Biochimica et Biophysica Acta 1864 (2017) 843–849

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Review

Calcium remodeling in colorectal cancer☆

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ARTICLE INFO

Article history:
Received 31 October 2016
Received in revised form 5 January 2017
Accepted 7 January 2017
Available online 10 January 2017

Keywords:
Colorectal cancer
Store-operated Ca^{2+} entry
Orai1
TRPC1
STIM1
STIM2
Aspirin
Non-steroidal anti-inflammatory drugs

ABSTRACT

Colorectal cancer (CRC) is the third most frequent form of cancer and the fourth leading cause of cancer-related death in the world. Basic and clinical data indicate that aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) may prevent colon cancer but mechanisms remain unknown. Aspirin metabolite salicylate and other NSAIDs may inhibit tumor cell growth acting on store-operated Ca^{2+} entry (SOCE), suggesting an important role for this pathway in CRC. Consistently, SOCE is emerging as a novel player in different forms of cancer, including CRC. SOCE and store-operated currents (SOCs) are dramatically enhanced in CRC while Ca^{2+} stores are partially empty in CRC cells. These features may contribute to CRC hallmarks including enhanced cell proliferation, migration, invasion and survival. At the molecular level, enhanced SOCE and depleted stores are mediated by overexpression of Orai1, Stromal interaction protein 1 (STIM1) and Transient receptor protein channel 1 (TRPC1) and downregulation of STIM2. In normal colonic cells, SOCE is mediated by Ca^{2+}-release activated Ca^{2+} channels made of STIM1, STIM2 and Orai1. In CRC cells, SOCE is mediated by different store-operated currents (SOCs) driven by STIM1, Orai1 and TRPC1. Loss of STIM2 contributes to depletion of Ca^{2+} stores and enhanced resistance to cell death in CRC cells. Thus, SOCE is a novel key player in CRC and inhibition by salicylate and other NSAIDs may contribute to explain chemoprevention activity.

Summary: Colorectal cancer (CRC) is the third most frequent form of cancer worldwide. Recent evidence suggests that intracellular Ca^{2+} remodeling may contribute to cancer hallmarks. In addition, aspirin and other NSAIDs might prevent CRC acting on remodeled Ca^{2+} entry pathways. In this review, we will briefly describe 1) the players involved in intracellular Ca^{2+} homeostasis with a particular emphasis on the mechanisms involved in SOCE activation and inactivation, 2) the evidence that aspirin metabolite salicylate and other NSAIDs inhibits tumor cell growth acting on SOCE, 3) evidences on the remodeling of intracellular Ca^{2+} in cancer with a particular emphasis in SOCE, 4) the remodeling of SOCE and Ca^{2+} store content in CRC and, finally, 5) the molecular basis of Ca^{2+} remodeling in CRC. This article is part of a Special Issue entitled: ECS Meeting edited by Claus Heizmann, Joachim Krebs and Jacques Haiech.

1. Introduction

Colorectal cancer (CRC) is one of the most frequent forms of cancer and the fourth leading cause of cancer-related death in the world, with an estimated incidence of 1.250.000 new cases every year and more than 600.000 deaths annually. Unfortunately, despite new developments of targeted therapy and other improvements, the prognosis for patients with metastatic CRC remains very limited [1]. This reality highlights the need for efficient chemoprevention and new therapies. Interestingly, there is a considerable body of pre-clinical, epidemiological and randomized data supporting the hypothesis that aspirin prevents CRC and has the potential to be an effective adjuvant cancer therapy [2]. A series of clinical trials show that aspirin reduces the risk of colorectal adenomas [3,4] but the action mechanism remains elusive. Although anti-inflammatory activity may contribute to chemoprevention, evidence suggests that action mechanism is largely independent of anti-inflammatory activity. Aspirin irreversibly inhibits cyclooxygenase 1 (COX-1) activity by acetylating the enzyme at Ser530 and inducible COX-2 at Ser516. In vivo, aspirin is quickly deacetylated to salicylic acid, which remains in plasma for much longer. Salicylic acid does not directly inhibit COX activity because it lacks the acetyl group, although it may inhibit COX-2 gene expression at the transcription level [5]. Thus, inhibition of both COX-2 activity and COX-2 gene expression has been proposed to contribute to the anti-tumoral effects of aspirin. However, aspirin and COX-2 inhibitors block proliferation through a prostaglandin-independent pathway as they inhibit cell proliferation in both COX-2 expressing cells (HT29 and HCA-7) as well as in cells not expressing COX-2 (SW480 and HTC-116) [6,7]. In the same line, the anti-proliferative effects of NSAIDs are independent of the level of COX-2 expression [8] or prostaglandin E2 production [9] but related to cell

☆ This article is part of a Special Issue entitled: ECS Meeting edited by Claus Heizmann, Joachim Krebs and Jacques Haiech.

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http://dx.doi.org/10.1016/j.bbamcr.2017.01.005
0167-4889/

cycle quiescence. Microarray analysis has shown that aspirin represses many cell-cycle-related genes and modulates multiple signaling pathways suggesting that an early mitotic signal may be the key target for the anti-proliferative effect [10].

