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Store-refilling involves both L-type calcium channels and reverse-mode sodium–calcium exchange in airway smooth muscle

S. Hirota and L.J. Janssen

ABSTRACT: The aim of the present study was to examine the relative contributions to store-refilling of the two following voltage-regulated calcium ion influx pathways: 1) L-type-Ca^{2+} channels; and 2) the reverse-mode of the sodium–calcium exchanger (NCX).

Successive acetylcholine-induced contractions, triggered in bovine tracheal smooth muscle strips, were measured to determine the effect of intervention on contractions as an indication of the extent of store-refilling.

Pre-treating the tissues with cromakalim significantly reduced the magnitude of successive contractions. Zero-Ca^{2+} bathing media abolished the contractions, an effect that was completely reversed upon reintroduction of Ca^{2+}. Inhibition of L-type Ca^{2+} channels, with nifedipine, significantly reduced the magnitude of the contractions. Similarly, inhibition of the reverse-mode of the NCX, with KB-R7943, significantly reduced the magnitude of the contractions. However, neither nifedipine nor KB-R7943 alone reduced contractions to the same extent as observed under zero-Ca^{2+} conditions. Concurrent treatment with nifedipine and KB-R7943 almost abolished successive contractions. Furthermore, concurrent treatment with nifedipine and zero-Na^{+} bathing media displayed a significantly greater effect than nifedipine alone. Probing the expression of NCX1 isoforms by Western blotting revealed the presence of three bands of 160, 120 and 110 kDa. The 120- and 110-kDa bands were identified as variably spliced NCX isoforms, NCX1.1 and NCX1.3, respectively.

Taken together, these data suggest that influx of calcium ions through both L-type calcium channels and the reverse-mode of the sodium–calcium exchanger is necessary for complete store-refilling in airway smooth muscle.

KEYWORDS: Airway smooth muscle, calcium-handling, L-type calcium channel, membrane depolarisation, reverse-mode sodium–calcium exchanger, sarcoplasmic reticulum

Contraction of airway smooth muscle (ASM) is mediated in large part through agonist-induced mobilisation of Ca^{2+} from the sarcoplasmic reticulum (SR). Following Ca^{2+}-mobilisation, mechanisms must exist to reduce intracellular Ca^{2+} levels, leading to relaxation. Cytosolic Ca^{2+} can be sequestered into the SR through the active pumping of the sarco/endoplasmic reticulum calcium-adenosine triphosphatase (ATPase) for subsequent mobilisation or extruded to the extracellular domain by the plasma membrane calcium-ATPase and the forward-mode of the sodium–calcium exchanger (NCX). The Ca^{2+} deficit resulting from the extrusion of Ca^{2+} from the cell must be reversed in order for cells to store adequate Ca^{2+} for subsequent mobilisation and contraction.

Indeed, agonist stimulation can activate Ca^{2+} influx pathways that contribute to the overall intracellular Ca^{2+} pool. Mobilisation of Ca^{2+} from the SR can activate depolarising membrane chlorine ion currents that trigger voltage-dependent Ca^{2+} influx [1–3]. L-type Ca^{2+} channels have been viewed as the primary conduit for voltage-dependent Ca^{2+} influx, and there are several reports that these appear to play a role in Ca^{2+} store-refilling [4–6], although inhibition of this pathway did not completely mimic the effect of complete removal of external Ca^{2+}. Ca^{2+} influx *via* the reverse-mode of the NCX is also driven by membrane depolarisation, in conjunction with increased intracellular Na^{+} levels mediated by the gating of store-operated nonselective cation channels (NSCCs) [7–11, reviewed in 12]. An

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STATEMENT OF INTEREST
None declared.

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additional contribution of reverse-mode NCX activity to partial refilling of the internal store in ASM following agonist stimulation has recently been documented [13]. Finally, a membrane conductance that is activated upon store depletion, and which might play a role in store refilling, has also been described [14]. The relative contributions of the two voltage-dependent Ca^{2+}-influx pathways (the reverse-mode of the NCX and L-type Ca^{2+}-channels) and the voltage-independent receptor/store-operated cation channel (ROC/SOC) current to store refilling in ASM are still the subject of much investigation and debate.

The aim of the present study was to address this question by comparing the effects of selective blockers of the reverse-mode of the NCX (KB-R7943) and/or L-type Ca^{2+}-channels (nifedipine) with those of suppressing voltage-dependent mechanisms in general using a potassium channel opener (cromakalim) and suppressing Ca^{2+} influx in general by omitting Ca^{2+} from the bathing medium.

MATERIALS AND METHODS

Animals

All experimental procedures were approved by the McMaster University Animal Care Committee (McMaster University, Hamilton, ON, Canada) and conformed to the guidelines set out by the Canadian Council on Animal Care (Ottawa, ON, Canada). Tracheae were obtained from cows (136–454 kg) euthanised at the local abattoir and transported to the laboratory in ice-cold modified Krebs buffer (116 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11 mM D-glucose, 0.01 mM indomethacin, 0.0001 mM propranolol and 0.1 mM N^{ω}-nitro-L-arginine (L-NNA), saturated with 95% oxygen/5% carbon dioxide to maintain pH at 7.4). Upon receipt of a trachea, the epithelium was removed and tracheal strips (~2–3 mm wide and ~10 mm long) were excised and used immediately or stored at 4°C for up to 48 h.

Study design

In order to examine the extent of store-refilling in smooth muscle, successive agonist-induced contractions of isolated ASM strips were measured using a standard organ bath preparation. Interventions aimed at inhibiting Ca^{2+} influx pathways (using Ca^{2+}-free Krebs buffer, composed as modified Krebs buffer but with the omission of $CaCl_2$ and addition of 1 mM ethyleneglycol bis-(2-aminoethylether)-*N*,*N*,*N*′*N*′-tetra-acetic acid (EGTA)), namely the reverse-mode of the NCX (using KB-R7943 (0.1 M in dimethylsulphoxide diluted in Krebs buffer as appropriate) and Zero-Na^+ Krebs buffer, composed as modified Krebs buffer but with *N*-methyl-D-glucamine in place of NaCl and KH_2PO_4 and $KHCO_3$ instead of NaH_2PO_4 and $NaHCO_3$, with the pH adjusted to 7.4 using 5 N HCl and saturated with 95% oxygen/5% carbon dioxide to maintain this pH) and L-type Ca^{2+} channels (using 0.1 M nifedipine in ethanol diluted in Krebs buffer as appropriate), were employed after obtaining a control acetylcholine (Ach) response (using 0.1 M ACh in distilled deionised water diluted in Krebs buffer as appropriate), and the magnitude of each subsequent contractile response was normalised to this control response in order to determine what effect inhibition of various Ca^{2+} influx pathways might have on store-refilling, reflected by a reduction in successive contractile responses.

