

Review

NSAIDs, Mitochondria and Calcium Signaling: Special Focus on Aspirin/Salicylates

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Abstract: Aspirin (acetylsalicylic acid) is a well-known nonsteroidal anti-inflammatory drug (NSAID) that has long been used as an anti-pyretic and analgesic drug. Recently, much attention has been paid to the chemopreventive and apoptosis-inducing effects of NSAIDs in cancer cells. These effects have been thought to be primarily attributed to the inhibition of cyclooxygenase activity and prostaglandin synthesis. However, recent studies have demonstrated unequivocally that certain NSAIDs, including aspirin and its metabolite salicylic acid, exert their anti-inflammatory and chemopreventive effects independently of cyclooxygenase activity and prostaglandin synthesis inhibition. It is becoming increasingly evident that two potential common targets of NSAIDs are mitochondria and the Ca²⁺ signaling pathway. In this review, we provide an overview of the current knowledge regarding the roles of mitochondria and Ca²⁺ in the apoptosis-inducing effects as well as some side effects of aspirin, salicylates and other NSAIDs, and introducing the emerging role of L-type Ca²⁺ channels, a new Ca²⁺ entry pathway in non-excitabile cells that is up-regulated in human cancer cells.

Keywords: aspirin; calcium; mitochondria; nonsteroidal anti-inflammatory drug (NSAID); reactive oxygen species

1. Introduction

Aspirin (acetylsalicylic acid) is a well-known nonsteroidal anti-inflammatory drug (NSAID) that has long been used as an anti-pyretic and analgesic drug. Other NSAIDs are also generally used to treat pain, inflammation and fever. The anti-inflammatory actions of NSAIDs have been thought to be primarily attributed to inhibition of prostaglandin (PG) synthesis [1]. Aspirin acetylates Ser-530 of cyclooxygenase (COX) I and II, thereby blocking PG and thromboxane A₂ synthesis, while therapeutic concentrations of aspirin and salicylates inhibit COX II protein expression [2]. However, there is also evidence that certain NSAIDs, including aspirin, salicylates, sulindac, ibuprofen and flurbiprofen have anti-inflammatory and anti-proliferative effects independent of COX activity and PG synthesis inhibition (for a comprehensive review, see [3]). The doses of aspirin used to treat chronic inflammatory diseases are much higher than those required to inhibit PG synthesis. Moreover, salicylate reduces inflammation, although it lacks the acetyl group and is ineffective as a COX inhibitor at therapeutic doses [4–6]. In addition, most of these effects have only been observed at high concentrations of the respective NSAIDs, which are 100- to 1000-fold higher than those required to inhibit PG synthesis [3]. Thus, individual NSAID may utilize intrinsic COX-independent mechanisms to exert their anti-inflammatory effects. These effects are mediated through inhibition of certain transcription factors such as nuclear factor- κ B (NF- κ B), AP-1 and nuclear factor of activated T cells [7–9]. Another possible important mechanism of the anti-inflammatory effects may be modulation of the activation of mast cells and basophils, since these cells play pivotal roles in allergic inflammatory reactions. Aspirin has been shown to modulate mast cell degranulation, COX-2 expression and release of pro-inflammatory cytokines [11]. We recently reported that aspirin and salicylates modulate proinflammatory mediator release in mast cells through a COX-independent mechanism in which Ca²⁺ signaling plays a key role [11,12]. Since this issue is close to the main theme of this review, we will discuss it in more detail in the section 2.

In addition to their anti-inflammatory actions, NSAIDs are emerging as promising antineoplastic drugs. Numerous studies have suggested that the use of NSAIDs, primarily aspirin, decreases the risks of several cancers, including, cancers of the colon and other gastrointestinal organs as well as those of the breast, prostate, lung, ovary and skin [13–19]. Since PGs inhibit apoptosis and induce the formation of new blood vessels, thereby contributing to tumor growth [20–22], COX inhibition may explain a part of the antineoplastic activities of certain NSAIDs. However, NSAIDs have growth inhibitory effects on colon cancer cell lines that do not express the COX-1 and COX-2 enzymes [23,24], and also on mouse embryo fibroblasts that are null for both the COX-1 and COX-2 genes [25]. Such observations are inconsistent with the conventional hypothesis that NSAIDs act primarily or exclusively by inhibiting PG synthesis. NSAIDs have also been shown to induce apoptosis and necrosis in cancer cells (for reviews, see [3,26]), which may be potential mechanisms for their chemopreventive effects. In addition, NSAIDs exhibit multiple effects on a variety of intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) cascade, ribosomal S6 kinase, signal transducer and activator of transcription 1 and transforming growth factor β . They also modulate several processes, such as cell cycle progression and the activities of nuclear receptor family members, including peroxisome proliferator-activated receptor γ . It remains unclear whether these effects are direct or indirect [3]. These biological effects may also play roles in tumor growth

inhibition and/or cell death induction. Thus, the molecular mechanisms underlying the chemopreventive effects of NSAIDs remain a matter of debate. In this review, we will focus on the COX-independent mechanisms of NSAID-induced cell death with special attention to the roles of mitochondria in Section 3.

Aspirin has various side effects on the gastro-intestinal tract, and primarily causes gastric lesions, ulcerations and erosions [27]. Aspirin also induces immunological side effects, which are collectively referred to as aspirin intolerance (see Section 2). Aspirin intolerance is a disorder that induces urticaria, asthma and anaphylaxis in response to oral administration of the drug [28,29]. Aspirin also potentiates some acute allergies such as food-dependent exercise-induced anaphylaxis (FDEIA), which is a food allergy induced by physical exercise. Recently, aspirin was shown to act as a powerful trigger of anaphylaxis in FDEIA patients [30].

2. COX-Independent Modulation of Mast Cell Activation by NSAIDs

Mast cells play critical roles in allergic inflammatory reactions. These cells express the high-affinity IgE receptor (FcεRI) on their cell surface and cross-linking of IgE-bound FcεRI by multivalent antigens induces aggregation of the receptor, which triggers biochemical cascades that lead to cell activation. Upon antigen stimulation, mast cells release various preformed granular substances, such as histamine and serotonin (a process referred as to degranulation), and synthesize and secrete arachidonate (AA) metabolites such as leukotrienes (LTs) and PGs as well as cytokines and chemokines [31]. These chemical mediators cause various pathophysiological events that contribute to acute and chronic inflammation. Therefore, inhibition of the proinflammatory mediator release is a potential mechanism for the anti-inflammatory effects of NSAIDs. Recent studies have revealed that NSAIDs modulate mast cell degranulation, COX-2 expression and release of pro-inflammatory cytokines by affecting heat shock protein and Toll-like receptor-mediated responses [11,32]. In addition, several studies have shown that an atopic background (high levels of serum IgE) is a risk factor for NSAID sensitivity [33]. One key feature of aspirin intolerance is overproduction of cysteinyl LTs (cys-LTs) such as LTC₄, LTD₄ and LTE₄, which are all sequentially synthesized from arachidonic acid. These cys-LTs are potent proinflammatory mediators and cause smooth muscle contraction and increased vascular permeability. Patients with aspirin intolerance have significantly higher levels of cys-LTs in their bronchoalveolar lavage fluid and urine before and after oral aspirin challenge [34]. Moreover, cys-LT synthase activity is predominantly detected in mast cells, which are the major producers of cys-LTs [35,36]. These observations suggest that mast cells may play roles in both the anti-inflammatory effects and side effects of certain NSAIDs, primarily aspirin. To understand the molecular mechanisms underlying aspirin intolerance, we investigated the possible effects of aspirin on cys-LT production in mast cells. Aspirin alone at concentrations ranging from 0.1 to 3 mM had minimal effects on LTC₄ secretion. However, aspirin had dual effects on antigen-induced LTC₄ secretion depending on the concentration used. At therapeutic levels (≤ 0.3 mM), representing the concentrations observed *in vivo* for antipyretic and analgesic use, aspirin enhanced LTC₄ secretion, while at higher concentrations (>1 mM), it suppressed LTC₄ secretion [11]. Essentially similar effects were observed with salicylates, which lack inhibitory effects on COX-1 and COX-2 activities [37], thereby indicating that aspirin exerts these effects independently of COX activity. Cytosolic

phospholipase A₂ (cPLA₂) mediates agonist-induced AA release in most cell types (for reviews, see [38,39]). The catalytic activity of cPLA₂ is phosphorylation-dependent. Phosphorylation of Ser-505 in cPLA₂ by extracellular signal-regulated kinase 1/2 (ERK1/2) is necessary for cPLA₂-mediated AA release following stimulation of various cell types by many different agonists [39,40]. Aspirin stimulates phosphorylation of Ser-505 in cPLA₂ at concentrations that augment LTC₄ secretion [11]. Antigen stimulation leads to ERK1/2 activation, as evidenced by increased dual phosphorylation of Thr-202 and Tyr-204, while the MAPK kinase inhibitor U0126 reduces LTC₄ secretion. These data suggest that ERK1/2 is activated by the upstream kinase MEK1/2, as reported in a variety of cell types [38,39]. Ser-727 in cPLA₂ is another important site for activation of the enzyme, which is mediated by p38MAPK activated via dual phosphorylation of Thr-180 and Tyr-182 [41]. Unexpectedly, it was found that aspirin at concentrations ranging from 0.1 to 3 mM dose-dependently reduces the activation of ERK1/2 and had no significant effects on the activation of p38MAPK. Collectively, these data indicate that aspirin enhances cPLA₂ activation independently of the ERK and p38MAPK pathways, thereby suggesting the involvement of another mechanism.

