

Review

Kca3.1-Related Cellular Signalling Involved in Cancer Proliferation

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Key Words

Cell cycle • Cancer • Ion channel • KCa3.1 • Proliferation • Signaling

Abstract:

Anomalous expression of potassium channels in cancer tissues is associated with several cancer hallmarks that support deregulated proliferation and tumor progression. Ion channels seem to influence cell proliferation; however, the crucial molecular mechanisms involved remain elusive. Some results show how extracellular mitogenic signals modulate ion channel activity through intracellular secondary messengers. It is relevant because we are beginning to understand how potassium channels can affect the proliferative capacity of cells, either in normal mitogen-dependent proliferation or in mitogen-unresponsive proliferation. Calcium-dependent potassium channels have been implicated in cell cycle signaling in many cancerous cell lines. In particular, the so-called intermediate conductance KCa3.1 (IKCa) is reported to play a significant role in uncontrolled cell cycle signaling, among other malignant processes driven by cancer hallmarks. In addition to these features, this channel can be subjected to specific pharmacological regulation, making it a promising cornerstone for understanding the signaling behavior of several types of cancer and as a target for chemotherapeutic approaches. This review is dedicated to the connection of KCa3.1 activity, in canonical and non-canonical ways, to the cell cycle signaling, including the cooperation with calcium channels to generate calcium signals and its role as a mediator of proliferative signals.

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Introduction

Cancer diseases are characterized by genetic instability, resulting in a series of changes in cell physiology called hallmarks [1, 2], that provoke malignant uncontrolled proliferation [3]. Many of those alterations involve the activity of ion channels [4]. Specifically, potassium channels are involved in the modulation of cell cycle progression, leading to uncontrolled

proliferation [5]. Potassium channels are abnormally expressed in several types of cancer tissues and cell lines [6]. They are also involved in controlling the progression of cell cycle, both through permeation-related phenomena and non-canonical permeation-independent mechanisms [5]. Regarding permeation-dependent mechanisms, membrane potential changes seem relevant to determine cell cycle progression. Potassium channels determine membrane potential (V_m) changes through the cell cycle [7, 8]. Non-canonical mechanisms generally involve the interaction of cytoplasmic domains of potassium channels with other proteins to exert some functional roles during the oncological process, independently of their permeating capacity [9, 5]. Potassium channels are relevant in intracellular signaling pathways by any of the two mechanisms mentioned above. K^+ channels are also relevant to cancer because of their interaction with many physiological variables. Some cancer-involved K^+ channels are responsive to transmembrane voltage changes (Kv) [10], while others respond to some signaling molecules and physiological variables such as oxygen tension, pH, and mechanical stretch as the two-pore-domain channels (K2P) [11, 12]. In this sense, calcium concentration sensible K^+ channels (KCa) are involved in cancer signal transduction in more ways than in their interaction with calcium [13], as mentioned below. Remarkably, this kind of channel has been found co-localized with calcium channels and appears to contribute to some of their hallmarks [14]. KCa channels are significant in proliferation-associated signaling because cell cycle progression depends on Ca^{2+} signal changes [15, 16].

All eukaryotic cells require Ca^{2+} signaling for cell proliferation, but some transformed cells and tumor cell lines exhibit a reduced Ca^{2+} dependence. Growing evidence shows that Ca^{2+} signaling pathways are often remodeled or deregulated in cancerous processes to sustain proliferation [17, 18, 19]. KCa channels are significant pieces in the deregulated proliferation via interaction with signaling molecules, as is the case with the big-conductance calcium-activated channel (BKCa) [20], or KCa3.1, which is the subject of this review.

KCa3.1 is involved in cell cycle signaling of various cell types, either through permeating evoked calcium signals or non-canonical ways. The Ca^{2+} -activated K^+ channel KCa3.1, also known as SK4, IK1, and IKCa1, encoded by the KCNN4 gene, has been implicated in physiologically relevant processes related to cancer hallmarks such as abnormal proliferation, metastasis, avoidance of apoptosis, support of angiogenesis and epithelial-to-mesenchymal transition [13]. Besides, this channel is up-regulated and promotes proliferation in some cell lines and their respective cancerous tissues, compared to their adjacent noncancerous surroundings. In particular, KCa3.1 takes part in the control of cell cycle progression in several tissues, such as: human pancreatic cancer cell lines [21], human endometrial cancer [22], human breast cell line MCF-7 [23], breast cancer murine model [24], prostate cancer cells [25], mesenchymal stem cells [26], colorectal [27], and finally liver, where it has also a role in cancer progression [28]. Although this channel is involved in cell cycle control, the complete mechanisms are unknown. This channel is involved in physiological mechanisms of cancerous or normal cells that allow proliferation, such as energy management [29], membrane potential and volume control [30, 31, 32, 33].

This review addresses the elucidation of the intermediary steps between either the expression or activity of this channel and the signals that promote cell proliferation [34, 35, 36, 32, 28, 37]. This channel is also relevant in cancerous progression and as a marker and prognostic tool [38]. The goal of this review is to relate the functioning of this channel within the cell proliferation signals in both non-cancerous and tumoral cells. It addresses the participation of this channel in some mitogen proliferative pathways, including calcium-related signaling and the non-canonical interactions. In particular this review states some details about deregulated proliferating pathways involving KCa3.1 in cancerous cells. The contrasting differences between cancerous and normal signaling involving this channel may aid in the development of anticancer therapies. Before discussing signaling processes, we first address some characteristics of KCa3.1.

Some features of the KCa3.1 and its functional control

KCa3.1 structure and general functional features

KCa3.1 is a homo-tetrameric voltage-independent K⁺ channel with six transmembrane segments, which is constitutively associated with calmodulin (CaM) at the intracellular C-terminal CaM-binding domain, and it has the pore motif between the 5th and 6th segment. The CaM C-lobe binds in Ca²⁺-independent manner to two alpha helices (A and B) at the C-terminal cytoplasmic region, just beside S6 in the C-terminal direction (CAMBD1, K(312)-T(329) or C-L-CaMBD in Fig. 1). During channel gating when [Ca²⁺]_i rises, the CaM N-lobe binds to an N-Lobe CaM binding domain in a nearby subunit of the channel, different from that subunit binding the C-lobe. That N-Lobe CaM binding domain is one of the two helices conforming the S4-S5 linker proximal to S4 (S45A helix: N-L-CaMBD in the Fig. 1). A detail structural description is given by Lee and MacKinnon [39].

