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REVIEW

# Ionic mechanisms and $Ca^{2+}$ handling in airway smooth muscle

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**ABSTRACT: Asthma is a disease characterised by reversible contraction of airway smooth muscle. Many signalling pathways are now known to underlie that contraction, almost all of which revolve around $Ca^{2+}$ handling. $Ca^{2+}$ homeostasis in turn is governed by a wide variety of ionic mechanisms, which are still poorly understood. The present review will briefly summarise those mechanisms that have been recognised for decades, but will then devote considerable attention to several novel ionic signalling mechanisms such as capacitative $Ca^{2+}$ entry, the reverse mode of the $Na^+/Ca^{2+}$ exchanger, the role of $Cl^-$ channels in the release of internal $Ca^{2+}$ and that of ryanodine receptors in the refilling of the sarcoplasmic reticulum, as well as the regulation of the monomeric G-protein Rho by ionic mechanisms. Lastly, evidence will be provided that $Ca^{2+}$-dependent contraction may be driven by spatial and temporal heterogeneities in the intracellular $Ca^{2+}$ concentration (*i.e.* $Ca^{2+}$ waves/oscillations) rather than by an increase in the global steady state intracellular $Ca^{2+}$ concentration.**

**KEYWORDS: Airway physiology, airway smooth muscle, calcium, contraction, ion channels, membrane potential**

Asthma is characterised by variable increases in airway resistance. Irrespective of the aetiology of this disease, inappropriate contraction of the smooth muscle of the airways is a major factor that contributes to the increase in resistance. Thus, understanding the mechanisms surrounding force generation in airway smooth muscle (ASM) is paramount in the quest for novel treatments for this and many other respiratory diseases.

Advances in the understanding of excitation–contraction (EC) coupling in ASM have borrowed from work carried out using other muscle preparations (usually vascular smooth muscle or even skeletal/cardiac muscles). In some respects this is justified, given the similarities between these different cell types. However, a growing body of literature attests to the marked differences between ASM and those other cell types, as previously reviewed by JANSSEN [1]. Thus, in order to accurately understand ASM physiology, it is important to synthesise and integrate data collected specifically from ASM and not to rely on findings made in cardiac or vascular smooth muscle, as so often seems to be the case.

The present review will begin with a brief presentation of the generally accepted working knowledge of EC coupling in ASM, focusing on $Ca^{2+}$-dependent contractions that are triggered following agonist stimulation. In addition to the traditional mechanisms governing $Ca^{2+}$ handling in ASM, evidence of other events which have been uncovered more recently in ASM will be summarised, such as capacitative $Ca^{2+}$ entry and the reverse mode of the $Na^+/Ca^{2+}$ exchanger (NCX), which may contribute to agonist-induced elevations in intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). Lastly, evidence will be provided that $Ca^{2+}$-dependent contraction may be driven by spatial and temporal heterogeneities in $[Ca^{2+}]_i$ (*i.e.* $Ca^{2+}$ waves/oscillations) rather than an increase in the global steady state $[Ca^{2+}]_i$.

## OVERVIEW OF EC COUPLING IN ASM

The contractile apparatus consists of actin and myosin filaments and accessory and regulatory proteins. During excitation, phosphorylation of the light chain of smooth muscle myosin by myosin light chain kinase (MLCK) leads to enhancement of its intrinsic adenosine triphosphatase (ATPase) activity and subsequent mechanical interactions with actin. This results in the sliding of the myosin molecule along the