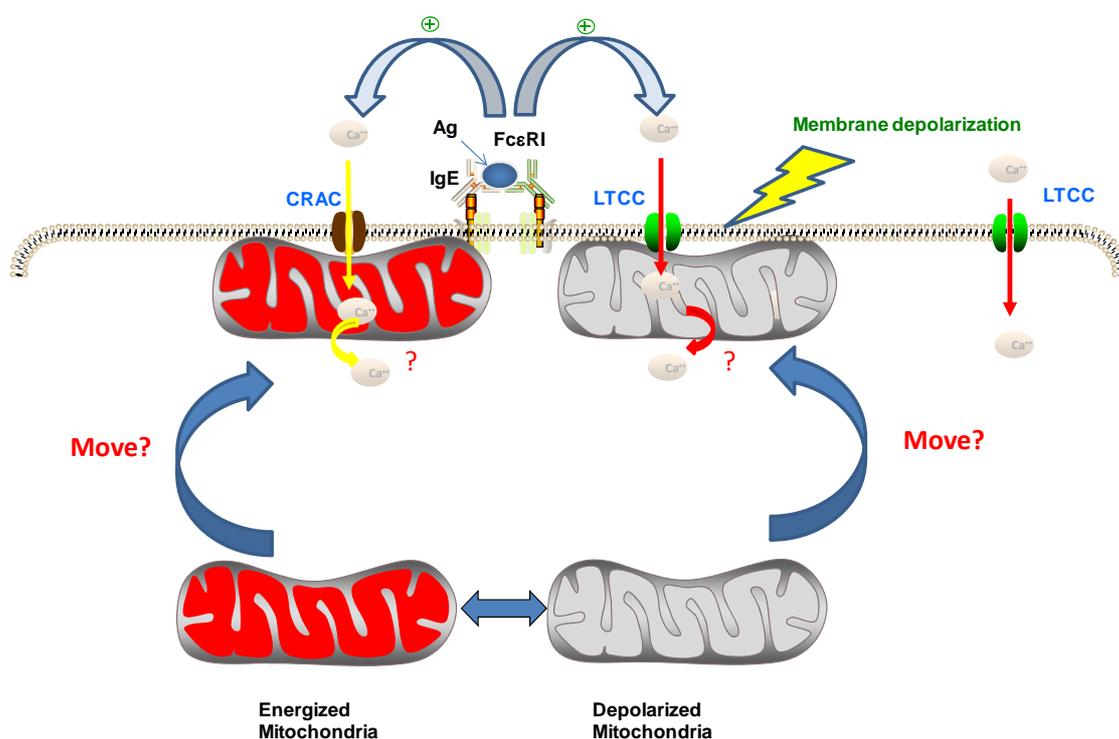
3. Modulation of Ca²⁺ Channel Activities by NSAIDs

Ca²⁺ is a highly versatile intracellular second messenger in many cell types, and regulates many complicated cellular processes, including cell activation, proliferation and apoptosis. Elevation of the intracellular Ca²⁺ concentration, mainly through Ca²⁺ entry from the extracellular space, is necessary for the new synthesis and secretion of cys-LTs [31]. Ca²⁺ binds to the amino-terminal C2 domain of cPLA₂ and leads to its translocation to the nuclear envelope and endoplasmic reticulum (ER) and activation [38,39]. Ca²⁺ is also an important regulator of 5-lipoxygenase, which catalyzes the addition of molecular oxygen to AA. Analyses of Ca²⁺ influx have revealed that aspirin has dual effects on this process depending on the concentration used, similar to the observations for LTC₄ secretion. Specifically, at low concentrations (≤0.3 mM), aspirin enhanced Ca²⁺ influx, while at high concentrations (>1 mM), it suppressed Ca²⁺ influx [11]. It is widely accepted that store-operated Ca²⁺ entry (SOCE) is the main mode of Ca²⁺ influx in electrically non-excitable cells, including mast cells [42]. SOCE is mediated by store-operated Ca²⁺ (SOC) channels like Ca²⁺ release-activated Ca²⁺ (CRAC) channels, which are activated by depletion of intracellular Ca²⁺ stores. Despite its stimulatory effect on Ca²⁺ influx at low concentrations, aspirin reduces CRAC channel activity. These data suggest that aspirin may stimulate another Ca²⁺ entry pathway. It has long been thought that long-lasting voltage-gated L-type Ca²⁺ channels (LTCCs) represent a characteristic feature of excitable cells. However, pharmacological, molecular and genetic approaches have recently revealed the existence of functional LTCCs or LTCC-like channels in a variety of hematopoietic cells such as B cells, dendritic cells, natural killer cells, neutrophils, mast cells and T cells (for reviews, see [43,44]). Among these, the Ca²⁺ channels in T cells have been the most extensively studied. These cells express a channel (or channels) sharing elements of the molecular structure and drug-sensitivity pattern of conventional LTCCs in electrically excitable cells. A common feature of these channels is their sensitivity to dihydropyridine (DHP) derivatives, such as nifedipine. The DHP receptor is well known originally as the α₁-subunit of LTCCs in excitable cells [45]. LTCCs in neurons and myocytes are heterotetrameric polypeptide complexes consisting of a channel-forming α₁-subunit, and at least three auxiliary

subunits (α_2/δ , γ and β) that specifically modulate the activity and allow depolarization-induced Ca^{2+} influx into the cytosol [45]. The predicted topology of the α_1 -subunit contains four repeated motifs (I–IV) and an inward-dipping loop between the S5 and S6 transmembrane segments that forms the channel pore, while the S4 transmembrane segment contains conserved positively charged amino acids that are voltage sensors and move outward upon membrane depolarization and open the Ca^{2+} channel by analogy with the voltage-gated K^+ channel [46]. The spectrum of DHP derivatives, which specifically bind with high affinities to the α_1 -subunits of LTCCs and regulate their functional state from closed to open or vice versa, allows both the identification and functional analyses of this class of molecules. Human and rodent T cells express transcripts and/or proteins of the α_{1S} ($\text{Ca}_v1.1$), α_{1C} ($\text{Ca}_v1.2$), α_{1D} ($\text{Ca}_v1.3$) and/or α_{1F} ($\text{Ca}_v1.4$) subunits [47,48]. In addition, various splicing variants and isoforms of $\text{Ca}_v1.2$, $\text{Ca}_v1.3$ and $\text{Ca}_v1.4$, together with auxiliary β -subunits, have been detected in human and mouse lymphocytes [47–49]. However, the issue of whether these channels are voltage-gated (gated by membrane depolarization) remains a matter of debate. It has been shown that LTCC agonists such as BayK8644 evoke robust Ca^{2+} influxes in Jurkat T cells and human peripheral blood T cells, which are blocked by the LTCC antagonist nifedipine [47]. On the other hand, in most experiments, high K^+ loading alone evokes minimal Ca^{2+} influxes in these cells [48]. It should be noted that some variants lack the voltage-sensing S4 transmembrane segment [49], which may explain why the activation of LTCC-like channels is independent of membrane depolarization. Mast cells express $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ and the LTCC activity is activated by antigen stimulation to regulate mediator release in a distinct manner from CRAC channels [50]. The lower expression of $\text{Ca}_v1.4$ can only be observed by nested PCR. Similar to the conventional LTCCs in excitable cells and T cells, the LTCC activity observed in mast cells is activated independently of Ca^{2+} store emptying and is sensitive to DHP derivatives and other Ca^{2+} channel blockers. We recently reported that, similar to the case for antigen stimulation [51], high K^+ loading evokes a robust Ca^{2+} influx in mast cells [50] that have been depleted of the ER Ca^{2+} stores, although thapsigargin induces no Ca^{2+} influx in these cells [51]. Moreover, both K^+ and antigen stimulation induce substantial Ca^{2+} influxes into mitochondria in unmanipulated cells, and these Ca^{2+} responses are blocked by nifedipine, diltiazem and verapamil [50,51] or by gene knockdown of $\text{Ca}_v1.2$ (unpublished data). Collectively, these observations suggest that certain LTCCs such as $\text{Ca}_v1.2$ are activated by membrane depolarization and contribute to Ca^{2+} influx into mast cells. Thus, an emerging view is that LTCCs comprise alternative Ca^{2+} entry pathways in immune cells. Specifically, aspirin at low concentrations (≤ 0.3 mM) augments the LTCC activity, whereas at higher concentrations (>1 mM), it suppresses the LTCC activity [11]. More recently, we found that in mast cells with knocked down of $\text{Ca}_v1.2$ gene expression, aspirin failed to affect the LTCC activity as well as Ca^{2+} influx, thereby indicating that $\text{Ca}_v1.2$ mediates the effects of aspirin (unpublished data). Despite the essential role of external Ca^{2+} entry in generating LTC_4 secretion, attention has only recently been paid to the Ca^{2+} channels involved in this entry. Biochemical analyses revealed that CRAC channels play key roles in AA release, cPLA₂ activation and LTC_4 secretion [52,53]. Recently, it has been revealed that the mammalian proteins stromal interaction molecule 1 (STIM1) and Orai1/CRAC modulator 1 (CRACM1) mediate the functions of CRAC channels (for reviews, see [54,55]). STIM1 senses the Ca^{2+} concentration in the ER and activates CRAC channels, while Orai1 is the pore-forming subunit of CRAC channels. The discovery of these molecules has enabled genetic analyses of the role of CRAC channels in LTC_4 secretion in mast cells. It was revealed

that LT secretion is strongly inhibited in mast cells derived from Orai1-knockout mice [56]. Thus, CRAC channels seem to be the major routes of Ca^{2+} entry involved in LTc_4 secretion. Our data are apparently inconsistent with that view, since aspirin impairs CRAC channel activity but facilitates Ca^{2+} influx and LTc_4 secretion. We found that even when CRAC channel activity is impaired, antigen stimulation still evokes robust LTc_4 secretion and that aspirin augments this secretion [11]. Taken together with the aspirin-mediated facilitation of LTCC activity, these data support the view that an LTCC-mediated, CRAC channel-independent LTc_4 secretion pathway exists, and that aspirin (and possibly salicylates) targets this pathway (Figures 1 and 2).