This channel has a selectivity for K⁺ more than ten times higher than for Na⁺, and its cooperative sensitivity to [Ca²⁺]_i establishes a Hill relationship with the maximal open probability of 0,44 above 1 μM [Ca²⁺]_p, and the following affinity and Hill coefficient parameters respectively: K_d = 188 nM and n_H = 3.2 [40]. The single conductance of this channel ranges between 20 to 80 pS, hence intermediary (IK) among single-channel conductance of other Ca²⁺ activated K⁺ channels (KCa): e.g., small conductance KCa (SK, 5–20 pS) and big-conductance KCa (BK, 100–300 pS) [41]. In addition to Ca²⁺, other signaling molecules modulate channel gating, which will be discussed in the following section.

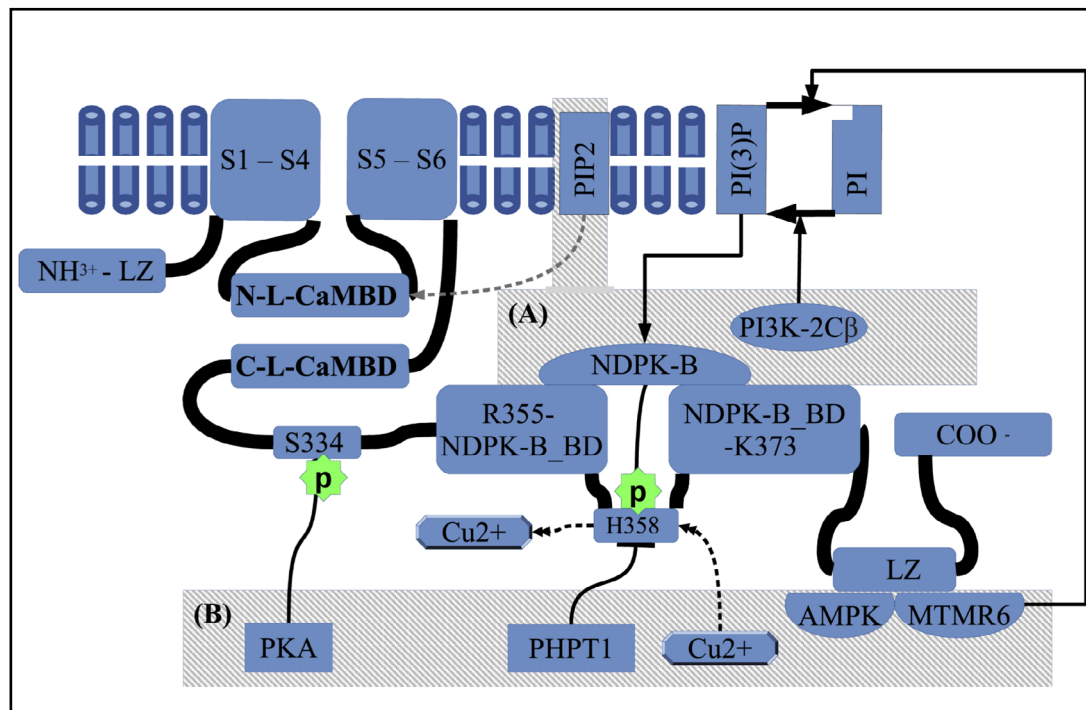


Fig. 1. Regulation of KCa3.1 activity by natural signaling molecules. The general structure of KCa3.1 consist of an intracellular N-terminal domain, a transmembrane segment that contains six transmembrane helices (S1 to S6). The selectivity filter is included between the 5th and the 6th transmembrane helices (THs). Between S4 and S5 there are two linker helices, the closer to S4 is called S₄₅A, which is the N-Lobe CaM binding domain (N-L-CaMBD). Coming from the THs containing segment the C-terminal has one C-Lobe-CaM binding domain (C-L-CaMBD), followed by an intermediate inhibitory phosphorylation site on S334 residue. On the way to C-terminal there is a NDPK-B binding domain (NDPK-B BD), which includes the H358 phosphorylating site, involved in activation. The copper inhibition is mutually exclusive with H358-phosphorylation. Also, the PI(3)P-PI equilibrium modulates the NDPK-B activating action. Finally, closer to the C-terminal there is a LZ domain working as an anchor site for AMPK and MTMR6. The (A) box summarizes the activating kinases and the recently reported of PIP2 activator which acts at the N-Lobe-CaMBD, and the (B) box corresponds to inhibitory factors.

KCa3.1 activity regulation

The positive modulators tend to increment the open state channel probability (PO), while negative ones do the opposite. Some molecules are known to directly regulate the channel activity through phosphorylation of specific sites [42, 43, 38]. Others may include interaction through intermolecular forces [44, 45] or upstream regulation of the direct interacting molecule [46].

KCa3.1 positive modulators

In addition to Ca^{2+} , phosphorylation of the His358 residue (h358) can activate KCa3.1. The nucleoside diphosphate kinase B NDPK-B phosphorylates h358 and releases a coordinated Cu^{2+} that causes channel inhibition [43] (see Fig. 1). NDPK-B binds to KCa3.1 through C-terminal domain (R(355)-M(368)) (Fig.1) and seems to act as a downstream target of phosphatidylinositol-(3)-phosphate (PI(3)-P), generated by the PI3 kinase subclass type II β (PI3K-2C β) [47, 48, 49]. Finally, recently has been reported the phosphatidylinositol 4, 5-bisphosphate (PIP2) as a potent KCa3.1 activator that acts at the interface of the CaM-binding domain [50] (see Fig. 1). ATP also activates KCa3.1 through its C-terminal domain, with residues from Arg(355) to Ala(413) being necessary and sufficient. Direct ATP binding is unlikely as the involved mentioned region lacks consensus ATP binding sites [49]

KCa3.1 negative modulators:

Negative regulation of the channel can be achieved by dephosphorylation of the p-h358 residue, which is operated by the phosphohistidine phosphatase-1 (PHPT1: see Fig. 1) [51] or by dephosphorylation of PI(3)-P by myotubularin-related protein 6 (MTMR6) [52]. In addition, intracellular AMP activates a 5'AMP-activated protein kinase (AMPK) that seems to regulate KCa3.1 through protein-protein interactions [44]. MTMR6 and AMPK have protein-protein interaction in the C-terminal Leucine Zipper motifs (LZ, Fig 1). Finally, the cAMP-activated protein kinase A (PKA) can phosphorylate Ser334(s334), which decreases the open probability of KCa3.1 by reducing CaM binding [42].

