Cellular Na+ handling mechanisms involved in airway smooth muscle contraction (Review)

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Abstract. A decrease in bronchial diameter is designated as bronchoconstriction (BC) and impedes the flow of air through the airway. Asthma is characterized by inflammation of the airways, reversible BC and nonspecific hyperreactivity. These last two symptoms are dependent on airway smooth muscle. Stimuli that trigger contraction can be characterized as chemical (neurotransmitters, cytokines and terpenoids) and physical (volume inspired, air pressure). Both stimuli activate signaling pathways by acting on membrane proteins and facilitating the passage of ions through the membrane, generating a voltage change and a subsequent depolarization. Na+ plays an important role in preserving the resting membrane potential; this ion is extracted from the cells by the Na+/K+ ATPase (NKA) or introduced into the cytoplasm by the Na+/Ca2+ exchanger (NCX). During depolarization, Na+ appears to accumulate in specific regions beneath the plasma membrane, generating local concentration gradients which determine the handling of Ca2+. At rest, the smooth muscle has a basal tone that is preserved by the continuous adjustment of intracytoplasmic concentrations of Ca2+ and Na+. At homeostasis, the Na+ concentration is primarily dependent on three structures: the NKA, the NCX and non-specific cation channels (NSCC). These three structures, their functions and the available evidence of the probable role of Na+ in asthma are described in the present review.

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1. Introduction

Airway bronchoconstriction (BC) and hyperresponsiveness are hallmarks of asthma, a chronic, irreversible inflammatory disease (1). Asthma-related BC can be induced by exercise, infection or allergen exposure according to the phenotypic characteristics of each patient (2) and is frequently relieved by inhaled corticosteroids and β_2 adrenergic agonists (3). Basically, it can be described as the consequence of the airway smooth muscle (ASM) contraction developed by increases in intracellular Ca2+ concentration ([Ca2+]$_i$) in response to agonists or membrane depolarization (4). On the other hand, asthma patients also show airway hyperresponsiveness commonly evaluated by inhalation of methacholine or histamine and this exacerbated ASM response also involves cellular Ca2+ handling mechanisms (5-8). In addition to Ca2+, Na+ is paramount in ASM contraction. The literature available, although not yet conclusive, indicates a possible link between altered ASM cell Na+ handling mechanisms and BC and this evidence is discussed in the present review.

It is well know that excitable cells possess a characteristic resting membrane potential, a state of equilibrium between inner and outer ionic concentrations. In airway myocytes, resting membrane potential fluctuates between -40 and -50 mV (9) and reflects many membrane mechanisms that keep the balance between internal and external ionic concentrations. In this tissue, stimuli that trigger contraction can be characterized as chemical (neurotransmitters, cytokines and terpenoids) and physical (inspired air volume, air pressure and temperature). Although different in nature, both types of stimuli act on membrane proteins that facilitate the passage of ions in and out, causing a current and a voltage change. Na+ transit through the cell membrane has been known to play the leading role

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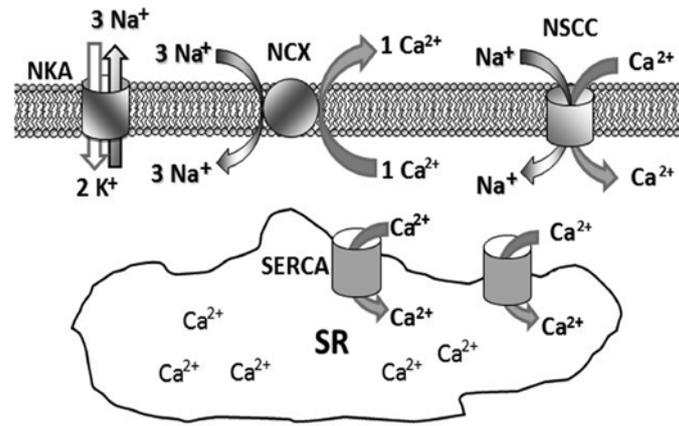
Figure 1. Cellular Na$^+$ homeostatic mechanisms in airway smooth muscle. The Na$^+$/K$^+$ ATPase (NKA) introduces 2 K$^+$ and extracts 3 Na$^+$ ions spending 1 ATP. The Na$^+$/Ca^{2+} exchanger (NCX), by following an electrochemical gradient, introduces 3 Na$^+$ and extrudes 1 Ca^{2+}. Non-specific cation channels (NSCC) allow Na$^+$ and Ca^{2+} entrance and the resting membrane potential in healthy airway smooth muscle is maintained by NSCC recognized as TRPC3 (56). SR, sarcoplasmic reticulum; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase.