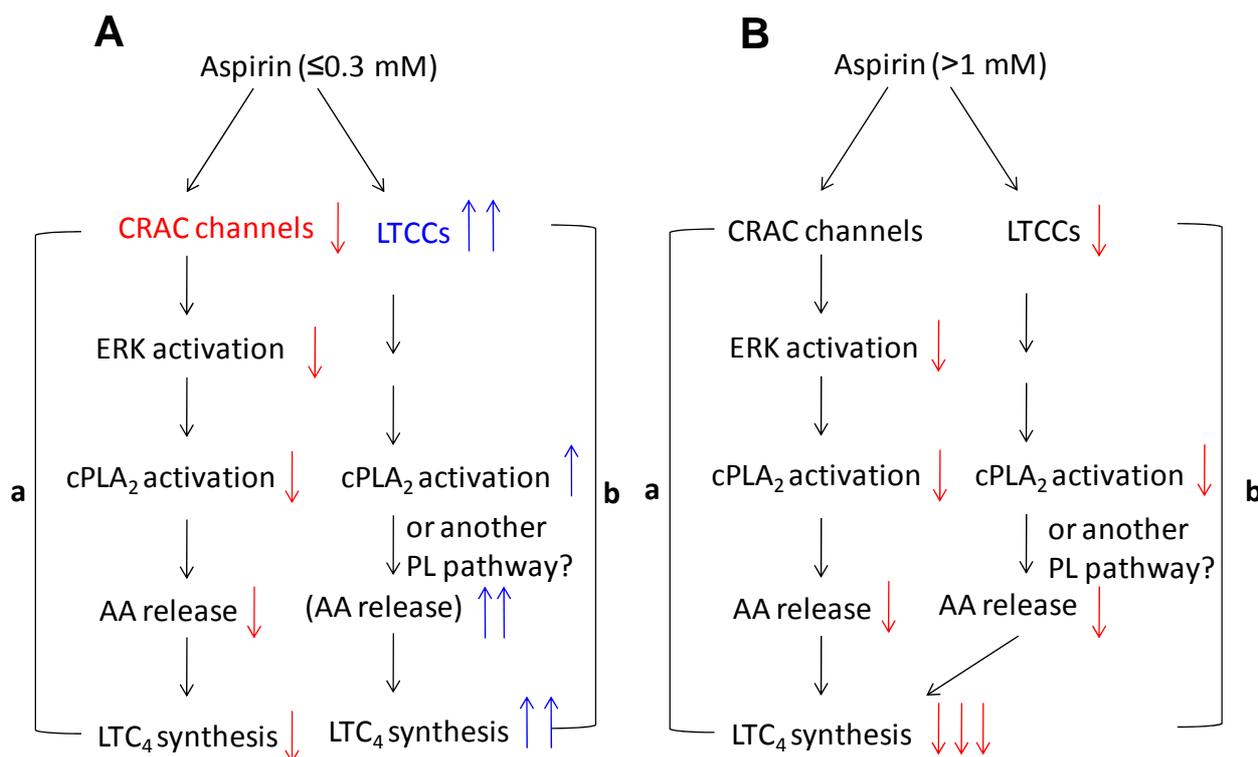
Figure 1. A model for Ca^{2+} signaling in mast cells.



Cross-linking of IgE-bound FcεRI by multivalent antigens results in Ca^{2+} store depletion followed by the activation of store-operated Ca^{2+} (SOC) channels such as CRAC channels. Energized mitochondria, which are physically associated with the ER, take up Ca^{2+} via the mitochondrial membrane potential-driven Ca^{2+} uniporters, thereby promoting the Ca^{2+} store depletion and CRAC channel activation. In addition, mitochondria may move to the cell membrane and enhance Ca^{2+} -dependent processes by increasing the local Ca^{2+} concentration through their Ca^{2+} uptake and release. It is becoming increasingly evident that LTCCs comprise an alternative pathway of Ca^{2+} influx and play a role in mediator release and cell survival. Some of these LTCCs may be voltage-gated or at least activated in a voltage-dependent manner, and participate in the maintenance of mitochondrial Ca^{2+} homeostasis, because high K^+ loading (membrane depolarization) can evoke a robust mitochondrial Ca^{2+} rise in an external Ca^{2+} -dependent manner and this Ca^{2+} signal is completely abolished by LTCC inhibitors and gene knockdown. This Ca^{2+} uptake also could be accelerated by the approach of the mitochondria to the cell membrane. It should be noticed that LTCC activation is facilitated in depolarized mitochondria, in which the CRAC channel activation is strongly impaired.

Although further studies are necessary to establish this view and the biological significance of such an alternative pathway, it should be noted that the LTCC-mediated LTC₄ secretion pathway is facilitated by mitochondrial depolarization, which strongly impairs the CRAC channel-mediated Ca²⁺ influx and LTC₄ secretion [11,52,57]. In the inflammatory milieu, mast cells may be exposed to oxidative stress, the major cause of mitochondrial depolarization, leading to inactivation of CRAC channel-mediated LTC₄ secretion. It is likely that under such conditions, low doses of aspirin facilitate LTC₄ secretion through the LTCC pathway, thereby leading to the exacerbation of allergic reactions, while high doses of aspirin block both of the two Ca²⁺ channel pathways, thereby strongly dampening LTC₄ secretion (Figure 1). This scenario is consistent with the clinical observations that aspirin intolerance is induced by low doses of aspirin and that patients with aspirin intolerance can be desensitized to aspirin by oral challenges with high doses of aspirin, which results in reduced LT secretion [37,58–60]. Thus, unveiling the molecular mechanisms underlying NSAID modulation of Ca²⁺ channel activities could contribute to better understanding of their anti-inflammatory actions as well as their immunological side effects.

Figure 2. Dual effects of NSAIDs on the novel LTCC-mediated LTC₄ synthesis pathway.



Ca²⁺ influx through CRAC channels in concert with ERK1/2 or p38MAPK activation causes increased LTC₄ synthesis via PLA₂ activation (pathway a). Activation of Ca_v1.2 LTCCs also causes activation of PLA₂ and possibly different types of phospholipases (PLs), thereby increasing LTC₄ synthesis (pathway b). Unlike the CRAC channel-mediated pathway, this pathway seems to be activated independently of the activation of ERK1/2 or p38MAPK. At low concentrations (≤0.3 mM), aspirin augments the LTCC-mediated LTC₄ synthesis pathway, whereas at higher concentrations (>1 mM), it inhibits this pathway. Aspirin at concentrations of ≥0.3 mM substantially inhibits the CRAC channel pathway in a concentration-dependent manner. Similar dual effects on the LTCC-mediated LTC₄ synthesis pathway were observed with salicylates.

4. Roles of ROS, Ca²⁺ and Mitochondria in the Chemopreventive Effects of NSAIDs

Much attention has been paid to the antineoplastic and chemopreventive effects of NSAIDs. Some clinical observations and epidemiological studies on numerous populations have revealed that prolonged use of aspirin and other NSAIDs reduces the risks of cancers of the colon and other gastrointestinal organs as well as those of the breast, prostate, lung and skin [13–19]. By definition, cancer chemoprevention is slowing, reversing or inhibiting carcinogenesis by the use of chemical agents, thereby lowering the risk of developing cancer. A growing list of agents including NSAIDs have been reported to have cancer chemopreventive activities, and many of them behave as apoptosis-inducing agents in animal and human cancer cells (for reviews, see [3,26,61–63]), consistent with the view that the commitment of these cells to cell death is an important mechanism underlying the chemopreventive effects. Different COX-independent mechanisms have been proposed to be involved in the chemopreventive and/or apoptosis-inducing effects of NSAIDs [3,26]. These mechanisms involve downregulation of NF- κ B activity [8,64], inhibition of the protein kinase B/Akt pathway [65,66], alterations in the levels of proapoptotic- and antiapoptotic proteins [67–69], activation of extrinsic and intrinsic pathways of apoptosis [70–73] and modulation of glucose and energy metabolisms [74,75]. Among these, we focus on the activation of the intrinsic or mitochondrial apoptosis pathway, since the vast majority of putative chemopreventive agents, including retinoids (e.g., all-*trans* retinoic acid, 9-*cis*-retinoic acid, *N*-(4-hydroxyphenyl)retinamide), vanilloids (e.g., capsaicin and resiniferatoxin), rotenoids (rotenone and deguelin) and polyphenols (curcumin, epigallocatechin gallate and resveratrol) appear to initiate apoptosis via this pathway [61,63,64]. Besides their well-known role as the power plants in eukaryotic cells, mitochondria are now recognized as central gateway controllers of the intrinsic or mitochondrial apoptotic pathway. Permeabilization of the outer mitochondrial membrane (OMM) by proapoptotic Bcl-2 family proteins promotes the release of a number of apoptogenic factors, such as cytochrome c, endonuclease G, second mitochondrial activator of caspases, Omi/HtrA2 and apoptosis-inducing factor (AIF), from the inner mitochondrial membrane (IMM) space into the cytosol, and these apoptogenic proteins promote the activation of the caspase cascade, thereby leading to apoptosis. Cytochrome c interacts with the apoptotic peptidase activating factor 1, leading to the formation of the multimeric apoptosome in the presence of ATP/dATP [76,77].

