Chapter 3

The Role of Sodium-Calcium Exchanger in the Calcium Homeostasis of Airway Smooth Muscle

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1. Introduction

ASM is a widespread component of the respiratory system. The lung parenchyma, like the airways, is a contractile tissue that responds to agonists like histamine and its muscular behavior highly impacts respiratory physiology. Asthma, for example, is a common disorder characterized by an excessive narrowing of the airways and inflammation in response to certain stimulants. Although the relative contribution of each element in this pathology is not precisely known, it is clear that smooth muscle relaxants alleviate acute asthmatic episodes. All of these points to ASM as an important target for study and therapy related to asthma.

The contractility of ASM highly depends on intracellular Ca^{2+} concentration and sensitization to Ca^{2+}, which in turn depend on several transport and signaling mechanisms. Ca^{2+} homeostasis can be understood as a balance between Ca^{2+} entry and exit pathways governed by a dynamic web of physical and chemical signals. Ca^{2+} entry pathways in ASM include: voltage activated Ca^{2+} channels, non-selective cationic channels, IP_3 activated Ca^{2+} channel and Ryanodine receptor-channel. On the other hand, Ca^{2+}- ATPase pumps located in the plasma membrane and the Sarcoplasmic Reticulum account for the Ca^{2+} exit pathways. A very peculiar transporter, the Na^{+}-Ca^{2+} exchanger (NCX), which is the main subject of this chapter, accounts for both Ca^{2+} exit and entry pathways due to its dual mode of operation. The many features and regulation of the NCX have been described mainly for the cardiac isoform which was the first to be cloned and characterized. Nevertheless, new research has been directed to other isoforms found in several tissues, including ASM, since a physiological role in contractility is now evident.

In this chapter, several aspects of ASM and the NCX will be addressed including: its role in Ca^{2+} homeostasis, contraction and proliferation; history of research related to the NCX; molecular and functional characteristics; and clinical implications.

2. The role of Calcium in airway smooth muscle

As with other smooth muscles, cytosolic Ca^{2+} concentration underlies the most important features of ASM: contractility, proliferation and phenotype. Various complex mechanisms regulate cytosolic Ca^{2+} concentration and are strongly influenced by neurotransmitters, cytokines and physical forces, to name a few. Since the ASM cell interacts closely with tissues such as nervous terminals, epithelium and lymphocytes that secrete all such substances, it is not surprising that pathologies such as asthma and COPD are strongly linked to alterations in ASM Ca^{2+} homeostasis. In this section, the role of Calcium in contraction, phenotype acquisition and proliferation will be reviewed.

2.1. ASM Contraction

ASM contraction induced by agonist stimulation results mainly from two phenomena: elevation in cytosolic Ca^{2+} concentration and sensitization of the contractile machinery to Ca^{2+} [1]. This tissue is constricted directly by agonists such as histamine, cysteinylleukotrienes, thromboxanes and acetylcholine released by mast cells or airway nerves [2]. The primary signaling mechanism coupled to most contractile receptors is the activation of phospholipase Cγ (PLCγ) via a pertussis toxin-insensitive $G_{q/11}$-protein [3]. Activation of PLC leads to hydrolysis of phosphatidylinositol-bis-phosphate to inositol-1, 4, 5-trisphospate (IP_3) and diacylglycerol (DAG) to, respectively, cause Ca^{2+} release from the sarcoplasmic reticulum (SR) and activate protein kinase C (PKC). It is known that prolonged stimulation by histamine causes intracellular Ca^{2+} increase in ASM strips which correlates with a sustained contraction [4]. As stated above, this is the sum of both sensitization and activation mechanisms involved in the contraction of smooth muscle.

Two key events in smooth muscle contraction are the phosphorylation and dephosphorylation of the regulatory light chains of myosin II (rMLC). These reactions are partly catalyzed by the Ca^{2+} and calmodulin-activated myosin light-chain kinase (MLCK) and the type 1 myosin phosphatase (MLCP), respectively. The balance of the activity of these enzymes results in the extent of contraction or relaxation of smooth muscle. After agonist stimulation, intracellular Ca^{2+} binds to calmodulin and changes its conformation, enabling it to activate MLCK. MLCK then phosphorylates rMLC, predominantly at Ser-19, allowing the myosin ATPase to be activated by actin. This leads to crossbridge formation between myosin and actin, and generates muscle contraction. The coupling between force and rMLC phosphorylation is quite variable and non-linear, however dephosphorylation of rMLC generally produces relaxation.

In addition to their effects of Ca^{2+} concentration changes, contractile agonists increase Ca^{2+} sensitivity of contraction. There are two ways to modulate such Ca^{2+} sensitivity: 1) altering the balance between the activities of MLCK and MLCP at a constant Ca^{2+} concentration, and 2) by rMLC phosphorylation-independent mechanisms as in the case of calponin, caldesmon and heat shock proteins.

MLCP is an heterotrimeric enzyme that contains a regulatory subunit referred to as the myosin phosphatase target subunit (MYPT) which helps to form the active heterotrimer as

well as to increase the substrate specificity of MLCP toward myosin. This subunit is known to be phosphorylated by Rho Kinase becoming inhibited and thus favoring contraction. On the other hand, CPI-17 is an endogenous inhibitory protein of MLCP expressed in smooth muscle tissues which is itself regulated by RhoK and PKC phosphorylation [5-7].

On the other hand there are other mechanisms independent of phosphorylation of rMLC and pertain only to the thin filament actin. Calponin and caldesmon interact with F-actin and myosin and inhibit actomyosin ATPase activity. Both are regulated by PKC and ERK-activities [8, 9]. Altogether, the specific state of sensitization and intracellular Ca^{2+} concentration during agonist stimulation result in force development or relaxation of smooth muscle.