KCa3.1 inhibitors and activators

Inhibitor compounds have been tested in model systems and clinical trials for several pathological conditions. They are powerful tools to interact with malignant cell cycle progression and thus tumor growth, cell migration, and apoptosis scape, among other hallmark-related phenomena [13]. Charybdotoxin (ChTx) and maurotoxin (MTx) are high-affinity peptide toxins against KCa3.1. However, MTx has the highest affinity for KCa3.1 while being highly specific below $1\mu\text{M}$ [53]. Among the small synthetic inhibitors, TRAM-34 is an agent with improved properties (1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole), which is a modified triarylmethane pyrazole analog of the clotrimazole molecule with higher affinity [54] although, it inhibits some human isoforms of cytochrome P450 (CYP) [55]. Finally, senicapoc is the highest affinity (IC50: 11 nM) known inhibitor [56], as shown in Table 1. Regarding KCa3.1 activators, there are two non-selective ones: 1-EBIO (1-ethyl-2-benzimidazolinone) [57], DC-EBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one) [58], the highly potent NS-309(EC50: 20 nM) (3-Oxime-6,7-dichloro-1H-indole-2, 3-dione) with very short *in vivo* half-life and low selectivity [59]; SKA-31 which is more selective [60], with relatively high affinity (EC50: 250 nM) [61]; and finally the most selective and potent to date is SKA-121(EC50: 111 nM) [62], as shown in Table 2. Inhibitors and activators are available to interact with KCa3.1, making it possible to dissect its functionality in cancer cells.

KCa3.1 trafficking

The assembly and trafficking of KCa3.1 to the cell surface require CaM binding in addition to gating [63]. This process is inhibited by cAMP kinase (PKA) acting on a single phosphorylation site [46]. The LZ consensus regions, which are composed of repeating

Table 1. Senicapoc has the highest affinity among the synthetic small molecule inhibitors, and ChTx has highest affinity of all (Affinities for Mtx, ChTx, TRAM-34, clotrimazole, extracted from Wei et al., 2005; Senicapoc from Stocker et al., 2003)

Inhibitors		
Small synthetic molecule	Peptides	Affinity/EC50
	Charybdotoxin(ChTx)	5 nM
	Maurotoxin(MTx)	1 nM
Clotrimazole		70 nM
TRAM-34		20 nM
Senicapoc		11 nM

heptads containing leucine at the C(L399/L406) and N(L18/L25) termini are required for proper anterograde trafficking to the plasma membrane [64, 65], in addition to the S4-S5 linker, and Lys197 (K197) necessary for the release from ER [66]. According to Schwab et al [67], retrograde trafficking, which involves endocytosis from the plasma membrane, is facilitated by clathrin and a C-terminal dileucine motif. At the plasma membrane, KCa3.1 is mildly ubiquitylated; after endocytosis, it is polyubiquitylated. Finally, the rate of lysosomal degradation determines deubiquitylation through UPS8 (ubiquitin-specific protease) [68]. The process is accomplished through the Rab7 small-molecular-weight guanine nucleotide-binding protein and the endosomal sorting complexes required for the transport (ESCRT) pathway [69]. Before trafficking to the plasma membrane, the expression of this channel is controlled by molecular mechanisms discussed in the following chapter.

Table 2. SKA-111 combines high affinity with high specificity among all activators (Affinities for activators were obtained: 1-EBIO from Wulff, and Köhler 2013; DC-EBIO from Wei et al., 2005; SKA-31 from Sankaranarayanan et al., 2009; NS-309 from Strøbaek et al., 2004; SKA-121 from Coleman et al., 2014)

Activators	
Small synthetic molecule	Affinity/EC50
1-EBIO	30 μM
DC-EBIO	1 μM
NS-309	10 nM
SKA-31	250 nM
SKA-121	111 nM

Control of KCa3.1 expression in cancerous cells

Basic control of KCa3.1 expression

The transcription of KCa3.1 is regulated by at least two factors: Activator Protein (AP-1) and Repressor Element 1 (RE1)-Silencing Transcription factor (REST) [70]. In T-lymphocytes the continuing cell proliferation and the transcription of KCa3.1 require Activator Protein (AP-1) and Ikaros-2 activity, triggered by the PKC mitogenic pathway [71]. REST binds to the RE-1 site in the KCNN4 gene in vascular smooth muscle cells (VSMCs), repressing its transcription. The decrease in REST expression allows KCa3.1 expression and facilitates

proliferation. That is consistent with the detected KCa3.1 activity in proliferating VSMCs but not in quiescent VSMCs. AP-1 is a potential transcriptional activator that may be coordinated with REST to regulate KCNN4 expression in VSMC [72] (See Fig. 2: F).

Deregulation of KCa3.1 expression

Abnormal expression of K⁺ channels can occur in tumors at various levels, including genomic, transcriptional, post-translational, or epigenetic [73]. In gliomas, e.g., the KCa3.1 potassium channel intervene in several malignancy aspects, including proliferation [74]. In glioblastoma cells, GL-15, the expression of KCa3.1 is regulated by the ERK1/2 pathway, which is often deregulated in cancerous cells [40]. Consistent with this, as found by Park et al [75], in mouse aortic endothelial cells (MAECs), the KCa3.1 expression is promoted by AP-1 via ERK (see Fig 2 A, Label 1) and repressed by REST. KCa3.1 is typically overexpressed in many cancerous cell lines, including human breast cancer cells MDA-MB-231 [76] and MCF-7. Its expression varies as the cell cycle advances, as evidenced by the latter [23]. Even though mechanisms that give rise to altered expression of ion channels are not well understood, it is believed that maintained expression of specific potassium channels in cancerous cells may confer a selective advantage [77, 6].