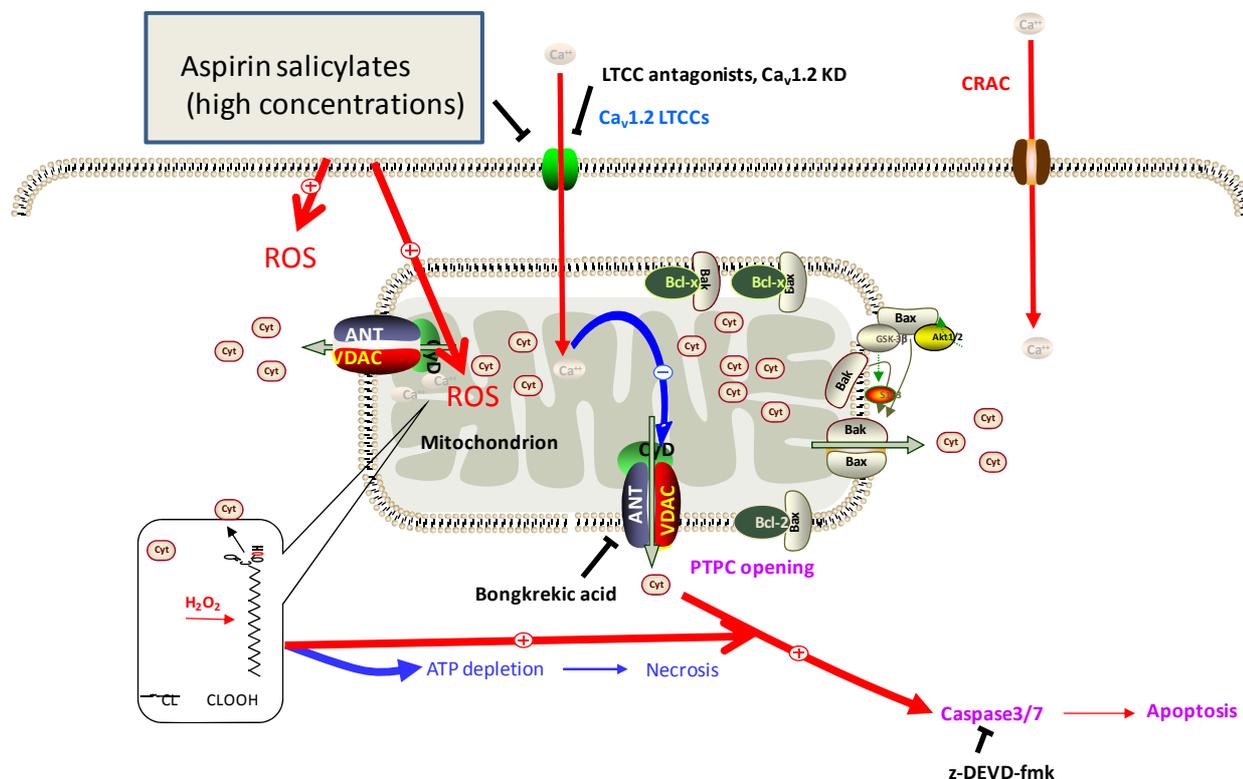
The apoptosome then activates the initiator caspase (caspase 9), which subsequently cleaves and activates the effector caspases (caspases 3 and 7). A cytochrome c-independent apoptosis pathway has also been defined, and this pathway requires proteins such as endonuclease G and AIF to carry out apoptosis. Hence, in this paradigm, mitochondrial integrity disruption and downstream apoptogenic protein release and caspase activation play pivotal roles. Although the molecular mechanisms underlying the OMM permeabilization are poorly understood, there is general agreement in the literature that the mitochondrial permeability transition (MPT), which was originally defined as a sudden increase in the IMM permeability to solutes with molecular masses of ~1500 Da, is involved. It is now believed that opening of a putative megachannel referred as to the mitochondrial permeability transition complex (PTPC) occurs [78,79]. The PTPC is a high-conductance non-specific pore in the IMM that is composed of proteins that link the IMM and OMM. Several mitochondrial proteins localized in the IMM and OMM, such as voltage-dependent anion channels (VDACs), adenine

nucleotide translocase (ANT), hexokinase, peripheral benzodiazepine receptors and cyclophilin-D are thought to constitute the PTPC. Under physiological conditions, the proteins in the OMM and IMM that constitute the PTPC are believed in close proximity to one another and in a closed or low conductance formation, although the PTPC has not been isolated and the components of this complex remain controversial [78–80]. When the PTPC changes to an open conformation, water and solutes with molecular masses of up to 1500 Da enter into the mitochondrial matrix, resulting in osmotic swelling of the mitochondrion. It has been believed that when multiple PTPCs open concurrently and extensive mitochondrial swelling takes place, physical disorganization of the OMM occurs and mitochondrial apoptogenic proteins are released, thereby triggering apoptosis [81]. Therefore, much attention has been paid to the potential role of PTPCs as a target for anticancer chemopreventive agents including NSAIDs [26,81,82]. For several reasons, reactive oxygen species (ROS) are believed to play a key role in MPT induction by affecting the PTPC conformation. First, ROS are byproducts of oxidative phosphorylation and excessive ROS generation is potentially deleterious to mitochondrial and cellular functions. Second, ANT has three cysteine residues whose oxidation is critical for PTPC open-closed transitions and Ca^{2+} release from the mitochondrial matrix, and PTPCs are believed to be particularly vulnerable to ROS [78–80]. Consequently, the MPT can be triggered by excessive mitochondrial ROS generation and/or disruption of the mitochondrial redox homeostasis [83–85]. Third, within mitochondria, cytochrome c is bound to the outer surface of the IMM by its association with the mitochondrial phospholipid cardiolipin, and oxidation of cardiolipin is thought to decrease this contact [86]. Thus, oxidation of cardiolipin may also be required to liberate sufficient cytochrome c to trigger caspase activation and induce apoptosis. The MPT also results in dissipation of the mitochondrial membrane potential and enhances ROS production via disintegration of the electron transport chain, thereby progressively shutting down oxidative phosphorylation and impairing energetic metabolism [87]. Hence, the MPT is a rate-limiting and self-amplifying process for apoptosis in which ROS play key roles.

Another biochemical change that has been associated with the induction of apoptosis in several cell types is deregulation of the intracellular Ca^{2+} concentrations. Excessive intracellular Ca^{2+} levels, such as those induced by Ca^{2+} ionophores have been shown to induce apoptosis [88,89]. Moreover, apoptosis appears to involve a Ca^{2+} -dependent endonuclease [90], and intracellular Ca^{2+} increases have been linked to apoptosis of both activated T cell hybridomas [91] and immature thymocytes [92]. In addition to its pro-apoptotic effects, Ca^{2+} has also been shown to act as an anti-apoptotic factor. IL-3-dependent primary cultured mast cells and cell lines can be protected against growth factor withdrawal-mediated apoptosis by the addition of Ca^{2+} ionophores [93], and programmed neuronal death is also suppressed by an increase in intracellular Ca^{2+} [94]. Collectively, Ca^{2+} appears to be necessary for both inducing and protecting against cell death, and the roles of Ca^{2+} in regulating cell death therefore seems to be more complex than initially thought. There is no general model that can depict the dual effects of Ca^{2+} . It is now widely accepted that mitochondria play a key role in regulating intracellular Ca^{2+} concentrations. It is quite likely that an appropriate elevation in the mitochondrial Ca^{2+} concentration ($[\text{Ca}^{2+}]_m$) supports energy metabolism, cell activation and cell survival, whereas $[\text{Ca}^{2+}]_m$ overload causes increased cell death [95,96]. There is general agreement in the literature that $[\text{Ca}^{2+}]_m$ overload can damage mitochondrial integrity, thereby inducing PTPC opening [97,98] and resulting in the release of apoptogenic proteins. On the other hand, it has been

shown that maintenance of $[Ca^{2+}]_m$ homeostasis is essential for cell survival, and that loss of $[Ca^{2+}]_m$ is closely correlated with cell death in cultured cells [99]. Collectively, ROS and Ca^{2+} are excellent targets for NSAIDs in regulating mitochondrial cell death. In fact, certain NSAIDs including aspirin, salicylates and aspirin analogs such as phosphoaspirin and nitric oxide (NO)-generating aspirin have been shown to exert proapoptotic effects on cancer cells via oxidative stress and/or ROS/NO generation [100–102]. However, it remains unclear whether the effects of NSAIDs on ROS generation are direct or indirect, and the molecular mechanisms of the oxidative responses are poorly understood.

