Reverse-mode NCX current in mouse airway smooth muscle: \(\text{Na}^+\) and voltage dependence, contributions to \(\text{Ca}^{2+}\) influx and contraction, and altered expression in a model of allergen-induced hyperresponsiveness

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Abstract
Aim: We examined the electrophysiological properties of reverse-mode \(\text{Na}^+{/}\text{Ca}^{2+}\) exchange (NCX) in mouse airway smooth muscle (ASM), assessing its contributions to regulation of \([\text{Ca}^{2+}]_c\), and its expression in acute and chronic airway hyperresponsiveness (AHR).

Methods: Membrane currents were studied in single murine ASM cells under voltage clamp at \(-60\) mV using ramp depolarizing commands to \(+80\) mV. Confocal fluorimetric and RT-PCR techniques were used to monitor changes in cytosolic \([\text{Ca}^{2+}]_c\) and NCX expression, respectively.

Results: With standard KCl-containing electrode, 30 \(\mu\)M KB-R7943 (an inhibitor of reverse-mode NCX activity) exhibited variable effects on membrane current, indicating modulation of more than one conductance. KB-R7943 activated outwardly rectifying current that was inhibited by 100 \(\mu\)M iberiotoxin (blocker of large-conductance \(\text{Ca}^{2+}\)-dependent \(\text{K}^+\) channels), indicating a direct enhancing effect of KB-R7943 on those \(\text{K}^+\) channels. After obviating \(\text{K}^+\) currents, we found that a current sensitive to 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid (blocker of \(\text{Ca}^{2+}\)-dependent \(\text{Cl}^-\) channels) was markedly increased by elevating \([\text{Na}^+]_e\) in the electrode solution to 13, 15.5 and 18 \(\mu\)M and suppressed by KB-R7943, indicating \(\text{Ca}^{2+}\) influx via reverse-mode NCX activity. With conditions preventing \(\text{Ca}^{2+}\) influx through voltage-dependent \(\text{Ca}^{2+}\) channels but promoting that through NCX, we found that introduction of \(\text{Ca}^{2+}\) led to marked but transient KB-R7943-sensitive elevation of \([\text{Ca}^{2+}]_c\). Additionally, KB-R7943 suppressed cholinergically evoked \(\text{Ca}^{2+}\) waves. Finally, NCX1 expression was not significantly changed in allergen-induced AHR acute model but increased approx. 2.5-fold in a chronic model.

Conclusion: Reverse-mode NCX activity leads to a physiologically relevant increase in \([\text{Ca}^{2+}]_c\) even under control conditions, and this may be exaggerated in allergen-induced AHR and asthma.

Keywords airway hyperresponsiveness, airway smooth muscle, KB-R7943, reverse-mode sodium calcium exchanger.
Bronchial asthma is a major world health problem and is associated with bronchospasm, airway hyperresponsiveness (AHR) and inflammation (Bateman et al. 2008). Airway narrowing is caused in part by hypertrophy and functional changes in the airway smooth muscle (ASM). Therefore, ASM is critical for asthma development. Increased intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in ASM cells is responsible for airway contraction, and abnormalities of [Ca\(^{2+}\)]\(_i\) may play a key role in AHR (Jude et al. 2008). Non-selective cation channels, particularly those of the transient receptor potential (TRP) family, and voltage-dependent Ca\(^{2+}\) channels contribute directly to the elevation of [Ca\(^{2+}\)]\(_i\). Along with the plasma membrane Ca\(^{2+}\) ATPase, Na\(^{+}\)/Ca\(^{2+}\) exchange (NCX) was originally thought to respond to elevated [Ca\(^{2+}\)]\(_i\) and contribute to Ca\(^{2+}\) extrusion (Blaustein et al. 1991, Blaustein & Lederer 1999). However, under certain conditions, NCX can also operate in the ‘reverse mode’ to mediate Ca\(^{2+}\) entry. That is, given that NCX reversibly exchanges three Na\(^{+}\) ions for each Ca\(^{2+}\) ion and thereby produces a net ion current, the direction of ionic flux through NCX is determined by not only the Ca\(^{2+}\) gradient, but also the Na\(^{+}\) gradient and the membrane potential. This has tremendous physiological implications. For example, bronchoconstrictors activate chloride and non-selective cation current (Janssen & Sims 1992, 1993, 1995, Janssen 1996), thereby depolarizing the membrane and allowing Na\(^{+}\) entry: the resultant elevation of subplasmalemmal [Na\(^{+}\)]\(_s\), in conjunction with low-to-moderate intracellular [Ca\(^{2+}\)]\(_i\), can bring the reversal potential for NCX (E\(_{NCX}\)) into a range of potentials more negative than that of the depolarized membrane, thereby forcing the NCX into reverse-mode operation (Blaustein et al. 1991, Hirota & Janssen 2007a, Hirota et al. 2007a, Bateman et al. 2008). In fact, we have postulated elsewhere (Janssen & Daniel 1991, Hirota & Janssen 2007a, Hirota et al. 2007a) that electrical slow waves, which are present in ASM of every species during excitation (Janssen & Daniel 1991, Janssen et al. 1998), may actually sweep the membrane potential back and forth through the reversal potential for NCX, causing the latter to oscillate back and forth between Ca\(^{2+}\)-efflux and Ca\(^{2+}\)-influx modes. Two recent studies using cultured human cells (Liu et al. 2010, Sathish et al. 2011) showed that NCX-mediated inward current is reduced upon removing external Na\(^{+}\); however, a more precise characterization of the internal [Na\(^{+}\)] dependence of this current in native (non-cultured) ASM is yet to be established. Pharmacological tools to selectively manipulate reverse-mode NCX activity are available: One of these – KB-R7943 – reduces agonist-induced contraction of ASM (Dai et al. 2006, Hirota & Janssen 2007a). Another selective antagonist SEA-0400, which was widely tested in cardiac (Takahashi et al. 2004), vascular (Ogata et al. 2003) and renal (Ogata et al. 2003, Iwamoto et al. 2004) tissues, has never been tested in ASM. The interaction of NCX with other membrane currents such as Ca\(^{2+}\)-dependent K\(^+\) or Cl\(^-\) currents (BK\(_{Ca}\) and Cl\(_{Ca}\), respectively) is also unknown.