2.2. ASM phenotype and proliferation

ASM remodeling is an important aspect of many atopic respiratory diseases. Among the structural changes that the airways undergo include epithelial fibrosis, increase in ASM mass, mucous gland hyperplasia and edema. Wall thickening due to ASM hyperplasia and hypertrophy are a common hallmark of asthma and constitute the mayor obstruction for air flow during a crisis. It is believed that these changes occur as a response to chronic airway inflammation and mechanical stretch in which ASM cells take an active role by migrating to the epithelium and secreting various adhesion molecules and cytokines. Airway remodeling is proposed to begin with ASM cell phenotype change from a contractile to a synthetic and migrating type. It is still not very clear how Ca^{2+} homeostasis is associated with the many features of ASM remodeling. It has been reported that the activity of Cav1.2 channels and the SERCA pump may underlie this process by an up-regulation of intracellular Ca^{2+}. Downstream signaling mechanisms that lead to phenotype change include: Ca^{2+} /calmodulin-dependent protein kinase IV (CaMK.IV), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-α), nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (mtTFA). Among these, mtTFA up-regulates mitochondrial DNA replication and biogenesis probably leading to ASM cell proliferation. Tissues from asthmatic patients tend to show increased intracellular Ca^{2+} levels, which may render ASM cells hyper-proliferative as well as hyperreactive to contractile stimuli [10, 11].

3. Calcium transport mechanisms

Ca^{2+} is a fundamental second messenger in the mechanisms of remodeling and contraction of smooth muscle cells. Under basal conditions, the intracellular Ca^{2+} concentration in smooth muscle cells ranges from 100 to 200 nM [6]. Upon activation by agonists such as acetylcholine or histamine, there is a biphasic intracellular Ca^{2+} response in ASM cells consisting of an initial Ca^{2+} rise followed by a fast decline to a steady-state level that remains above basal concentration until agonist is washed out [12]. This biphasic profile reflects the Ca^{2+} release from the SR as well as Ca^{2+} influx from the extracellular space. On the other hand, there are mechanisms that remove intracellular Ca^{2+} such as the Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase(SERCA), the plasmalemal Ca^{2+} ATPase (PMCA) and the mitochondria which become evident once agonist is removed and Ca^{2+} entry is abolished.

3.1. Ca^{2+} release from the SR

In airway smooth muscle, Ca^{2+} release from SR depends on IP_3 and RyR receptor-channels [13, 14]. These channels belong to two different families and share significant homology, especially in the sequences that are proposed to form the channels´ pore. These Ca^{2+} release channels are large oligomeric structures formed by association of either four IP_3R proteins (300 KDa each) or four RyR proteins (565 KDa each) [15-17].

The IP_3R channel requires binding of IP_3 for Ca^{2+} release with each monomer of the channel binding one molecule of IP_3 in a non-cooperative fashion with a K_D around 50 nM. An endogenous ligand for the RyR channels has remained elusive, and so far it has been proposed that in smooth muscle tissue, Ca^{2+} released by IP_3R activates RyR channels; an event referred to as Ca^{2+} -Induced Ca^{2+} Release (CICR). Ca^{2+} release channels are regulated by various factors. Cytoplasmic Ca^{2+} shows a biphasic effect on the IP_3 -induced Ca^{2+} release with a maximum rate at 300 nM. From that concentration on, the channel is inhibited by Ca^{2+}. This inhibitory effect is also shared by the RyR channel, although it is not physiologically relevant, since millimolar concentrations of Ca^{2+} (1 – 10 mM) are required to inhibit the channel. Therefore, the Ca^{2+} release from the RyR channel shows only Ca^{2+}-dependent activation in the physiological range of Ca^{2+} concentration (1 – 10 μM). Both channels are activated by free ATP, around 10 μM for the IP_3R and 300 μM for the RyR channel.

An important modulator of the RyR channel is the plant alkaloid ryanodine which binds to each monomer with high affinity (Kd ‹ 50 nM). Low doses of ryanodine (around 10 nM) are reported to increase the frequency of single RyR channel opening. Intermediate ryanodine doses (around 1 μM) are reported to induce very long – duration open events and simultaneously reduce ion conductance through the pore. High doses of ryanodine (around 100 μM) are reported to lock the channel in a closed configuration.

3.2. Ca^{2+} uptake into the SR

The SR is continually re-filled with Ca^{2+} with the aid of the SERCA pumps located in its membrane. The SERCA are 110 kDa proteins that belong to the P-type ion pumps family. Their activity is a cycle of chemical reactions that lead to conformational changes and Ca^{2+} transport powered by phosphorylation. The activity of SERCA is largely regulated by phospholamban which in its phosphorylated state increases Ca^{2+} uptake. Even in the absence of an agonist, SR Ca^{2+} uptake appears to be critical for ASM basal tone maintenance. This has been explored in experiments where the SERCA pumps are inhibited by Cyclopiazonic acid (CPA) or Thapsigargin (TG) in resting smooth muscle. Under such conditions, ASM spontaneously contracts following an increase in intracellular Ca^{2+}, which can be explained as a leak from the SR which cannot be handled by the impaired SERCA function. SERCA function is also evident when its inhibition is followed by incubation of ASM in Ca^{2+}- fee solutions and agonist stimulation. Upon Ca^{2+} re-addition in these circumstances, a transitory contraction can be observed which is characteristic of a depleted SR and reflects the store-operated Ca^{2+} entry (SOCC) which is discussed below.

3.3. Ca^{2+} influx from the extracellular space

Besides the SR, the other source for Ca^{2+} in ASM is the extracellular media, but in this respect the involved mechanisms are unclear. Three main sources of Ca^{2+} have been proposed to be active in ASM: voltage activated $Ca_V1.2$ channels, transient receptor potential channels (TRP) and the NCX in reverse mode. The signaling mechanisms that activate each of these channels and transporters have not been completely understood, given that some of them have been just recently described in this tissue. Another interesting feature is the functional interaction among these systems as well as interactions with other channels such as the Ca^{2+}-activated chloride channels (Cl_{Ca}) and the Ca^{2+} activated potassium channels (BK_{Ca}). As will be discussed later, these channels regulate membrane potential and thus alter the activity of $Ca_V1.2$ channels, TRPC channels and the NCX. On the other hand, the ionic concentration reached within the vicinity of these channels upon stimulation, has also been observed to alter their function regardless of whole membrane potential.

