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► **To cite this version:**

Anne-Sophie Borowiec, Gabriel Bidaux, Natascha Pigat, Vincent Goffin, Sophie Bernichtein, et al.. Calcium channels, external calcium concentration and cell proliferation. *European Journal of Pharmacology*, 2014, 739, pp.19-25. 10.1016/j.ejphar.2013.10.072 . hal-03060345

HAL Id: hal-03060345

<https://hal.science/hal-03060345v1>

Submitted on 13 Dec 2020

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Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Calcium channels, external calcium concentration and cell proliferation

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ARTICLE INFO

Article history:

Received 5 July 2013

Received in revised form

28 September 2013

Accepted 17 October 2013

Keywords:

Calcium channels

Cell cycle

Cell proliferation

Calcium sensing receptor

ABSTRACT

Evidence for a role for calcium channel proteins in cell proliferation is numerous suggesting that calcium influx is essential in this physiological process. Several studies in the past thirty years have demonstrated that calcium channel expression levels are determinant in cell proliferation. Voltage-gated, store-operated, second messengers and receptor-operated calcium channels have been associated to cell proliferation. However, the relationship between calcium influx and cell proliferation can be uncoupled in transformed and cancer cells, resulting in an external calcium-independent proliferation. Thus, protein expression could be more important than channel function to trigger cell proliferation suggesting that additional channel functions may be responsible to reconcile calcium channel expression and cell proliferation. When needed, external calcium concentration is obviously important for calcium channel function but it also regulates calcium sensing receptor (CaSR) activity. CaSR can up- or down-regulate cell proliferation depending on physiological conditions. CaSR sensitivity to external calcium is within the 0.5 to 5 mM range and therefore, the role of these receptors in cell proliferation must be taken into account. We therefore suggest here that cell proliferation rates could depend on the relative balance between calcium influx and CaSR activation.

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Contents

1. Introduction	1
2. Evidence for a role for calcium influx in cell proliferation	2
3. Calcium-sensing receptors	3
4. Calcium channels and cell cycle distribution	3
5. Additional functions for calcium channels and possible link to cell proliferation	4
6. Conclusion and perspectives	5
References	5

1. Introduction

The relation between external Ca^{2+} concentration and cell proliferation is not as straightforward as originally thought in mammalian cells. First, several cell lines can proliferate even when external Ca^{2+} is reduced to a few tens of μM (Capiod, 2013). Second, when external Ca^{2+} is required, we need to clarify whether proliferation rate increase results from the activation of plasma membrane calcium channels or calcium-sensing receptors (CaSR) that are also present in many cell

types (Riccardi and Gamba, 1999). CaSR are G-protein coupled receptors (GPCR) (Brown et al., 1998) and intracellular Ca^{2+} transients were recorded (Breitwieser and Gama, 2001; Rey et al., 2010) following activation by increasing external Ca^{2+} concentrations in the 0.5–5 mM range. Variations in cytosolic calcium $[\text{Ca}^{2+}]_{\text{cyt}}$ transient frequency have been proposed as being responsible for cellular responses modulation. Indeed, cytosolic calcium transients are dependent on external calcium in the long term suggesting the need for Ca^{2+} influx for their maintenance (Putney and Bird, 2008; Shuttleworth and Mignen, 2003). However, recent evidence against a role for calcium influx in $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations came from experiments performed in the presence of siRNA against ORAI1 or ORAI3. $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations were recorded even in ORAI1 (Wedel et al., 2007) and

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Orai3 (Thompson and Shuttleworth, 2011) knock-downs suggesting that neither I_{CRAC} (calcium release activated calcium current) or I_{arc} (arachidonic related calcium current), the archetype currents for store-operated (SOCE) and store-independent calcium (SICE) entries respectively, were needed to maintain this cellular response, at least on a short time scale. Ca^{2+} transients were recorded in mouse and human stem cells (Ferreira-Martins et al., 2009; Kapur et al., 2007) and they were thought to be involved in cell growth. It was claimed that these Ca^{2+} transients did not require SOCE or plasma membrane Ca^{2+} ATPase (PMCA) but rely on Ca^{2+} cycling between ER and cytosol. Therefore, in the case of mammalian cells, calcium channels activation during cell cycle, hence in cell proliferation, remains to be clearly established (Capiod, 2011, 2013).