There is much less available information regarding the effects of NSAIDs on cellular and mitochondrial Ca^{2+} concentrations. As mentioned above (Section 3), we recently found that aspirin modulates both CRAC channel and $Ca_v1.2$ LTCC activities. One of the most attractive properties of $Ca_v1.2$ LTCCs is their anti-apoptotic function. $Ca_v1.2$ LTCCs protect mast cells against activation-induced cell death by preventing mitochondrial integrity collapse and the mitochondrial cell death pathway [103]. Pharmacological (e.g., LTCC antagonists) or genetic (gene knockdown) blockade of $Ca_v1.2$ LTCC activity causes substantial apoptosis in activated cells. Moreover, activation (K^+ loading) or augmentation (e.g., LTCC agonists) of $Ca_v1.2$ LTCC activity protects mast cells against thapsigargin-induced apoptosis [103]. This prevention is accompanied by significant maintenance of the $[Ca^{2+}]_m$ levels (unpublished data). Taken together with our data that $Ca_v1.2$ LTCCs are necessary for mitochondrial Ca^{2+} uptake, it is quite possible that Ca^{2+} introduced via $Ca_v1.2$ LTCCs is important for the maintenance of $[Ca^{2+}]_m$, thereby conveying a pro-survival signal. Consequently, blockade of LTCC-mediated anti-apoptotic Ca^{2+} signaling by relatively high concentrations of aspirin and salicylates may be a novel mechanism underlying their apoptosis-inducing effects (Figure 3). Specifically, we found that inhibition of $Ca_v1.2$ LTCC activity affects the survival of tumor mast cells more markedly than that of primary mast cells [103], thereby suggesting that tumor cells rely more heavily on the LTCC-mediated pro-survival pathway than normal cells. In this regard, it should be noted that LTCC expression is up-regulated and/or LTCC activity is elevated in human cancer cells such as colon cancer and leukemia cells compared with their normal counterparts [104–106]. Moreover, the flavonoid wogonin has been shown to kill malignant T cells (in T cell leukemia), but not peripheral blood T cells by affecting LTCCs [106]. These observations are consistent with the view that cancer cells are more sensitive to the interference of LTCC activity than normal cells. We previously reported that NO generation via NO synthase (NOS) activity is necessary for the maintenance of cell mitochondrial integrity and cell survival in rat basophilic leukemia cells [107].

Figure 3. Proposed model for the apoptosis-inducing effects of NSAIDs.

Besides permeabilization of the OMM by proapoptotic Bcl-2 family proteins such as Bax and Bak, which are believed to form a putative pore, the PTPC opening is important to trigger the mitochondrial integrity disruption and subsequent the release of apoptogenic proteins such as cytochrome c. Once this megachannel changes to an open conformation, water and solutes with molecular masses of up to 1500 Da enter into the mitochondrial matrix, resulting in osmotic swelling of the mitochondrion. When multiple PTPCs open concurrently and extensive mitochondrial swelling takes place, physical disorganization of the OMM and release of mitochondrial apoptogenic proteins occur, thereby triggering apoptosis. In series of recent studies, we have revealed that activation of Ca_v1.2 LTCCs primarily causes Ca²⁺ entry into the mitochondria, and this Ca²⁺ response is necessary for preventing the collapse of the mitochondrial transmembrane potential and the opening of PTPCs. NSAIDs such as aspirin and salicylates block this anti-apoptotic pathway when used at high concentrations, thereby exerting an apoptosis-inducing effect. The generation of ROS by NSAIDs may also be important for exerting their apoptosis-inducing properties. ROS are byproducts of oxidative phosphorylation, and excessive ROS generation is potentially deleterious to mitochondrial and cellular functions and a potential cause of PTPC opening, because ANT, the critical component of PTPCs, is particularly vulnerable to ROS. In addition, the generation of ROS, especially in mitochondria, causes the oxidation of cardiolipin (CL) to CL hydroperoxide (CLOOH), which decreases its contact with cytochrome c, thereby facilitating the release of cytochrome c from the IMM and activating the mitochondrial death pathway.

Subsequent studies revealed that endothelial NOS (eNOS) is essential for the generation of NO and activation of Ca_v1.2 LTCCs [108]. Importantly, knockdown of the expression of Ca_v1.2 LTCC [103] or eNOS [108] has minimal effects on cell survival in the resting state, thereby indicating that eNOS

and $\text{Ca}_v1.2$ LTCCs are specifically required for the survival of activated cells. Given that eNOS is activated by the PI3K-Akt pathway [107], it is most likely that NO generated by the PI3K-Akt-dependent eNOS activation pathway positively regulates the $\text{Ca}_v1.2$ LTCC activity. Interestingly, the PI3K-Akt pathway and/or eNOS have been shown to play key roles in the survival of various cell types as well as in chronic inflammation and cancer [109–115]. Since the absence of $\text{Ca}_v1.2$ LTCCs is significantly compensated for by blocking PTPC opening or inhibiting the downstream caspase cascade pathway, this type of Ca^{2+} channel may prevent extensive PTPC opening, thereby playing a key role in the maintenance of mitochondrial integrity. Taken together with several of the above-mentioned lines of evidence that (i) gene expression of LTCCs is up-regulated in cancer cells and LTCC activities are elevated compared with normal cells, (ii) cancer cell survival seems to rely more heavily on this type of Ca^{2+} channel than normal cell survival, (iii) these Ca^{2+} channel activities are necessary for the maintenance of mitochondrial integrity and prevention of apoptosis and (iv) several chemopreventive agents such as aspirin, salicylates and wogonin commonly affect these Ca^{2+} channel activities, LTCCs may be promising target molecules for cancer prevention and therapy.

5. Conclusions and Perspectives

Recent studies have revealed unequivocally that certain NSAIDs exert their anti-inflammatory and cancer chemopreventive effects, as well as certain side effects, independently of COX activity and PG synthesis inhibition. It is very clear in the literature that multiple pathways are involved in these effects, but they are not shared by all NSAIDs. In this review, we have discussed the molecular basis of an emerging view that Ca^{2+} and mitochondria are novel and potentially more generalized targets for the biological effects of NSAIDs, as well as their side effects. If induction of apoptosis is the final goal of cancer chemopreventive drugs, better understanding of the molecular mechanisms underlying the aspirin-mediated modulation of PTPCs and LTCCs may help toward the development of cancer-selective drugs and/or therapies, since cancer cells seem to more sensitive to the modulation of these two types of channels than normal cells.

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References

1. Vane, J.R. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat. New Biol.* **1971**, *231*, 232–235.
2. Wu, K.K. Aspirin and other cyclooxygenase inhibitors: New therapeutic insights. *Semin. Vasc. Med.* **2003**, *3*, 107–112.

3. Tegeder, I.; Pfeilschifter, J.; Geisslinger, G. Cyclooxygenase-independent actions of cyclooxygenase inhibitors. *FASEB J.* **2001**, *15*, 2057–2072.
4. Chiabrande, C.; Castelli, M.G.; Cozzi, E.; Fanelli, R.; Campoleoni, A.; Balotta, C.; Latini, R.; Garattini, S. Antiinflammatory action of salicylates: Aspirin is not a prodrug for salicylate against rat carrageenin pleurisy. *Eur. J. Pharmacol.* **1989**, *159*, 257–264.
5. April, P.; Abeles, M.; Baraf, H.; Cohen, S.; Curran, N.; Doucette, M.; Ekholm, B.; Goldlust, B.; Knee, C.M.; Lee, E.; *et al.* Does the acetyl group of aspirin contribute to the antiinflammatory efficacy of salicylic acid in the treatment of rheumatoid arthritis? *Semin. Arthritis Rheum.* **1990**, *19*, 20–28.
6. Preston, S.J.; Arnold, M.H.; Beller, E.M.; Brooks, P.M.; Buchanan, W.W. Comparative analgesic and anti-inflammatory properties of sodium salicylate and acetylsalicylic acid (aspirin) in rheumatoid arthritis. *Br. J. Clin. Pharmacol.* **1989**, *27*, 607–611.
7. Tegeder, I.; Niederberger, E.; Israr, E.; Gühring, H.; Brune, K.; Euchenhofer, C.; Grösch, S.; Geisslinger, G. Inhibition of NF-kappaB and AP-1 activation by R- and S-flurbiprofen. *FASEB J.* **2001**, *15*, 2–4.
8. Kopp, E.; Ghosh, S. Inhibition of NF-kappa B by sodium salicylate and aspirin. *Science* **1994**, *265*, 956–959.
9. Román, J.; de Arriba, A.F.; Barrón, S.; Michelena, P.; Giral, M.; Merlos, M.; Bailón, E.; Comalada, M.; Gálvez, J.; Zarzuelo, A.; Ramis, I. UR-1505, a new salicylate, blocks T cell activation through nuclear factor of activated T cells. *Mol. Pharmacol.* **2007**, *72*, 269–279.
10. Mortaz, E.; Redegeld, F.A.; Nijkamp, F.P.; Engels, F. Dual effects of acetylsalicylic acid on mast cell degranulation, expression of cyclooxygenase-2 and release of pro-inflammatory cytokines. *Biochem. Pharmacol.* **2005**, *69*, 1049–1057.
11. Togo, K.; Suzuki, Y.; Yoshimaru, T.; Inoue, T.; Terui, T.; Ochiai, T.; Ra, C. Aspirin and salicylates modulate IgE-mediated leukotriene secretion in mast cells through a dihydropyridine receptor-mediated Ca^{2+} influx. *Clin. Immunol.* **2009**, *131*, 145–156.
12. Suzuki, Y.; Yoshimaru, T.; Inoue, T.; Terui, T.; Ochiai, T.; Ra, C. Analysis of the mechanism for the development of allergic skin inflammation and the application for its treatment: Aspirin modulation of IgE-dependent mast cell activation: Role of aspirin-induced exacerbation of immediate allergy. *J. Pharmacol. Sci.* **2009**, *110*, 237–244.
13. Gupta, R.A.; DuBois, R.N. Aspirin, NSAIDS, and colon cancer prevention: mechanisms? *Gastroenterology* **1998**, *114*, 1095–1098.
14. Thun, M.J.; Henley, S.J.; Patrono, C. Nonsteroidal anti-inflammatory drugs as anticancer agents: Mechanistic, pharmacologic, and clinical issues. *J. Natl. Cancer Inst.* **2002**, *94*, 252–266.
15. Ulrich, C.M.; Bigler, J.; Potter, J.D. Non-steroidal anti-inflammatory drugs for cancer prevention: promise, perils and pharmacogenetics. *Nat. Rev. Cancer* **2006**, *6*, 130–140.
16. Akre, K.; Ekström, A.M.; Signorello, L.B.; Hansson, L.E.; Nyrén, O. Aspirin and risk for gastric cancer: A population-based case-control study in Sweden. *Br. J. Cancer* **2001**, *84*, 965–968.
17. Schreinemachers, D.M.; Everson, R.B. Aspirin use and lung, colon, and breast cancer incidence in a prospective study. *Epidemiology* **1994**, *5*, 138–146.