Methods

Organ bath studies

Contractile studies were performed as described previously by HIROTA and co-workers [13, 15]. Briefly, ASM strips were mounted in 4-mL organ baths, using silk thread to anchor one end of the strip and attach the other to a Grass FT.03 force transducer (Grass Technologies, Longueuil, QC, Canada). Isometric tension was recorded on-line using the DigiMed System Integrator program (MicroMed, Louisville, KY, USA). Tissues were bathed in modified Krebs buffer saturated with 95% oxygen/5% carbon dioxide, and heated to 37°C. During the 1-h equilibration, tissues were repeatedly washed with modified Krebs buffer and tested for viability using 60 mM KCl. The KCl was then washed out, and tissues were allowed to recover before experiments were conducted. Successive ACh-induced contractions were triggered using the concentration of ACh previously found to be half-maximal (3×10^{-7} M) [13, 15]. Each tissue strip underwent only one intervention.

Western blot assay for NCX isoform 1

Tissues were homogenised in ice-cold magnesium lysis buffer (50 mM tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 750 mM NaCl, 5% Igepal CA-630 (Sigma–Aldrich Canada, Oakville, ON, Canada), 50 mM $MgCl_2$, 10% glycerol, 10 $\mu g \cdot mL^{-1}$ aprotinin, 10 $\mu g \cdot mL^{-1}$ leupeptin, 1 mM phenylmethylsulphonyl fluoride, 1 mM 4-(2-aminoethyl)-benzenesulphonyl fluoride and 2 mM sodium orthovanadate), total protein content determined (Bradford method) and protein concentration adjusted to 100 $\mu g \cdot mL^{-1}$ using 2× Laemmli sample/loading buffer (62.5 mM Tris–HCl (pH 6.8), 2% sodium dodecylsulphate, 10% glycerol, 50 mM dithiothreitol, 0.1% 2-mercaptoethanol and 0.01% bromophenol blue). Samples were boiled for 5 min in this buffer, subjected to electrophoresis and then transferred to nitrocellulose membrane (blocked with 5% bovine serum albumin and 5% skimmed milk in Tris-buffered saline). NCX isoform 1 (NCX1) was visualised using a polyclonal rabbit primary antibody directed against NCX1 (Chemicon, Billerica, MA, USA) and a mouse anti-immunoglobulin G secondary antibody preparation (Upstate Biotechnology, Waltham, MA, USA).

Analysis

The effect of interventions targeting Ca^{2+} influx pathways is expressed as the percentage change from within-tissue control ACh responses (3×10^{-7} M). All responses are reported as mean ± SEM. Comparisons were performed using an unpaired t-test (for single pairwise comparisons) or two-way ANOVA (for multiple comparisons of means) followed by the appropriate *post hoc* test (Newman–Keuls). A p-value of <0.05 was considered significant.

RESULTS

In order to probe the relative involvement of Ca^{2+} influx pathways in store-refilling in ASM, a standard organ bath preparation was employed. Using this technique, successive agonist-induced contractions were triggered using ACh (3×10^{-7} M). Reproducible contractions could be triggered at 15-min intervals (fig. 1). Removal of Ca^{2+} from the bathing

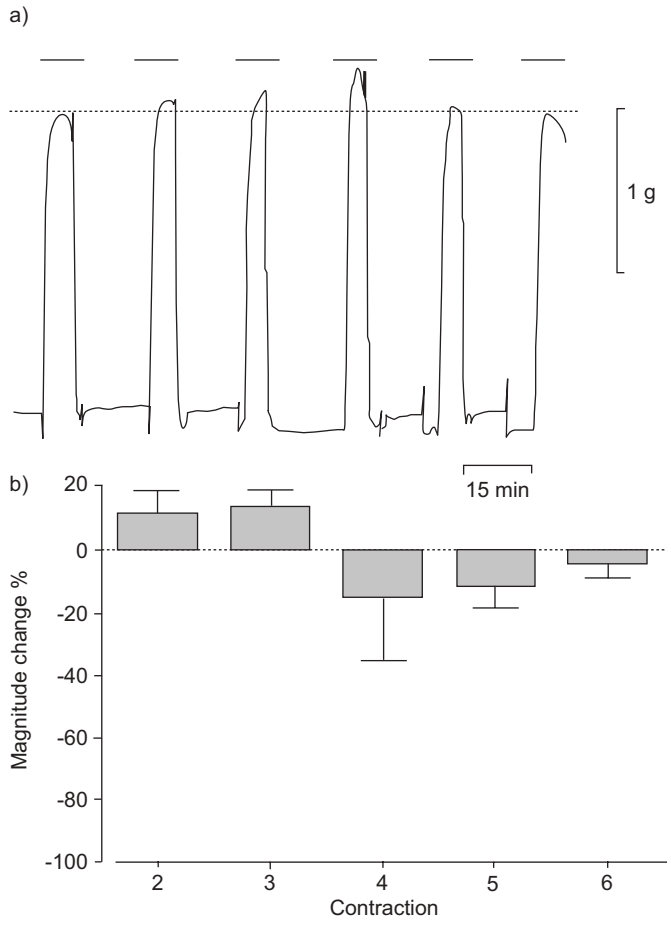

FIGURE 1. a) Representative 3×10^{-7} M acetylcholine (ACh; horizontal bars)-induced contractions of bovine airway smooth muscle (n=10). .....: Magnitude of first contraction. b) Mean change in peak magnitude of the ACh-induced contractions. Data are presented as mean±SEM.

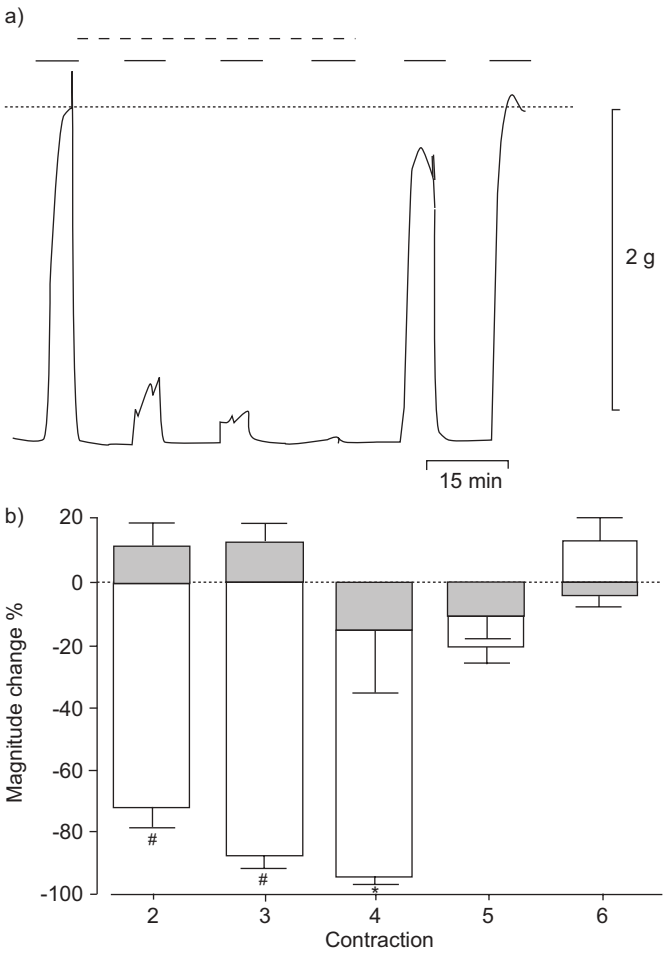

FIGURE 2. a) Representative 3×10^{-7} M acetylcholine (ACh; horizontal bars)-induced contractions of bovine airway smooth muscle (ASM) before, during and after removal of extracellular calcium ions (n=5). At the end of the zero-Ca^{2+} period (- - - -), the ASM was washed with standard Ca^{2+}-containing buffer. .....: Magnitude of first contraction. b) Mean change in peak magnitude of these ACh-induced contractions (□) compared with control responses (■; n=10). Data are presented as mean±SEM. *: $p<0.05$; #: $p<0.005$.

solution led to a rapid and complete but reversible reduction in the magnitude of the agonist-induced contractions (fig. 2).