Evidence for an essential role of calcium channel expression in cell proliferation are numerous (Capiod, 2013). It is interesting to note that sensitivity to external Ca^{2+} is rarely controlled as it seems obvious that calcium influx must be involved as it is the primary function for these proteins. But it is also known that some of these channels have additional functions and a few of them were named as chanzymes (Montell, 2003; Touyz, 2008). If a role for these proteins in cell proliferation independently of external Ca^{2+} remains to be proven, it is still interesting to reconsider the published data from a new perspective.

The role of calcium channels in immune cells proliferation will not be discussed here but it is indeed clear that these cells rely on calcium influx following T-cell receptor activation (Feske, 2011; Shaw and Feske, 2012). The present review is focused on the role of external Ca^{2+} concentration, calcium channel proteins and calcium influx in cell proliferation. The question here is to discuss how calcium channel protein can influence cell proliferation in the absence of external Ca^{2+} , and when external Ca^{2+} is needed, to discriminate between calcium influx and CaSR activation.

2. Evidence for a role for calcium influx in cell proliferation

We have already discussed in recent reviews (Capiod, 2011, 2013) the need for external Ca^{2+} need in cell growth and division in mammalian cells. On one hand, several studies in the seventies

and early eighties clearly demonstrated that sensitivity to external calcium depends on the degree of transformation with neoplastic cells being less dependent on external calcium (Boynton et al., 1976; Hazelton et al., 1979; Whitfield, 1992; Whitfield et al., 1979). On another hand, many other cell types required external Ca^{2+} to proliferate but it can then be questioned whether this divalent cation acts either through calcium fluxes or through the activation of calcium-sensing receptors.

Two main approaches were used to establish the role for calcium influx in cell proliferation. However, it seems that in several studies, the dependence on external Ca^{2+} in the presence of serum was not always assessed. This basic experience in the absence of external Ca^{2+} should have been conducted to demonstrate definitively the importance of calcium entry in the cell line of interest.

As described in a previous review (Capiod, 2013), several types of calcium channels seem to play a major role in cell proliferation and a non exhaustive list is shown on Fig. 1. Several other types of calcium channels also play a major role in the process of cell proliferation. For instances, purinergic P2X receptors (Burnstock, 2009; Burnstock and Kennedy, 2011), NMDA ionotropic receptors (Nacher and McEwen, 2006) and voltage-gated T type channels (Lory et al., 2006; Panner and Wurster, 2006), all subjects of recent reviews, are also involved in cell proliferation. Evidence for the involvement of cyclic nucleotide gated channels in cell proliferation is sparse but a recent paper suggested that blocking hyperpolarization-activated cyclic nucleotide-gated in embryonic stem cells resulted in an increase in S phase and a decrease in G_0/G_1 phase of the cell cycle (Lau et al., 2011).

The use of calcium channels blockers was one of the strong arguments in favor of a role of calcium influx in cell proliferation. The blockade of L- and T-type voltage-dependent, SOCE or SICE channels have potential antiproliferative effects in several tissues. The list of blockers includes verapamil, diltiazem, nifedipine, amlodipine, mibefradil, TH-1177, 2-APB, SK&F 96365 and carboxyamidotriazole (CAI) (Chung et al., 1994; Enfissi et al., 2004; Haverstick et al., 2000; Nel et al., 1986; Panner and Wurster, 2006; Taylor and Simpson, 1992; Taylor et al., 2008). Other I_{CRAC} blockers have been identified and were the subject of another