18. Jacobs, E.J.; Thun, M.J.; Bain, E.B.; Rodriguez, C.; Henley, S.J.; Calle, E.E. A large cohort study of long-term daily use of adult-strength aspirin and cancer incidence. *J. Natl. Cancer Inst.* **2007**, *99*, 608–615.
19. Schildkraut, J.M.; Moorman, P.G.; Halabi, S.; Calingaert, B.; Marks, J.R.; Berchuck, A. Analgesic drug use and risk of ovarian cancer. *Epidemiology* **2006**, *17*, 104–107.
20. Sheng, H.; Shao, J.; Morrow, J.D.; Beauchamp, R.D.; DuBois, R.N. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res.* **1998**, *58*, 362–366.
21. Tsujii, M.; DuBois, R.N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell* **1995**, *83*, 493–501.
22. Masferrer, J.L.; Leahy, K.M.; Koki, A.T.; Zweifel, B.S.; Settle, S.L.; Woerner, B.M.; Edwards, D.A.; Flickinger, A.G.; Moore, R.J.; Seibert, K. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. *Cancer Res.* **2000**, *60*, 1306–1311.
23. Hanif, R.; Pittas, A.; Feng, Y.; Koutsos, M.I.; Qiao, L.; Staiano-Coico, L.; Shiff, S.I.; Rigas, B. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem. Pharmacol.* **1999**, *52*, 235–245.
24. Lai, M.Y.; Huang, J.A.; Liang, Z.H.; Jiang, H.X.; Tang, G.D. Mechanisms underlying aspirin-mediated growth inhibition and apoptosis induction of cyclooxygenase-2 negative colon cancer cell line SW480. *World J. Gastroenterol.* **2008**, *14*, 4227–4233.
25. Zhang, X.; Morham, S.G.; Langenbach, R.; Young, D.A. Malignant transformation and antineoplastic actions of nonsteroidal antiinflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts. *J. Exp. Med.* **1999**, *190*, 451–459.
26. Jana, N.R. NSAIDs and apoptosis. *Cell Mol. Life Sci.* **2008**, *65*, 1295–1301.
27. Sung, J.; Russell, R.I.; Nyeomans, Chan, F.K.; Chen, S.; Fock, K.; Goh, K.L.; Kullavanijaya, P.; Kimura, K.; Lau, C.; Louw, J.; Sollano, J.; Triadialafalopulos, G.; Xiao, S.; Brooks, P. Non-steroidal anti-inflammatory drug toxicity in the upper gastrointestinal tract. *J. Gastroenterol. Hepatol.* **2000**, *15* (Suppl.), G58–G68.
28. Grattan, C.E. Aspirin sensitivity and urticaria. *Clin. Exp. Dermatol.* **2003**, *28*, 123–127.
29. Ying, S.; Corrigan, C.J.; Lee, T.H. Mechanisms of aspirin-sensitive asthma. *Allergol. Int.* **2004**, *53*, 111–119.
30. Morita, E.; Kunie, K.; Matsuo, H. Food-dependent exercise-induced anaphylaxis. *J. Dermatol. Sci.* **2007**, *47*, 109–117.
31. Kinet, J.P. The high-affinity IgE receptor (FcεRI): From physiology to pathology. *Annu. Rev. Immunol.* **1999**, *17*, 931–972.
32. Mortaz, E.; Engels, F.; Nijkamp, F.P.; Redegeld, F.A. New insights on the possible role of mast cells in aspirin-induced asthma. *Curr. Mol. Pharmacol.* **2009**, *2*, 182–189.
33. Bae, J.S.; Kim, S.H.; Ye, Y.M.; Yoon, H.J.; Suh, C.H. Nahm, D.H.; Park, H.S. Significant association of FcεRIα promoter polymorphisms with aspirin-intolerant chronic urticaria. *J. Allergy Clin. Immunol.* **2007**, *119*, 449–456.

34. Sullivan, S.; Dahlén, B.; Dahlén, S.E.; Kumlin, M. Increased urinary excretion of the prostaglandin D₂ metabolite 9 α , 11 β -prostaglandin F₂ after aspirin challenge supports mast cell activation in aspirin-induced airway obstruction. *J. Allergy Clin. Immunol.* **1996**, *98*, 421–432.
35. Mita, H.; Endoh, S.; Kudoh, M.; Kawagishi, Y.; Kobayashi, M.; Taniguchi, M.; Akiyama, K. Possible involvement of mast-cell activation in aspirin provocation of aspirin-induced asthma. *Allergy* **2001**, *56*, 1061–1067.
36. Wang, X.S.; Wu, A.Y.; Leung, P.S.; Lau, H.Y. PGE₂ suppresses excessive anti-IgE induced cysteinyl leucotrienes production in mast cells of patients with aspirin exacerbated respiratory disease. *Allergy* **2007**, *62*, 620–627.
37. Stevenson, D.D.; Hankammer, M.A.; Mathison, D.A.; Christiansen, S.C.; Simon, R.A. Aspirin desensitization treatment of aspirin-sensitive patients with rhinosinusitis-asthma: Long-term outcomes. *J. Allergy Clin. Immunol.* **1996**, *98*, 751–758.
38. Leslie, C.C. Properties and regulation of cytosolic phospholipase A₂. *J. Biol. Chem.* **1997**, *272*, 16709–16712.
39. Gijón, M.A.; Leslie, C.C. Regulation of arachidonic acid release and cytosolic phospholipase A₂ activation. *J. Leukoc. Biol.* **1999**, *65*, 330–336.
40. Nemenoff, R.A.; Winitz, S.; Qian, N.X.; Van Putten, V.; Johnson, G.L.; Heasley, L.E. Phosphorylation and activation of a high molecular weight form of phospholipase A₂ by p42 microtubule-associated protein 2 kinase and protein kinase C. *J. Biol. Chem.* **1993**, *268*, 1960–1964.
41. Hefner, Y.; Borsch-Haubold, A.G.; Murakami, M.; Wilde, J.I.; Pasquet, S.; Schieltz, D.; Ghomashchi, F.; Yates, J.R., III; Armstrong, C.G.; Paterson, A.; Cohen, P.; Fukunaga, R.; Hunter, T.; Kudo, I.; Watson, S.P.; Gelb, M.H. Serine 727 phosphorylation and activation of cytosolic phospholipase A₂ by MNK1-related protein kinases. *J. Biol. Chem.* **2000**, *275*, 37542–37551.
42. Parekh, A.B.; Putney, J.W., Jr. Store-operated calcium channels. *Physiol. Rev.* **2005**, *85*, 757–810.
43. Kotturi, M.F.; Hunt, S.V.; Jefferies, W.A. Roles of CRAC and Cav-like channels in T cells: More than one gatekeeper? *Trends Pharmacol. Sci.* **2006**, *27*, 360–367.
44. Suzuki, Y.; Inoue, T.; Ra, C. L-type Ca²⁺ channels: A new player in the regulation of Ca²⁺ signaling, cell activation and cell survival in immune cells. *Mol. Immunol.* **2010**, *47*, 640–648.
45. Bodi, I.; Mikala, G.; Koch, S.E.; Akhter, S.A.; Schwartz, A. The L-type calcium channel in the heart: the beat goes on. *J. Clin. Invest.* **2005**, *115*, 3306–3317.
46. Jiang, Y.; Ruta, V.; Chen, J.; Lee, A.; MacKinnon, R. The principle of gating charge movement in a voltage-dependent K⁺ channel. *Nature* **2003**, *423*, 42–48.
47. Kotturi, M.F.; Carlow, D.A.; Lee, J.C.; Ziltener, H.J.; Jefferies, W.A. Identification and functional characterization of voltage-dependent calcium channels in T lymphocytes. *J. Biol. Chem.* **2003**, *278*, 46949–46960.
48. Stokes, L.; Gordon, J.; Grafton, G. Non-voltage-gated L-type Ca²⁺ channels in human T cells. *J. Biol. Chem.* **2004**, *279*, 19566–19573.
49. Brereton, H.M.; Harland, M.L.; Froschio, M.; Petronijevic, T.; Barritt, G.J. Novel variants of voltage-operated calcium channel alpha 1-subunit transcripts in a rat liver-derived cell line: Deletion in the IVS4 voltage sensing region. *Cell Calcium* **1997**, *22*, 39–52.
50. Yoshimaru, T.; Suzuki, Y.; Inoue, T.; Ra, C. L-type Ca²⁺ channels in mast cells: activation by