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actin filament. The details of this complex interaction are beyond the scope of the current discussion, but have been reviewed elsewhere [2]. MLCK is activated by the $Ca^{2+}$/calmodulin complex: thus, $Ca^{2+}$ plays a key and central role in EC coupling in ASM (fig. 1). A great many ionic events within the ASM cell culminate in changes in $[Ca^{2+}]_i$, as outlined below. It will first be described how $[Ca^{2+}]_i$ is maintained at, or restored to, resting levels through the actions of several $Ca^{2+}$ pumps and exchangers (see $Ca^{2+}$ homeostasis: $Ca^{2+}$ pumps and exchangers section). Next, it will be examined how $[Ca^{2+}]_i$ is elevated through the release of internally sequestered $Ca^{2+}$ (see Release of internally sequestered $Ca^{2+}$ section) as well as the influx of external $Ca^{2+}$ across the membrane (see Voltage-dependent influx of external $Ca^{2+}$ and Voltage-independent influx of $Ca^{2+}$ sections); the latter will necessitate a brief diversion in order to explore a variety of other ionic events and mechanisms that contribute to $Ca^{2+}$ influx *via* voltage-gated $Ca^{2+}$ channels (VGCC; see Voltage-dependent $Ca^{2+}$ influx section). Finally, some very recent work will be summarised describing how these mechanisms have been assembled and integrated into very elaborate yet fundamental cell functions (see Novel mechanisms section).

Several other signalling pathways involved in mediating contraction will not be discussed in the present review. Some of these involve a change in $Ca^{2+}$ sensitivity of the contractile apparatus *via* a suppression of myosin light chain phosphatase (MLCP) activity, mediated by RhoA-associated kinase or by protein kinase (PK)C. Excellent reviews have been provided by SOMLYO and SOMLYO [3]. Alternatively, others have described contractions that are $Ca^{2+}$ independent, and indeed that appear to be independent of ionic mechanisms in general, and these will also not be considered in detail in the present review, although excellent reviews have been given by GUNST and co-workers [4, 5].

## $Ca^{2+}$ HOMEOSTASIS: $Ca^{2+}$ PUMPS AND EXCHANGERS

In general, $[Ca^{2+}]_i$ is maintained at low levels by: 1) extrusion of $Ca^{2+}$ from the cytoplasm *via* a $Ca^{2+}$ ATPase and the NCX on the plasmalemma; 2) sequestration of $Ca^{2+}$ into intracellular