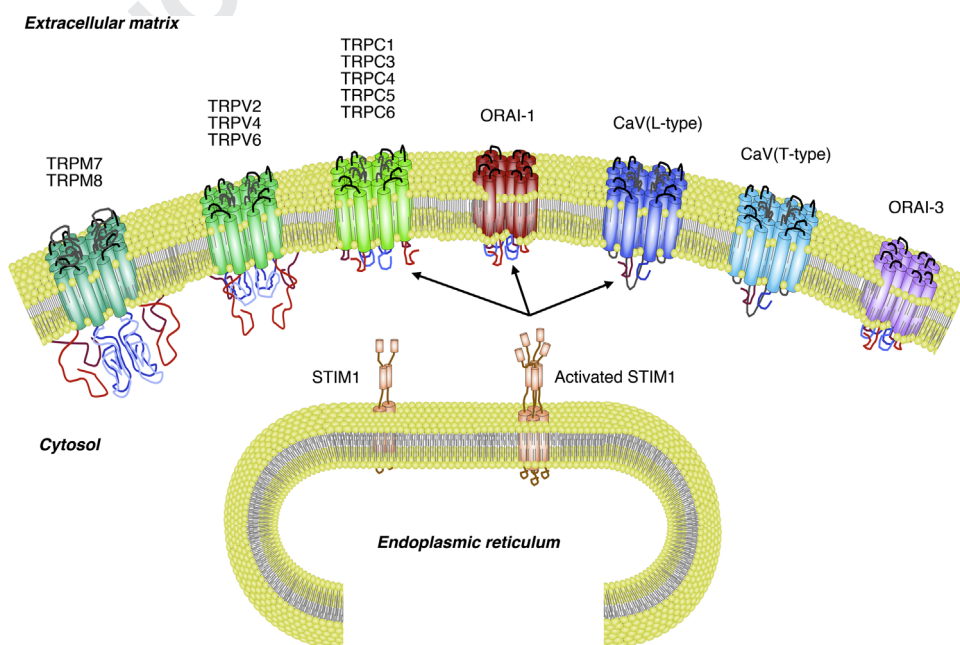


Fig. 1. Illustration of some of the calcium channels playing a role in cell proliferation. Interactions between STIM1 located on ER membrane and L-type, Orai1 and TRPC channels are indicated by the arrows.

recent review (Parekh, 2010). Although none of these drugs are entirely selective to one type of channel, they all emphasize the role of calcium channels in the control of cell proliferation. A considerable effort is now made to discover selective activators and blockers of SOCE and SICE channels of use for therapeutic purposes as these channels play a major in the immune response and cancer development.

The link between calcium channel expression and cell proliferation was emphasized in knock-down experiments. In almost all cases, knocking down transient receptor potential canonical (TRPC), transient receptor potential vanilloid (TRPV), transient receptor potential melastatin (TRPM) or ORAI expression induces decrease in cell proliferation as well as calcium influx in almost all cases, strongly suggesting that reduced calcium entry and cell proliferation are directly linked (Capiod, 2013). However, there are a few exceptions to this above scheme. Reducing TRPC1 and TRPC4 expression decreased cell proliferation with no effect on SOCE or I_{CRAC} activity in endothelial cells (Abdullaev et al., 2008). This is rather unexpected but provides evidence for additional functions for these proteins. TRPV4 behavior is rather different from other TRP channels. TRPV4 activation by synthetic phorbol-derivative, 4 α -phorbol 2,13-didecanoate (4 α PDD), induced an increase in calcium influx and a decrease in proliferation of cholangiocyte in polycystic liver diseases (Gradilone et al., 2010) and human oesophageal epithelial cells (Ueda et al., 2011). The last two sets of data provide arguments against a role of calcium influx in cell proliferation in some particular cases. In addition, knocking down (stromal interacting molecule) STIM1, the ER membrane counterpart for SOCE channels (Cahalan, 2009; Varnai et al., 2009), has no effect on cell proliferation in vascular smooth muscle cells (Li et al., 2008), human myoblasts (Darbellay et al., 2009), HEK293 cells (El Boustany et al., 2010), human umbilical vein endothelial cells (HUVEC) derived cell line EA.hy926 (Antigny et al., 2012) and probably normal HUVEC (Abdullaev et al., 2008). At the same time I_{CRAC} is greatly reduced, which suggested strongly that, in some cells, this current and cell proliferation can be uncoupled. Another exception to the direct relation between calcium influx and proliferation refers to the increase in psoriatic keratinocyte proliferation, coupled to a reduced TRPC6 expression (Leuner et al., 2011). Results with TRPM8 are controversial. TRPM8 is needed for pancreatic adenocarcinoma proliferation (Yee et al., 2010) but TRPM8 over-expression reduced human prostate PC-3 cells proliferation (Yang et al., 2009b).