A few years ago, we reported that salicylate, the major aspirin metabolite, inhibits CRC cell proliferation acting on store-operated Ca^{2+} entry (SOCE), a Ca^{2+} entry pathway involved in cell proliferation in different cell types [11,12]. This pathway is activated by physiological agonists that induce phospholipase C activity and synthesis of IP_3. In turn, IP_3 activates ligand-gated, Ca^{2+} release channels at the endoplasmic reticulum (ER) inducing the emptying of the intracellular Ca^{2+} stores and SOCE activation. This pathway, first envisioned by James W. Putney [13,14], is responsible for agonist-induced Ca^{2+} entry in most types of cells, being particularly abundant and relevant in non-excitable cells that lack voltage-gated Ca^{2+} influx [15]. The functional role of this pathway was originally believed to be intended solely to support the refilling of intracellular Ca^{2+} stores after cell stimulation. However, it is becoming increasingly clear that SOCE subserves multiple physiological roles in different cells types including control of cell proliferation [15]. This view is supported by a large series of papers showing that pharmacological inhibition of this pathway or knockdown of molecular players involved results almost invariably in inhibition of cell proliferation [15]. Therefore, as this pathway is prevented by the aspirin metabolite salicylate, it follows that many pharmacological actions attributed to aspirin and dietary salicylates, including the prevention of CRC, could be mediated in fact by inhibition of this important Ca^{2+} entry pathway.

Recent data suggest an unexpected role for SOCE in cancer including CRC [16–19]. Fortunately, the molecular basis of SOCE emerged recently [20,21], thus providing the tools to investigate the role of SOCE in cancer on solid grounds. Soon, it become clear that many of the molecular players involved in SOCE are expressed differentially in multiple tumor cells and may contribute significantly to some cancer hallmarks in a series of forms of cancer including hepatoma, glioblastoma, breast cancer and CRC [22–24]. Some of these molecular players are presently under scrutiny to ascertain whether they may actually be considered as markers of cancer progression. Moreover, even more recent evidence indicate that mutations in oncogenes and tumor suppressors commonly found in different forms of cancer are able to promote changes in intracellular Ca^{2+} homeostasis and contribute to Ca^{2+} remodeling in cancer [25].

In this review, we will briefly describe 1) the players involved in intracellular Ca^{2+} homeostasis with a particular emphasis on the mechanisms involved in SOCE activation and inactivation, 2) the evidence that aspirin metabolite salicylate and other NSAIDs inhibits tumor cell growth acting on SOCE, 3) evidences on the remodeling of intracellular Ca^{2+} in cancer with a particular emphasis in SOCE, 4) the remodeling of SOCE and Ca^{2+} store content in CRC and, finally, 5) the molecular basis of Ca^{2+} remodeling in CRC.

2. Intracellular Ca^{2+} homeostasis: the players

Intracellular Ca^{2+} is a versatile second messenger involved in the control of many different cell and physiological functions. Ca^{2+} is not synthesized or metabolized inside cells like the rest of second messengers. Instead, it is transported down and up electrochemical gradients through specific channels and pumps, respectively, located at plasma membranes or endomembranes of the ER, mitochondria and other organelles. The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is very low, in the nM range, so that small changes in Ca^{2+} channel conductance promote quick changes in $[Ca^{2+}]_{cyt}$. These changes are sensed by calmodulin and by many other Ca^{2+}-operated enzymes that start a signaling cascade or promote cell responses in virtually all types of cells. Changes in $[Ca^{2+}]_{cyt}$ may be global in the whole cell or restricted in time and space resulting in elementary events named Ca^{2+} microdomains that regulate cellular functions restricted in special subcellular regions such as exocytosis in plasma membrane, cell respiration and ATP synthesis in mitochondria or gene transcription in the nucleus. Whereas Ca^{2+} pumps and transporters contribute largely to the maintenance of basal or resting $[Ca^{2+}]_{cyt}$ and to the recovery of basal $[Ca^{2+}]_{cyt}$ after stimulation, most increases in $[Ca^{2+}]_{cyt}$, are induced by activation of Ca^{2+} channels at the plasma membrane and Ca^{2+} release channels at the ER. IP_3 and ryanodin receptors are ligand-gated Ca^{2+} channels involved in Ca^{2+} release from stores after agonist stimulation. These Ca^{2+} release channels induce transient rises in $[Ca^{2+}]_{cyt}$ but their activity may secondary activate Ca^{2+} channels in plasma membrane that are gated by emptying of intracellular Ca^{2+} stores, the so-called SOCE or capacitative Ca^{2+} entry [15].