3.4. ASM resting membrane potential

It has been established that ASM membrane potential relies mainly on the activity of Cl_{Ca}, BK_{Ca} and TRPC channels. Cl_{Ca} and BK_{Ca} channels are activated by intracellular Ca^{2+} showing slightly different sensitivities between the ranges 100 to 900 nM [18, 19], and influence the membrane potential in opposite ways. Cl_{Ca} channels allow Cl^- to exit the cell, therefore depolarizing the membrane, while BK channels allow K^+ to exit causing membrane hyperpolarization. It has been reported that Ca^{2+} released by the SR during histamine stimulation causes activation of Cl_{Ca} channels and membrane depolarization [20]. TRPC have also been proposed to impact membrane potential at rest and after agonist stimulation causing depolarization. Histamine evokes an inward Na^+ current in equine tracheal myocytes together with an outward Cl^- current. In that work, it was suggested that NSCC of the TRPC family were responsible for the cationic current observed [21].

3.5. $Ca_V1.2$ channels

In many tissues, smooth muscle dihydropyridine-sensitive channels activated by membrane depolarization comprise an important source for external Ca^{2+} [22]. Interestingly, blockade of these channels has not served as a therapeutic tool in asthma, and thus their physiological relevance remains unclear. These channels are composed of pore-forming (α subunit) and accessory subunits that regulate expression, gating and channel kinetics. The α subunit carries the Ca^{2+} current and provides the voltage- and DHP-sensitivity of these channels [23]. Research has shown that voltage-related Ca^{2+} currents in ASM reflect $Ca_V1.2$ channel activity [12] which is the main isoform expressed in this tissue [24, 25]. Electrophysiological studies have found the threshold potential for these currents to be around -40mV and the peak activation between +10 and +20 mV. Although no precise value for resting ASM membrane potential has been described, several studies have reported values ranging between -60 and -30 mV [26, 27]. Thus, depolarization must occur before $Ca_V1.2$ channels could participate in agonist ASM induced contraction.

3.6. Non-selective cationic channels of the TRP family

It has been reported that agonists like histamine and carbachol provoke a small and inward cationic current through non-selective cationic channels (NSCC) in tracheal smooth muscle of different species [28, 29] .There is evidence that points to the transient receptor potential channels (TRP) as candidates for this conductance. TRP channels were first described in *Drosophila melanogaster* and then, homologues for these channels in at least 20 mammalian species were found to the point that almost all mammalian TRP channels are now known. Unlike most ion channels, TRP channels are identified by their homology rather than by ligand function or selectivity, because their functions are diverse and mostly unknown. The canonical (TRPC) subfamily of these channels comprises seven isoforms: TRPC1-7, and which have been detected in guinea pig and human ASM [30, 31]. It is generally accepted that TRPC channels are activated downstream by agonist-stimulated PIP_2 hydrolysis, but still their exact mode of activation and operation is unclear. Both store-dependent and independent mechanisms of activation have been proposed, in cases, even for the same channel in different preparations. All mammalian TRPC channels can be activated by GPCRs including muscarinic type 1 receptors (TRPC1, TRPC4, TRPC5 heteromers or TRPC4 and TRPC5 homomers); histaminergic type 1 receptors (TRPC3, TRPC6) and purinergic receptors (TRPC7) [32].

It is important to note at this point that Ca^{2+} release from the SR results in lowering of the Ca^{2+} content in intracellular stores to a certain degree. This lowering in turn activates a signaling mechanism that allows Ca^{2+} entry from the extracellular space. This mechanism was originally called [33] store-operated Ca^{2+} entry (SOCE). Since then, this phenomenon has received much attention, but still the complete mechanism and molecular identity of SOC channels remain unclear [34]. Experiments performed in our laboratory on guinea pig epithelial-free tracheal rings suggest that SOCs are non-selective cation channels that mainly permit Na^+ entry causing depolarization (unpublished results). We proposed that such depolarization and increased levels in Na^+ induce the NCX to allow Ca^{2+} influx which in turn activates Cl_{Ca}, and opening of the $Ca_v1.2$ channels [35]. The molecular identity of SOC channels points to the protein ORAI1 as well as to TRPC channels [36, 37]. ORAI1 is a four-transmembrane spanning protein that forms a pore with high selectivity for Ca^{2+}. More recent advances have been made regarding the signaling mechanisms that induce SOC current. The stromal interacting molecule (STIM) 1 has been found to sense Ca^{2+} concentration within the SR. STIM1 contains an EF-hand Ca^{2+} binding domain on the N-terminal ER luminal portion. When Ca^{2+} diminishes in the SR, STIM1 suffers a change in its distribution on the SR membrane and forms discrete clusters called puncta that interact with the plasma membrane. It is now clear that STIM1 couples to Orai1 to refill SR in some cell types. Nevertheless, interaction between STIM1 and other channels such as TRPC has also been observed and could account for SR refilling [38, 39].

3.7. The Na^+/Ca^{2+} exchanger in ASM

NCX is a membrane associated protein that catalyzes electrogenic exchange of 3 Na^+ ions and 1 Ca^{2+} ion across the plasma membrane in a high capacity, and low Ca^{2+} affinity fashion.

This transporter can operate in either the Ca^{2+} -efflux or Ca^{2+} -influx mode depending on the electrochemical gradients of the substrate ions. Its physiological relevance became apparent as the role of extracellular Na^+ in regulating contraction of smooth muscle was studied. Experiments performed in our laboratory pointed to the NCX as a crucial transporter involved in ASM contraction. In our hands, Na^+ substitution by N-Methyl D-glucamine (NMDG) or inhibition of Na^+/K^+ pump with Ouabain produced an increase in intracellular Ca^2 in cultivated ASM cells [40]. Also, Na^+ substitution with NMDG or inhibition of Na^+/K^+ pump with Ouabain increase muscle tension [41], and histamine-pre-contracted guinea-pig tracheal rings show decreased relaxation rate when washed in a Na^+ -free solution [42]. The role of NCX in ASM is still unclear, although much evidence has been mounting towards its importance. In further sections of this chapter, various aspects of this transporter will be reviewed in detail.