Several groups have reported an association between Na\(^{+}/K\(^+\) pump inhibition (leading to intracellular Na\(^{+}\) accumulation, as measured in leucocytes (Agrawal et al. 2005, Chhabra et al. 1999) and platelets (Skoner et al. 1991, Tribe et al. 1994) and increased severity of airway dysfunction: it has been hypothesized, therefore, that elevated intracellular [Na\(^{+}\)] in ASM of asthmatics triggers Ca\(^{2+}\) influx through reverse-mode NCX and contributes in part to ASM hyperresponsiveness. It is well known that intracellular [Ca\(^{2+}\)] is important in the pathogenesis of asthma (Thomas 2001) and chronic obstructive airway disease (Barnes et al. 2003).

A recent study using the inflammatory cytokines in cultured human ASM cells showed that TNF\(_\alpha\) and IL-13 enhance the expression of NCX1 and thereby increases [Ca\(^{2+}\)]\(_i\) (Sathish et al. 2011). Moreover, RT-PCR and WESTERN blotting show that NCX1 is the only isoform present in ASM (Liu et al. 2010). We hypothesized that chronic exposure to house dust mite (HDM) allergen increases the expression of NCX1 protein in ASM.

The focus of this study, then, was to characterize the NCX activity in native (non-cultured) ASM, particularly its dependence upon voltage and internal [Na\(^{+}\]); the latter has not yet been examined in detail nor using physiological interventions (i.e. aside from simply removing external Na\(^{+}\)) and is central to understanding the relationship of this membrane current to membrane potential and electrical slow wave activity. We also sought to examine its interactions with other ion transport pathways (BK\(_{Ca}\) and Cl\(_{Ca}\)), and to compare the actions of SEA-0400, none of which has yet been done in ASM. We also assessed the contribution of reverse-mode NCX activity to changes in [Ca\(^{2+}\)]\(_i\) and contraction in control mice. Finally, we examined whether NCX expression was increased in HDM allergen-treated mice in acute and chronic models of AHR.

**Method**

**Animals**

10 to 12-week-old BALB/c female mice were housed in environmentally controlled, specific pathogen-free conditions for 1-week acclimatization period and throughout the study period. The procedures were approved by the Animal Research Ethics Board at...
House dust mite allergen exposure model (HDM) and intervention design

For acute intervention, two groups of mice \( n = 20 \) in each) were studied: (i) acute saline exposed and (ii) acute HDM exposed. Group (ii) mice received 15 \( \mu \)g of HDM extract (Greer Laboratories, Lenoir, NC, USA) prepared as described previously (Southam et al. 2007) in 25 \( \mu \)L phosphate-buffered saline volume on days 1–5, 8–12 and 15–16 under gaseous anaesthesia via the intranasal (IN) route. Group (i) animals received IN saline exposure on those days. Outcome measurements were made 24 h post-final HDM exposure (day 17).