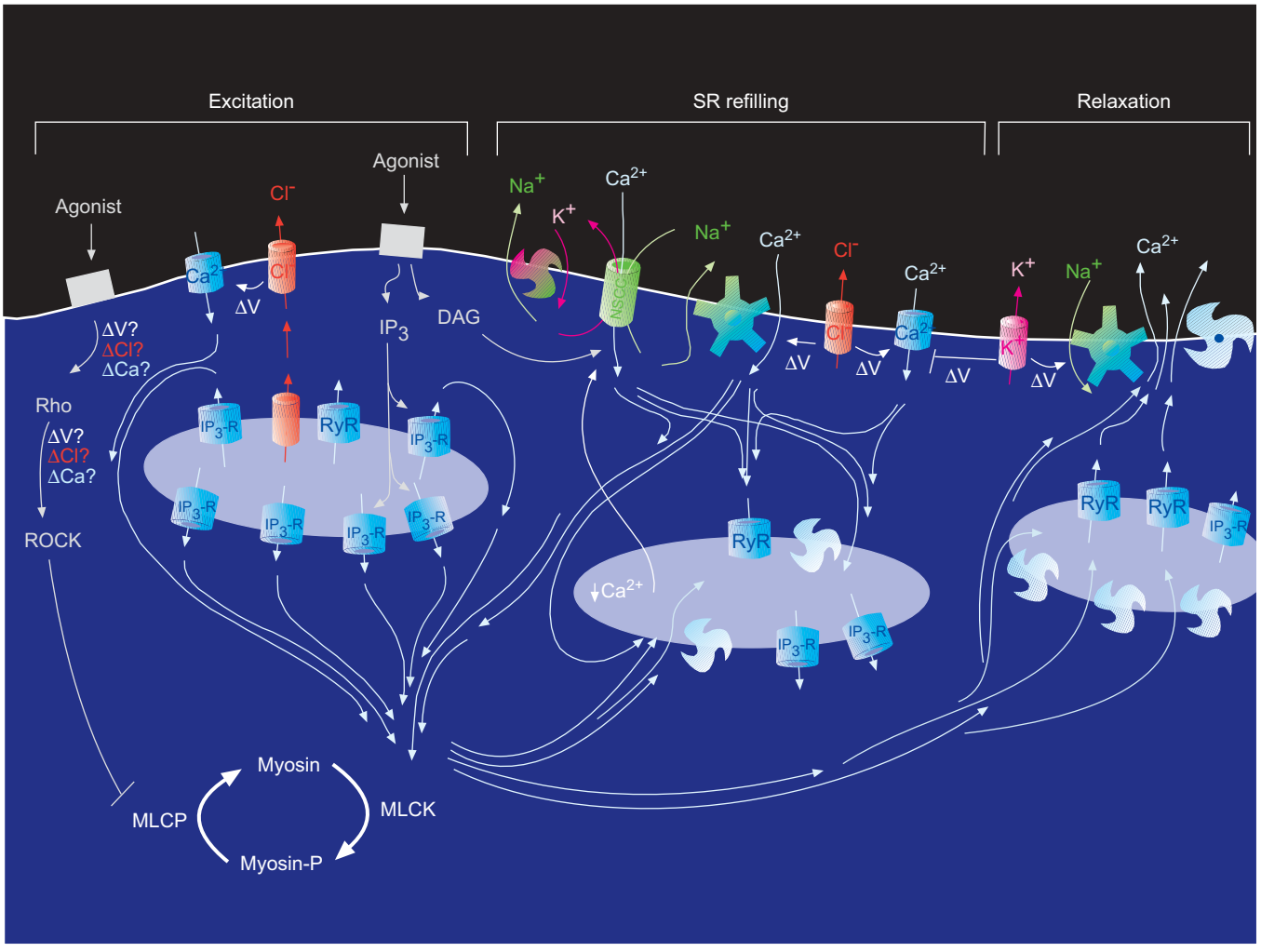


**FIGURE 1.** Overview of ionic pathways in airway smooth muscle. Fluxes of $Ca^{2+}$ (blue), $Cl^-$ (red), $Na^+$ (green) and $K^+$ (pink) during excitation, refilling of the sarcoplasmic reticulum (SR) and relaxation. Details of these fluxes are given within the text. ΔV: change in membrane potential; ΔCl: change in $[Cl^-]$; ΔCa: change in $[Ca^{2+}]$. NSCC: nonselective cation channel; ROCK: Rho-associated kinase; MLCP: myosin light chain phosphatase; MLCK: myosin light chain kinase; Myosin-P: phosphorylated myosin; $IP_3$: inositol-1,4,5-trisphosphate; $IP_3$-R: $IP_3$ receptor; DAG: diacylglycerol; RyR: ryanodine receptor. Truncated arrows indicate inhibition.

organelles; and 3) buffering of $[Ca^{2+}]_i$ by various cytosolic $Ca^{2+}$-binding proteins (fig. 1).

Several organelles are known to sequester $Ca^{2+}$, including the sarcoplasmic reticulum (SR), mitochondria and the nuclear envelope [6, 7]. Of these, the SR is widely agreed to play the major role in regulating contractile function in ASM. The mitochondria seem to play a supportive or modulatory role in the regulation of $[Ca^{2+}]_i$ in vascular smooth muscle, but this has not been adequately investigated in ASM [6, 8]. There is widespread agreement that sarco-endoplasmic $Ca^{2+}$ ATPase (SERCA) plays a key role in maintaining resting $[Ca^{2+}]_i$, because blockers purported to be selective for this enzyme, such as cyclopiazonic acid (CPA) or thapsigargin (TG), typically evoke a rise in $[Ca^{2+}]_i$ and contractions. This suggests that there is a constant ''leak'' of $Ca^{2+}$ out of the SR that is driven by the tremendous concentration gradient across the SR membrane (10,000-fold, comparable to that existing across the plasmalemma). Inhibition of SERCA leads to functional depletion of the SR, indicated by loss of the $Ca^{2+}$-transient or $Ca^{2+}$-dependent membrane currents in response to cholinergic stimulation [9, 10]. This $Ca^{2+}$ leak may account, in part, for the spontaneous $Cl^-$ currents (spontaneous transient inward currents) and $K^+$ currents (spontaneous transient outward currents (STOCs)) recorded in ASM [11–15].