- membrane depolarization and distinct roles in regulating mediator release from store-operated Ca^{2+} channels. *Mol. Immunol.* **2009**, *46*, 1267–1277.
51. Suzuki, Y.; Yoshimaru, T.; Inoue, T.; Nunomura, S.; Ra, C. The high-affinity immunoglobulin E receptor (Fc ϵ RI) regulates mitochondrial calcium uptake and a dihydropyridine receptor-mediated calcium influx in mast cells: Role of the Fc ϵ RI β chain immunoreceptor tyrosine-based activation motif. *Biochem. Pharmacol.* **2008**, *75*, 1492–1503.
 52. Chang, W.C.; Parekh, A.B. Close functional coupling between Ca^{2+} release-activated Ca^{2+} channels, arachidonic acid release, and leukotriene C_4 secretion. *J. Biol. Chem.* **2004**, *279*, 29994–29999.
 53. Chang, W.C.; Nelson, C.; Parekh, A.B. Ca^{2+} influx through CRAC channels activates cytosolic phospholipase A_2 , leukotriene C_4 secretion, and expression of c-fos through ERK-dependent and -independent pathways in mast cells. *FASEB J.* **2006**, *20*, 2381–2383.
 54. Feske, S. Calcium signalling in lymphocyte activation and disease. *Nat. Rev. Immunol.* **2007**, *7*, 690–702.
 55. Vig, M.; Kinet, J.P. Calcium signaling in immune cells. *Nat. Immunol.* **2009**, *10*, 21–27.
 56. Vig, M.; DeHaven, W.I.; Bird, G.S.; Billingsley, J.M.; Wang, H.; Rao, P.E.; Hutchings, A.B.; Jouvin, M.H.; Putney, J.W., Jr.; Kinet, J.P. Defective mast cell effector functions in mice lacking the CRACM1 pore subunit of store-operated calcium release-activated calcium channels. *Nat. Immunol.* **2008**, *9*, 89–96.
 57. Makowska, A.; Zablocki, K.; Duszyński, J. The role of mitochondria in the regulation of calcium influx into Jurkat cells. *Eur. J. Biochem.* **2000**, *267*, 877–884.
 58. Szczeklik, A.; Stevenson, D.D. Aspirin-induced asthma: advances in pathogenesis, diagnosis, and management. *J. Allergy Clin. Immunol.* **2003**, *111*, 913–921.
 59. Arm, J.P.; Austen, K.F. Leukotriene receptors and aspirin sensitivity. *N. Engl. J. Med.* **2002**, *347*, 1524–1526.
 60. Juergens, U.R.; Christiansen, S.C.; Stevenson, D.D.; Zuraw, B.L. Inhibition of monocyte leukotriene B_4 production after aspirin desensitization. *J. Allergy Clin. Immunol.* **1995**, *96*, 148–156.
 61. Hail, N., Jr.; Lotan, R. Cancer chemoprevention and mitochondria: Targeting apoptosis in transformed cells via the disruption of mitochondrial bioenergetics/redox state. *Mol. Nutr. Food Res.* **2009**, *53*, 49–67.
 62. Scatena, R.; Bottoni, P.; Botta, G.; Martorana, G.E.; Giardina, B. The role of mitochondria in pharmacotoxicology: A reevaluation of an old, newly emerging topic. *Am. J. Cell Physiol.* **2007**, *293*, C12–C21.
 63. Sun, S.Y.; Hail, N., Jr.; Lotan, R. Apoptosis as a novel target for cancer chemoprevention. *J. Natl. Cancer Inst.* **2002**, *96*, 662–672.
 64. Yin, M.J.; Yamamoto, Y.; Gaynor, R.B. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I κ B kinase β . *Nature* **1998**, *396*, 77–80.
 65. Hsu, A.L.; Ching, T.T.; Wang, D.S.; Song, X.; Rangnekar, V.M.; Chen, C.S. The cyclooxygenase-2 inhibitor celecoxib induces apoptosis by blocking Akt activation in human prostate cancer cells independently of Bcl-2. *J. Biol. Chem.* **2000**, *275*, 11397–11403.
 66. Lincová, E.; Hampl, A.; Pernicová, Z.; Starsichová, A.; Krcmár, P.; Machala, M.; Kozubík, A.;

- Soucek, K. Multiple defects in negative regulation of the PKB/Akt pathway sensitise human cancer cells to the antiproliferative effect of non-steroidal anti-inflammatory drugs. *Biochem. Pharmacol.* **2009**, *78*, 561–572.
67. Zhou, X.M.; Wong, B.C.; Fan, X.M.; Zhang, H.B.; Lin, M.C.; Kung, H.F.; Fan, D.M.; Lam, S.K. Non-steroidal anti-inflammatory drugs induce apoptosis in gastric cancer cells through up-regulation of bax and bak. *Carcinogenesis* **2001**, *22*, 1393–1397.
 68. Gu, Q.; Wang, J.D.; Xia, H.H.; Lin, M.C.; He, H.; Zou, B.; Tu, S.P.; Yang, Y.; Liu, X.G.; Lam, S.K.; Wong, W.M.; Chan, A.O.; Yuen, M.F.; Kung, H.F.; Wong, B.C. Activation of the caspase-8/Bid and Bax pathways in aspirin-induced apoptosis in gastric cancer. *Carcinogenesis* **2005**, *26*, 541–546.
 69. Ho, C.C.; Yang, X.W.; Lee, T.L.; Liao, P.H.; Yang, S.H.; Tsai, C.H.; Chou, M.Y. Activation of the caspase-8/Bid and Bax pathways in aspirin-induced apoptosis in gastric cancer. *Eur. J. Clin. Invest.* **2003**, *33*, 875–882.
 70. Zimmermann, K.C.; Waterhouse, N.J.; Goldstein, J.C.; Schuler, M.; Green, D.R. Aspirin induces apoptosis through release of cytochrome c from mitochondria. *Neoplasia* **2000**, *2*, 505–513.
 71. Piqué, M.; Barragán, M.; Dalmau, M.; Bellosillo, B.; Pons, G.; Gil, J. Aspirin induces apoptosis through mitochondrial cytochrome c release. *FEBS Lett.* **2000**, *480*, 193–196.
 72. Bellosillo, B.; Piqué, M.; Barragán, M.; Castaño, E.; Villamor, N.; Colomer, D.; Montserrat, E.; Pons, G.; Gil, J. Aspirin and salicylate induce apoptosis and activation of caspases in B-cell chronic lymphocytic leukemia cells. *Blood* **1998**, *92*, 1406–1414.
 73. Redlak, M.J.; Power, J.J.; Miller, T.A. Role of mitochondria in aspirin-induced apoptosis in human gastric epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2005**, *289*, G731–G738.
 74. Spitz, G.A.; Furtado, C.M.; Sola-Penna, M.; Zancan, P. Acetylsalicylic acid and salicylic acid decrease tumor cell viability and glucose metabolism modulating 6-phosphofructo-1-kinase structure and activity. *Biochem. Pharmacol.* **2009**, *77*, 46–53.
 75. Biban, C.; Tassani, V.; Toninello, A.; Siliprandi, D.; Siliprandi, N. The alterations in the energy linked properties induced in rat liver mitochondria by acetylsalicylate are prevented by cyclosporin A or Mg²⁺. *Biochem. Pharmacol.* **1995**, *50*, 497–500.
 76. Danial, N.N.; Korsmeyer, S.J. Cell death: Critical control points. *Cell* **2004**, *116*, 205–219.
 77. Yan, N.; Shi, Y. Mechanisms of apoptosis through structural biology. *Annu. Rev. Cell Dev. Biol.* **2005**, *21*, 35–56.
 78. Lemasters, J.J.; Theruvath, T.P.; Zhong, Z.; Nieminen, A.L. Mitochondrial calcium and the permeability transition in cell death. *Biochim. Biophys. Acta* **2009**, *1787*, 1395–1401.
 79. Halestrap, A.P.; Brennerb, C. The adenine nucleotide translocase: A central component of the mitochondrial permeability transition pore and key player in cell death. *Curr. Med. Chem.* **2003**, *10*, 1507–1525.
 80. Zhivotovsky, B.; Galluzzi, L.; Kepp, O.; Kroemer, G. Adenine nucleotide translocase: A component of the phylogenetically conserved cell death machinery. *Cell Death Differ.* **2009**, *16*, 1419–1425.
 81. Hail, N., Jr. Mitochondria: A novel target for the chemoprevention of cancer. *Apoptosis* **2005**, *10*, 687–705.