For chronic intervention, two other groups of mice were treated with IN saline or HDM (groups iii and iv, respectively; \( n = 20 \) each): in this case, allergen exposure was also done on days 1–5 and 8–12, but then also for the next 6 weeks on the 1st, 3rd and 5th day of each week. Outcome measurements were made 48 h post-final exposure of total 8 weeks (day 57). Bronchoalveolar lavage and serum analyses were performed as described previously (Leigh et al. 2002).

Preparation of airway smooth muscle cells from mouse

Cells were isolated using a two-step enzymatic digestion method as described previously (Wang et al. 2003). In short, mice were killed with 95% CO\(_2\) (7 L min\(^{-1}\)). Then, the trachea and main stem bronchi were removed and transferred in ice cold balanced salt solution (HBSS; Sigma-Aldrich, St Louis, MO, USA) and supplemented with 10 mg mL\(^{-1}\) papain, 10 mg mL\(^{-1}\) DTT and 50 mg mL\(^{-1}\) BSA and incubated at 37°C for 20 min. The tissue was then incubated with fresh HBSS containing 10 mg mL\(^{-1}\) collagenase II, 10 mg mL\(^{-1}\) collagenase F, 10 mg mL\(^{-1}\) DTT, 50 mg mL\(^{-1}\) BSA (Sigma-Aldrich). Finally, the tissue was gently agitated with a fire polished wide bore glass pipette to release the cells. Only single cells that were spindle shaped with a length of around 150 \( \mu \)m were used (within 6 h of digestion) at 25°C; we have found such cells to contract in response to ACh or caffeine, and they are easily distinguished from the other major cell type – epithelial cells – which are smaller, round and often ciliated.

RNA isolation and RT-PCR

Airway smooth muscles were homogenized in 1 mL of TRIzol\(^\text{®}\) (Invitrogen, Carlsbad, CA, USA); RNA was extracted using standard methods. RNA concentration and integrity were determined with a microgel bioanalyzer (Agilent Bioanalyzer 2100; Agilent, Mississauga, ON, Canada). One microgram of RNA was DNase treated, and quantitative real-time PCR was conducted using the ABI Prism 7500 PCR system (Applied Biosystems, Foster City, CA, USA). RT-PCR probe and primer sets (gene expression assays) were purchased from Applied Biosystems. Results were normalized to β2-microglobulin. Relative gene expression was calculated using the \( \Delta\Delta CT \) method (Applied Biosystems 1997).

Electrophysiology

The patch-clamp technique was used in the whole-cell configuration as previously described (Janssen et al. 1997), using glass electrodes with a resistance of 3–6 MΩ. Briefly, currents were monitored using an Axopatch-B current–voltage amplifier, filtered at 1 kHz, digitized at 2 kHz and analysed using standard software (AXOSCOPE 9.0; Axon Instruments, Sunnyvale, CA, USA). Current–voltage relationships were investigated using voltage ramps (~80 to +80 mV, 400 ms duration; preceded by a 30 ms step to ~80 mV to allow for settling of the capacitive transients), delivered at 15s intervals. Reversal potentials quoted in the text, but not current–voltage relationships in the figures, were corrected for a measured liquid junction potential of 25 mV.

Lung slices preparation and measurement of \( \text{Ca}^{2+} \) Signalling

Lung slices (130 \( \mu \)m thickness) were prepared as previously described (Khan et al. 2007). They were loaded with 20 \( \mu \)M Oregon Green-AM in HBSS solution (5–10 slices mL\(^{-1}\)) containing 0.1% Pluronic F-127 and 100 \( \mu \)M sulforhodamine for 1 h at 30°C, followed by an additional 30 min in shHBSS containing 100 \( \mu \)M sulforhodamine for the de-esterification of the dye. Final DMSO concentration was 1.25%. Fluorescence images were recorded at video rate 30 Hz by VIDEO SAVANT software (London, ON, Canada) and a custom-built confocal microscope, as described previously (Tazzeo et al. 2008). The average fluorescence intensity of a region of interest (5 × 5 pixels) inside a single SMC was analysed frame by frame with SCION IMAGE software (NIH, Bethesda, MD, USA) and custom-written macros. Fluorescence values (F) were expressed as a fraction of the initial fluorescence (\( F_0 \)).