3.8. The PMCA and Ca^{2+} extrusion

Similar to SERCA function during SR Ca^{2+} uptake, the PMCA is constantly extruding Ca^{2+} outside the cell. The PMCA is a membrane protein that also belongs to the P-type pump family. It operates with high Ca^{2+} affinity and low transport capacity with a Kd ranging from 10-30 nM at rest to 0.2-0.5 μM at its optimal activation. It is thus considered to be the fine tuner of cytosolic Ca^{2+} concentration [43]. The PMCA is inhibited by Lanthanum ions and Vanadate, as many other transport systems, and for a long time there was no specific inhibitor available. Recently, some peptides such as caloxin have been synthesized which bind to the extracellular domains of the pump significantly reducing its activity.

4. Brief history of the NCX

The existence of the NCX exchanger was proposed in 1963 [44] as a result of studies in cardiac muscle contraction in low Na^+ concentrated solutions. However the proposal was the result of reports from many other investigators who had previously documented the important role that Ca^{2+} and Na^+ played in cardiac contraction. For example, it was reported [45, 46] that cardiac muscle contraction depended on extracellular Ca^{2+}. On the other hand it was [47] described that the force of cardiac contraction increased in the presence of low concentrated sodium solutions and years later other group [48] reported that the increase in the cardiac contraction force was associated with the quotient between the extracellular Ca^{2+} concentration and the extracellular Na^+ concentration. Also, it was reported [44] that the decrease in the extracellular Na^+ concentration is related to an increase in the Ca^{2+} content in cardiac muscle cells. The aforementioned papers and others which will not be mentioned here due to lack of space, lead to the conclusion [44] which says that Na^+ and Ca^{2+} ions had to be transported by a transporter. During the same decade several papers were published, which allowed to suggest the presence of a Na^+-Ca^{2+} transport mechanism in other muscular tissues: skeletal muscle [49, 50], vascular smooth muscle [51] and intestinal muscle [52]. It was not until the end of the sixties when two groups of researchers working separately proposed the existence of a contratransport system coupled for Na^+ and Ca^{2+} [53-58]. Similarly the existence of the Na^+-Ca^{2+} exchanger in the smooth airway muscle was suggested by [59, 60].

The strongest evidence of the existence of the Na^+-Ca^{2+} exchanger was given by the partial purification of the protein [61] and their posterior molecular cloning [62] from cardiac muscle. Other exchangers have been completely or partially cloned in other tissues such as photoreceptors [63], airway smooth muscle [64, 65], brain [66], kidney [67], etc.

We should point out that thanks to the discovery of the Na^+-Ca^{2+} exchanger it was possible to give a rational explanation of the inotropic effect of cardiac glycosides and, also, lead the group of M.P. Blaustein and J. M. Hamlyn to the discovery of the endogenous ouabain [68] a compound that is indistinguishable from plant ouabain, a Na^+/K^+ ATPase specific blocker.

5. Molecular aspects of the NCX

The mammalian Na^+-Ca^{2+} exchanger belongs to a family of at least 3 genes NCX1, NCX2 and NCX3 which share a high degree of homology at the DNA and protein sequence level. NCX1 is the best characterized and is expressed in most tissues but mainly in heart brain and kidney, whereas NCX2 is expressed mainly in brain and NCX3 in brain and skeletal muscle [62, 69, 70].

An additional member of the mammalian NCX gene family recently identified is the mitochondrial Na^+- Ca^{2+} exchanger (NCLX). This gene is expressed in various tissues, such as pancreas, skeletal muscle and stomach smooth muscle and encodes a 70 KDa protein distinct from the other members of the family. This molecule is localized to the inner mitochondrial membrane and mediates the mitochondrial Ca^{2+} efflux in exchange for Na^+ or Li^+, contributing apparently to intracellular Ca^{2+} homeostasis [71].

NCX 1 is the best characterized at the molecular level and its gene consists of a coding region of 12 exons that encode a protein of 938 amino-acids, and a large upstream regulatory region of more than 2000 bp that contains binding sites for several transcription factors such as GATA 4 SRF, NF-Y, CREB, C/EBP and AP1, among others [72, 73]. The H1 promoter regulates the expression in the heart, K1 in the kidney and Br1 in the brain. The use of each of these promoters in a tissue specific manner produces transcripts with different length of exon 1 and might enable the response to different stimuli. Exon 1 is part of the 5′-untranslated regions (5′-UTR) and each of these alternate exons is spliced to the common coding exon 2. Although this process does not change the coding sequence, it changes the length of the 5′-UTR which might be important for a posttranscriptional regulation.

In cardiac hypertrophy NCX expression is induced by α-adrenergic stimulation mediated in part by p38 MAPK activation and this is dependent on the presence of the proximal CArG promoter. Moreover it seems possible that the activation of the ERK kinase induced by hypertrophic stimuli plays a role in the transcriptional up-regulation of cardiac NCX. Within this region there are at least 3 alternate promoters that confer tissue specificity for NCX expression [73-75].

The major isoform of NCX1 encodes a protein of 120 KDa and NCX2 and NC3 encode proteins of approximately 100 kDa, respectively. The actual topological model suggests five transmembrane helices followed by a large intracellular loop of about 550 amino-acids,

flanked by 2 α-repeats, and then the last four transmembrane segments. Spanning the large intracellular loop of NCX1 there is an alternatively spliced region that encompasses 6 exons (A, B, C, D, E, and F), which are expressed in a relatively tissue specific manner. Exons A and B are mutually exclusive and the others are combined with either of these two to produce at least 17 spliced isoforms of the exchanger [69-71]. Exon A appears in excitable tissues (heart, brain, skeletal muscle) and exon B mainly in no-excitable tissues (76-78). The longest spliced isoform is expressed in heart with exon A, C, D, E, F (NCX1.1) and the shortest in brain with the B, D (NCX1.3) isoform. Adjacent to this region there are 2 Ca^{2+} binding sites, CBD1 and CBD2, and close to the latter there is a alternatively spliced variable region. The interaction between CBD2 and the variable region seems to influence the sensitivity of the NCX isoforms to the regulation by intracellular Na^{+} and Ca^{2+} (79).