In mitochondria, the main Ca^{2+} channel is the mitochondrial Ca^{2+} uniporter (MCU), a Ca^{2+}-activated, Ca^{2+} channel recently characterized at the molecular level [26,27]. Activation of this channel requires large $[Ca^{2+}]_{cyt}$. At variance with the rest of channels, activation of the MCU removes Ca^{2+} from cytosol into mitochondria and helps clearing large cytosolic Ca^{2+} loads [28]. Resting mitochondrial $[Ca^{2+}]$ is similar to resting cytosolic $[Ca^{2+}]$. However, Ca^{2+} influx into mitochondria is empowered by the huge mitochondrial potential ($\Delta\Psi$), close to −180 mV and negative inside the inner mitochondrial membrane [12]. However, the mitochondrial Ca^{2+} uniporter (MCU), the channel responsible for mitochondrial Ca^{2+} uptake is normally closed, thus not allowing the influx of Ca^{2+} into mitochondria unless surrounding Ca^{2+} is large enough to activate this Ca^{2+} operated, Ca^{2+} channel. Ca^{2+} microdomains at the mouth of the MCU can be formed during activation of voltage-gated Ca^{2+} channels in excitable cells like chromaffin [29] and anterior pituitary cells [30]. In addition, large Ca^{2+} microdomains may be formed during release of Ca^{2+} from the ER at close contact sites between the ER and mitochondria enabling efficient ER-mitochondria cross talk [31].

At the plasma membrane there are many different types of Ca^{2+} channels, including receptor-operated Ca^{2+} channels (ROCCs) and voltage-operated Ca^{2+} channels (VOCCs) widely expressed in excitable cells together with voltage-independent channels particularly relevant in the non-excitable cells. In these latter cells, the most important Ca^{2+} entry pathway is the above mentioned SOCE. As many other Ca^{2+} channels, the Ca^{2+} channel responsible for SOCE is usually inactivated by Ca^{2+} leading to a transient Ca^{2+} entry. This mechanism is prevented by mitochondria located nearby these channels that are able to sense high Ca^{2+} microdomains and remove Ca^{2+} to prevent the Ca^{2+}-dependent inactivation of these channels [32,33] (Fig. 1). Thus, mitochondria play a pivotal role in sustaining Ca^{2+} signals initiated by SOCE. This is the case, for instance, of the activation of the nuclear factor of activated T cells (NFAT) during the immunological synapse. T cell receptor activation induced by antigen presentation, promotes Ca^{2+} release and Icrac activation in T cells. Nearby located mitochondria take up entering Ca^{2+} and prevent the slow, Ca^{2+}-dependent Icrac inactivation, thus leading to a sustained entry of Ca^{2+}. The sustained and moderate increase in $[Ca^{2+}]_{cyt}$ activates calcineurin, a Ca^{2+}-dependent phosphatase that removes NFAT phosphorylation revealing an NFAT nuclear import signal. The sustained presence of NFAT in the nucleus allows the expression of interleukin 2 that promotes finally the clonal expansion of the activated T cell [34]. Therefore, the role of mitochondria in removing the Ca^{2+}-dependent inactivation of Icrac channels is responsible for sustaining SOCE and cell proliferation in T cells. We have shown that salicylate, the major aspirin metabolite, depolarizes partially mitochondria and limits largely the ability of mitochondria of Jurkat T cells to take up Ca^{2+}, thus promoting SOCE inactivation and inhibition of cell proliferation in T cells [11].

Since the first description of SOCE by James W. Putney [13,14], the molecular basis of SOCE remained elusive for nearly 20 years. However, the molecular players involved in SOCE began to be crack after the discovery of the TRP superfamily of ion channels first, and the subsequent discovery of STIM and Orai proteins [20,21]. At the molecular level, SOCE starts with the emptying of intracellular Ca^{2+} stores from resting