In order to investigate the pathways through which Ca^{2+} enters the cell on its way to the SR, a series of pharmacological interventions were employed. First, successive contractions were evoked in the presence of cromakalim (30 μM (0.01 M in distilled deionised water diluted in Krebs buffer as appropriate)), a compound that increases the open probability of adenosine triphosphate (ATP)-dependent potassium channels (K_{ATP}) [6, reviewed in 16]. Activation of K_{ATP} interferes with membrane depolarisation, thereby inhibiting voltage-dependent downstream events (*e.g.* the reverse-mode of the NCX and L-type Ca^{2+} channels). Cromakalim treatment led to a significant reduction in the magnitude of successive ACh-induced contractions (figs 3a and c). This effect was abolished by concurrent treatment with glibenclamide

Since hyperpolarisation of the membrane reduced the magnitude of successive contractions, an approach that targeted the two downstream Ca^{2+} influx pathways that can be activated by membrane depolarisation was employed to determine their relative contribution to store-refilling. In order to examine the contribution of the NCX, tissues were treated with KB-R7943 (10 or 20 μM), a selective inhibitor of the reverse-mode (*i.e.* Ca^{2+} influx mode) of the NCX [17–21]. Both 10 and 20 μM KB-R7943 significantly reduced successive ACh-induced contractions by ~50% (fig. 4). Similarly, successive responses from tissues treated with nifedipine (1 μM), a selective inhibitor of L-type Ca^{2+} channels, were significantly reduced by ~50% (fig. 5). Thus neither KB-R7943 nor nifedipine alone reduced successive contractions to the same extent as observed when tissues were exposed to zero-Ca^{2+} conditions (fig. 6). However, concurrent treatment with KB-R7943 (10 or 20 μM) and nifedipine (1 μM) effectively abolished successive ACh-induced contractions (fig. 7) to the extent observed during zero-Ca^{2+} treatment (fig. 8).

In addition to pharmacological inhibition of the NCX, an ionic substitution protocol was employed to generate Na^+-free Krebs buffer. It has previously been shown that long-term bathing in zero-Na^+ buffer reduces refilling through inhibition of the reverse-mode of the NCX [13]. Treating tissues with nifedipine

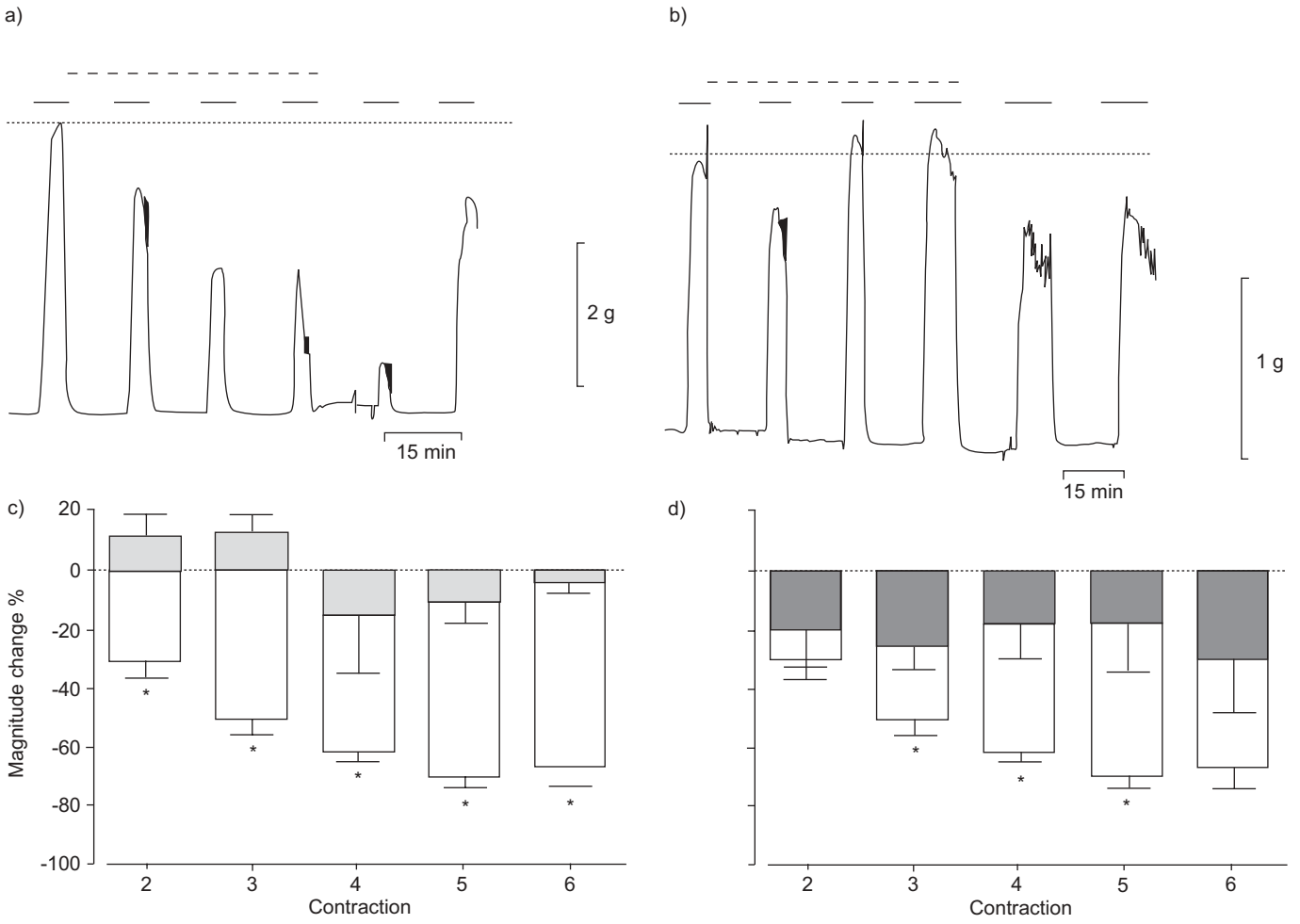

FIGURE 3. Representative 3×10^{-7} M acetylcholine (ACh; horizontal bars)-induced contractions of bovine airway smooth muscle (ASM) before, during and after exposure to 30 μM cromakalim: a) alone (n=12) and b) in combination with 10 μM glibenclamide (n=5). After exposure (- - - -), the ASM was washed with standard Ca^{2+}-containing buffer. .....: Magnitude of first contraction. c, d) Mean change in peak magnitude of these ACh-induced contractions (□: cromakalim; ■: cromakalim plus glibenclamide) compared with control responses (■; n=10). Data are presented as mean±SEM. *: $p<0.05$.