Solutions and drugs

Electrode solution contained (in mM): 130 CsAsp or 135 KCl (as indicated in text), 0–20 NaCl (as indicated), 1 MgCl\(_2\), 0.1 EGTA, 4 Na\(_2\)ATP, 10 HEPES, 20 HEPES, and 0.2 HEPES/balanced salt solution (HBSS; Sigma-Aldrich, St Louis, MO, USA).
PES, pH adjusted to 7.2 using CsOH. Osmolarity was confirmed to be approx. 290 mOsm, and free \([\text{Ca}^{2+}]\) estimated to be 61 nM using WinMaxC v2.10 software (http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm) and assuming contaminating Ca\(^{2+}\) in our deionized water to be 1 ppm (i.e. \(2.5 \times 10^{-5}\ \text{M}\)). Standard Ringer’s solution contained (in mM): 135 NaCl, 5 KCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 10 glucose, pH 7.4 with NaOH. For fluorimetric studies, high-K’/high-Ca\(^{2+}\) solution contained (mM): 100 KCl, 20 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, pH 7.4 with KOH. This solution and the standard Ringer’s solutions (osmolarity approx. 300 mOsm) were supplemented with nifedipine (10\(^{-5}\) M, to block voltage-dependent Ca\(^{2+}\) currents), ouabain (10\(^{-5}\) M, to block Na’/K’-ATPase activity), and either niflumic acid (10\(^{-4}\) M) or its isothiourea methanesulfonate (KB-R7943) and iberiotoxin were purchased from Tocris (Minneapolis, MN, USA). All other reagents were obtained from Sigma-Aldrich. KB-R7943 was first dissolved in DMSO; the final concentration of DMSO was 0.1% or less, which we have previously found to have no effect. SEA-0400 was kindly provided by Dr Lorand Kiss, (Institute of Pharmaceutical Chemistry, University of Szeged, Hungary).

Data analysis

All experiments were performed at room temperature. In fluorimetric experiments, \(n = 4–12\) animals. In electrophysiology, \(n\) indicates the numbers of isolated cells tested. In HDM allergen model, \(n\) represent the number of animals in each group. Data are presented as means ± SEM and significant differences were detected using the Student’s \(t\)-test (\(P < 0.05\)).

Results

Inhibition of NCX currents by KB-R7943 in airway smooth muscle

We first assessed the effect of two selective inhibitors of reverse-mode NCX activity on membrane currents after obviating K’ currents (equimolar replacement of K’ with Cs’ and blocking Ca\(^{2+}\)-dependent Cl\(^-\) currents and voltage-dependent Ca\(^{2+}\) currents as well as Na’/K’ activity (refer to Solutions and drugs). Under these conditions, and when intracellular [Na’] was 18 mM (electrode containing 10 mM NaCl and 4 mM Na\(_2\)ATP) and free [Ca\(^{2+}\)], estimated to be 61 nM, ramp depolarizing commands evoked a linear current that was significantly reduced in magnitude by KB-R7943 (30 \(\mu\)M; Fig. 1a,e) or by SEA-0400 (3 \(\mu\)M; Fig. 1b,e). The difference currents are displayed in Figure 1c,d and reveal an outwardly rectifying current, especially in the case of SEA-0400; this suggests SEA-0400 may be more selective than KB-R7943 against reverse-mode (Ca\(^{2+}\) influx) NCX. The magnitude of the KB-R7943-sensitive current (\(I_{\text{KBR}}\)) was dependent upon cytosolic [Na’], being significantly reduced when cytosolic [Na’] was reduced to 13 mM (5 mM NaCl plus 4 mM Na\(_2\)ATP) and undetectable with [Na’] = 8 mM (electrode containing 4 mM Na\(_2\)ATP but no NaCl; Fig. 1f). The mean reversal potentials for \(I_{\text{KBR}}\) when [Na’] in the pipette was 18 mM, 15.5 mM, or 13 mM were \(-49 \pm 11\ \text{mV (}\ n = 9\), \(-42 \pm 12\ \text{mV (}\ n = 4\)) and \(-39 \pm 10\ \text{mV (}\ n = 5\), respectively (values corrected for a measured liquid junction potential of 25 mV).