The epigenetic control of *KCNN4* expression is performed by modification of the pattern in chromatin acetylation or methylation and DNA methylation close to the gene. In the advanced-stage tumors of non-small cell lung cancer, KCa3.1 mRNA over-expression is due to gene promoter hypomethylation, compared to normal tissue [78]. A posterior study in cervical tissue suggests that over-expression results from positive feedback in which Ca²⁺ influx, promoted by KCa3.1-operated hyperpolarization, activates AP-1 and post-translational histone acetylation. Thus, further expression of the channel. Histone acetylation within the promoter enhances transcription factor binding, thereby maintaining an open DNA conformation for the transcription factor to access [79]. Furthermore, histone deacetylase inhibitors (HDACis) down-regulates KCa3.1 transcription in breast (YMB-1) and prostate (PC3) cancer cell lines [80]. In addition, other up-regulation mechanisms have been uncovered: e.g., in neointimal hyperplasia produced by proliferating vascular cells, the KCa3.1 *de novo* transcription is promoted through the reduced expression of REST acting on RE1 sequence in *KCNN4* [82]. REST plays different roles in normal and cancerous proliferation depending on cell type considered. That is to say, in the nervous system and its tumors, REST acts as an oncogene, whereas it acts as a tumor suppressor in some carcinomas of breast, lung, and colon [83, 84]. Another feature is that in HaCaT keratinocytes and C6 glioma cells, the expression of the human KCa3.1 channel seems to be regulated by a feedback mechanism since after three days of treatment with 1-EBIO, mRNA levels and activity of human KCa3.1 were substantially diminished, accompanied by loss of mitogenic activity and an increase in cell size, all in a reversible way [85]. Intracellular signals may determine the expression of this channel and its feedback mechanisms of expression. Understanding those processes may lead someday to the regulation of cancer proliferation. In particular, studying the interaction of this channel within the signaling network may improve the understanding of its proliferative role in both cancer and normal cells.

Control of proliferation by extracellular signals through expression and/or activity of KCa3.1

Depending on the cell line, KCa3.1 expression has different influences upon proliferation. For instance, *in vitro* assays show that over-expression or activation of this channel increases proliferation in human prostate cancer cell lines [25], whereas it seems innocuous for proliferation in human breast cancer cell line MDA-MB-231. Moreover, KCa3.1 activation decreased proliferation in non-tumorigenic immortalized breast cell line MCF-10A, even in control or KCa3.1 over-expressing cells, but not by KCa3.1 over-expression alone. Surprisingly, KCa3.1 over-expression increased the primary tumor growth of MDA-MB-231