(1 μM) in the absence of extracellular Na^+ significantly reduced successive ACh-induced contractions (figs 9a and b). Furthermore, removal of extracellular Na^+ during nifedipine treatment led to a significantly greater reduction in responses than observed when tissues were treated with nifedipine alone (fig. 9c). However, this treatment did not reduce contractions to the extent observed under zero-Ca^{2+} conditions (fig. 9d).

In addition to examining the functional importance of the NCX in store-refilling, NCX1 expression was examined in protein isolates from bovine tracheal smooth muscle. In tracheal strips from six different animals, Western blot analysis revealed the existence of three predominant proteins of 160, 120 and 110 kDa (fig. 10).

DISCUSSION

The aim of the present study was to examine the relative roles of two Ca^{2+} influx pathways, both regulated by membrane potential, in the process of refilling the SR. A role for the NCX in store-refilling has recently been described, whereby its reverse-mode provides substantial Ca^{2+} influx for the refilling of the SR following agonist-induced Ca^{2+} mobilisation [13]. In this earlier report, inhibition of the reverse-mode of the NCX, through removal of extracellular Na^+ or treatment with KB-R7943, reduced the magnitude of successive agonist-induced contractions. This was associated with a reduction in agonist-induced Ca^{2+} mobilisation, measured *via* Ca^{2+} fluorescence imaging, in freshly isolated ASM cells. In addition to the reverse-mode of the NCX, others have reported that voltage-dependent Ca^{2+} influx, *via* activation of L-type Ca^{2+} channels, plays a significant role in store-refilling [4–6]. Interestingly, these conclusions have often been based on experimental protocols designed to manipulate membrane potential in order to alter Ca^{2+} influx *via* L-type Ca^{2+} channels. However, the reverse-mode of the NCX is also driven by membrane depolarisation, in conjunction with local increases in intracellular Na^+ concentration that can be triggered through gating of NSCCs [7–11], reviewed in [12]. The previous studies examining voltage-dependent Ca^{2+} influx have overlooked the role of the reverse-mode of the NCX, attributing influx entirely to L-type Ca^{2+} channels.

Influx pathways activated during agonist-induced contraction must exist to provide the cell with an intracellular Ca^{2+} pool

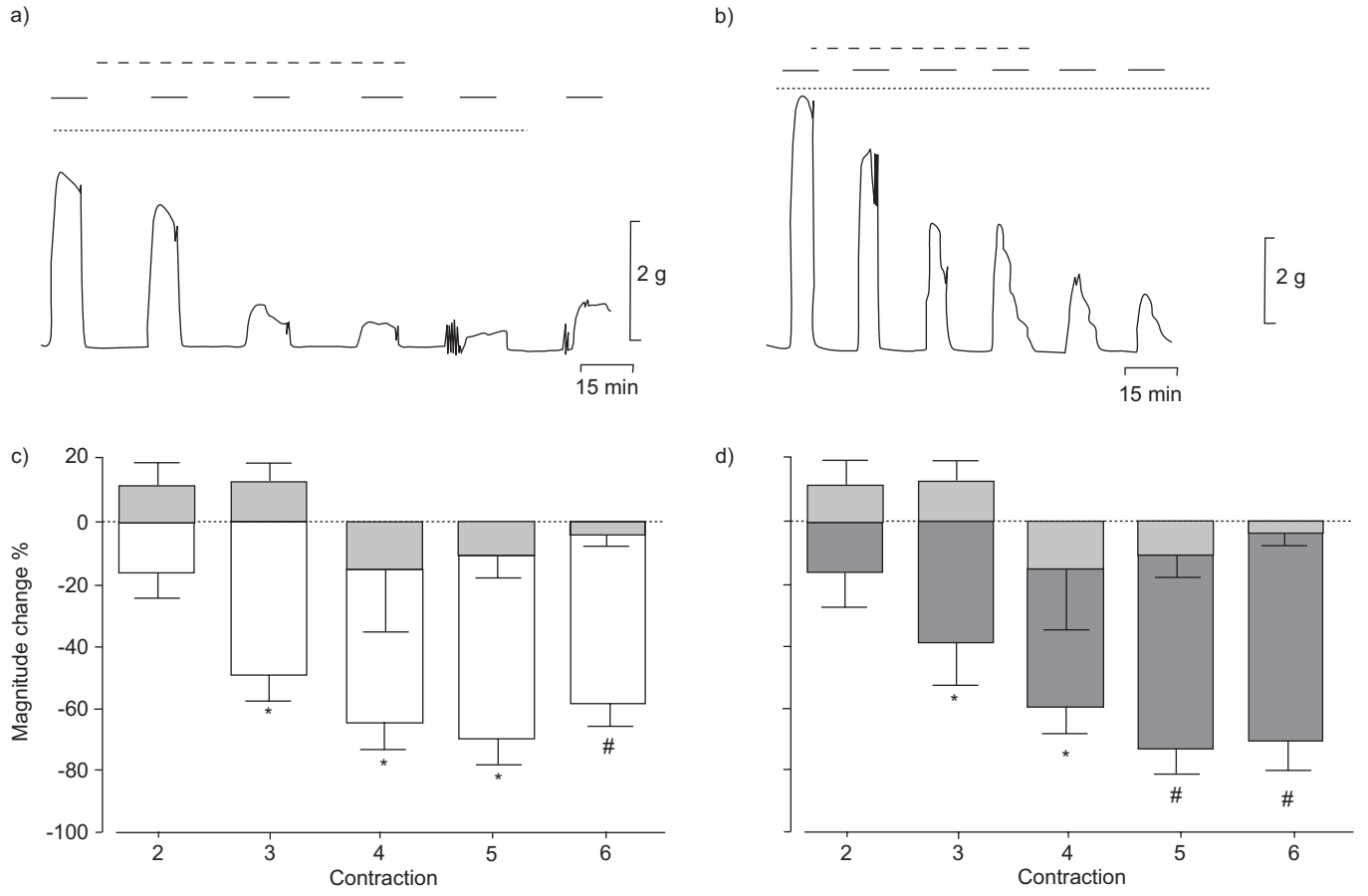

FIGURE 4. Representative 3×10^{-7} M acetylcholine (ACh; horizontal bars)-induced contractions of bovine airway smooth muscle (ASM) in the presence of KB-R7943 (- - - -). a) 10 μM (n=10) and b) 20 μM (n=7). .....: Magnitude of first contraction. c, d) Mean change in peak magnitude of these ACh-induced contractions (□: 10 μM KB-R7943; ■: 20 μM KB-R7943) compared with control responses (■; n=7). After exposure, the ASM was washed with standard Ca^{2+}-containing buffer. Data are presented as mean±SEM. *: $p<0.05$; #: $p<0.005$.

from which the SR can be appropriately refilled. The importance of Ca^{2+} influx in SR refilling is especially evident when tissues are repetitively stimulated in Ca^{2+}-free bathing solutions. Under these conditions, successive agonist-induced contractions are almost abolished, but return to control levels following the reintroduction of extracellular Ca^{2+} (fig. 2).