in preserving membrane potential at rest. It is extracted from the cells by the Na$^+$/K$^+$ ATPase (NKA) pump or introduced into the cytoplasm by the Na$^+$/Ca^{2+} exchanger (NCX) (Fig. 1). At rest, ASM has a basal tension (tone) which results from the continuous adjustment of Ca^{2+} and Na$^+$ intracytoplasmic concentrations.

During membrane depolarization, Na$^+$ seems to accumulate in regions beneath the plasma membrane, generating local concentration gradients that influence cell Ca^{2+} handling mechanisms. The local Na$^+$ concentration increases rely on three proteins: the NKA, the NCX and non-specific cation channels (NSCC). These proteins are located in precise membrane regions and their function reflects as an augmentation in Na$^+$ concentration and a consequent change in the amount of Ca^{2+} (10). Increases in [Ca^{2+}]$_i$ regulate diverse processes depending on magnitude, duration and cytoplasmic region; in ASM, such cellular processes range from contraction, including protein synthesis, to apoptosis (programmed cell death) (11). Therefore, complex and refined methods to handle [Ca^{2+}]$_i$ have evolved. In this regard, the sarcoplasmic reticulum (SR) stores most of the ASM cellular Ca^{2+}. This ion is released during contraction, but the SR is never completely depleted from its content. After its emptying, the SR Ca^{2+} refilling seems to involve increases in Na$^+$ concentrations in sites nearby. This refilling is also related to smooth muscle sustained contraction (12-18). Recent findings in our laboratory, demonstrate that Na$^+$ permeates through the L-type voltage dependent Ca^{2+} channels when ASM is stimulated with carbachol (19). It is feasible that this Na$^+$ entrance contributes to depolarization, favoring Ca^{2+} entry for SR refilling and sustained contraction maintenance (Fig. 2).

2. Na$^+$/K$^+$ATPase

The NKA on the plasma membrane extracts 3 Na$^+$ ions from the cytoplasm and simultaneously introduces 2 K$^+$ against both ions' concentration gradient. Spending ATP is required, hence the name of ATPase. This pump is very sensitive to inhibition by cardioactive steroids, particularly the group of digitalis and ouabain used to treat congestive heart failure