Activation of Ca\(^{2+}\)-dependent currents by NCX in airway smooth muscle

We examined whether \(I_{\text{KBR}}\)-mediated Ca\(^{2+}\) influx could be detected by endogenous Ca\(^{2+}\)-dependent ion currents: large-conductance Ca\(^{2+}\)-dependent K’ (BK\(_{\text{Ca}}\)) and Cl (Cl\(_{\text{Ca}}\)) currents (Janssen & Sim’s 1992, 1993, 1995, Janssen 1996). In cells in which Ca\(^{2+}\) and Cl- currents were obviated pharmacologically and that were dialysed internally with 18 mM Na\(^{+}\), ramp depolarizing commands evoked large outwardly rectifying currents that were reduced partially by the subsequent application of KB-R7943 (30 \(\mu\)M) and reduced further by the subsequent addition of the selective BK\(_{\text{Ca}}\) blocker iberiotoxin (100 nM; Fig. 2a,c). Similar observations were made when the order of these two blockers was reversed (Fig. 2b,d).

Likewise, when K’ currents were obviated (using Cs’) and the cytosol dialysed with 18 mM Na\(^{+}\), ramp depolarizing commands evoked a large non-rectifying current that was reduced considerably by KB-R7943 (30 \(\mu\)M) and then nearly abolished by further addition of niflumic acid (100 \(\mu\)M; Fig. 3a,d). Similar observations were obtained using another Cl- channel blocker DIDS (Fig. 3b–d).

All three sets of observations are consistent with a mechanism by which Ca\(^{2+}\) influx via reverse-mode NCX activity augments BK\(_{\text{Ca}}\) or Cl\(_{\text{Ca}}\).

Inhibition of NCX current by Ni\(^{2+}\) in airway smooth muscle

As Ni\(^{2+}\) inhibits NCX activity (Wongcharoen et al. 2006), we examined its effect upon the current evoked by ramp depolarizing commands after first blocking K’ currents (using Cs’), Cl- currents
We found 10 mM Ni2+ did indeed suppress those currents (Fig. 4) to the same extent as KB-R7943 or SEA-0400 (Figs 1c, 2c and 3d). Moreover, the reversal potential of the Ni2+-inhibited current was -44 ± 7 mV (n = 6), which is not statistically significantly different from the KB-R7943-inhibited current (-49 ± 11 mV; corrected for liquid junction potential).

**Chronic HDM-induced AHR and NCX expression**

Next, we examined the expression of NCX in a model of AHR, comparing that expression in mice that had been sensitized acutely or chronically to HDM (see Method) and then challenged with the allergen vs. those in which saline alone (without allergen) was used during the sensitization/challenge periods.

Acute and chronic HDM exposure resulted in marked airway inflammation (Fig. 5a,b) that was...
primarily eosinophilic in nature (Fig. 5c,d), much as we have shown several times before (Southam et al. 2007). We did not quantify AHR per se, because the cholinergic stimulation, which this intervention entails, might otherwise influence the subsequent electrophysiological and fluorimetric measurements: however, we have shown several times in the past that these interventions do produce statistically significant AHR (Southam et al. 2007). More importantly, using RT-PCR techniques, we found NCX1 expression to be not significantly changed in the acute AHR model but statistically significantly increased (approx. 2.5-fold) in the chronic AHR model (Fig. 5e,f).

**High-K⁺-induced Ca²⁺ signalling**

We further studied the potential role for Ca²⁺ influx mode NCX activity as a potential source of intracellular Ca²⁺ signalling using Oregon Green. Baseline fluorescence was first obtained while bathing with standard Ringers solution, then following introduction of a modified Ringer’s solution designed to favour reverse-mode NCX by depolarizing the membrane (using 100 mM K+) and raising the external concentration of Ca²⁺ (20 mM) while blocking voltage-dependent Ca²⁺ currents and Na⁺/K⁺-ATPase activity (see Method for composition). Introduction of this modified high-K⁺/high-Ca²⁺ solution was accompanied by a marked but transient elevation of [Ca²⁺]i (Fig. 6a–f). In a separate group of cells, pretreatment with KB-R7943 had no significant effect on baseline fluorescence but abrogated the high-K⁺/high-Ca²⁺-induced elevation of [Ca²⁺]i (Fig. 6f). In addition, we studied the role of KB-R7943 on the agonist-induced elevation of [Ca²⁺]i in the presence of normal extracellular Ca²⁺ concentration. 10 μM acetylcholine (Ach) enhanced the baseline fluorescence up to 62 ± 24%, which was inhibited by KB-R7943 (Fig. 6g,h).