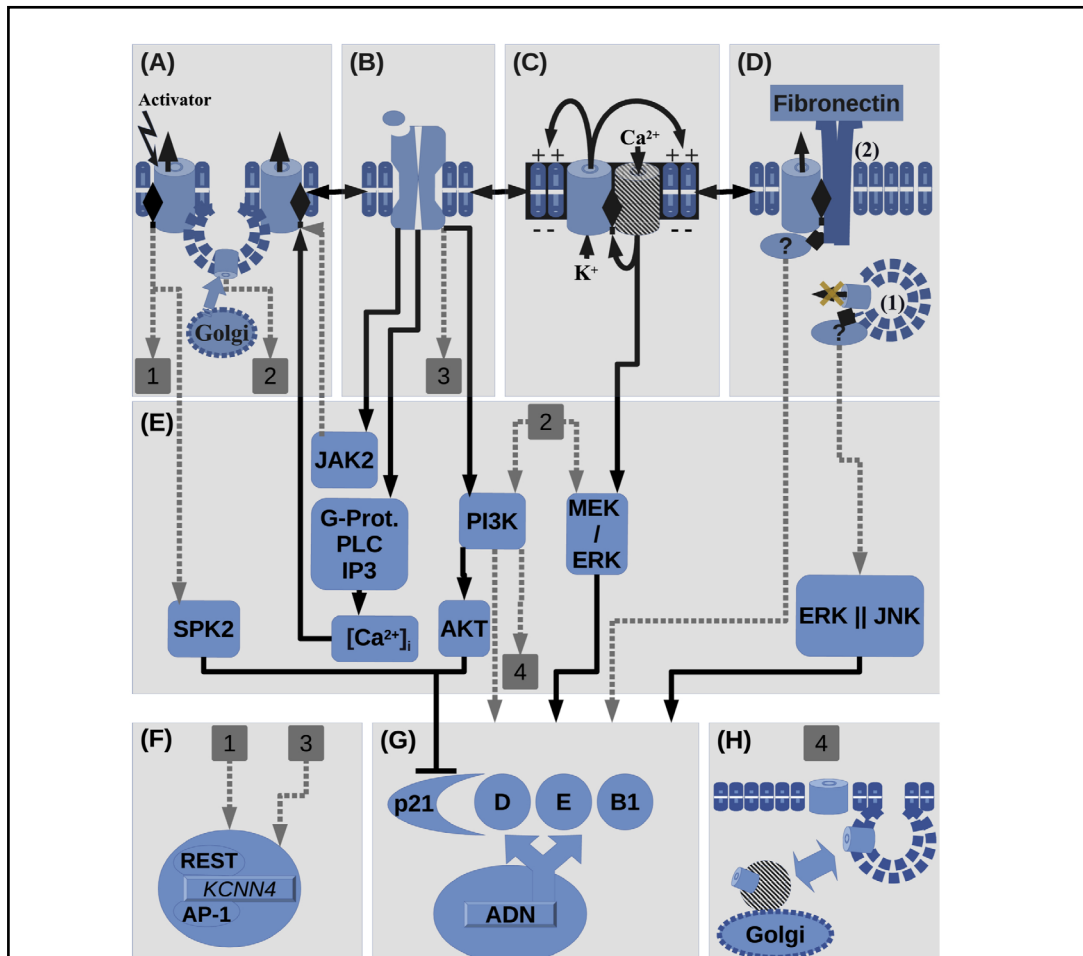


Fig. 2. Summary of interactions involving KCa3.1 in proliferation. (A) Activation (e.g. 1-EBIO) of KCa3.1 and stimulation of its surface expression, by e.g. interaction with plasma membrane (PM) receptors (PMR, shown in B). KCa3.1 activation site is represented in the channel cartoon as a lateral diamond symbol. KCa3.1 can be activated by Ca^{2+} , JAK2 and its regulating kinases (shown in Fig. 1). KCa3.1 activation can promote its transcription (Shown by square label 1) or SKP2 activation, which promotes cell cycle by inhibiting p21. Also KCa3.1 surface up-regulation/expression, which can be regulated by receptors in B, can promote proliferation through PI3K/Akt and MEK/ERK pathways (Shown by square label 2: which corresponds to label 1 in F). (B) Histamine/Hormone/Growth factor Receptors (PRL, GFRs: IGF-1, EGF, TGF- β , HGF) can regulate activity of A or C (cooperating channels). Vice versa relation among A, B and C, is also possible. Mitogens can up-regulate KCa3.1 transcription (in the case of TGF- β , shown by square label 3) or activate KCa3.1 (in the case of PRL) through JAK2, which also promotes proliferation (omitted pathway because of its indetermination and graphical overload). Also PMR can regulate KCa3.1 trafficking through IP3K pathway (shown by square label 4). (C) Positive feedback between KCa3.1 and Ca^{2+} channels (CaC). KCa3.1 drives membrane potential (V_m) while CaC drives increments in $[\text{Ca}^{2+}]_i$, including SOCE. CaC can be: TRPC, TRPV, Orai, or CD20. This phenomenon may interact with B (interaction with PMR) and D (non-canonical functions). Ca^{2+} entry can regulate ERK phosphorylation. (D) Non-canonical functions: in case (1), KCa3.1 expression seems to promote proliferation while in traffic to the PM, through ERK and JNK pathways in parallel. In the case (2), interaction between KCa3.1 and β -1 integrins in presence of fibronectin promotes proliferation. As TRPC1 expression is also stimulated by interaction with fibronectin the cooperation with KCa3.1 is possible. KCa3.1, TRPC1 and integrin expression is also promoted in presence of fibronectin. (E) Summary of intracellular signaling molecules that involve KCa3.1 in proliferation. (F) KCa3.1 gene expression can be regulated by undetermined pathways involving PMR and KCa3.1 activation at the membrane. (G) Expression and activity of cyclins or its CDKIs. As far as can be pointed on this review KCa3.1 can stimulate cell cycle advancement by MEK/ERK, and by SPK and PI3K/AKT pathways through p21 inhibition. (H) KCa3.1 expression and anterograde/retrograde traffic can be regulated through signaling pathways, in particular PI3K.

in orthotopic xenografts. In breast cancer cells, evidence suggests that microenvironmental factors influence KCa3.1 activation and its signaling pathways, which may be significant for extracellular signal-dependent proliferation [76]. Different proliferative influences of KCa3.1 should correspond to the particular signaling network of each cell line and the environmental differences, which might be related to extracellular factors: e.g., in breast cancer murine models, this channel is involved in mitogenic-dependent cell cycle promotion, since serum-containing growth factors evoked Ca^{2+} signals and the transition to S phase was suppressed by pharmacological blockade or genetic ablation of KCa3.1 [24]. Old data also suggest that KCa channels are involved in the response of breast cancer cells to extracellular factors such as EGF (Fig. 2, A-B) and insulin [86, 87].

KCa3.1 channel activity can increase by upstream changes, such as the activation by hormones/growth factor receptors (Fig. 2, A-B) [74]. This channel plays roles at different levels within the mitogen-activated signaling chain. In breast cancer cells, Ca^{2+} -activated K^+ channels may play their role in cell cycle physiology through the mitogen-initiated electrical signals. EGF treatment of mouse mammary cells induced transient hyperpolarizations of the membrane potential mediated by KCa channels [86]. Since the last decade of the 20th century, evidence shows that in the estrogen receptor-positive (ER+) breast cancer cell line MCF-7, a channel with all the characteristics of KCa3.1 is upregulated during its proliferative phase compared to cells in the plateau phase or tamoxifen-treated cells. However, not all cells rely on this channel to proliferate, as it is absent in some regularly proliferative cell batches [88]. There are alternative pathways for proliferation, and the KCa3.1 channel may play a role in at least one of these signaling pathways mediating proliferation.

Besides, in the same cell line insulin-like growth factor 1 (IGF-1) mitogenic effect, mediated by Akt signaling pathway, depends on the Kv10.1 voltage-dependent K^+ channel [89]. More recently, Kv10.1 and KCa3.1 have been involved in MCF-7 basal proliferation but surprisingly not in estrogen-stimulated proliferation [90]. As MCF-7 cells express both estrogen receptors, ER-alpha and ER-beta [91], proliferation is much faster when treated with estrogens [92], but at least this mitogen's associated signaling does not seem to involve those channels. Interestingly, in this cell line (MCF-7), TRAM-34, a supposedly specific KCa3.1 blocker, has been reported as a novel non-steroidal ER agonist [90]. It remains to see whether there are any K^+ channels involved in MCF-7 estrogen-triggered proliferation. Regarding the proliferative role, KCa3.1 mRNA expression in tumor cells is significantly correlated, on one side by the absence of estrogen receptor (ER-)/progesterone receptor (PR) status and the P53 abnormal gene on the other side. Those features are unfavorable breast cancer parameters [93]. On the other side, another well-known mitogen in breast cancer cells is prolactin (PRL), which induces proliferation by increasing KCa3.1 activity through the Janus Kinase 2 (JAK2) signaling pathway (See Fig 2. A-E-B). Human KCa3.1 channels constitute a target of PRL, leading to breast cancer cell proliferation [34]. The JAK2/STAT5 pathway is involved in MCF-7 cell proliferation by activating the cyclin D1 gene promoter [94]. Multiple studies indicate that PRL, which could be within circulating plasma or expressed locally by mammary tissue, among others, promotes proliferation and metastasis in various breast cancer models, *in vitro* and animal models [95]. Specifically in non-PRL expressing MCF-7 derived cells, the PRL receptor (PRL-R) stimulates the cell cycle by increasing cyclin D1 and B1 expression and facilitates the G1/S transition by decreasing the p21(WAF1) levels [96]. The relationship of KCa3.1 to these cell cycle regulators is unknown in breast cancer cells but has been reported in other cell types, as discussed in Section 5.

Growth factor-induced proliferation also appears to be associated with KCa3.1 expression. For instance, in respiratory smooth muscle, KCa3.1 channel upregulation is mitogen stimulated: in human airway smooth muscle (HASM) cells by transforming growth factor-beta (TGF- β) and in human bronchial smooth muscle (BSM) by platelet-derived growth factor (PDGF), as evidenced by increases in mRNA, protein expression and activity [97, 98]. Furthermore, the expression of KCa3.1 also switches BSM cells from a contractile to a proliferative phenotype through activation of ERK and PI3K/Akt pathways (Fig. 2, A-E, Label 2) [98]. In lung carcinoma, KCa3.1 expression promotes lung adenocarcinoma cell

proliferation through the PI3K/Akt and MEK/ERK signaling pathways (Fig. 2, A-E, Label 2) [37]. These pathways are also active in non-small cell lung carcinoma cell lines (NSCLC) and determine EGF-dependent proliferation [99].