and arrhythmias (20). Abundant sources report the presence of a 'serum factor' (most probably ouabain) that inhibits the function of NKA (21-26). Endogenous ouabain has been characterized as a steroidal glycoside found in blood plasma and is synthesized by the adrenal glands, hypothalamus and pituitary (27-33). *In vitro*, the release of ouabain in primary cultures of bovine adrenocortical cells is favored by the adrenocorticotropic hormone (ACTH), angiotensin II, vasopressin and phenylephrine (34,35). In ASM, exogenous ouabain induces contraction by blocking the NKA as it promotes a Na$^+$ concentration increase that causes the Na$^+$/Ca^{2+} exchanger (NCX) to reverse (NCX$_{REV}$). When in this reverse state, NCX$_{REV}$ introduces Ca^{2+} into the cytoplasm, favoring contraction. This mechanism is identical to that exerted by inotropic glycosides in cardiac muscle (36), indicating that this steroid could induce BC *in vivo* (37). The NKA consists of αβ heterodimers; the α catalytic subunit contains the binding sites for Na$^+$, K$^+$, ATP and ouabain and is phosphorylated during each pump cycle. The β subunit is indispensable for the function of NKA; it stabilizes the conformation of the α subunit and is the chaperone of the αβ complex in its transit between the SR and the plasma membrane. Each one of the four mammalian isoforms of the α subunit (α1-α4) is the product of a different gene and they show ~90% sequence identity. Functionally, they are different in their correspondent kinetics, expression pattern and regulatory mechanisms. In most cells, NKA isoform α1 is expressed with another isoform; for instance, muscles express α1 and α2 subunits. It has recently been found that the affinity of ouabain differs for each NKA α subunit isoform. Thus, its affinity for the α2 subunit is ~2.5 times less than for the α1 subunit (38). Furthermore, each NKA subtype is located in specific membrane domains depending on its isotype. While NKAs formed by α1 subunits are distributed ubiquitously throughout the membrane, the α2/α3 isoforms are confined to membrane nanodomains adjacent to the SR. The NCX and many canonical transient receptor potential (TRPC) channels that belong to the NSCC are located there, as well; both NCX and NSCC are also described herein. The low affinity for ouabain and the membrane location of the NKAs with α2 subunit compared to those formed by α1, may indicate
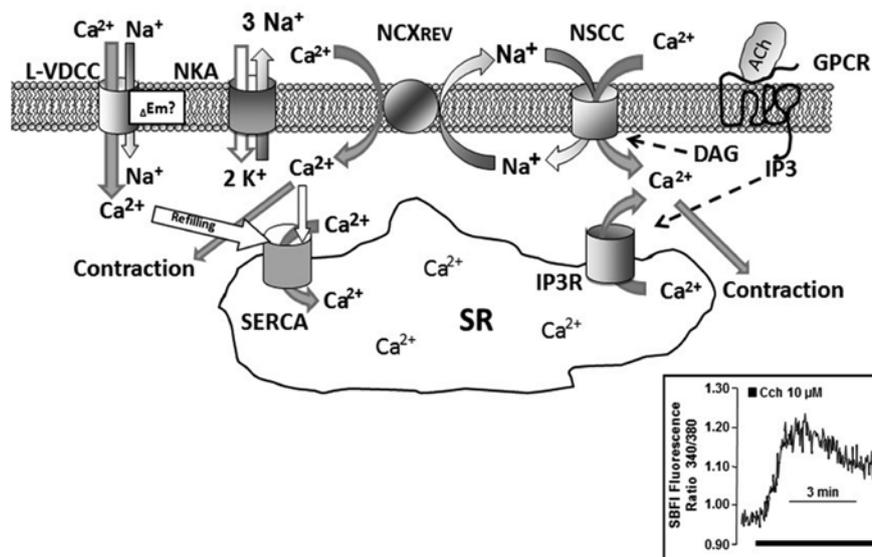
Figure 2. Airway smooth muscle cell Na$^+$ management during stimulation with a cholinergic agonist. Non-selective cationic channels (NSCC) activated by the lowering of the sarcoplasmic reticulum (SR)-Ca^{2+} content can be denominated store operated channels (SOC), while NSCC corresponding to receptor operated channels (ROCs) are activated by IP3 cascade secondary messengers (i.e., DAG, diacylglycerol). Localized Na$^+$ gradients originated by this cation's flux through either NSCC induce the Na$^+$/Ca^{2+} exchanger's reversal (NCX$_{REV}$). NCX$_{REV}$ introduces 1 Ca^{2+} and extrudes 3 Na$^+$, contributing to regulate intracellular Na$^+$ concentrations and SR Ca^{2+} refilling. Ionic currents flowing through NSCC depolarize the membrane, promoting the opening of the voltage-dependent L-type Ca^{2+} channel (L-VDCC). This channel permeates both Ca^{2+} and Na$^+$. This Na$^+$ entrance probably contributes to membrane depolarization (ΔEm) to maintain Ca^{2+} entrance and SR refilling. Inset: representative fluorometric recording of a bovine tracheal myocyte stimulated with carbachol (CCh, 10 μM) in normal Na$^+$ containing Krebs solution (2 mM Ca^{2+}). Na$^+$-specific fluorophore (SBFI) signal was augmented when the cell received CCh, indicating the augmentation of this cation in the cytoplasm (19). SERCA, SR Ca^{2+} ATPase; NKA, Na$^+$/K$^+$ATPase; ACh, acetylcholine; GPCR, G protein-coupled receptor; IP3, inositol trisphosphate; IP3R, inositol trisphosphate receptor.