We also found the elevation of [Ca²⁺]i, evoked by high-K⁺/high-Ca²⁺ media was equivalent to that of Ach (10⁻⁸ to 10⁻⁵ M) with F/F₀ of 1.27 ± 0.06 and 1.29 ± 0.09, respectively. On the other hand, the reduction in diameter resulting from contraction by high-K⁺/high-Ca²⁺ media was almost half of the mechanical response to Ach (12 ± 3% and 25 ± 8%, respectively, Fig. 7). This finding suggests that the Ca²⁺ entry by reverse-mode NCX increases intracellular Ca²⁺ but not all of these Ca²⁺ are available to the contractile apparatus. Although the contractile response to Ach was sustained, that to high-K⁺/high-Ca²⁺ media was transient, consistently reverting back to baseline within 2–3 min.
In this study, we provide direct electrophysiological and pharmacological evidence of a reverse-mode NCX current in ASM. We demonstrate directly for the first time that the NCX current is critically dependent upon intracellular [Na+] and that Ca2+ influx via this pathway elevates [Ca2+], augmenting other Ca2+-dependent

**Table 1**

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<thead>
<tr>
<th>Electrolytes (mM)</th>
<th>Intracellular</th>
<th>Extracellular</th>
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<tr>
<td>Na+ 135, Ca+ 1, K+ 5, Cl- 144</td>
<td>Na+ 18, Ca+ 0, K+ 135, Asp 100</td>
<td>Ouabain, Nifedipine</td>
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**Figure 3** Reverse-mode Na+/Ca2+ exchange (NCX) and Ca-activated Cl- current. Representative current traces showing the reduction in membrane current by 30 μM KB-R7943 plus/minus 100 μM niflumic acid (a) or 300 μM 4,4’-disorbiophenylstilbene-2,2’-disulfonic acid (DIDS) (b). (c) shows the effect of first blocking Cl- current using 300 μM DIDS and then blocking NCX current using 300 μM DIDS. Average blocking effects are shown in (d) (n = 4–6); P < 0.05 (*), relative to control.

**Figure 4** Inhibition of reverse-mode Na+/Ca2+ exchange by Ni2+. Representative current traces showing the blocking of membrane current by 10 mM Ni2+ after first blocking Cl-, voltage-dependent Ca2+ and Na+/K+-ATPase currents (a); average blocking effect is shown in (b) (n = 6); *P < 0.05, relative to control.

**Discussion**

In this study, we provide direct electrophysiological and pharmacological evidence of a reverse-mode NCX current in ASM. We demonstrate directly for the first time that the NCX current is critically dependent upon intracellular [Na+] and that Ca2+ influx via this pathway elevates [Ca2+], augmenting other Ca2+-dependent

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controls. We also provide the first evidence that the expression of NCX protein in ASM is enhanced in a chronic model of allergen sensitization in NCX protein in ASM is enhanced in a chronic model of allergen sensitization. We also provide the first evidence that the expression of mRNA for Na+/Ca2+ exchange has recently been proposed to provide a Ca2+ entry pathway in ASM (Janssen 2007a, b, Hirota et al. 2007, Sims 1993, Blaustein & Lederer 1999, Algarasuarez et al. 2007, Hirota & Janssen 2007a, b, Hirota et al. 2007a, b, Liu et al. 2010). However, the majority of these studies did not control or manipulate the physiological driving forces that are necessary for Ca2+ entry through NCX: that is, elevated cytosolic [Na+] and membrane depolarization. Only one other study examined the voltage dependence of NCX activity (Liu et al. 2010) but had several limitations, as outlined later (next paragraph). Also, none of those earlier studies examined whether NCX was altered in tissues in hyper-responsive airways. Our recording conditions (obviating K+, Cl−, voltage-dependent Ca2+ and Na+/K+-pump currents, as well as controlling membrane potential and transmembrane Na+ and Ca2+ gradients) allow us to isolate NCX much more effectively than previous studies that used calcium fluorimetry or mechanical activity as indirect indices of [Ca2+]i. We observed that the amplitude of the KB-R7943-sensitive current (i.e. Ca2+-influx mode NCX current) was critically dependent on the elevation of internal [Na+]i: it was non-measurable at [Na+]i of 8 mM, while the elevation of [Na+]i by only a few millimolar to 13 or 18 mM, well within the physiological range, was sufficient to markedly increase the magnitude of this current (Fig. 1f) and shift the measured reversal potential into a range in which reverse-mode Ca2+ influx would be triggered and be modulated by electrical slow wave activity. The latter have been described in ASM of the human (Honda & Tomita 1997), guinea-pig (Honda et al. 1996), dog (Janssen & Daniel 1991), cow (Hirota & Janssen 2007a) and ferret (Murlas & Doupnik 1989) and comprise oscillations in membrane potential centred around −40 to −30 mV with amplitudes of up to 25 mV and a frequency of approx. 1 Hz. These would sweep the membrane potential back and forth across the reversal potential for NCX, allowing the latter to alternate between the Ca2+-influx and Ca2+-efflux modes and to be exquisitely modulated by changes in cytosolic [Na+]i and/or [Ca2+]i. Interestingly, earlier reports have described an intimate relationship between the NCX and TRP proteins and suggested that NCX and the Na+/K+ pump are co-localized in smooth muscle caveolae in close apposition to the sarcoplasmic reticulum (SR) (Matsuda et al. 2001, Namekata et al. 2009). Thus, it is possible that reverse-mode NCX, driven by membrane depolarization and elevated [Na+]i, in the region between the SR and the plasmalemma, may contribute to Ca2+ influx in the ASM cell and refilling of its SR.