Other domains in the intracellular loop include an XIP site (eXchanger Inhibitory Peptide), an α-catenin homology region and a putative binding site, within the alternatively spliced region, for the ganglioside GM1. This interaction seems to be specific for exon B expressing isoforms and allow the localization of NCX1 to the nuclear envelope, where might influence not only nuclear Ca^{2+} but endoplasmic reticulum lumen stores as well, through its vicinity with the nuclear envelope. This interaction has been observed in neuronal and non-neuronal cells, and it has been suggested that the presence of 4 arginine residues in exon B, instead of 1 in exon A could favor a major interaction of the negatively charged ganglioside with these NCX1 isoforms (80).

Apparently NCX may be phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) but it is still unclear whether these posttranslational modifications confer physiological effects directly or indirectly through the interaction with other proteins. It has been reported evidence that the large intracellular loop forms a complex with the PKC and PKA kinases subunits, PP1 and 2 phosphatases, and the PKA-anchoring protein AKAP and although there are compelling evidence for in vitro NCX phosphorylation by PKA and PKC, debate about the functional significance of these findings, still remains. It seems that the intracellular loop is necessary for agonist stimulation of NCX activity, but not necessarily the direct phosphorylation (81-83).

Airway smooth muscle cells and tissue express mainly NCX1, and absence of expression of the NCX2 and NCX3 isoforms. The first molecular evidence of NCX1 expression in ASM was realized in human trachea smooth muscle tissue where the alternatively spliced isoform revealed through reverse transcription coupled polymerase chain reaction (RT-PCR) method was the alternatively spliced isoform NCX1.3 (64).

This same isoform was later found in guinea pig tracheal tissue, showing a high grade homology in the alternatively spliced region among both species, with only minor aminoacid conservative changes. This isoform is predominantly expressed in kidney and contains a 102 amino-acids B exon linked to an 8 aminoacids D exon (84, 65).

At the protein level, NCX1 expression was demonstrated in bovine tracheal smooth muscle, where apparently a 120 kDa and a 110 kDa proteins corresponding to NCX1.1 and NCX1.3 isoforms, respectively, were identified by Western blotting [85]. Functional and comparative

studies of the major NCX1.1 and the NCX1.3 isoforms have shown aminoacid differences within these variable exons that influence the inhibitory sensitivity de NCX to intracellular Na^+ (77-79).

Recently, an advanced molecular approach based on protein expression knocking down at the messenger RNA (mRNA) level by interference with small RNA molecules (siRNA), has been successfully applied to human airway smooth muscles allowing a better correlation of expression level with function in this tissue. Interestingly, these studies show that histamine and cytokines, like TNFα and IL-13, are able to induce the expression of NCX. When these cells are transfected with siRNA specific for NCX, the protein levels of the exchanger are decreased, as well as the Ca^{2+} influx elicited by these stimuli [86]. Moreover, in one of these studies it has been shown that cytokine induction of NCX1 is at the transcription level, mediated apparently by a mitogen activated protein kinase (MAPK) and NFκB pathways [87].

6. Functional aspects of the NCX

In this section, the basic aspects of NCX function will be reviewed considering the molecular mechanics of ion transport, activation of ion transport, interaction between NCX and ionic channels and pharmacology of the NCX.

6.1. Mechanism of Na^+/Ca^{2+} transport

The mechanism of transport of NCX1 has been widely studied and reveals a consecutive mechanism in which only 1 substrate ion is translocated at a time. Interaction of NCX1 with Na^+ or Ca^{2+} is asymmetric since the apparent affinity for intracellular Ca^{2+} is several hundred times higher than that for extracellular Ca^{2+}, although affinities for Na^+ differ little. Besides being transported substrates, Ca^{2+} and Na^+ regulate the NCX1 activity. In both modes of operation, the NCX1 is activated only when regulatory intracellular Ca^{2+} binds to a high-affinity site showing $K_{1/2}$ values of 0.1 to 0.4 μM [88]. In contrast, intracellular Na^+ exerts an inhibitory process upon NCX that occurs when the transport sites in NCX1 are fully loaded with Na^+ from the cytoplasmic side. This inactivation process is influenced by a variety of factors: it is enhanced at low pH but attenuated by intracellular Ca^{2+}, millimolar ATP or PIP_2. The steady-state activity of the NCX1 also exhibits intracellular pH dependence. At pH 6, activity is almost null; whereas at pH 9, activity is maximal [89]. Also, NCX activity shows voltage dependence, attributed mostly to voltage dependence on behalf of the Na^+ translocation step, or Na^+ binding to the NCX, which is rate limiting in overall reaction [90].

A very interesting effect occurs when alkali metal ions such as Na^+, K^+ or Li^+ are present on the extracellular side: all of them increase NCX activity 2 to 3 times with low affinity. Apart from Na^+, these cations bind to sites which are different from the transport sites and are not transported by the NCX [91]. Intracellular metal cations also stimulate the NCX, but apparently they need to be present as well in the internal side of NCX to show such effect. Gadsby et al. [92] found a striking difference between the outward NCX current-voltage relationships obtained in isolated guinea-pig myocytes when extracellular Na^+ was completely replaced with

Li^+ as compared to replacing with $NMDG^+$. This increase in outward current observed when Li^+ replaced Na^+ suggested that the voltage sensitivity and the magnitude of Na^+-Ca^{2+} exchange depend on the nature of the extracellular monovalent cation present. Our group previously observed that Na^+ substitution with Li^+ in force experiments performed on guinea pig tracheal rings produced a small reproducible increase in tension (Figure 1. upper trace). Once histamine was added, a further sustained contraction was observed and the peak tension, measured from the previous basal level, showed no significant decrease as compared to control. We suggested that histamine stimulation produces Li^+ influx through TRPC, membrane depolarization and activation of Cav1.2 channels. This depolarization is apparently enough to completely explain contraction, as observed when verapamil was added causing almost complete relaxation [35]. It is worth noting that Li^+ is not transported through the NCX in either direction [93] and therefore, NCX function under this condition is expected to be null.