82. Brenner, C.; Grimm, S. The permeability transition pore complex in cancer cell death. *Oncogene* **2006**, *25*, 4744–4756.
83. Skulachev, V.P. Why are mitochondria involved in apoptosis? Permeability transition pores and apoptosis as selective mechanisms to eliminate superoxide-producing mitochondria and cell. *FEBS Lett.* **1996**, *397*, 7–10.
84. Orrenius, S.; Gogvadze, V.; Zhivotovsky, B. Mitochondrial oxidative stress: implications for cell death. *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 143–183.
85. Ralph, S.J.; Rodríguez-Enríquez, S.; Neuzil, J.; Moreno-Sánchez, R. Bioenergetic pathways in tumor mitochondria as targets for cancer therapy and the importance of the ROS-induced apoptotic trigger. *Mol. Aspects Med.* **2010**, *31*, 29–59.
86. Ott, M.; Zhivotovsky, B.; Orrenius, S. Role of cardiolipin in cytochrome c release from mitochondria. *Cell Death Differ.* **2007**, *14*, 1243–1247.
87. Kroemer, G.; Petit, P.; Zamzami, N.; Vayssière, J.L.; Mignotte, B. The biochemistry of programmed cell death. *FASEB J.* **1995**, *9*, 1277–1287.
88. Kizaki, H.; Tadakuma, T.; Odaka, C.; Muramatsu, J.; Ishimura, Y. Activation of a suicide process of thymocytes through DNA fragmentation by calcium ionophores and phorbol esters. *J. Immunol.* **1989**, *143*, 1790–1794.
89. Tadakuma, T.; Kizaki, H.; Odaka, C.; Kubota, R.; Ishimura, Y.; Yagita, H.; Okumura, K. CD4⁺CD8⁺ thymocytes are susceptible to DNA fragmentation induced by phorbol ester, calcium ionophore and anti-CD3 antibody. *Eur. J. Immunol.* **1990**, *20*, 779–784.
90. Ribeiro, J.M.; Carson, D.A. Ca²⁺/Mg²⁺-dependent endonuclease from human spleen: Purification, properties, and role in apoptosis. *Biochemistry* **1993**, *32*, 9129–9136.
91. Mercep, M.; Noguchi, P.D.; Ashwell, J.D. The cell cycle block and lysis of an activated T cell hybridoma are distinct processes with different Ca²⁺ requirements and sensitivity to cyclosporine A. *J. Immunol.* **1989**, *142*, 4085–4092.
92. McConkey, D.J.; Hartzell, P.; Amador-Perez, F.J.; Orrenius, S.; Jondal, M. Calcium-dependent killing of immature thymocytes by stimulation via the CD3/T cell receptor complex. *J. Immunol.* **1989**, *143*, 1801–1806.
93. Rodriguez-Tarduchy, G.; Prupti, M.; Lopez-Rivas, A.; Collins, M.K.L. Inhibition of apoptosis by calcium ionophores in IL-3-dependent bone marrow cells is dependent upon production of IL-4. *J. Immunol.* **1992**, *148*, 1416–1422.
94. Lampe, P.A.; Cornbrooks, E.B.; Juhasz, A.; Johnson, E.J.; Franklin, J.L. Suppression of programmed neuronal death by a thapsigargin-induced Ca²⁺ influx. *J. Neurobiol.* **1995**, *26*, 205–212.
95. Nicotera, P.; Zhivotovsky, B.; Orrenius, S. Nuclear calcium transport and the role of calcium in apoptosis. *Cell Calcium* **1994**, *16*, 279–288.
96. Dowd, D.R. Calcium regulation of apoptosis. *Adv. Second Messenger Phosphoprotein Res.* **1995**, *30*, 255–280.
97. Green, D.R.; Reed, J.C. Mitochondria and apoptosis. *Science* **1998**, *281*, 1309–1312.
98. Zhu, L.P.; Yu, X.D.; Ling, S.; Brown, R.A.; Kuo, T.H. Mitochondrial Ca²⁺ homeostasis in the regulation of apoptotic and necrotic cell deaths. *Cell Calcium* **2000**, *28*, 107–117.
99. Kuo, T.H.; Zhu, L.; Golden, K.; Marsh, J.D.; Bhattacharya, S.K.; Liu, B.F. Altered Ca²⁺

- homeostasis and impaired mitochondrial function in cardiomyopathy. *Mol. Cell Biochem.* **2002**, *272*, 187–199.
100. Vad, N.M.; Yount, G.; Moridani, M.Y. Biochemical mechanism of acetylsalicylic acid (Aspirin) selective toxicity toward melanoma cell lines. *Melanoma Res.* **2008**, *18*, 386–399.
 101. Zhao, W.; Mackenzie, G.G.; Murray, O.T.; Zhang, Z.; Rigas, B. Phosphoaspirin (MDC-43), a novel benzyl ester of aspirin, inhibits the growth of human cancer cell lines more potently than aspirin: A redox-dependent effect. *Carcinogenesis* **2009**, *30*, 512–519.
 102. Tesei, A.; Zoli, W.; Fabbri, F.; Leonetti, C.; Rosetti, M.; Bolla, M.; Amadori, D.; Silvestrini R. NCX 4040, an NO-donating acetylsalicylic acid derivative: Efficacy and mechanisms of action in cancer cells. *Nitric Oxide* **2008**, *19*, 225–236.
 103. Suzuki, Y.; Yoshimaru, T.; Inoue, T.; Ra, C. Ca_v1.2 L-type Ca²⁺ channel protects mast cells against activation-induced cell death by preventing mitochondrial integrity disruption. *Mol. Immunol.* **2009**, *46*, 2370–2380.
 104. Wang, X.T.; Nagaba, Y.; Cross, H.S.; Wrba, F.; Zhang, L.; Guggino, S.E. The mRNA of L-type calcium channel elevated in colon cancer: Protein distribution in normal and cancerous colon. *Am. J. Pathol.* **2000**, *157*, 1549–1562.
 105. Zawadzki, A.; Liu, Q.; Wang, Y.; Melander, A.; Jeppsson, B.; Thorlacius, H. Verapamil inhibits L-type calcium channel mediated apoptosis in human colon cancer cells. *Dis. Colon Rectum.* **2008**, *51*, 1696–1702.
 106. Baumann, S.; Fas, S.C.; Giaisi, M.; Müller, W.W.; Merling, A.; Gülow, K.; Edler, L.; Krammer, P.H.; Li-Weber, M. Wogonin preferentially kills malignant lymphocytes and suppresses T-cell tumor growth by inducing PLCgamma1- and Ca²⁺-dependent apoptosis. *Blood* **2008**, *111*, 2354–2363.
 107. Inoue, T.; Suzuki, Y.; Yoshimaru, T.; Ra, C. Nitric oxide protects mast cells from activation-induced cell death: The role of the phosphatidylinositol-3-kinase-Akt-endothelial nitric oxide synthase pathway. *J. Leukoc. Biol.* **2008**, *83*, 1218–1229.
 108. Suzuki, Y.; Inoue, T.; Ra, C. Endothelial nitric oxide synthase is essential for nitric oxide generation, L-type Ca²⁺ channel activation and survival in RBL-2H3 mast cells. *Biochim. Biophys. Acta* **2010**, *1803*, 372–385.
 109. Martelli, A.M.; Faenza, I.; Billi, A.M.; Manzoli, L.; Evangelisti, C.; Fala, F.; Cocco, L. Intranuclear 3'-phosphoinositide metabolism and Akt signaling: New mechanisms for tumorigenesis and protection against apoptosis? *Cell Signal.* **2006**, *18*, 1101–1107.
 110. Dimmeler, S.; Zeiher, A.M. Nitric oxide-an endothelial cell survival factor. *Cell Death Differ.* **1999**, *6*, 964–968.
 111. Choi, B.M.; Pae, H.O.; Jang, S.I.; Kim, Y.M.; Chung, H.T. Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. *J. Biochem. Mol. Biol.* **2002**, *35*, 116–126.
 112. Parcellier, A.; Tintignac, L.A.; Zhuravleva, E.; Hemmings, B.A. PKB and the mitochondria: AKTing on apoptosis. *Cell Signal.* **2007**, *20*, 21–30.
 113. Furuke, K.; Burd, P.R.; Horvath-Arcidiacono, J.A.; Hori, K.; Mostowski, H.; Bloom, E.T. Human NK cells express endothelial nitric oxide synthase, and nitric oxide protects them from activation-induced cell death by regulating expression of TNF-alpha. *J. Immunol.* **1999**, *163*, 1473–1480.

114. Ho, F.M.; Lin, W.W.; Chen, B.C.; Chao, C.M.; Yang, C.R.; Lin, L.Y.; Lai, C.C.; Liu, S. H.; Liao, C.S. High glucose-induced apoptosis in human vascular endothelial cells is mediated through NF-kappaB and c-Jun NH2-terminal kinase pathway and prevented by PI3K/Akt/eNOS pathway. *Cell Signal.* **2006**, *18*, 391–399.
115. Ying, L.; Hofseth, L.J. An emerging role for endothelial nitric oxide synthase in chronic inflammation and cancer. *Cancer Res.* **2007**, *67*, 1407–1410.

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