Even after obviating membrane currents through K+ channels (complete replacement of internal K+ with Cs+), chloride channels (100 μM niflumic acid), voltage-dependent Ca2+ channels (1 μM nifedipine) and the Na+/K+ pump (10 μM ouabain), there still appeared to be some other conductance(s) in addition to the NCX current, because a substantial portion of the remaining current was resistant to KB-R7943, SAE-0400 or Ni2+. Also, our observed value for NCX reversal potential when internal Na+ was 13 mM
deviated somewhat from the value predicted from the Nernst equation (−39 and −67 mV, respectively), and this discrepancy grew larger as internal [Na+] was increased (shifting the NCX reversal potential to much more negative values). These findings collectively suggest the presence of another contaminating current.

**Figure 6** Ca²⁺ signalling induced by high K⁺/high Ca²⁺ in mouse airway smooth muscle (ASM) without or with pre-application of KB-R7943 (30 μM). Fluorescence images show lung slices before or after the application of high-K⁺/high-Ca²⁺ Ringers solution (a and b, respectively) in the absence of KB-R7943; arrows indicate ASMs. In presence of KB-R7943, image (c) and (d) show ASMs before and after the application of high-K⁺/high-Ca²⁺ Ringers solution respectively. (e) The transient elevation of [Ca²⁺]i, evoked by high-K⁺/high-Ca²⁺ buffer is significantly smaller following pre-treatment with KB-R7943 (P < 0.005). Each column represents the mean ± SEM, n > 5 in each group. (f) Region of interests were defined in ASMs and Ca²⁺ changes in response to high K⁺/high Ca²⁺ with (right panel) or without (left panel) pre-applied KB-R7943 (bars) expressed as a fluorescence ratio. (g, h) With normal extracellular Ca²⁺ concentration, the transient elevation of [Ca²⁺]i, evoked by acetylcholine (Ach) is abrogated by the presence of KB-R7943 (30 μM). P < 0.05 (*) relative to Ach.
Figure 7 Changes in \([Ca^{2+}]\), and lumen area evoked by Ach or high-K*/high-Ca\(^{2+}\) media. Fluorescence ratio (a) and (b) reduction in lumen area evoked by Ach \((10^{-8} \text{ to } 10^{-5} \text{ M})\) or by the introduction of high-K*/high-Ca\(^{2+}\) media. \(n > 5\) in each group.