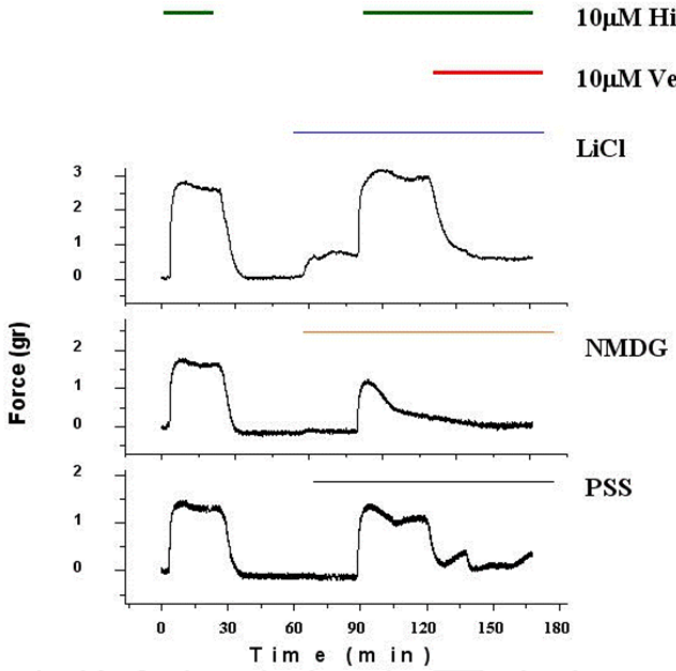

Figure 1. Representative traces of isometric force measurements of histamine-stimulated guinea-pig tracheal rings in Na^+ -free with LiCl (upper trace), Na^+ - free with $NMDG^+$ (middle trace) and PSS (lower trace). PSS = Saline Solution. Force measurements were considered 5 min after histamine stimulation for comparison between contractions.

6.2. Functional relationship between NCX and ion channels.

As mentioned before, the NCX is a transporter whose activity and mode of operation can be finely modulated by the electrochemical gradient for Na^+ and Ca^{2+}. It has also been suggested that its localization and physical association with ion channels and cell organelles

(as the SR) might determine its modulation [94]. Activation of NCX in the Ca^{2+} influx mode after agonist stimulation has been observed by different groups. Rosker et al. reported that in HEK 293 cells over-expressing TRPC3 channels, stimulation with carbachol was associated with an increase of intracellular Ca^{2+} concentration, which depended on extracellular Na^{+} since its substitution or the NCX inhibition with KB-R7943 reduced such effect. In the same cell line they also reported that NCX and TRPC3 are physically associated after cellular fractionation in low-density sucrose gradients and co-immunoprecipitation. The same group using glutathione S-transferase pull-down technique, which revealed that NCX interacts with the carboxy-terminal of TRPC3, confirmed these data. They also showed by co-immunoprecipitation experiments that NCX and TRPC3 are physically associated [95].

Later, this group tested a similar hypothesis on rat cardiomyocytes, and found that inhibition of the NCX in reverse mode by KB-R7943 also diminished the Ca^{2+} entry associated with agonist stimulation [96]. Other groups have also observed this functional association using models that better resemble in vivo conditions. It was reported that in rat aortic smooth muscle cells, the NCX inhibitor KB-R7943 as well as the TRPC inhibitor SKF-96365 abolished Ca^{2+} influx after ATP stimulation. They also observed a similar effect when cells were transfected with a dominant negative transcript for TRPC6 [97].

Hirota et al. [98] have also suggested a functional association between the NCX and TRPC activated by store depletion in dog ASM. In that work, several agonists were tested on tracheal rings together with NCX inhibitor KB-R7943 and sensitivity to this drug was observed. Also, contraction depended on extracellular Na^{+}, corresponding to our own observations. Dai et al. [99] observed in porcine tracheal smooth muscle bundle that Ca^{2+} waves typically obtained by acetylcholine stimulation were sensitive to TRPC blocker SKF-96365 as well as to KB-R7943. They also observed this sensitivity at the level of muscle contraction, where these drugs, together with nifedipine completely relaxed the muscle.

In our own experience, this relationship between the NCX and TRPC channels became evident in guinea pig ASM. We performed several force experiments on guinea pig tracheal rings and observed that histamine contraction depends on extracellular Na^{+} and is sensitive to the non-specific NCX blocker KBR-7943 as well as to TRPC non-specific blockers SKF-96365 and 2-APB. These findings have led us to propose that histamine causes Ca^{2+} entry mediated by the NCX operating in its reverse mode secondary to a cationic influx (primarily Na^{+}) through TRPC. We then proposed that histamine stimulates Na^{+} influx through TRPC, cell depolarization and the increase in subplasmalemmal Na^{+} concentration. These conditions might favor first the reverse mode of the NCX and later the activation of Cav1.2 channels which have been characterized in this tissue previously [24]. On the other hand, as Na^{+} is replaced by $NMDG^{+}$, neither depolarization nor Na^{+} influx through TRPC could be possible. Thus Ca^{2+} release from SR, Ca^{+2} entries through TRPC and Ca^{+2} entries through reverse mode NCX would provide for the small histamine-induced contraction observed [35]. Pre-incubation with KB-R7943 allowed us to explore the role of NCX during the beginning of histamine contraction as well as throughout tonic force development. In the presence of KB-R7943 a significant diminishment in maximal force developed as observed, whereas pre-

incubation with 70 nM nifedipine had no effect. We thus suggest that NCX is active in its reverse mode at an early stage of contraction after Ca^{2+} release from the SR, while Cav1.2 channels participate somewhat later during stimulation. In addition, this contraction is also similar to the one observed when Na^{+} is substituted for NMDG. Our interpretation for this is that when NCX has been inhibited, not only Ca^{2+} entry is blocked but also membrane depolarization does not reach the threshold for Cav1.2 channel activation.