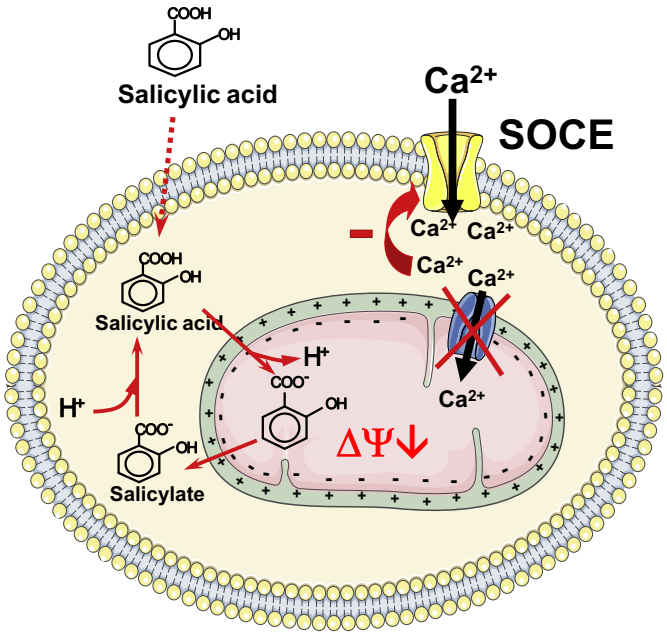

Fig. 1. Aspirin metabolite salicylate depolarizes mitochondria, thus preventing mitochondrial Ca^{2+} uptake and promoting the Ca^{2+} dependent inactivation of Store-operated Ca^{2+} entry. Aspirin protects against CRC by mechanism independently of its anti-inflammatory activity. Aspirin is quickly deacetylated in vivo to salicylate. As many other non-steroidal anti-inflammatory drugs (NSAIDs), salicylate behaves a mild mitochondrial uncoupler able to reduce mitochondrial potential, the driving force for mitochondrial Ca^{2+} uptake. This effect inhibits mitochondrial Ca^{2+} uptake and compromises the ability of mitochondria to prevent the slow, Ca^{2+}-dependent inactivation of store-operated channels. The end result is inhibition of SOCE and downstream signaling pathways like, for instance, cell proliferation in CRC cells. This mechanism may contribute to explain the anti-tumoral effects of aspirin and other NSAIDs.

levels around 700 μM to about 200 μM after agonist-induced Ca^{2+} release, as revealed by ER targeted probes with very low affinity for Ca^{2+} [35]. This "emptying" is detected by a sensor named Stromal Interaction Molecule 1 (STIM1) which, upon dissociation of Ca^{2+} ions from Ca^{2+} binding sites, undergoes oligomerization that promotes its interaction with Orai1, a Ca^{2+} channel located in specific places of the plasma membrane. This interaction opens Ca^{2+} release activated channels (Icrac) enabling the influx of Ca^{2+} and refilling of the store [20,21]. There is another Ca^{2+} sensor at the ER called STIM2 with lower affinity for Ca^{2+} but its function is less known [36]. In addition, two other Orai proteins named Orai2 and Orai3 have been discovered but their roles in SOCE are also poorly known [37]. Finally, some members of the TRP superfamily of cation channels, particularly TRPC channels, may contribute to SOCE as well by forming channel complexes with STIM1 and Orai1 or simply forming alternative store-operated channels less selective for Ca^{2+} [38].

3. The aspirin metabolite salicylate and other NSAIDs inhibit SOCE and prevent colorectal cancer

As stated above, a large series of basic, epidemiological and clinical evidences suggest that aspirin and other NSAIDs prevent colorectal cancer and other forms of cancer. For instance, a recent meta-analysis showed that NSAIDs decreased the frequency of adenomas, CRC and deaths because of CRC in 57 out of the 59 studies carried out between 1988 and 2006 [39]. Recently, a few clinical trials have been completed in high risk individuals including Lynch syndrome patients, a type of familial cancer with 100% chances of developing CRC. Aspirin protected largely (63%) against CRC in these high risk patients which has resulted in the recommendation of aspirin use for high risk patients of CRC [40]. Ongoing clinical trials suggest that combinations of aspirin or other NSAIDs with additional chemoprevention compounds may be even more efficient in preventing polyp formation and cancer death in patients that had undergone surgery for tumor removal. Moreover, the U.S. Preventive Services Task Force recommended recently initiating low-dose aspirin use for the primary prevention of CRC in adults aged 50 to 59 years who are not at increased risk for bleeding, have a life expectancy of at least 10 years, and are willing to take low-dose aspirin daily for at least 10 years [41].

Interest on the aspirin's antitumoral action mechanism is growing since the realization that a large part of the effects may be independent of the anti-inflammatory activity of these compounds. This view is based in that antitumor activity remains in tumor cells lacking expression of COX, the classic target of anti-inflammatory compounds. Moreover, structural analogues like R-flurbiprofen that lack ant-inflammatory activity are also efficient in preventing tumor cell growth. Therefore, even though COX-mediated synthesis or prostanoids may contribute to inflammation and colon tumorogenesis and NSAIDs may act partially by preventing inflammation, other targets of aspirin and other NSAIDs are likely involved in the antitumor actions of these drugs [6–10].

Weiss et al. reported for the first time that inhibition of SOCE contributed to the anti-proliferative effect of NSAIDs in human colon cancer cells [42]. Specifically, sulindac and mefenamic acid, but not aspirin, inhibited SOCE and cell proliferation of HTR18 CRC cells. They proposed SOCE to be involved in CRC cell proliferation as several SOCE antagonists also prevented CRC cell proliferation. However, no mechanism of action was provided. A few years ago, we reported that salicylate, the main aspirin metabolite, inhibited also SOCE and cell proliferation in HT29 cells [11,12]. In search for a mechanism we realized that salicylate is considered a mild mitochondrial uncoupler [43] and the important role of mitochondria in controlling SOCE [32,33]. Salicylic acid uncouples partially mitochondria as it enters mitochondria down its chemical gradient. There, the basic mitochondrial pH favors its dissociation into salicylate that should be trapped into mitochondria. However, the negative charge of the carboxylic residue is delocalized in the salicylate aromatic ring making the salicylate anion permeable and able to exit the mitochondrial matrix. In cytosol, salicylate can be protonated back to salicylic acid that can enter freely again into the mitochondrial matrix. The net result is the release of one proton in each uncoupling cycle leading to mitochondrial depolarization, thus limiting the electromotive force for mitochondrial Ca^{2+} uptake [11,12,43]. Inasmuch as SOCE is strongly regulated by mitochondria, we proposed that salicylate uncouplying effect promotes the Ca^{2+}-dependent inactivation of Icrac and inhibition of SOCE (Fig. 1).