Agonist stimulation of ASM triggers membrane depolarisation, primarily through the activation of Ca^{2+}-dependent Cl^- channels. These responses have been characterised in various airway preparations [1–3, 22, 23]. Thus it is plausible that agonist-induced membrane depolarisation activates the Ca^{2+} influx pathways required for appropriate store-refilling. In order to probe the role of membrane depolarisation in store-refilling, tissues were treated with cromakalim (30 μM), a compound that selectively activates K_{ATP} [16]. Cromakalim is known to cause relaxation of many smooth muscle types, including ASM, and this effect is universally attributed to its action on glibenclamide-sensitive K_{ATP} [24–27]. By triggering K^+ efflux *via* the K_{ATP}, cromakalim hyperpolarises the membrane potential towards the potassium equilibrium potential (*i.e.* ~80 mV) [6]. Cromakalim treatment significantly reduced the magnitude of successive agonist-induced contractions (figs 3a and c), an effect that was abolished in the presence of glibenclamide (10 μM); a selective blocker of the K_{ATP} (figs 3b and d) [16].

The importance of this finding is three-fold. First, the effect of cromakalim was almost abolished in the presence of glibenclamide, a selective inhibitor of the K_{ATP}, supporting the selectivity of the intervention. Secondly, inhibition of membrane depolarisation through pre-treatment with cromakalim caused a graded reduction in the magnitude of successive contractions, suggesting a gradual reduction in SR refilling throughout the protocol, in response to inhibition of voltage-dependent Ca^{2+} influx pathways. If voltage-dependent Ca^{2+} influx were directly involved in excitation–contraction coupling, cromakalim's effect would have been immediate. Instead the gradual and progressive reduction suggests that agonist-induced Ca^{2+} mobilisation is the primary mechanism driving Ca^{2+}-dependent contraction, and that the SR is being refilled, in part, through voltage-dependent Ca^{2+}-influx. Finally, these data call into question any major role of ROCs/SOCs in the store-refilling process in ASM. When examining the electrophysiological properties of NSCCs, the current–voltage relationship is such that the inward current is greatest under negative membrane potentials [14]. Thus Ca^{2+} influx through ROCs/SOCs should be increased by membrane hyperpolarisation,

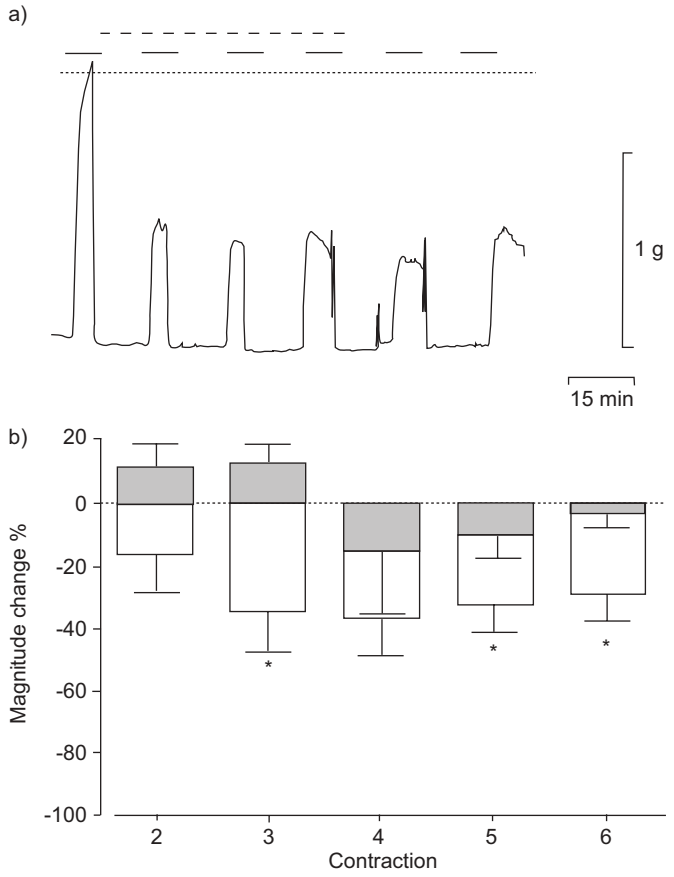

FIGURE 5. a) Representative 3×10^{-7} M acetylcholine (ACh; horizontal bars)-induced contractions of bovine airway smooth muscle (ASM) in the presence of 1 μM nifedipine (- - - -; n=6). .....: Magnitude of first contraction. b) Mean changes in peak magnitude of these ACh-induced contractions (□) compared with control responses (■; n=10). After exposure, the ASM was washed with standard Ca^{2+}-containing buffer. Data are presented as mean±SEM. *: $p<0.05$.