that they are involved in different cellular mechanisms, besides the regulation of the membrane potential at rest. At nanomolar concentrations, ouabain partially inhibits arterial smooth muscle contraction by augmenting the Na$^+$ concentration in the microdomains containing NKAs with α2 subunit well above that in the rest of the cytoplasm. This is due to the low affinity of α2 pumps for ouabain (38,39) and could have implications in cell physiology. The wide distribution of NKAs with subunit α1 indicates that they are responsible of controling Na$^+$ concentration in the whole cell, while the α2/α3 subunits regulate only the concentrations of the nanodomains where the electrochemical gradient of Na$^+$ reverses the NCX function to increase regional [Ca^{2+}]$_i$. In contrast, when the ASM is stimulated by an agonist, the NCX is reversed by the local increase in Na$^+$ entry through the NSCC (Fig. 2).

3. Na$^+$/Ca^{2+}exchanger

When the [Ca^{2+}]$_i$ increases, it is promptly lowered by various calcium handling proteins. One of them is the NCX (40). Although its activity is determined by the surrounding Na$^+$ concentration, it contributes significantly to the removal of intracellular Ca^{2+}. In a smooth muscle at rest, its function is to extract one Ca^{2+} ion from the cytoplasm and introduce 3 Na$^+$. In mammals, the NCX is encoded by three genes: NCX1, 2 and 3 (41) and in various tissues, its cDNA is subjected to alternative splicing. This splicing implies the elimination of certain regions of the cDNA and as a result, at least 5 exchanger isoforms are generated (42). It has been reported that the NCX1 isoform has at least 12 splicing variants that define the exchanger's ionic sensitivity and regulation (43). Their tissue

distibution could be related to this characteristic; for instance the NCX1.1 has been found in the heart, while the NCX1.3 is located in the kidney and tracheal smooth muscle (44-46). As illustrated on Fig. 1, the NCX1.3 is located in certain cytoplasmic membrane regions. Local increases in Na$^+$ in the areas next to the NCX1.3 induce its function to reverse, favoring the entry of Ca^{2+}; under these circumstances, the NCX is said to be in reverse mode (NCX$_{REV}$). NCX$_{REV}$ extracts Na$^+$ and introduces Ca^{2+} to the cytoplasm (41). In ASM, the NCX$_{REV}$ provides Ca^{2+} for SR refilling, a physiological process indispensable for ASM sustained contraction induced by agonists (47,48) (Fig. 2). In this sense, using Patch Clamp techniques, an outwardly rectifying Na$^+$ current was characterized in cultured human bronchial smooth cells loaded with Na$^+$ and in the presence of extracellular Ca^{2+}. siRNA against NCX1 or knockdown of the stromal cell interaction molecule, (STIM1, an SR Ca^{2+} sensor) significantly diminished this response, suggesting a functional link between these calcium handling proteins. Visibly, STIM seems to promote SR Ca^{2+} refilling through NCX$_{REV}$ activity (49).

4. Non-specific cation channels

This group is constituted by ion channels of varied nature that permeate mainly ions such as Ca^{2+} and Na$^+$ and are designated as NSCC. They can be clasified in two groups based on the cellular mechanisms that activate them: store operated (SOC) channels permate cations in response to a decrease in the SR Ca^{2+} content and receptor operated (ROC) channels which, once the agonist (e.g., acetylcholine) ocupies its receptor activating a signaling pathway, open in response to secondary