In support of the above view we have shown that salicylate depolarizes mitochondria at very low concentrations, inhibits mitochondrial Ca^{2+} uptake and inactivates SOCE [11,12]. We have also shown that this mechanism may contribute to explain also the antiproliferative effects of salicylate in T cells as well as in vascular smooth muscle cells [11,44,45].

We also tested the effects of salicylate on Rat Basophilic Leukemia (RBL) cells where mitochondrial control of SOCE and Icrac is well established. We found that salicylate and other NSAIDs cells promoted the Ca^{2+} dependent inactivation of Icrac in physiological buffer, having no effect in strong Ca^{2+} buffer, a condition that remove Ca^{2+} dependent inactivation of Icrac [44]. In short, salicylate and other NSAIDs do not inhibit Icrac directly but promote their Ca^{2+} dependent inactivation secondary to the impairment of mitochondrial Ca^{2+} uptake [44]. However, the effects of salicylate and other NSAIDs on SOCs in normal colonic and CRC cells remain to be characterized.

In summary, the above data strongly suggest an important role for SOCs and SOCE in cell proliferation in different types of cells including CRC cells. Consistently with this view, in the last few years, emerging evidence is accumulating that suggest an important role of SOCE and intracellular Ca^{2+} remodeling in different forms of cancer including CRC. This evidence is reviewed next.

4. Ca^{2+} remodeling in cancer

A series of recent reports suggest that changes in intracellular Ca^{2+} homeostasis (remodeling) may contribute to critical cancer hallmarks. In fact, cell activities such as proliferation, migration and invasion capabilities as well as survival are functions that are regulated by intracellular Ca^{2+} [46–48]. Early reports showed evidence of changes in the expression of VOCCs in prostate, gastric and colorectal cancers [49–50] and TRP channels in breast, ovary and kidney cancers and glioma [24]. Recent data suggest also a role for store-operated Ca^{2+} entry (SOCE) and its underlying currents and molecular players in different forms of cancer [16–19]. For example, STIM1 and Orai1, the most important proteins involved in Icrac and SOCE, play a role in breast cancer cell migration and metastasis [51]. STIM1 is involved also in cell growth and cell migration in cervical cancer [52]. In fact, STIM1 has been reported to be overexpressed in around 70% of all cervical cancers and this increase has been linked to metastasis. Consistently with an important role of STIM1 in glioblastoma, Orai1 and STIM1 are involved in glioblastoma invasion and its silencing promotes G0/G1 phase arrest and inhibits proliferation of human glioblastoma cells [53,54]. Orai3 could contribute to prostate and breast cancer [55,56]. Thus, multiple evidences suggest aberrant expression of molecular players involved in SOCE and its contribution to cancer hallmarks in different forms of cancer.

In addition, different proto-oncogenes and tumor suppressors may exploit the central role of Ca^{2+} signaling by targeting the IP_3R. Activation of proto-oncogenes or inactivation of tumor suppressors modulates IP_3R function and ER Ca^{2+} homeostasis, thereby decreasing mitochondrial Ca^{2+} uptake and mitochondrial outer membrane permeabilization which is critical for resistance to apoptotic cell death, another cancer hallmarks [57]. Specifically, several major oncogenes, including Bcl-2, Bcl-XL, Mcl-1, PKB/Akt, and Ras, and tumor suppressors, such as p53, PTEN, PML, BRCA1, and Beclin 1, have been identified as direct and critical regulators of Ca^{2+}-transport systems located at the ER membranes, including IP_3 receptors and SERCA Ca^{2+} pumps. Therefore, oncogenes and tumor suppressors may execute part of their functions by controlling ER-mitochondrial Ca^{2+} fluxes promoting survival (oncogenes) or cell death tumor suppressors. Oncogenic mutations, gene deletions or amplifications critically involved in oncogenesis may alter the expression and/or function of these proteins, thus influencing the delicate equilibrium between oncogenes and tumor suppressors and favoring malignant cell function and behavior [25]. Next we will review the evidence of intracellular Ca^{2+} remodeling in a particularly widespread and deadly form of cancer, CRC.