Some extrusion pathway must also be involved in $Ca^{2+}$ homeostasis; otherwise, the internal $Ca^{2+}$ pool would eventually become overloaded. The ubiquitous plasmalemmal pump, termed the plasma membrane $Ca^{2+}$ ATPase (PMCA), is most probably involved, although there are no selective blockers or knock-out models available to test this hypothesis. $Ca^{2+}$ can also be extruded *via* the NCX, which uses the energy resident within the $Na^+$ gradient to move $Ca^{2+}$ against its electrochemical gradient, extruding one $Ca^{2+}$ ion in exchange for three $Na^+$. NCX appears to be important in ASM of some species such as the cow [16], pig [17] and guinea pig [18], but apparently not at all in that of the dog [19]. The ability of NCX to operate in a capacity of $Ca^{2+}$ extrusion is widely and easily accepted; however, under certain conditions it can equally operate in the reverse, $Ca^{2+}$-influx, mode (see NCX and $Ca^{2+}$ store refilling section).

## RELEASE OF INTERNALLY SEQUESTERED $Ca^{2+}$

Activation of the contractile apparatus in ASM is initiated by agonist-induced mobilisation of internally sequestered $Ca^{2+}$ from the SR. G-protein-coupled receptor (GPCR)-mediated activation of phospholipase C triggers the cleavage of membrane-bound phosphatidylinositol-4,5-bisphosphate, liberating inositol-1,4,5-trisphosphate ($IP_3$), which in turn acts on specific ligand-activated $Ca^{2+}$ channels on the SR ($IP_3$ receptors). There are three subtypes of $IP_3$ receptor which differ in their gating kinetics: although there have been many descriptions of $IP_3$-induced release of $Ca^{2+}$ in ASM [20–23], the subtype(s) present and/or involved in ASM are as yet unclear. $IP_3$ receptors are also regulated by autacoids other than $IP_3$. When $[Ca^{2+}]$ at the cytosolic face of the $IP_3$-receptor rises into the micromolar range, the channel enters an inactivated state [24, 25], a property which is generally deemed to be important in explaining repetitive $Ca^{2+}$ waves (see $Ca^{2+}$ oscillation frequency determines contractile function section). Its activity is also modulated by phosphorylation of the receptor/channel by several different kinases, including PKA and PKC. As in other cell types, these $IP_3$ receptors are inhibited by xestospongins, 2-aminoethoxydiphenyl-borate (2-APB) or heparin.

The SR also expresses another class of $Ca^{2+}$ channel, referred to as the ryanodine receptor (RyR) because of its sensitivity to certain plant alkaloids. Once again, there are three subtypes of RyR. Using the experimental tools currently available, it would appear that human ASM expresses only RyR3 and not the other two subtypes [26], while RyR1 and RyR2 were found in rat ASM [27] and all three subtypes were found in murine ASM [28]. Even more interestingly, in the latter study, RyR1 were localised towards the periphery of the cells whereas RyR3 were more centrally located around the nucleus [28]. Such regiospecific heterogeneity would hint at physiologically important mechanisms (discussed later on and in the Transition from pharmacomechanical coupling to electromechanical coupling section). The endogenous ligand for the RyR is debated, with many proposing it to be cyclic ADP ribose [29–33], perhaps acting through FK506 binding protein of 12.6 kDa [31]. The RyR also shows substantial sensitivity to $[Ca^{2+}]_i$, being enhanced by a range of $[Ca^{2+}]_i$ above baseline (a phenomenon referred to as $Ca^{2+}$-induced $Ca^{2+}$ release, or CICR) and then suppressed as $[Ca^{2+}]_i$ rises into potentially cytotoxic ranges [7, 34–36]. Caffeine, a pharmacological tool often used to evoke $Ca^{2+}$ release, acts by enhancing the $Ca^{2+}$ sensitivity of the RyR such that basal levels of $[Ca^{2+}]_i$ are sufficient to trigger CICR [36]. Ryanodine, on the other hand, has several binding sites on the RyR with widely ranging affinities: relatively low concentrations of ryanodine bind to high-affinity sites which lock the RyRs in a subconductance state (leading to $Ca^{2+}$ release), whereas higher concentrations bind to other sites and completely block $Ca^{2+}$ conduction [36]. RyRs are also regulated by endogenous signalling pathways, generally *via* phosphorylation by $Ca^{2+}$/calmodulin-dependent kinase II or PKA [35, 36].