In Daudi lymphoma B cells, the intermediate conductance KCa channel was upregulated on the cell surface during cell cycle progression in response to fetal bovine serum (FBS) via a phosphatidylinositol 3-kinase (PI3K)-dependent pathway, possibly through the IGF-1 receptor, as it is involved in PI3K-associated pathways. The trafficking of this channel can be mediated by PI3K, as has been shown for other channels (Fig. 2, B-E, Label 4) [100]. PI3K controls intracellular vesicular traffic, specifically class II and class III regulate membrane traffic in endosomal recycling (Fig. 2, pathway E-H, Label 4) [101]. Besides, in human gastric adenocarcinoma cell line SC-M1 cells, Ca^{2+} activated and voltage-independent K^+ current, and therefore possibly carried by KCa3.1, is involved in hepatocyte growth factor (HGF)-induced cell proliferation, even though it did not correlate with the density of HGF receptors [102]. Finally, another external factor is histamine, which has a crucial role in cancer progression, stimulating cancer hallmarks in cell lines derived from breast, pancreas, and hepatoma [103], particularly modulating the proliferation and migration of glial cell lines derived from brain tumors [104, 105]. Histamine selectively activates the KCa3.1 channel in human glioblastoma GL-15 cells by histamine receptor (H1) activation and G protein/PLC/IP3/[Ca]ⁱ pathway (Fig. 2, B-E-A). The high Ca^{2+} affinity of KCa3.1 confers its selectivity, although there are other Ca^{2+} -sensitive channels [106]. As mentioned above, the evidence points to KCa3.1 as a participant in the proliferative signaling pathways that depend on diffusible mitogenic factors. The following section discusses the proliferative processes that regulate the cell cycle through intracellular signaling involving KCa3.1.

KCa3.1 regulates some kinase proteins within cell cycle signaling

The cell cycle course is controlled by cyclin proteins through its Cyclin-Dependent Kinases (CDKs) [107] and also by Cyclin-Dependent Kinase Inhibitors (CDKIs) [108]. In particular, cyclins D1 and E allow getting through the G1 and G1/S transition. In human normal BSM and murine mesenchymal stem cells (MSCs), KCa3.1 activation promotes cyclin D1 expression [109; 98]. Moreover, blocking KCa3.1 expression or activity in cancerous cells, such as angiosarcoma cell line ISO-HAS and human endometrial carcinoma (EC) cells HEC-1-A and Ishikawa, reduce expression levels of cyclin D1 and in the two last ones also cyclin E decreases [22, 110].

In human prostate cancer cells (PCa), KCa3.1 channel inhibition leads to an accumulation of p21^{Cip1} CDKI [111], a cell cycle regulator involved in cell cycle arrest in response to stressful stimuli, transcriptional regulation, modulation, or inhibition of apoptosis and differentiation [112, 113]. A recent report on hepatocellular carcinoma (HCC) cells suggests that KCa3.1 promotes cell cycle progression through post-transcriptional regulation of S-phase protein kinase 2 (SKP2) expression, which subsequently promotes p21/p27 ubiquitin-mediated degradation (Fig. 2, pathway A-E-G) [28]. KCa3.1 may participate in cell-cycle control by positively regulating G1 cyclins and negatively regulating CDKIs. Besides, the PRL pathway in breast cells interacts with D1 cyclin and KCa3.1, as seen above. It remains to know what is the causal sequence and whether it is the same as in other cell lines, such as HCC. Finally, in glioblastoma tumor cells, KCa3.1 regulates the passage through the G2/M checkpoint via the cdc25C (cell division cycle 25C) phosphatase activity [32]. This phosphatase triggers entry into the M(mitosis) cell cycle phase by dephosphorylating the cyclin B-Cdk1 [114]. The relationship between the KCa3.1 channel and the phosphorylation dynamics that control the cell cycle is beginning to be elucidated. Similarly, the electrophysiological effects on the cell cycle are also evident, as shown below.

Nonspecific effects of KCa3.1 inhibitors upon proliferative capacity

Some cancer cells express the KCa3.1 channel, which is not involved in proliferation, although KCa3.1 inhibitors affect proliferation. In glioblastoma cell lines (U251 and U67) and primary glioma cultures, where KCa3.1 was found functionally expressed, it is not involved in proliferation, as demonstrated by knocking down this channel with siRNA. Nevertheless, using KCa3.1 inhibitors (clotrimazole and TRAM-34), an antiproliferative effect was obtained, but only at concentrations higher than those necessary to inhibit the channel. Therefore, the authors concluded that the effect on proliferation is due to off-target actions [115]. Coincidentally, in human IGR1 melanoma cells expressing KCa3.1, the proliferation was not affected by channel inhibition [116]. Other studies on melanoma and pancreatic cancer cell lines also suggest that inhibitors of this channel (clotrimazole or senicapoc) may act on targets outside the plasma membrane or perhaps block IKCa3.1 channels in intracellular organelles [117]. As a matter of fact, TRAM-34 has been reported as an agonist of ER nuclear receptor, in the breast cancer cell line MCF-7 [90].

In addition to the plasma membrane, functional expression of mitochondrial KCa3.1 (mtKCa3.1) also has been identified on the inner mitochondrial membrane of several cancer cell lines: non-small cell lung cancer (NSCLC) cells [118], pancreatic ductal adenocarcinoma (PDAC) cells [119], colon cancer cells [120], melanoma cells [121] and cervical cancer cells. Although there is some evidence that mtKCa3.1 may be involved in proliferation, it is inconclusive [122] and cannot exclude the off-target effect [117]. Targeting this channel with novel mitochondria-targeting TRAM-34 derivatives reduced tumor growth and metastasis in orthotopic melanoma and pancreatic ductal adenocarcinoma models [123]. In pancreatic ductal adenocarcinoma cell lines, there is evidence that mtKCa3.1 is involved in mitochondrial respiration and proliferation. However, the authors do not exclude that the effect on proliferation could be due to plasma membrane-expressing channels or off-target effects of the KCa3.1 channel inhibitors. This mitochondrial expression of the channel, in contrast to normal cells, may be another reason to address the therapy using this target. Nevertheless, it is unclear whether the proliferation of pancreatic cancer cells depends on mtKCa3.1 [119]. Instead, the mtKCa3.1 channel seems to play a role in the response of melanoma cells to apoptotic stimuli [124]. In melanoma cells (WM266-4) and pancreatic cells (Panc-1), clotrimazole and senicapoc decreased viability equally, even though the former expresses the KCa3.1 channel depending on the cell cycle phase and the latter poorly expresses it. The reduction in viability by the KCa3.1 inhibitor was not a direct result of its blockade at the plasma membrane. Therefore, effects through the channel expression/activity on intracellular membranes cannot be excluded. The use of the KCa3.1 inhibitor (BA6b9) did not affect the viability of Panc-1 cells, even at high concentrations. However, it had slight viability effects on WM266-4 cells, indicating potential secondary targets for the former KCa3.1 inhibitors or effects that depend on the expression of the channel at intracellular organelles. It is important to note that this mechanism strongly depends on the chemical nature of the drug [117]. The evidence linking the mtKCa3.1 channel to proliferation is limited and inconclusive.