5. Ca^{2+} remodeling in CRC

Early reports suggested altered expression of VOCC in CRC. For example, the mRNA for the cardiac isoform of the voltage-gated L-type Ca^{2+} channel is increased in CRC [58]. In fact, increased mRNA corresponding to α_1 subunit of the cardiac isoform of the L-type Ca^{2+} channel has been proposed as useful marker of CRC. The role of increased expression of VOCC in CRC is unknown. However, α_1 subunit protein expression is increased when colonic cells are non-confluent or dividing which may account for the rise in cancer. A few years later, we reported that salicylate, the most important aspirin metabolite, could inhibit CRC cell proliferation acting on SOCE, thus implying a role for SOCE in CRC [11,12]. Further evidence supporting a role of intracellular Ca^{2+} remodeling in CRC was reported by Wang et al. [59] who showed that plasma membrane Ca^{2+} ATPase 4 (PMCA4) is downregulated during progression of some CRC as tumor cells become less and less differentiated. Importantly, reversing the low expression of PMCA4 reduced cellular proliferation and down regulated transcription of the Ca^{2+} sensitive early response gene FOS. These data invite speculation that remodeling of the Ca^{2+} signal in CRC is associated with defective Ca^{2+} efflux that promotes proliferative pathways while avoiding sensitization to apoptotic stimuli [60]. We have recently confirmed that resting intracellular $[Ca^{2+}]$ is enhanced in CRC cell lines compared with normal cell lines of human colon mucosa [19]. These data are consistent with decreased expression of PMCA4. However, enhanced resting Ca^{2+} levels could be also mediated by enhanced influx of Ca^{2+}. In support of this view, plasma membrane resting voltage is more negative in CRC cells than in normal colonic mucosa cells [19].

We have shown recently that normal colonic mucosa cells are proliferating cells that reflected properly the normal phenotype [61]. As expected, CRC cells proliferated at a much higher rate than normal cells. Interestingly, there was a very good correlation between the rate of cell proliferation and the extent of SOCE in each cell type, thus demonstrating an important role for enhanced SOCE in CRC cell proliferation. Moreover, SOCE inhibition prevented cell proliferation in both normal and CRC cells [19]. SOCE inhibition also prevented HT29 cell invasion as tested by *in vitro* invasion assays [19]. Evidence indicates that enhanced SOCE also contributes to colon cancer cell migration. For example it has been shown that SK3, a K^+ channel previously involved in cell migration, may control cancer cell migration through an interaction with the Ca^{2+} channel Orai1 within lipid rafts [62]. The formation of the channel complex is essential as none of the individual proteins promoted the complete phenotype. In addition, removal of the complex from lipid rafts impaired cancer cell Ca^{2+} entry, cell migration and bone metastases [62] likely providing a novel target to inhibit bone metastases. Taken together, these data highlight our early data of inhibition of SOCE and tumor cell proliferation and motility by aspirin metabolite salicylate and provide strong support to the view that SOCE may be one of the actual targets of aspirin in CRC chemoprevention.

Interestingly, differences in SOCE between normal colonic and CRC cells become even more evident when it is induced in a more physiological manner by G-protein coupled receptor activation. We found that agonist-induced release of Ca^{2+} was much larger in colon cancer cells than normal colonic epithelium cells. Moreover, agonist-induced entry Ca^{2+} only in tumor cells indeed, but not in the normal colonic cells, despite that both agonists released Ca^{2+} from intracellular stores [19].

Agonist-independent experiments using caged-IP_3 compounds suggested that IP_3 released more Ca^{2+} from intracellular stores in tumor cells than in normal cells and/or Ca^{2+} store content was larger in tumor cells. Paradoxically, we found that Ca^{2+} store content assessed as the Ca^{2+} release induced by ionomycin and/or cyclopiazonic acid was much larger in normal cells than in tumor cells. Therefore, Ca^{2+} stores are partially empty in CRC cells, yet agonists release most Ca^{2+} stored in CRC cells. In contrast, in normal colonic cells, Ca^{2+} stores are loaded but agonists release just a minor fraction of the stored Ca^{2+}, probably not large enough to activate SOCE in physiological conditions [19]. This data might explain why agonist-induced Ca^{2+} entry is observed only in colon cancer cells but not in normal cells [19].

There is another important aspect of differential Ca^{2+} store content in normal and tumor cells. Inasmuch as Ca^{2+} store content contributes to mitochondrial Ca^{2+} overload-dependent apoptosis, differential store refilling should promote differential ability to undergo apoptosis. Consistently with this prediction, we found that H_2O_2 promoted apoptosis to a much larger extent in normal colonic cells than in CRC cells [19]. In other words, CRC cells are resistant to apoptosis, a common characteristic of most tumor cells and, therefore, critical cancer hallmark.

Electrophysiological analysis of SOCs in normal and tumor cells provided more details about the mechanisms for enhanced SOCE in tumor cells [19]. Ca^{2+} store depletion in normal cells using thapsigargin induced the appearance of a current that resembles closely the classic Ca^{2+}-release activated current (Icrac) reported first in mast and T cells. This current is small, voltage-independent, inward rectifying, highly selective for Ca^{2+} and sensitive to La^{3+} and low concentrations of 2-APB [19]. In CRC cells, however, thapsigargin promotes the appearance of two different SOCs. A first one similar to the current found in normal cells but showing a much enlarged current density and a second one, absent in normal cells, with a large outward component, not selective for Ca^{2+}, largely driven by Na^+ and sensitive to high concentrations

of 2-APB [19]. In summary, CRC cells display enhanced SOCE and SOCs and decreased Ca^{2+} store content and this remodeling may contribute to cancer hallmarks.