messengers (e.g., diacylglycerol) (50). The components of NSCCs have not been fully defined, but the TRPC channels are considered as an essential part of most of them. In general, all TRPs have six transmembranal regions (TM1-6) and oligomerize to form homotetramers or heterotetramers (51). The TRPC family consists of seven members (1-7) and TRPCs are present in excitable and non-excitable cells allowing nonspecific cation entry through the membrane. The current hence generated depolarizes the membrane, triggering the opening of voltage-dependent channels involved in diverse cellular functions. Thus, the TRPCs may cause action potentials in excitable cells (51). These channels also contribute to an increase in intracellular Na⁺ concentration to induce NCX_{REV} (Fig. 2). TRPCs were initially characterized in *Drosophila*; in this species, certain mutation prevents the passage of Ca^{2+} through the phospholipase C coupled channels. Normally, these photoreceptors remain depolarized when exposed to a continuous light source but those cells from specimens with the mutation show a transient response, therefore 'TRPC'. In mammals, the equivalent of the TRPC studied in *Drosophila* is classified into four subfamilies: TRPC1, TRPC2, TRPC3/6/7 and TRPC4/5 (sometimes TRPC1 is included in the subfamily TRPC4/5) (52-54). TRPC1 is considered paradigmatic for the study of SOCs as it has been shown to participate in SR Ca^{2+} refilling (capacitive entry) and in the sustained contraction. TRPC1 forms part of many types of NSCCs, from relatively selective to strictly non-selective channels (regarding Ca^{2+} permeability) and this diversity may be related to its heteromeric association with other TRPCs. Some studies even suggest that the presence of TRPC1 depends entirely on its interaction with another TRPC (TRPC4) (55). It has also been reported that the association of TRPC1 with two other proteins (STIM1 on the SR membrane and Orai on the plasma membrane) is highly selective for Ca^{2+} and that they only permeate a capacitive Ca^{2+} current known as calcium release-activated calcium current (ICRAC) (50,56-58).

With regard to those channels operated by the receptor, it has been found that TRPC3 (and most likely TRPC6 and TRPC7) are activated by inositol triphosphate (IP3) and/or the IP3 receptor (IP3R) (59-61). Therefore, TRPC3 is used as a model channel to study what is considered a conformational coupling between the channel and the IP3R; the latter requires at least its N-terminal portion to activate TRPC3 (62). Nevertheless, IP3 presence is not necessary to induce Ca^{2+} entry through these channels, as pharmacological methods other than agonist stimulation (thapsigargin or ionomycin) induce the same response. It has also been reported that the presence of TRPC3 is crucial to maintain the resting membrane potential in cells of healthy ASM, while in myocytes from asthmatic airways, TRPC1 seems to play an important role (63). Many of the functions of the different TRPC isoforms remain unclear; it has even been proposed that TRPCs are not indispensable for SOCs, but rather to ROCs due to their sensitivity to diacylglycerol (64).

5. Na⁺ voltage dependent currents in airway smooth muscle

The recent evidence presented by Bradley *et al* (65) regarding the expression of a voltage-dependent Na⁺ current in freshly dispersed rabbit bronchial myocytes seemingly mediated by the Nav1.5 α subunit, cannot be overlooked. When this current in bronchial myocytes was pharmacologically altered (blocked with veratridine), their action potential was prolonged. This finding opens new perspectives, as it is possible that airway inflammatory conditions like those prevailing in asthma could modify the voltage-dependent inactivation of the current promoting a bronchospasm.

Former studies (66,67) have already shown that cultured human bronchial smooth muscle cells express Na⁺ currents. Nakajima *et al* (68) were able to characterize and diminish Nav1.7 channel expression with dexamethasone, suggesting that these channels may be important in airway remodeling in asthma.

6. Evidence of Na⁺ participation in asthma

Multiple studies of patients with atopic asthma and/or allergic rhinitis report the presence of plasmatic, endogenous ouabain, indicating a probable intracellular Na⁺ accumulation (21-26). In fact, the study by Tribe *et al* (25) shows an increased intracellular Na⁺ concentration in mononuclear cells from healthy individuals incubated with plasma of asthmatic blood donors. Apparently, endogenous ouabain acts not only on ASM NKAs, but also on inflammatory cells perhaps contributing to consolidate airway hyperresponsiveness. Knox *et al* (69) postulate that the difference lies on the tissue, since ASM contracts *in vitro* in response to exogenous ouabain but does not predispose tissue to hyperresponsivenes. Moreover, Knudsen *et al* (70) demonstrated that ouabain addition to rat peritoneal mast cells induced histamine release while simultaneously blocking intracellular Rb⁺ increase (used as surrogate to evaluate K⁺ cell entry through Na⁺/K⁺ ATPase activity).