There are less examples coupling calcium channel overexpression and cell proliferation. Overexpression of ORAI1 alone reduces amplitude of the native I_{CRAC} (Peinelt et al., 2006; Soboloff et al., 2006) resulting in a clear and expected decrease in cell proliferation rate in A549 lung cancer cells (Hou et al., 2011). TRPC6-overexpression by itself increased both SOCE amplitude and cell proliferation rate in human hepatoma cells (El Boustany et al., 2008).

3. Calcium-sensing receptors

The concentrations of $[Ca^{2+}]_{ext}$ are kept at nearly constant levels with the help of a complex homeostatic system, which includes the parathyroid glands and calcitonin-secreting C cells of the thyroid gland, kidneys, bones, and intestines (Brown and MacLeod, 2001). If Ca^{2+} -selective ion channels enable Ca^{2+} to move across the cell membrane resulting in large and rapid increase in cytosolic calcium concentration, it was not clear how some cell types, such as the chief cells of the parathyroid glands, were extremely sensitive to the slightest variations in $[Ca^{2+}]_{ext}$. In 1993, a membrane-spanning, calcium-sensing receptor (CaSR) was cloned from the bovine parathyroid (Brown et al., 1993). This CaSR

belongs to the GPCR family and mainly exists in the form of a dimer (Bai et al., 1998). Cloning of the receptor enabled to reveal that CaSR are expressed in a wide variety of tissues outside the parathyroid gland (Hofer et al., 2004) and it was suggested that CaSR serves as a sensor of local $[Ca^{2+}]_{ext}$ in conditions in which this concentration is likely to fluctuate in situ. CaSR are not particularly sensitive to changes in $[Ca^{2+}]_{ext}$ as half-maximal activation of the human parathyroid receptor does not occur until $[Ca^{2+}]_{ext}$ reaches 3.5 mM (Quinn et al., 1997). However, CaSR is stimulated over a wide range of Ca^{2+} concentrations (threshold around 0.5 mM Ca^{2+} with maximal stimulation at 5.5 mM), and the binding of Ca^{2+} to the CaSR is known to be a highly cooperative process (Hill coefficient >3), making CaSR a good $[Ca^{2+}]_{ext}$ increment detector. The receptor is therefore able to sense small $[Ca^{2+}]_{ext}$ changes (± 0.2 mM) in the physiological range (i.e. around 1.8 mM). Cancer, calcium and cell proliferation are intimately linked and it was obvious that CaSR would play a role here. CaSR increases cell proliferation in a number of cancer cell lines (Brown et al., 1998; Riccardi and Gamba, 1999; Rodland, 2004; Saidak et al., 2009; Sarkar and Kumar, 2012) including prostate (Lin et al., 1998) and breast cells (Cheng et al., 1998).

Another mean for CaSR to control cytosolic Ca^{2+} concentration and cell proliferation is to modify calcium channel expression. It was recently shown that CaSR proliferative effect in breast cancer cell line MCF-7 was due to an up regulation of TRPC1 (Transient Receptor Potential Canonical 1) through the ERK1/2 pathway in a large part (El Hiani et al., 2009a, 2009b). As mentioned before, CaSR are able to detect small changes in external calcium concentration which probably would not dramatically change calcium influx amplitude. SOCE and SICE activation and increase in calcium channel expression could relay and amplify CaSR effects on cell proliferation in several cell types.