6. Molecular basis of Ca^{2+} remodeling in CRC

To address the molecular basis for Ca^{2+} remodeling in CRC we have investigated recently the molecular players involved in SOCE and SOCs in normal and colon cancer cells using conventional and quantitative RT-PCR. All members of STIM and Orai protein families are expressed in both normal and tumor cells. TRPC channels including TRPC1 and TRPC4 channels are expressed as well in both normal and tumor cells. In contrast, other TRP channels previously related to SOCE in some other cell types are expressed only in normal cells or missing in both cell types. Interestingly, the expression of these genes at the mRNA level showed a similar pattern in normal and CRC cells, except that most genes are increased significantly in CRC cells relative to normal cells [19]. Western blotting revealed some significant differences at the protein level as well. Specifically, all three Orai proteins Orai1, Orai2, and Orai3 are overexpressed in tumor cells relative to normal cells. Likewise, TRPC1 expression is also enhanced to the largest extent in tumor cells. In addition, STIM1 protein is overexpressed in tumor cells as well. Unexpectedly, the other Ca^{2+} sensor at the ER, STIM2 that was highly overexpressed at the mRNA level, was dramatically downregulated at the protein level in tumor cells [19]. Taken together, these data suggest that changes in expression of molecular players involved in SOCE should underlie differences in SOCE and SOCs between normal colonic and CRC cells [19]. Importantly, recent data suggest overexpression of STIM1 in CRC samples and correlation with prognosis features [63].

As stated above, in normal human colonic cells, thapsigargin induced activation of a Ca^{2+}-selective, inward rectifying current quite similar to Icrac. Consistently, silencing of TRPC1 has no effect on the current while Orai1 knockdown decreased significantly the current. Silencing of STIM proteins also decreased significantly SOCE in normal cells [19]. Therefore, SOCE in normal cells is likely driven by interaction between ER Ca^{2+} sensors and Orai1 while TRPC1 does not contribute to SOCE. The possible role of Orai2 and Orai3 remains to be established.

CRC cells displayed a much larger SOCE and SOCs are a mix of enhanced Icrac and a non-selective current with outward component. Interestingly, knockdown of either Orai1 and TRPC1 inhibit both the inward and the outward components of SOCs in CRC cells. Thus, at variance with normal cells, SOCs are driven by both Orai1 and TRPC1 providing evidence of formation of a channel complex specific for CRC rather than separate channels. This view is based in i)the ability of TRPC1 siRNA to inhibit the inward component of Icrac in CRC cells and ii) the unexpected ability of Orai1 siRNA to inhibit the outward component of Isoc in CRC cells [19]. Additional silencing experiments suggested no role for Orai2 and Orai3 in SOCE in CRC despite these proteins are overexpressed in CRC cells. Finally, our preliminary results suggest also that STIM1, but not STIM2, is involved in SOCE in CRC cells, consistently with STIM1 overexpression in CRC cells and downregulation of STIM2 in CRC. The emerging picture from these results is shown in Fig. 2. In normal colonic cells, SOCE is made of Icrac-like currents driven by interactions between STIM1 and Orai1 and where STIM2 may also play a role. This channel complex resembles the one found in rat hippocampal neurons that is also driven by interaction between Orai1, STIM1 and STIM2. In contrast, SOCE in CRC cells is mediated likely by a channel complex made of Orai1 and TRPC1 and regulated solely by STIM1.

The role played by TRPC1 in the channel complex and CRC remains unknown. However, since TRPC1 is essentially a Na^{+} channel, its presence in the channel complex may provide a way for self-limiting the rate of Ca^{2+} entry by modulating plasma membrane potential. Alternatively, as TRPC1 channels are stretch-sensitive and involved in volume control during cell proliferation, it is also likely that the channel may function in a non-store operated mode providing sensitivity for cell migration. Consistently with this view, Potier-Cartereau and cols. Have recently confirmed that SOC in CRC cells is made of a channel complex mediated by interactions between Orai1 and TRPC1 [64] that is relevant for cell migration in CRC cells. Interestingly, they also provide evidence that channel complex includes also SK3, a K^{+} channel that may keep negative membrane potential for enlarged influx of Ca^{2+} and has been involved in cancer cell migration [62,64]. Formation of this channel complex may depend on the type of CRC cell line or the type of tumor as, for instance, HT29 cells lack expression of SK3 channels. The formation of Orai1/TRPC1/SK3 channel complex depends on STIM1