KCa3.1 inhibitors affect proliferative capacity on irradiated glioma cells

Plasmalemma KCa3.1 regulates the cell cycle in irradiated tumor cells. In human glioblastoma (GB) cell lines (T98G, U87MG), KCa3.1 is upregulated in the plasma membrane and also in glioma during transformation and malignant progression, in contrast to its low expression or absence in astrocytes, a type of glia in the central nervous system. In these GB cell lines, ionizing radiation activates KCa3.1, which increases $[Ca^{+2}]_i$ and induces G2/M arrest. Presumably, the increase in $[Ca^{+2}]_i$ is involved in this activation. Furthermore, pharmacological inhibition of KCa3.1 (clotrimazole or TRAM-34) or its mRNA knockdown in irradiated cells reduced IR-induced G2/M arrest, DNA repair, and clonogenic survival. In addition, inhibition of these channels also radiosensitized GB cells grown ectopically

in mice during radiotherapy. The authors conclude that the KCa3.1-mediated response is required for the survival of irradiated cells [125]. The radiogenic activity of KCa3.1 has been reported in other cancer cell lines [126]. Pharmacological targeting of KCa3.1 interferes with cell cycle control, involving CamKII, a long-acting signaling mediator. CamKII inhibits cdc25 phosphatase, which prevents the activation of phosphorylated cdc2. Instead, the cyclin B-cdc2 complex, when activated, arrests the cell cycle in G2/M [127]. As TRAM-34 had a negligible effect on the cell cycle of unirradiated T98G cells, the above results are in agreement with Abdullaev et al. (2010). The presence and functional significance of KCa3.1 on gliomas, regarding proliferation as well as migration and invasion is also reviewed by Elias et al. [128].

KCa3.1 contribution to membrane potential changes and to cell cycle signaling by promoting Ca²⁺ influx

During the cell cycle advance, the membrane potential is driven by the activity of potassium channels [129]. More negative V_m or hyperpolarization is necessary to pass through the G1/S transition, while less negative V_m precedes G2/M advance, and hence depolarization is necessary for mitosis entry [130, 5, 131]. Progression through checkpoints of the cell cycle is associated also with changes in potassium channel activity and V_m [9]. Regulation of Ca²⁺ ion flux is one of the V_m effects upon cellular signaling, as part of the mechanism influencing the cell cycle. Studies with cancerous cells show that the activity of KCa3.1 promotes Ca²⁺ influx by hyperpolarizing the cell membrane, which increases the driving force for Ca²⁺ influx (Fig. 2, C), promoting proliferation [23, 111, 132]. Intracellular Ca²⁺ regulation allows appropriate cell cycle progression. In particular, nucleoplasmic Ca²⁺ regulates cell proliferation by activation of tyrosine kinase receptors (RTKs; Fig. 2, C-B), with subsequent translocation to the nucleus, where they are part of nuclear Ca²⁺ signaling. Finally, nuclear Ca²⁺ regulates the expression of genes involved in cell proliferation [133]. Cancer cells have different ways of regulating calcium signaling to proliferate and survive than normal cells [134]. Some transformed cells may proliferate at external Ca²⁺ concentrations as low as tens of micromolar [135], whereas normal cell proliferation appears to be more dependent on calcium influx from the external environment [136]. Nevertheless, some plasma membrane calcium channels are overexpressed or more active in cancer cells [137].

Cell cycle phases are characterized by Ca²⁺ transients activating multiple Ca²⁺ effectors downstream of the initial Ca²⁺ signal [138, 19]. Among those effectors that dynamically regulate Ca²⁺ entry are the Ca²⁺-sensitive potassium channels (KCa), which drive intracellular Ca²⁺ levels [16]. In various cell types, fluctuations in intracellular calcium concentration [Ca²⁺]_i determine cell cycle progression during the early G1 phase, G1/S, and G2/M transitions [138, 137, 19]. During the G1/S transition, hyperpolarization can increment the driving force for calcium entry through cooperation between potassium and calcium channels (Fig. 2, C). For example, KCa3.1 up-regulation in colorectal cancer cells HCT116 contributes with an electrochemical driving force to calcium entry mechanisms [27]. Calcium entry is recognized to be mediated either by Store Operated Calcium Entry (SOCE) or Store Independent Calcium Entry (SICE) in breast cancer cells [139], as well as in other cancer cells, in which calcium channels change their expression in plasma membrane along the cell cycle. Nevertheless, in transformed or cancer cells the causal relationship between proliferation and calcium entry is not generalized in all cell types and physiological conditions. Possible new functions for calcium channels besides calcium influx remain to elucidate: p.e. enzymatic activity [140]. Likewise, non-conducting functions promoting cell proliferation are also found in potassium channels, either voltage-dependent Kv1.3 or calcium-dependent KCa3.1 [141, 142]. Calcium-activated channels also appear to form complexes with Ca²⁺ channels. Thus, they functionally cooperate [16], as will be discussed here.

KCa3.1 promotes proliferation by its cooperation with Ca²⁺ channels

Ca²⁺ influx is essential for MCF-7 cell proliferation since it can be suppressed by a decrease in the extracellular Ca²⁺ concentration [Ca²⁺]_o [143]. In non-excitabile cells, KCa and Ca²⁺ channels make a low-energy cost-positive feedback loop in Ca²⁺ regulation. KCa- Ca²⁺ channel complexes found in cancer cells contribute to cancer-associated functions such as cell proliferation, among other hallmark-related phenomena, allowing a fine regulation of [Ca²⁺]_i [16]. Given the relatively high affinity of the channel (K_{d(Ca)}: 188 nM) [40] and the fact that the basal [Ca²⁺]_i is close to 100 nM [144], a change carrying a new level of up to 200 nM may well activate the channel by more than half of its operating capacity. For the reasons above and the small diffusion distances of the cell, this proximity is not necessary to activate the channel. This feature may reflect the importance of fine local regulation of the channel and the [Ca²⁺]_i signal for cellular processes, e.g. proliferation.