Cell proliferation needs to be precisely controlled and should not, of course, start spontaneously whenever an increase of Ca^{2+} concentration is detected in plasma. Cancer cell proliferation is sensitive to external Ca^{2+} while normal cells stay quiescent. It was recently proposed that a switch of G protein coupled to these CaSR could trigger cell proliferation in cancer cells (Mamillapalli et al., 2008). The CaSR expressed on normal mammary epithelial cells inhibited secretion of parathyroid hormone-related protein (PTHrP) and participate in the regulation of calcium and bone metabolism during lactation while in breast cancer cells, the CaSR was linked to PTHrP production enhancement. It was shown that CaSR are coupled to $G\alpha_i$ in normal mammary epithelial cells but coupled to $G\alpha_s$ in cancer cells and breast cancer cell line MCF-7 (Mamillapalli et al., 2008). Thus, the opposing effects of the CaSR on PTHrP production were because of alternate G-protein coupling of the receptor in normal versus transformed breast cells. Because PTHrP contributes to hypercalcemia and bone metastases, switching of G-protein usage by the CaSR may contribute to the pathogenesis of breast cancer. In this manner, PTHrP contributes to increased bone resorption and Ca^{2+} release during osteolysis. Elevated external Ca^{2+} concentration levels, at concentrations of 0.8 or 0.9 mM higher than serum Ca^{2+} concentrations, activate the CaSR, producing additional release of PTHrP from the cancer cells, thus feeding the vicious cycle. This phenomenon occurs in several cancer cell lines including breast, prostate, rat testicular, astrocytomas, and meningiomas (Saidak et al., 2009). This important finding clearly demonstrated how external Ca^{2+} and CaSR can contribute to cancer development.

4. Calcium channels and cell cycle distribution

The need for calcium influx in cell proliferation implies that calcium channel expression is a key point in cell cycle progression.

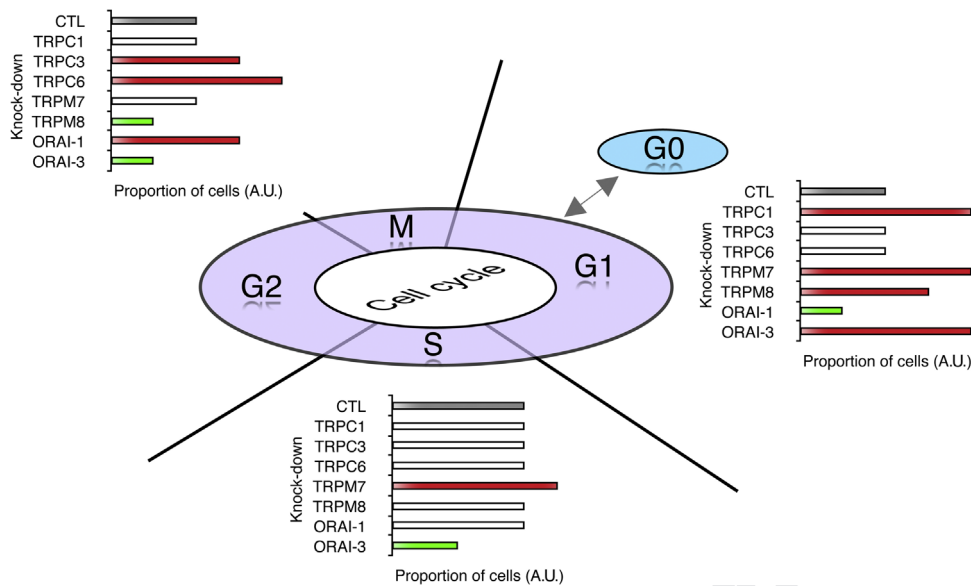


Fig. 2. Decreased calcium channel expression (arbitrary unit AU) and its consequence on cell cycle distribution. The figure summarizes data compiled from literature cited in the Text.