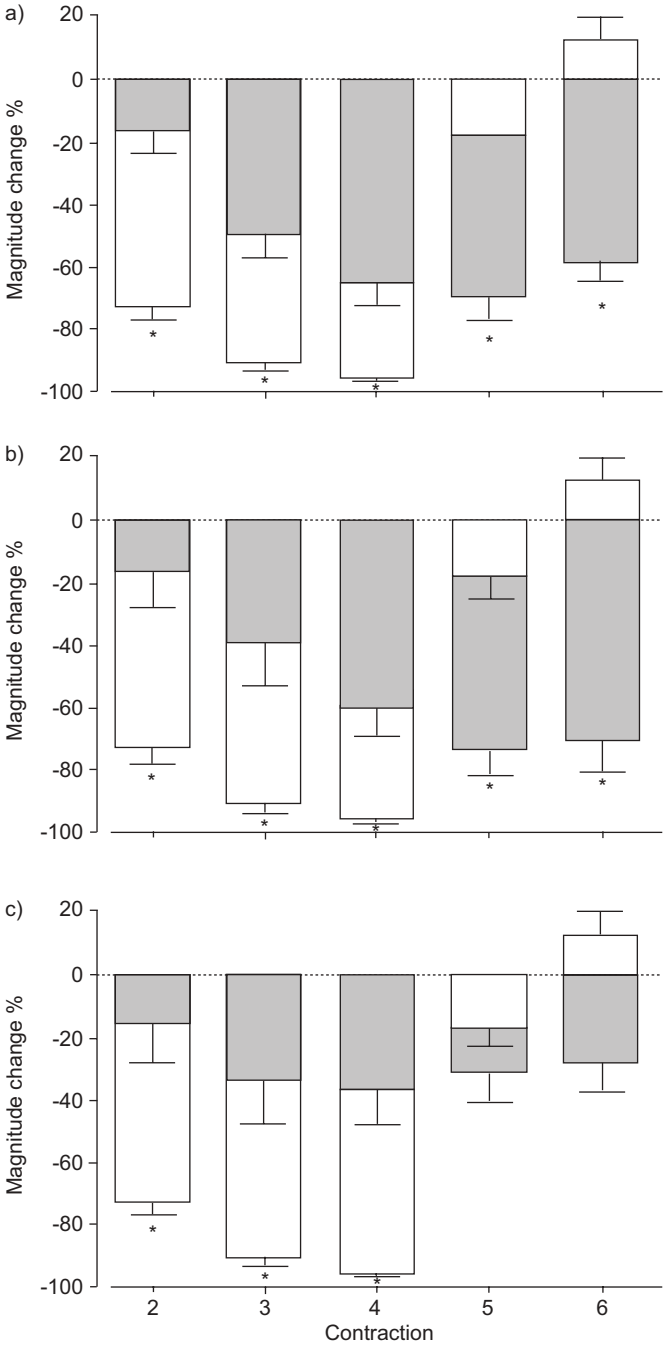

FIGURE 6. Mean changes in peak magnitudes of 3×10^{-7} M acetylcholine (ACh)-induced contractions in the presence (■) of: a) 10 μM KB-R7943 (n=10); b) 20 μM KB-R7943 (n=7); and c) 1 μM nifedipine (n=6) compared with responses in the absence of extracellular Ca^{2+} (□; n=5). After exposure (contractions 2–4), the ASM was washed with zero-Ca^{2+} buffer (contractions 5 and 6). Data are presented as mean±SEM. *: $p<0.05$.

or clamping in negative potentials, during cromakalim treatment. As such, any contribution of these channels to store-refilling should have been amplified by treatment with cromakalim. In contrast, the data suggest that store-refilling is dominated by voltage-dependent mechanisms, and that voltage-independent mechanisms, such as ROC/SOC currents, play only a minor role, if any.

Since it is apparent that voltage-dependent Ca^{2+} influx pathways play a role in store-refilling, successive agonist-induced contractions were examined in the presence of agents targeting the reverse-mode of the NCX and L-type Ca^{2+} channels, both voltage-regulated systems. KB-R7943 is a selective inhibitor of the NCX, and blocks the reverse-mode almost 60 times more strongly than the forward-mode [17–21]. When examining the pharmacology of KB-R7943, BRADLEY *et al.* [18] reported that it had no effect on the release of Ca^{2+} from the SR, and nor did it alter the inward current mediated by Ca^{2+}-dependent Cl^- channels. Furthermore, KAWANO *et al.* [28] reported that high concentrations of KB-R7943 (30–100 μM) reduced spontaneous Ca^{2+} oscillations in human mesenchymal stem cells, but had no effect on the lanthanum-sensitive component of these oscillations mediated by NSCCs. In intact tissue preparations, DAI *et al.* [7] reported that KB-R7943, at the concentration used in the present study, abolished agonist-induced asynchronous Ca^{2+} oscillations, an effect attributed to inhibition of Ca^{2+} influx *via* the reverse-mode of the NCX. More recently, DAI *et al.* [29] used KB-R7943 to identify a role for the reverse-mode of the NCX in the agonist-induced asynchronous Ca^{2+} oscillations observed in intact human ASM preparations.