Co-expression of KCa3.1 and the SOCE participant channel Orai1 are detected nearby in microglia [145] or evidenced by co-immunoprecipitation upon overexpression in HEK 293 cells [146]. Correspondingly, SOCE is important for normal or pathological cellular processes, also regulated by KCa3.1 activity, as in Colorectal cancer (CRC) [27] and neutrophils [147]. For instance, proliferation is promoted by KCa-activation including KCa3.1, which cooperates to sustain Ca²⁺ entry mainly through SOCE in normal cells such as T-Cells [148], cardiac progenitor cells (eCPCs), bone marrow (BM)-derived MSCs [149], and in a cancerous chondrocyte cell line (OUMS-27), in response to histamine release (Fig. 2, interaction between A, B and C) [150]. The cooperation between KCa3.1-mediated hyperpolarization and TRP channels produces the proliferating signal, e.g., in MCF-7, TRPC1 provides Ca²⁺ entry, which regulates cell proliferation via ERK1/2 phosphorylation (Fig. 2, pathway C-E-G) [151], cooperating functionally with KCa3.1 and regulating cell cycle progression in G1 phase and G1/S transition. Expression of both channels increases at the end of the G1 phase, and silencing of either KCa3.1 or TRPC1 induces G0/G1 cell cycle arrest [93]. In lung carcinoma NSCLC cell line, the TRPC1 participates in G1/S transition by mediating Ca²⁺ entry, which is crucial to EGFR signaling regulation involving cyclin D1 and D3 expression, as well as MAPK downstream pathways [99]. In this case, further study is needed to know whether KCa3.1 cooperates with TRPC1. However, the presence of these two channels in those lung tissue cancers is striking. On the other hand, in prostate cancer cell line LNCaP there is evidence of cooperation and co-expression with the TRPV6 channel. This channel has been proposed as a relevant means of passive Ca²⁺ influx in response to hyperpolarization associated with KCa3.1 activity. Besides, immunoprecipitation experiments indicated a close physical interaction between the KCa3.1 and TRPV6 in these cells, and that KCa3.1 channels are up-regulated in prostate cancer (PCa) cells vs non-PCa tissues [111], suggesting specific cancer calcium signaling alterations that promote Ca²⁺ entry through TRPV6. Also, TRPV4 regulates proliferation by co-activation with KCa3.1, in the following tissues: human melanoma cell lines [152], reactive astroglia, a pathologic cellular condition characterized by deregulated proliferation of astrocytes [153], and a proliferative phenotype of human bronchial smooth muscle (HBSM) [154]. Finally, co-activation was also found in Daudi lymphoma cells, where the intermediate conductance KCa activity depends on Ca²⁺ entry through a Ca²⁺-permeable channel CD20. CD20 channel activation and KCa3.1 expression were promoted by a serum factor, which could be IGF-1, as the authors suggested [100]. Then, a positive feedback mechanism is common among cancer cells through the functional cooperation between KCa and Ca²⁺ channels. There is much evidence of its close localization and physical interaction [155, 16, 93], although its proximity is not necessary to activate the channel due to its relatively high affinity. Moreover, another crucial physical intermolecular association in proliferative processes involving KCa3.1 is the non-canonical functionality, discussed below.

Non-canonical role of KCa3.1 channel involved in cell cycle signaling

Recombinant human KCa3.1 channels induce cell proliferation in human embryonic kidney 293 cells (HEK293), independently of its ion-conducting capabilities or derived consequences. The increased proliferative effect of nonconducting pore mutant KCa3.1(GYG/AAA) in those cells was similar to that of the wild KCa3.1 version. Moreover, the channel does not need to be located at the plasma membrane (PM) in similar quantities as in the wild KCa3.1-expressing cells to keep its proliferative function, as the trafficking mutant expressing KCa3.1(L18A/L25A) mostly outside of PM also increases proliferation. This flux-independent proliferative influence may occur through signaling ERK1/2 and JNK pathways (Fig. 2, pathway: D(1)-E-G) [141].

Aside from soluble extracellular factors, there is also proliferative information coming from the stiffness of the extracellular matrix (EM) [156], which activates ERK and PI3K pathways [157]. The cross-talking between growth factor receptors and EM adhesive receptors, such as integrins, in the plasma membrane activates proliferative pathways, migration, and survival in cancer cells. The expression of integrins is also upregulated in tumor cells [158, 159]. In primary cultured alveolar cells, KCa3.1 and β -1 integrin interact physically and cooperate to regulate proliferation in the presence of fibronectin, an EM structural component (Fig. 2, pathway: D(2)-E-G). Besides KCa3.1, TRPC4 and β -1 integrin plasma membrane expression were stimulated by fibronectin during a wound-healing assay. KCa3.1 and TRPC4 channels have complementary roles in modulating the interaction of β -1 integrin with fibronectin during lung epithelial regeneration [Girault et al., 2015]. Although regeneration in the aforementioned epithelium is not a pathological process, this cooperation between channels involved in proliferating signals resembles the cases of cancer cell lines in the previous sections. Interestingly, adhesion to fibronectin, mediated by β 1-integrin, also stimulates the formation of a macromolecular complex, also with Kv11.1(hERG1), and the ensuing current activation of the ion channel, in transfected cell lines from neuroblastoma (SH-SY5Y) and human embryonic kidney (293) [160]. Further studies on structure, function, up-regulation, and interaction between these ion channels and integrins may lead to some clues about the modulation of proliferative signaling networks, among other features relating to cancer hallmarks, and how these molecular interactions respond to extracellular signals; either diffusible, matrix related or a combination of both.

Conclusion

The KCa3.1 channel's electrophysiological or Vm-dependent and non-canonical capabilities are crucial in determining proliferation in many mitogen-dependent types of cancer. Maybe the relevance of each mechanism for proliferation depends upon the kind of cell expressing the channel and the environment to which the cell is exposed. KCa3.1 may involve basal or mitogen-regulated proliferation through Vm-dependent, non-canonical mechanisms, or both. Studies analyzing the cellular signaling pathway determining cancerous proliferation mechanisms may help to improve therapy approaches.

It is also important to identify the unspecific targets of some KCa3.1 inhibitors. In addition to its apoptotic function, mtKCa3.1 also plays a significant role in cellular energetics. Therefore, the use of membrane-permeable KCa3.1 inhibitors may have adjuvant effects in some cells by acting on both the plasma and mitochondrial membranes. Targeting KCa3.1 is a promising avenue for future cancer therapies, as it may impact more cancer hallmarks than just the related to proliferation.

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