The nature of this other putatively non-NCX current is not certain to us but could include one or more of the non-selective cation currents (e.g. TRP currents) and/or \(Ca^{2+}\)-store refilling currents (e.g. Orai1) that are known to be present in ASM and have reversal potentials close to 0 mV. However, store-operated \(Ca^{2+}\) entry in other cell types is highly sensitive to \(Ni^{2+}\) (Matsuoka et al. 2009, Ng et al. 2009), whereas the KB-R7943- and SEA-0400-insensitive current in our study was unaffected by \(Ni^{2+}\). The discrepancy between observed and predicted values in an earlier study of NCX in ASM (Liu et al. 2010) was considered more than \(23 \text{ mV vs. } -137 \text{ mV}\), respectively, given that their electrode solution contained 20 \(mM\) \(Na^{+}\) and 14 \(nM\) free \(Ca^{2+}\) to an extent that it was hard to interpret and was not explained. This study was unaffected by \(Ni^{2+}\). The discrepancy between observed and predicted values in an earlier study of NCX in ASM (Liu et al. 2010) was considered more than \(23 \text{ mV vs. } -137 \text{ mV}\), respectively, given that their electrode solution contained 20 \(mM\) \(Na^{+}\) and 14 \(nM\) free \(Ca^{2+}\) to an extent that it was hard to interpret and was not explained. That study was unaffected by \(Ni^{2+}\). The discrepancy between observed and predicted values in an earlier study of NCX in ASM (Liu et al. 2010) was considered more than \(23 \text{ mV vs. } -137 \text{ mV}\), respectively, given that their electrode solution contained 20 \(mM\) \(Na^{+}\) and 14 \(nM\) free \(Ca^{2+}\) to an extent that it was hard to interpret and was not explained. That study was unaffected by \(Ni^{2+}\). The discrepancy between observed and predicted values in an earlier study of NCX in ASM (Liu et al. 2010) was considered more than \(23 \text{ mV vs. } -137 \text{ mV}\), respectively, given that their electrode solution contained 20 \(mM\) \(Na^{+}\) and 14 \(nM\) free \(Ca^{2+}\) to an extent that it was hard to interpret and was not explained. That study was unaffected by \(Ni^{2+}\). The discrepancy between observed and predicted values in an earlier study of NCX in ASM (Liu et al. 2010) was considered more than \(23 \text{ mV vs. } -137 \text{ mV}\), respectively, given that their electrode solution contained 20 \(mM\) \(Na^{+}\) and 14 \(nM\) free \(Ca^{2+}\) to an extent that it was hard to interpret and was not explained. That study was unaffected by \(Ni^{2+}\).
study, we observed that the inhibitory effect of 3 μM SEA-0400 is equivalent to that of 30 μM KB-R7943 and appeared to not alter the inward component of the current–voltage relationship (Fig. 1), which pertains to Ca\(^{2+}\)-efflux mode NCX activity.

NCX1 exchange 1 protein was detected in cultured human bronchial and bovine tracheal smooth muscle cells, with NCX1 being the only mRNA transcript variant (Hirota & Janssen 2007a, Liu et al. 2010, Sathish et al. 2011). Sathish et al. (2011) found that NCX1 expression in cultured ASM cells was enhanced by the pro-inflammatory cytokines TNFα and IL-13. However, no one has yet examined whether this expression is modulated neither in native cells nor in standard models of AHR. Therefore, we assessed NCX1 expression in an acute model and a chronic model of allergen-induced airway hypersensitivity. Consistent with our previous studies of multiple allergen exposure (Leigh et al. 2002, DiGiovanni et al. 2009), we observed increases in total lung inflammatory cell and eosinophil counts in HDM-exposed mice compared with saline controls, both in our acute and chronic models, and that the chronic model is accompanied by markedly increased expression of NCX protein (approx. 2.5-fold) relative to β2-microglobulin (accounts for changes in ASM mass). Interestingly, the number of allergen-exposures in the acute model seems to be insufficient to also lead to such increased expression. The chronic allergen model is accompanied by remodelling of the airway wall, including ASM hypertrophy, goblet cell hyperplasia and increased matrix protein deposition. Moreover, the AHR observed after chronic allergen exposure can persist for up to 8 weeks, while that observed following brief exposure resolves typically within 2–4 weeks (Leigh et al. 2002, DiGiovanni et al. 2009). Possibly, the increased expression of the NCX protein may play a role in the sustained nature of AHR in these models. Our data do not allow us to determine the cellular localization of this increased NCX expression.

In conclusion, we provide direct electrophysiological and pharmacological evidence of reverse-mode NCX activity in ASM and find that Ca\(^{2+}\) influx via that pathway leads to a physiologically relevant increase in [Ca\(^{2+}\)]\(_{i}\) even under control conditions and which may be exaggerated in a chronic model of allergen-induced AHR and asthma.

Conflict of Interest

None of the authors have any conflict of interest pertaining to this work.

The authors would like to acknowledge Dr Martin Kolb for his collaboration and Fuqin Duan for helping in RT-PCR, as well as Jennifer Wattie and Tracy Tazzeo for the intranasal application of HDM and saline.

Author contributions

Conception, design, analysis and interpretation were performed by Mozibur Rahman and Luke Janssen. The manuscript for important intellectual content was also drafted by above-mentioned authors. SEA-0400 was synthesized by Dr Lorand Kiss. Animal Study designed by Mark Inman, Mozibur Rahman and Luke Janssen.

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