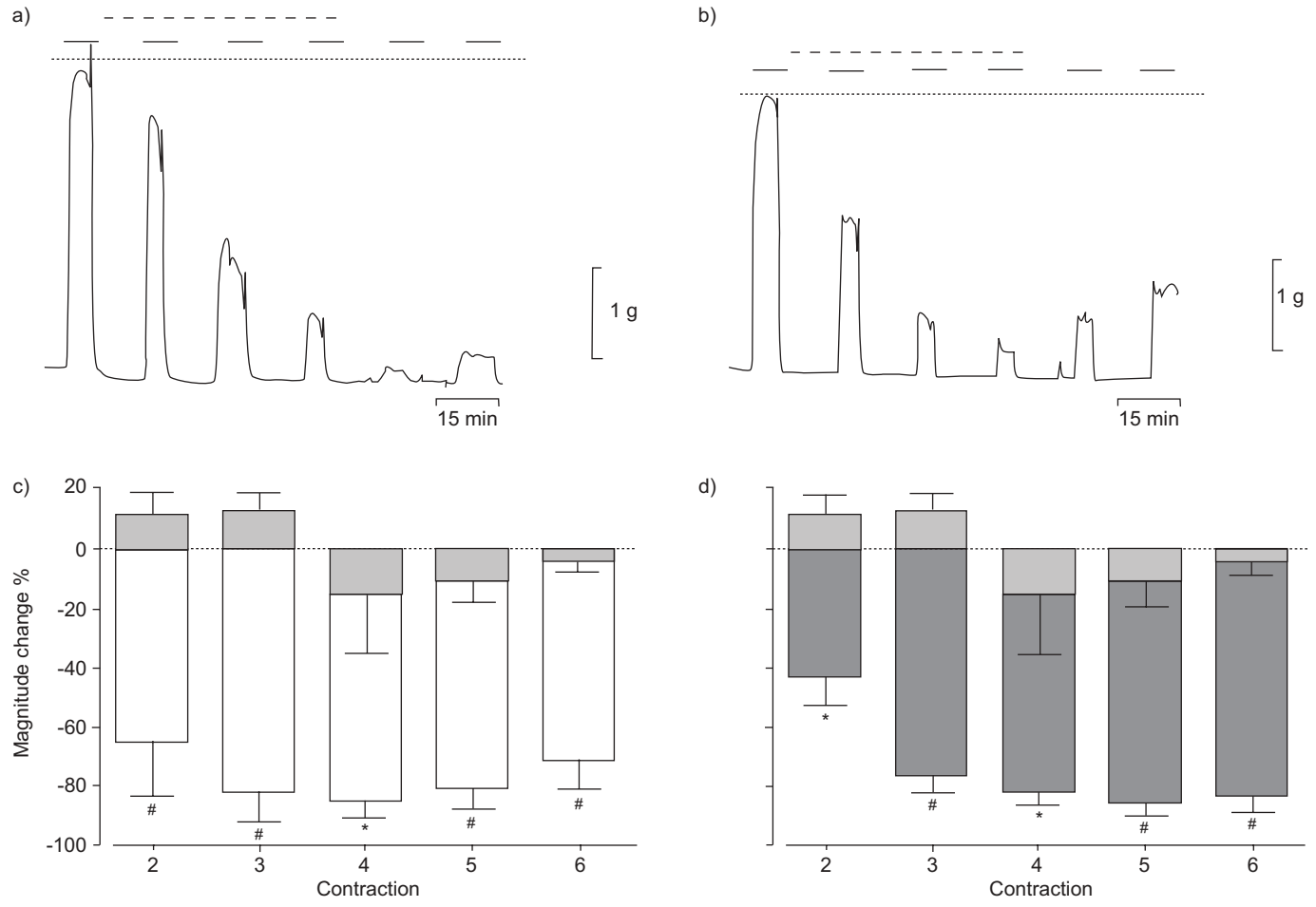

FIGURE 7. Representative 3×10^{-7} M acetylcholine (ACh; horizontal bars)-induced contractions of bovine airway smooth muscle (ASM) during concurrent treatment with 1 μM nifedipine and: a) 10 μM KB-R7943 (n=4); and b) 20 μM KB-R7943 (n=8). After exposure (- - - -), the ASM was washed with standard Ca^{2+}-containing buffer. .....: Magnitude of first contraction. c, d) Mean change in peak magnitude of these ACh-induced contractions (□: 1 μM nifedipine plus 10 μM KB-R7943; ■: 1 μM nifedipine plus 20 μM KB-R7943) compared with control responses (■; n=10). Data are presented as mean±SEM. *: $p<0.05$; #: $p<0.005$.

In the present study, both KB-R7943 and nifedipine, a prototypical L-type Ca^{2+} channel blocker, significantly reduced the magnitude of successive contractions (figs 4 and 5). However, neither KB-R7943 nor nifedipine alone reduced contractions to the extent observed in the absence of extracellular Ca^{2+} (fig. 6). Thus it seems that Ca^{2+} influx through both the NCX and L-type Ca^{2+} channels is necessary for complete refilling of the intracellular Ca^{2+} pool; concurrent treatment with both KB-R7943 and nifedipine displayed an additive effect in terms of reducing the magnitude of successive agonist-induced contractions (fig. 7). Indeed, simultaneous inhibition of the reverse-mode of the NCX and L-type Ca^{2+} channels practically abolished successive agonist-induced contractions, similar to the effect observed when tissues were bathed in Ca^{2+}-free buffering solution (fig. 8).

In addition to pharmacological interventions, the removal of extracellular Na^+ from buffering solutions can gradually inhibit the NCX. This approach was utilised to probe the role of the NCX in store-refilling following agonist-induced Ca^{2+} mobilisation [13]. In the present study, removal of extracellular Na^+ in the presence of nifedipine (to inhibit L-type Ca^{2+} channels) led to a significant reduction in the magnitude of successive responses, similar to that observed during treatment with KB-R7943 (figs 9a and b). However, the reduction in magnitude observed during concurrent zero Na^+ and nifedipine treatment was not as great as that observed under zero Ca^{2+} conditions (fig. 9d). This may reflect the nature of this intervention; its effect is reliant upon the gradual depletion of intracellular Na^+ throughout the series of successive responses. Inhibition of the NCX occurs when the intracellular Na^+ concentration is depleted, which is a gradual rather than immediate event. In contrast, pharmacological inhibition of the NCX is a more immediate event since it is dependent upon the direct interaction of KB-R7943 with the exchanger [19]. Interestingly, Na^+ depletion, in the presence of nifedipine, decreased responses beyond those observed during nifedipine treatment alone (fig. 9c). This observation highlights the existence of a Na^+-dependent Ca^{2+} influx pathway (*i.e.* the reverse-mode of the NCX), which plays a role in store-refilling following agonist-induced Ca^{2+} mobilisation.

In addition to the functional identification of a Ca^{2+}-influx pathway mediated through the reverse-mode activity of the

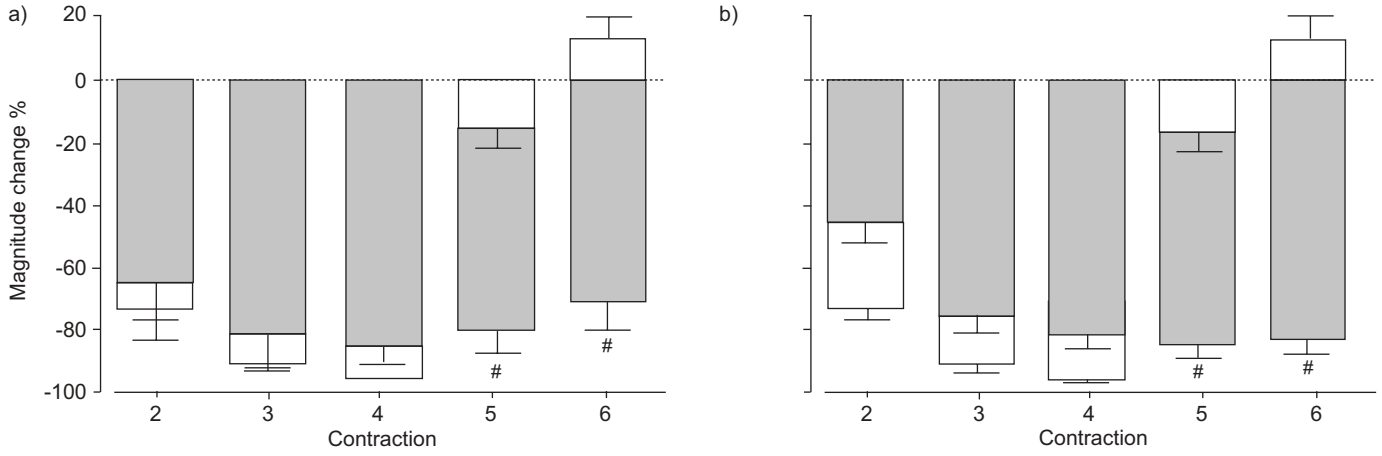

FIGURE 8. Mean changes in peak magnitudes of 3×10^{-7} M acetylcholine (ACh)-induced contractions in the presence (■) of 1 μM nifedipine plus: a) 10 μM KB-R7943 (n=4) and b) 20 μM KB-R7943 (n=8) compared with responses in the absence of extracellular Ca^{2+} (□; n=5). After exposure (contractions 2–4), the ASM was washed with zero-Ca^{2+} buffer (contractions 5 and 6). Data are presented as mean±SEM. #: $p<0.005$.