The origin of endogenous ouabain found in atopic asthmatic plasma has not been clarified completely, but documentary evidence indicates that mammals produce an endogenous ouabain analog. It is a hormone found in blood plasma, synthesized mainly by the adrenal, hypothalamus and pituitary glands (27-33). *In vitro*, the release of ouabain in primary cultures of bovine adrenocortical cells is favored by the addition of ACTH, angiotensin II, vasopressin and phenylephrine (34,35).

On the other hand, ouabain has been claimed to have anti-inflammatory capabilities by suppressing TNFα production (71). In this regard, the high ouabain generation seen in atopic asthma may be associated with a compensatory process intended to regulate circulating TNF concentrations (72).

7. The paradox of exercise-induced bronchoconstriction

Excercise-induced bronchoconstriction (EIB) is currently defined as a syndrome where exercise or an increase in ventilation triggers airflow obstruction that may last 30-90 min if not attended. Although it occurs most frequently in asthmatic patients, cross sectional studies show that only a portion of patients with asthma have EIB when tested with a specific challenge test (73). The indirect challenges commonly used to define EIB are exercise itself, eucapnic voluntary hyperpnea, hypertonic (4.5%) saline, mannitol and adenosine (74). These 'specific challenge tests' differ from conventional methacholine or histamine challenges. Theoretically, exercise

increases the amount of inhaled air but augments airway lining fluid osmolarity by dehydration, favoring lung mast cell activation and pro-inflammatory mediator release from the epithelium involving a soluble phospholipase A (75,76). Most of these pro-inflammatory mediators can induce ASM contraction directly or favor a primed response to other agonists (hyperactivity). It is conceivable that airway lining fluid Na^+ content could augment osmolarity in the airways during exercise. In this regard, Schmitt *et al* (77) established that, in airway epithelia from asthmatic patients with EIB, transepithelial nasal potential carried out by Cl^- and Na^+ is not modified during exercise, indicating that a Na^+ handling mechanism is at stake. Also associated with impaired cellular Na^+ homeostasis, is the inhibition of the NKA in atopy and EIB. Mast cells express NKAs and NCXs (78); the $[Ca^{2+}]_i$ increase induced by NCX_{REV} in these cells favors the release of inflammatory mediators, pointing out this exchanger's role in inflammation. In fact, the use of an NCX_{REV} inhibitor (KB-R7943) significantly reduced the amount of histamine released by stimulated peritoneal mast cells which correlated with a decrease in $[Ca^{2+}]_i$ (79).

Plenty of evidence suggests that Na^+ intake worsens EIB; a low-sodium diet improved spirometric results after physical activity in exercise-induced asthma patients, while subjects in each of the control groups (healthy individuals under high- or low-sodium diet) had no changes in their spirometric values (80,81). Furthermore, high serum concentrations of antioxidant vitamins, selenium, calcium, chloride and iron, were associated with better FEV_1 values, as compared with higher concentrations of potassium and sodium, that were correlated with lower FEV_1 (82). Asthmatic patients following a low-sodium diet showed reduced inflammation associated with EIB measured by eosinophilic cationic protein, IL-1β and IL-8 concentration in their sputum. This decrease correlated with an improvement in respiratory function assessed by spirometry. In contrast, the group of patients on a high-sodium diet showed a significant worsening of spirometric parameters that correlated with the amount of inflammatory cells and cytokine concentrations in sputum (83). Thus it appears that increasing dietary salt intake worsened inflammation of the airways in asthma patients. However, two separate Cochrane reviews established that there were no significant benefits of salt restriction on the control of asthma. In one of them, Pogson and McKeever (84) none the less claim that 'there was some evidence from the exercise-induced asthma studies that a low-sodium diet may improve lung function after exercise and possibly baseline lung function, but this is based on findings from a very small number of participants'. In the other, Ram and Arden (85) analyzed six randomized controlled trials and did not find any significant effect of dietary salt restriction on asthma treatment or management.

8. Conclusion

In conclusion, dietary sodium intake seems not to play a pivotal role in asthma nor in EIB, rather it is more plausible that Na^+ handling mechanisms are partially responsible for EIB and augmented inflammatory mediator release in both ailments. This argument warrants further and deeper research in ASM sodium handling mechanisms.

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