [54].

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 IGF-I, that induces its translocation from the intracellular pools to the plasmamembrane [26].

From influx to function

Regarding the role of these signals, available evidence refers mainly to the progression through G1 and the G1/S transition. In some cell lines, 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.* occuring 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 [2–4, 34].

Calcium channel blockers

Several inhibitors of mitogen-activated calcium entry have been identified, some of them employed to distinguish and classify different types of calcium currents. They include inorganic compounds, mostly ions (La^{3+}, Cd^{2+}, Co^{2+}, Gd^{3+}; see *e.g.* [2, 29]), and organic drugs (such as imidazole derivatives: econazole, miconazole, SK&F96365, SC38249; see *e.g.* [4, 55]). Notably, an imidazole derivative, carboxyamidotriazole (CAI), showed the ability to block calcium entry in several normal and transformed cell types, to inhibit their proliferation, and to significantly reduce growth, angiogenesis and metastasis in some solid tumors *in vivo*. For this reason it is under clinical trials as an anti-tumoral agent [56].

Unfortunately, the scarce information presently available about the structural properties of calcium channels activated by mitogens, together with the great complexity of the signal transduction pathways underlying calcium signals, render the identification of specific (both natural and synthetic) blockers a challenging goal.

One ion for several effects: the problem of specificity

The development of confocal microscopy has dramatically increased the spatial resolution of calcium measurements in living cells, giving the opportunity to reveal new intracellular domains. Local intracellular calcium increases (puffs and others, see [57]) have been detected where the ion can transiently reach higher concentrations (tens of μM) than those predicted by the conventional measurements. In some conditions, calcium channels on the plasmamembrane, mitochondria and ER interplay in the generation of localized calcium elevations with different amplitude and time course. Observations have been performed on excitable (neurons and muscle cells), secretory, HeLa and endothelial cells [58, 59]: even if these data are not referred to mitogenic stimulations, localized events are probably involved also in the calcium-dependent control of cell proliferation.

In addition to confocal approach, recombinant aequorin, a calcium sensitive photoprotein, specifically targeted to mitochondria, ER, Golgi and nucleus, has been used as a tool for monitoring calcium in the different cellular compartments: as an example, through this technique a critical role of mitochondria as cellular buffers of local calcium increases has been evidenced [60, 61].

Moreover, the association of calcium microdomains to the existence of stable and localized signaling machineries, like caveolae, could provide a key mechanism explaining the differential activation of calcium-sensitive intracellular substrates and then the specificity of the calcium-dependent response: InsP3 receptor-like proteins, Ca-ATPases, eNOS, PKC isoforms and several receptors colocalize with caveolae [62].

Interestingly, differential gene activation has been correlated to calcium signals with different spatiotemporal shapes [63]; even if the evidence is only preliminary, this issue will see significant developments in the near future.

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