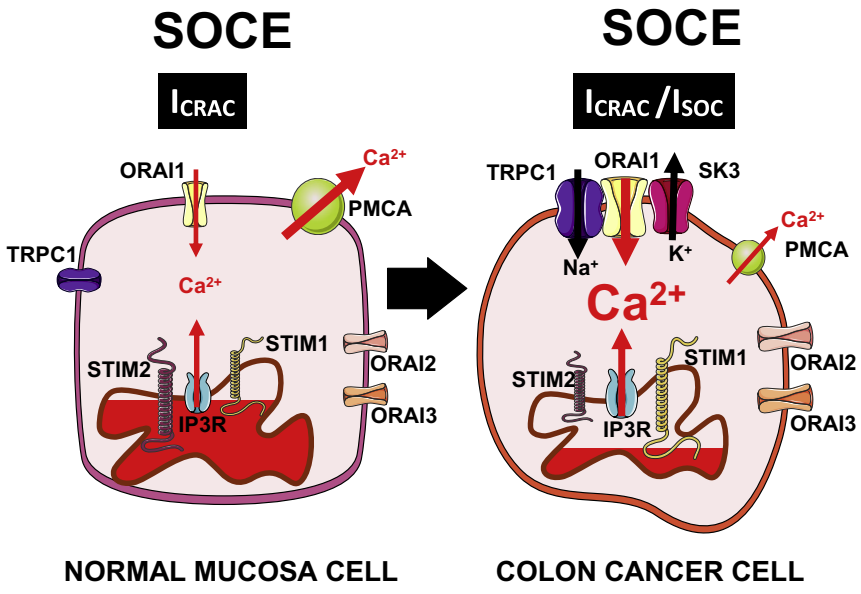

Fig. 2. Remodeling of store-operated Ca^{2+} entry (SOCE) and Ca^{2+} store content in colorectal cancer cells. Normal human colonic mucosa cells [left] show a small SOCE associated to classic Icrac mediated by Orai1, STIM1 and STIM2, and a large Ca^{2+} store content associated to high expression of STIM2. CRC cells [right] show enhanced SOCE associated to enlarged Icrac and the appearance of a non-selective Isoc mediated by channel complexes made of STIM1, Orai1, TRPC1 and SK3. Enhanced SOCE contributes to enhanced cell proliferation, migration and invasion. Stim2 downregulation contributes to the partial depletion of Ca^{2+} stores and resistance to apoptosis in CRC. Thus, intracellular Ca^{2+} remodeling contributes to cancer hallmarks in CRC.

phosphorylation by EGF and the activation of the Akt pathway [64]. Thus, SOCE activates both Akt pathway and SK3 channel activity leading to SOCE amplification forming a feed-forward loop. Interestingly, only 10–20% of patients with metastatic CRC benefit from the use of anti-EGFR monoclonal antibodies. The authors have shown that anti-EGFR mAbs can modulate SOCE and cancer cell migration through the Akt pathway [64]. Moreover, an alkyl-lipid Ohmline previously established as a SK3 channel inhibitor, is able to dissociate the lipid raft ion channel by preventing phosphorylation of Akt and modulation of mAbs action. Therefore, inhibition of the SOCE-dependent, colon cancer cell migration may be a novel strategy to modulate Anti-EGFR mAb action in CRC [64].

It has been reported that STIM2 is overexpressed in nearly 64% of all CRCs [65]. How is this result compatible with the our findings that STIM2 is downregulated in CRC cells. The likely explanation is that, although STIM2 mRNA is overexpressed also in CRC cells, it is indeed downregulated at the protein level so that it is barely detected in CRC cell samples [19]. Consistently, it has been reported that loss of STIM2 correlates with Gleason grade in prostate cancer [66]. STIM2 is a ER Ca^{2+} sensor with low affinity for Ca^{2+} that senses Ca^{2+} concentration at around 500 μM. A decrease in ER [Ca^{2+}] below this value should activate STIM2 and likely SOCE to keep the store filled. Consistently, silencing of STIM2 decreases SOCE and Ca^{2+} store content in normal cells [19]. Inasmuch as Ca^{2+} store content has been related to mitochondrial Ca^{2+} overload and apoptosis, STIM2 knockdown in normal cells increased their resistance to apoptosis induced by H_2O_2.

Taken together, these data suggest that STIM2 downregulation in CRC cells could contribute to decrease Ca^{2+} store content and increase apoptosis resistance, another critical hallmark of cancer. In CRC cells, the loss of STIM2, leaves STIM1 in charge of sensing and refilling the stores. STIM1 is a different ER Ca^{2+} sensor with higher affinity for Ca^{2+} than STIM2 (300 μM). Thus, in the absence of STIM2, STIM1 may contribute to keep Ca^{2+} stores in a partially depleted state thus providing survival advantages to tumor cells on one hand. On the other hand, partial depletion of Ca^{2+} stores may also favor activation of SOCE by physiological stimuli. Thus, loss of STIM2 may contribute to both enhanced SOCE and depleted Ca^{2+} stores in CRC cells [19].

In summary, Ca^{2+} remodeling in CRC is mediated by changes in molecular players involved in SOCE, Ca^{2+} channel complexes and Ca^{2+} store content. This remodeling appears to contribute largely to cancer hallmarks including enhanced cell proliferation, motility and survival, thus supporting the view that CRC chemoprevention afforded by aspirin and other NSAIDs is mediated by modulation of intracellular Ca^{2+} and likely providing novel targets for CRC diagnosis, prognosis and therapy.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

This work has been funded by a grant from Ministerio de Economía y competitividad, Spain (BFU2015-70131R) and Junta de Castilla y León, Spain [BIO/VA46/14].

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