Ca^{2+} imaging experiments performed on freshly isolated tracheal smooth muscle cells pointed to the same direction as the tension experiments. We first obtained isolated muscle cells able to contract and to show an increase in FURA-2 fluorescence ratio after histamine stimulation. Stimulation resulted in a peak in fluorescence ratio followed by a plateau of fluorescence just above the basal value (15% of the peak in Ca^{2+} rise), which persisted until the agonist was washed out. Addition of 100 μM KB-R7943 significantly decreased the change in peak fluorescence ratio in a second stimulation with histamine as well as during the sustained phase (Figure 2.). External Na^{+} substitution by $NMDG^{+}$ showed a significant decrease in fluorescence ratio in the sustained phase suggesting that the NCX is operating in the Ca^{2+} influx mode and that the KB-R7943-insensitive component is due to Ca^{2+} release from the stores and perhaps Ca^{2+} entry through TRPC. We also observed that application of SKF-93635 and thus inhibition of TRPC significantly lowers the peak fluorescence ratio and completely abolishes fluorescence in the sustained phase of the curve. This is in agreement with results reported by Dai et al. [99] where SKF-96365 inhibits contraction and Ca^{2+} waves in porcine tracheal smooth muscle cells.

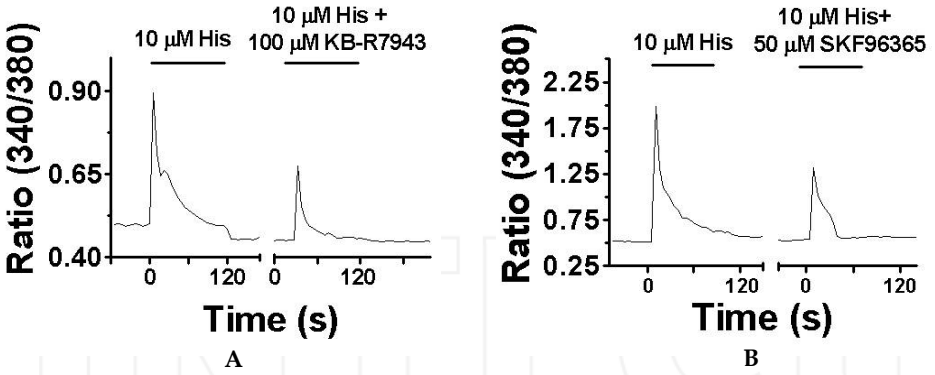

Figure 2. Representative traces from fluorescence ratio changes observed during stimulation of isolated smooth muscle cells with 10 μM histamine. Cells were stimulated twice with histamine and given a 20 min recovery time between stimulations. Fluorescence ratio was measured as indicated by arrows. During the second stimulation histamine was added together with 100 μM KB-R7943 in PS, A) or 50 μM SKF-96365 in PS, B).

These results led us to propose the following model: Activation of histamine receptors triggers a signaling cascade leading to formation of IP_3 and DAG which causes Ca^{2+} release from SR generating initial contraction. Emptying of the SR by such Ca^{2+} release, activates TRPC

channel opening leading to Na^+ influx. This Na^+ current in turn causes membrane depolarization as well as a local increase in $[Na^+]_i$ in the vicinity of NCX which promote its reverse mode of operation. Ca^{2+} entry mediated by the NCX may add to Ca^{2+} released from the SR and activate Ca^{2+}-activated Cl^- channels [20, 100]. This in turn should cause enough depolarization to activate a larger population of Cav1.2 channels and, together with sensitization events, give rise to a characteristic histamine contraction.

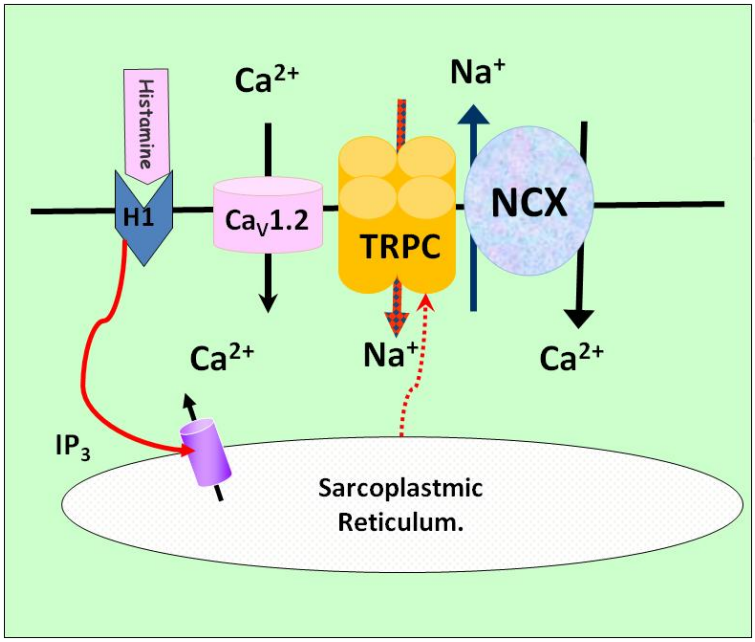

Figure 3. Proposed model that explains the functional interaction between NCX and TRPC during histamine stimulation. Histamine acts on its specific H1 receptor and initiates a signaling cascade leading to formation of IP_3. IP_3 produces Ca^{2+} release from SR and this in turn causes TRPC opening. The Na^+ current entering through these channels depolarizes the membrane and locally increases $[Na^+]_i$ in the vicinity of NCX. These conditions would then promote NCX operation in reverse mode as well as Cav1.2 channel activation. Ca^{2+} entry through NCX might activate Ca^{2+}-dependent Cl^- channels and cause even greater depolarization resulting in an activation of a greater Cav1.2 channel population.