NCX, protein expression was probed in bovine tracheal smooth muscle samples. Three major isoforms of the NCX exist: NCX1, NCX2 and NCX3 [12, 30]. NCX2 and NCX3 expression are mainly limited to the brain and skeletal muscle [12, 31–33], whereas NCX1 displays a more profuse expression profile [12, 34]. In addition to this major classification, the

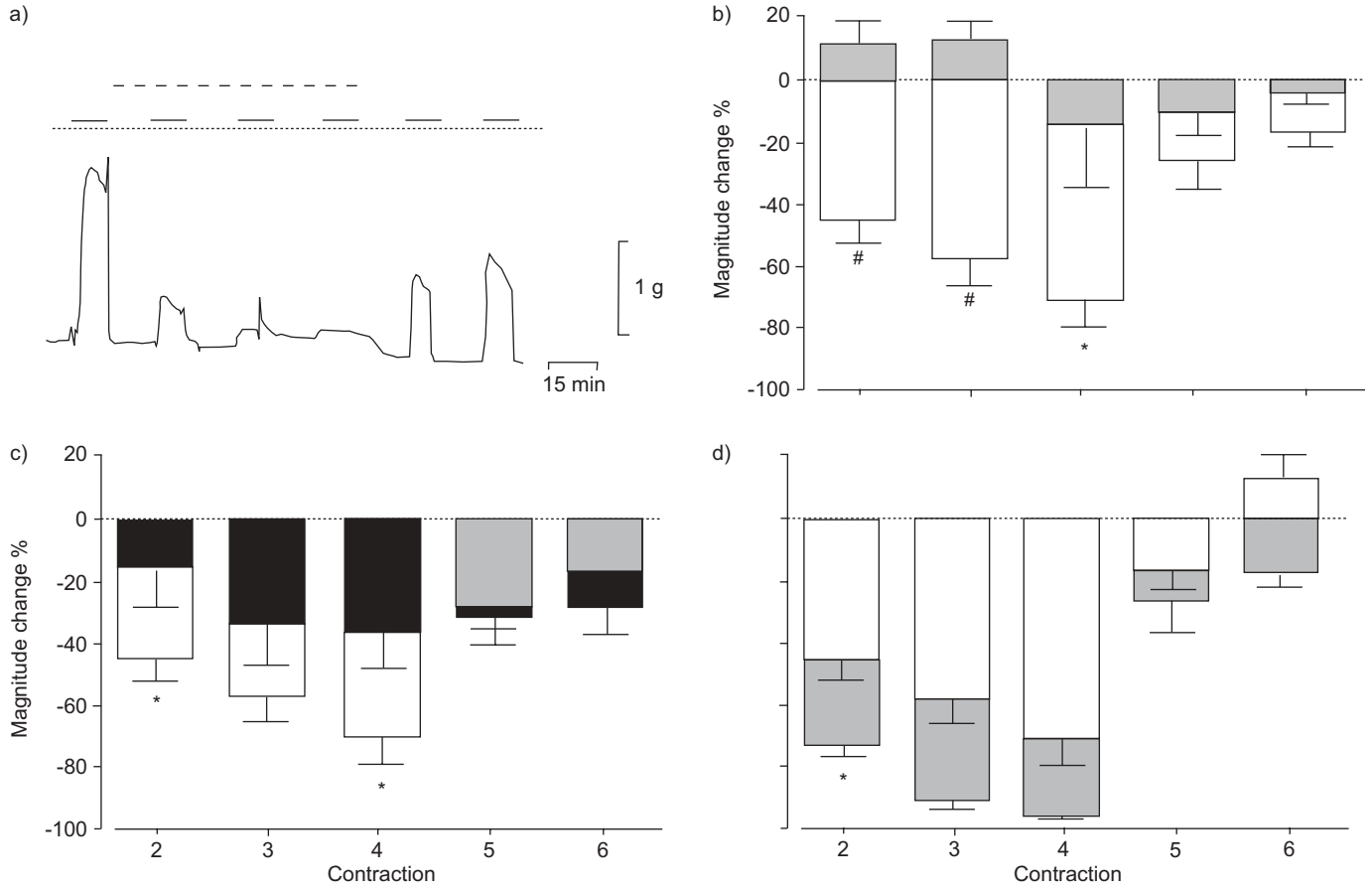

FIGURE 9. a) Representative 3×10^{-7} M acetylcholine (ACh; horizontal bars)-induced contractions of bovine airway smooth muscle (ASM) during concurrent treatment with 1 μM nifedipine and zero-Na^+ buffer (- - - -; n=5). ……..: Magnitude of first contraction. b–d) Mean changes in peak magnitude of these ACh-induced contractions in the presence of 1 μM nifedipine and zero-Na^+ buffer (□) compared with b) control responses (■; n=10); c) nifedipine treatment alone (■; n=6); and d) zero-Ca^{2+} conditions (■; n=5). After exposure (contractions 2–4), the ASM was washed with fresh buffer (contractions 5 and 6). Data are presented as mean±SEM. *: $p<0.05$; #: $p<0.005$.

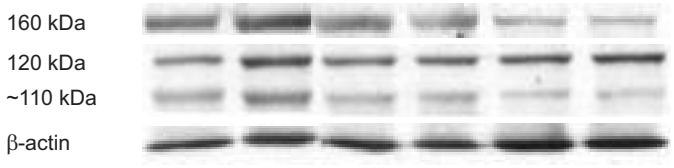

FIGURE 10. Western blot detection of sodium–calcium exchanger (NCX) isoform 1, performed in protein extracts isolated from bovine tracheal smooth muscle cells, indicating expression of NCX1.1 (~120 kDa) and NCX1.3 (~110 kDa). Each lane represents a protein isolate from a different animal.

NCX1 isoform can be expressed as a variety of splice variants which determine variable properties in terms of ionic sensitivity and regulation by intracellular processes [35–39]. When probing the present preparation for NCX1, three major bands corresponding to molecular masses of 160, 120 and 110 kDa were observed. The 160-kDa band has been reported in cases in which the reducing agents have insufficiently altered the protein structure prior to separation [39, 40]. The 120-kDa band corresponds to the unspliced NCX1.1 isoform reported in a number of different systems [12, 30, 36, 39, 40]. Alternative splicing of an intracellular loop of NCX1 can lead to a number of smaller isoforms [12, 30]. Indeed a band of ~110 kDa, which corresponds to the theoretical and reported size of the NCX1.3 isoform, was identified [12, 36, 41]. Interestingly, the NCX1.3 isoform has been associated with the aberrant Ca^{2+} influx observed in salt-dependent hypertension [37, 38, 42].

Taken together, the present data suggest that previous reports examining the role of voltage-dependent calcium ion influx in store-refilling may have overlooked the contribution of the reverse-mode of the sodium–calcium exchanger. It is apparent that inhibition of L-type calcium channels alone does not completely abolish successive agonist-induced contractions of airway smooth muscle. Indeed, this approach has failed in the clinical setting, whereby dihydropyridine compounds were proven ineffective in the management of obstructive airway diseases, such as asthma [43–45]. However, treatment aimed at inhibiting both L-type calcium channels and the reverse-mode of the sodium–calcium exchanger in airway smooth muscle may prove effective at reversing the increased contractile tone associated with airway obstruction. Interestingly, inhibition of the sodium–calcium exchanger is currently touted as a potential clinical strategy for treating hypertensive conditions associated with increased calcium ion influx through the reverse-mode of the sodium–calcium exchanger [37, 38, 42, 46, 47]. It is apparent that further *in vivo* study is required in order to determine whether or not the sodium–calcium exchanger plays a role in obstructive airway disease. Furthermore, animal models of airway hyperresponsiveness may prove the ideal setting in which to determine what effect concurrent inhibition of the reverse-mode of the sodium–calcium exchanger and L-type calcium channels might have on the airway hyperresponsiveness and reduced airway calibre associated with asthma.

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