Recording of voltage-gated calcium channels along the cell cycle revealed large variations in L-type and T-type currents (Cribbs, 2006; Kuga et al., 1996) and similar behavior was later observed in SOCE activity (Bodding, 2001; Tani et al., 2007). It was then obvious that a decrease in calcium channel expression would lead to cell cycle arrest and Fig. 2 compiles the data obtained with TRP and ORAI channels (Abdullaev et al., 2008; Cai et al., 2009; Ding et al., 2010a, 2010b; El Boustany et al., 2010; Faouzi et al., 2011, 2013; Ge et al., 2009; Hanano et al., 2004; Hou et al., 2011; Kuang et al., 2011; Shi et al., 2009; Wang et al., 2010; Yang et al., 2009a, 2009b; Yee et al., 2010, 2011).

Knocking down calcium channel expression often has a more dramatic effect on cell proliferation inhibition than on cell cycle blockade. Decreasing ORAI1 and ORAI3 expression resulted in a clearer effect on cell proliferation than on cell cycle arrest. Cell proliferation was much slower, though not totally inhibited, in the absence of both calcium channels. The small but significant rise in cell number in G₂/M phase of the cell cycle observed in the absence of ORAI1 and ORAI3 is probably underlying a bottleneck effect rather than a complete cell cycle arrest. It also suggested that the other cell cycle phases are probably equally affected by calcium channel extinction resulting in the same distribution using flow cytometry analysis.

The blockade in G₂/M observed in ORAI1 and TRPC6 knock-downs is interesting as there is evidence that SOCE inactivates during mitosis (M phase). The first suggestion that Ca²⁺ influx is inhibited during cell division was reported more than 20 years ago in a study of HeLa cells (Volpi and Berlin, 1988), probably through uncoupling of store depletion from SOCE (Preston et al., 1991). This was confirmed recently in HEK293, Cos-7, RBL-2H3 and HeLa cells where SOCE is dramatically reduced during mitosis (El Boustany et al., 2010; Russa et al., 2008; Smyth et al., 2009; Tani et al., 2007). STIM1 phosphorylation (Smyth et al., 2009), decrease in ORAI1 expression (El Boustany et al., 2010) and internalization of ORAI1 channel (Yu et al., 2009, 2010) during mitosis were recently suggested to be the reasons for the observed decrease in SOCE. The significance of this has already been discussed (Arredouani et al., 2010) and involves microtubule-network remodeling (Russa et al., 2008) as perturbation of the actin cytoskeleton resulted in a decrease in SOCE amplitude (Patterson et al., 1999).

5. Additional functions for calcium channels and possible link to cell proliferation

Recent work has demonstrated that many ion channels can directly influence biochemical events in ways that do not directly depend on their function as ion channels (Kaczmarek, 2006). As pointed out by Kaczmarek (2006), although many signaling molecules are known to associate with ion channels, the finding that an integral element of the channel also catalyzes an enzymatic reaction or participates in cell-cell interactions strongly suggests that changes in channel activity could influence these processes. Fragments or subunits of the voltage-gated Ca²⁺ channels probably provide additional functions by controlling Ca²⁺ homeostasis or gene regulation (Barbado et al., 2009; Rousset et al., 2005) or even acting as a transcription factor (Gomez-Ospina et al., 2006). Members of the TRPM subfamily have longer cytoplasmic C-terminal domains (Fig. 1) than other TRP channels and were the first ion channels shown to have enzymatic activity (Huang, 2004). As a result, members of this family were named 'chanzymes' (Montell, 2003). The C-terminus of TRPM2 encodes an ADP-ribose pyrophosphatase domain that binds ADP-ribose and NAD, both of which directly activate the channel (Sano et al., 2001; Zhang et al., 2006). TRPM6 and TRPM7 both contain protein kinases in their C-terminal domains (Schmitz et al., 2005). These kinases are capable of phosphorylating both the channels themselves and other substrates. It is therefore conceivable that new functions for TRPC and ORAI channels could be uncovered in near future and that a direct link between the channel structure and the transcriptional machinery would explain why some cells need the channel more than calcium influx to proliferate. TRPV1 can be directly phosphorylated by Cdk5 and inhibition of Cdk5 activity by roscovitine decreases TRPV1 function and Ca²⁺ influx (Pareek et al., 2007) or L-type Ca_v1.2 activity (Yarotsky and Elmslie, 2007). As a matter of fact, our preliminary results (Borowiec et al., unpublished data) show that HEK293 cell proliferation strictly depends on ORAI1 and ORAI3 expression and is totally independent of external calcium. The mechanisms by which these channels can control cell cycle and division are still unknown. But it is clear that they can interact with more than one single intracellular protein and we are currently investigating which domain of these