Recently, the functional interaction between NCX and the SOCC channel activator STIM1 was observed in human bronchial smooth muscle cells [86]. In this work, electrophysiological recordings of isolated cells revealed an outwardly rectifying current characteristic of the NCX in reverse mode which was completely abolished by KB.R7943. Interestingly, the current was activated by histamine addition and inhibited completely by STIM1 knockdown. STIM is proposed as a sensor of SR emptying which interacts with membrane channels, it is possible that the TRPC channels are activated by STIM1 causing Na^+ influx and NCX activation as was previously proposed. This evidence shows again the tight relationship between the NCX and other channels which are activated in response to agonist stimulation or SR emptying.

6.3. Role of the NCX during ASM relaxation

The NCX working in reverse mode promotes ASM contraction according to the evidence just described. Nevertheless, its mode of operation once agonist is washed seems to be different. In contrast to its alleged role in heart as a fundamental Ca^{2+} extrusion system [101], we observed that inhibition of the NCX with KB-R7943 during relaxation of guinea pig tracheal rings does not alter the process at all [65]. On the other hand, Na+ substitution by NMDG during washing does retard the relaxation of the rings, indicating that the NCX is turned to the Ca2+ influx mode. This delay in the relaxation process in Na+-free washing was abolished by KB-R943, suggesting the participation of the NCX under these conditions. In accordance with our results, it has also been shown that the NCX plays at most a minor role as a Ca^{2+} extrusion system during canine ASM relaxation [102]. This is in agreement with our results, suggesting that the NCX found in ASM is active during contraction in the Ca^{2+} entry mode, but not during relaxation.

6.4. Pharmacology of the NCX

Specific inhibitors of the NCX are not yet available for research or therapeutic use. Many divalent and trivalent cations such as La^{+}, Ni^{2+} and Cd^{2+}, as well as amiloride derivatives or the substituted pyrrolidineethanamine have long been used, although their lack of specificity remain a great handicap for their use. The isothiourea derivative KB-R7943 has been used as a potent inhibitor of the NCX [103]. It is 3-fold less potent on NCX1 and NCX2 than on NCX3 and has a preferential effect on the Ca^{2+} influx mode of NCX1 [104]. This drug seems to act on specific residues of the NCX1: Val 820, Gln 826 and Gly 833 which lie in a reentrant membrane loop [105]. An important handicap for the use of KB-R7943 is the lack of specificity for NCX, since it has been reported to block ion channels [106], neuronal nicotinic acetylcholine receptor [107], N-methyl-D-aspartate receptor [108] and norepinephrine transporter at relatively low doses. Another more potent and specific inhibitor of NCX is SEA0400. This drug has been reported to be 30 times more powerful than KB-R7943 and to block predominantly NCX1 in CCL39 cells [109,110]. Analysis performed with NCX1 and NCX3 chimeras showed that multiple amino acids are involved in SEA0400 sensitivity encompassing residues 73-108 and 193-230. Regarding its specificity, SEA0400 at 1 μM does not affect $Ca_V1.2$ channels, $Ca_V2.2$ channels or Na^{+} channels. The affinity reported for NCX in cultured neurons, astrocytes and microglia has IC_{50} values from 5 to 33 nM [111]. An important drawback for the use of SEA0400 is that it is not yet commercially available, limiting its use to the general research public. Two other NCX blockers are SN-6 and YM-244769 and are under investigation. The blockers mentioned before have the characteristic that they are poorly active when the exchanger is working in the forward mode under normal conditions (low intracellular Na^{+}) but very active when the exchanger is working in the reverse mode under pathological conditions [112].

7. Clinical implications

As it has been mentioned in previous sections, the NCX plays a critical role in the regulation of intracellular Ca^{2+} concentration. The direction of Na^{+} in exchange for Ca^{2+} depends on the

membrane potential and the Na^+ and Ca^{2+} transmembrane ionic gradient. Out of the two types of exchange, the reverse mode (Ca^{2+} influx mode) has received more attention due to the fact that its function determines extracellular Ca^{2+} influx into the cell. When this is given in normal conditions is used for either the contraction process or to refill the SR. However, there is experimental evidence that suggests that in certain pathological conditions such as essential hypertension [113], ischemia-reperfusion injury [113] and certain types of cardiac arrhythmia [114] the NCX transports a bigger amount of Ca^{2+} than necessary.

The usage of NCX blockers such as KB-R7943, SEA0400, and SN-6 [115] have helped to understand the physiological role that the exchanger plays in different tissues. Moreover, they are now seen as potential therapeutic drugs. Indeed, the experimental evidence accumulated over the past few years has allowed to establish that, at least in experimental models, the blockers of the reverse mode of the NCX are useful to diminish the high blood pressure, to abolish cardiac arrhythmias or to reduce the tissue damage after ischemia-reperfusion damage models.

However, as far as we know no reports regarding the use of the NCX blockers have been published in models of airway disease such as Asthma or Chronic Obstructive Pulmonary Disease. It is well known that Asthma is an inflammatory chronic disease characterized by reversible airflow obstruction and nonspecific airway hyperresponsiveness. In spite of the drugs for Asthma treatments available such systemic or local steroids, leukotrienes inhibitors and/or smooth muscle airway relaxants (B_2 adrenergic agonist) no absolute control of the disease is obtained. Thus, and because the prevalence of Asthma worldwide has increased in the past few years it challenges the discovery of new and better pharmacological treatments for it. Throughout the past years it has been suggested that the use of NCX blockers could be of some help in the therapeutic management of this disease but the experimental information is scarce. Our group reported [35] that the tonic phase of the contraction induced by histamine is partially blocked by KB-R7943 and its effect is not due to the Ca^{2+} voltage dependent channel blockage since these had been previously inhibited by Nifedipine. Other groups have reported similar results to ours [116-118]. Therefore, it is expected that in the next years different research groups will proceed to investigate if NCX blockers have any kind of therapeutic use in animal Asthma models.

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