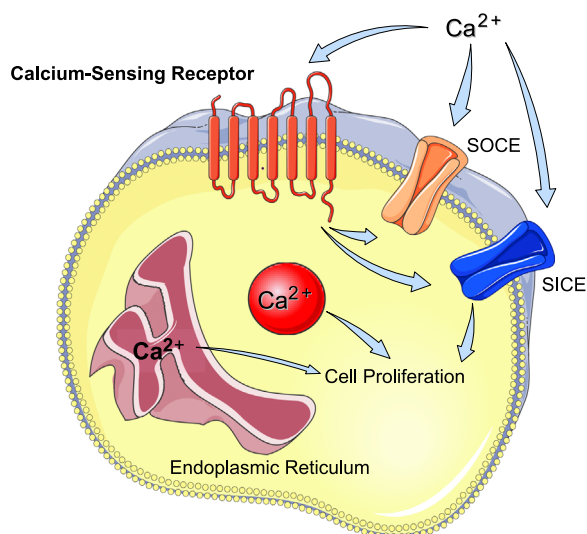


Fig. 3. Balance between calcium-sensing receptors, ER Ca²⁺ contents and calcium influx in the control of cell proliferation. CaSR and calcium channels External calcium induced cellular responses are relayed by CaSR or calcium channels. Calcium influx can activate intracellular kinases and phosphatases directly linked to cell proliferation. CaSR-induced cell proliferation involves second messengers such as 1,4,5 trisphosphate or diacylglycerol, direct activation of SOCE and SICE channels, increase in calcium channel expression at the plasma membrane and release from ER. Intraluminal Ca²⁺ concentration plays a major in controlling cell proliferation rate. Calcium influx is important in maintaining intraluminal Ca²⁺ concentration on the long term and during stimulation of the cells by extracellular agonists including growth factors.

channel-proteins is essential for this unexpected physiological response.

6. Conclusion and perspectives

We previously discussed the role of ER Ca²⁺ contents in cell proliferation (Capiod, 2013). Intraluminal Ca²⁺ concentration eventually decreases during repeated stimulation by extracellular agonists, including growth factors involved in cell proliferation. Fig. 3 illustrates external Ca²⁺ contribution to CaSR activity, activated calcium influx and sustained intraluminal Ca²⁺ concentration. It emphasizes the relative balance of CaSR, calcium channels and ER Ca²⁺ in controlling cell proliferation and cancer cell development.

To conclude, the relationship between calcium influx and cell proliferation should not be taken for granted in all cell types and in all physiological conditions. A better knowledge of the real nature of the calcium channels involved in each cell type should provide information on how calcium entry should control all the downstream events leading to cell proliferation. Establishing new functions for calcium channel in cell proliferation independently of calcium flux, looking for specific blockers and studying the calcium influx during cell cycle in the absence of any kind of chemically-induced block, are probably the next steps needed to clarify the role of calcium influx in cell proliferation and cell cycle progression.

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