In many nonairway smooth muscle cell types, RyRs contribute to contraction through CICR [7], or to relaxation of vascular smooth muscle through activation of $Ca^{2+}$-dependent $K^+$ channels [37]. The potential role of the RyR in ASM, on the other hand, is less clear. They are certainly functionally active: the RyR agonist caffeine typically evokes reproducible $Ca^{2+}$ transients which are sensitive to SERCA inhibitors as well as to high concentrations of ryanodine [9–11, 26, 38, 39] or anaesthetics [26]. However, agonist-evoked contractile responses in ASM show differential sensitivity to high concentrations of ryanodine: those in the airways of the mouse [28, 38, 39], rat [27] and dog [40] are suppressed, while in the airways of the human [26] and cow (unpublished data) they are not affected. This species-related difference may be due to the differential expression patterns (subtypes and regional heterogeneity) mentioned above. Conversely, relaxations in ASM evoked by β-agonists or nitric oxide donors are not inhibited by ryanodine (unpublished data). The current authors have hypothesised that RyRs function to discharge an overloaded $Ca^{2+}$ store (see Superficial buffer barrier section and fig. 1). This could account not only for the spontaneous transient currents mentioned above, but also for the observation that interleukin-4 inhibits cholinergic responses by activating RyRs and depleting the SR [41].

## VOLTAGE-DEPENDENT INFLUX OF EXTERNAL $Ca^{2+}$

The role for VGCCs in ASM is puzzling. Several lines of evidence suggest that they play a similar role in ASM as they do in other cell types. For example, many types of ion channels are involved in regulating membrane potential ($V_m$) at rest (see Resting membrane potential section) and in the generation of electrical slow waves (see Voltage-dependent $Ca^{2+}$ influx section); also, many bronchoconstrictors trigger membrane depolarisation (see Agonist-evoked membrane depolarisation section), while bronchodilators can cause membrane hyperpolarisation. Thus, it would seem self-evident that $Ca^{2+}$ channels play an important role in EC coupling in ASM, just as they do in other smooth muscle cell types. While this may be true under certain experimental conditions (see Transition from pharmacomechanical coupling to electromechanical coupling section), it is important to note that this is generally not true under normal physiological conditions: release of internally sequestered $Ca^{2+}$ alone is sufficient to mediate the contractile response, as attested to by the persistence of contraction during exposure to blockers of the $Ca^{2+}$ channels, removal of external $Ca^{2+}$, or during voltage-clamp at $V_m$ at which the $Ca^{2+}$ channels are not active [1, 9, 42–44]. Conversely, the present authors' group and others have shown dihydropyridine-sensitive $Ca^{2+}$ channels to play an important role in the refilling of the internal $Ca^{2+}$ store [9, 45–48].

### *Resting membrane potential*

Resting membrane potential ($V_R$) in ASM does not correspond to the equilibrium potential of any single ion species. For example, $V_R$ ranges from -70– -30 mV, while the equilibrium potential for potassium ($E_K$) is ~ -80 mV, that for chloride ($E_{Cl}$) ranges from -33– -6 mV [49, 50], and those for $Na^+$ and $Ca^{2+}$ are in the very positive range (fig. 2). This would suggest that $V_R$ is determined by membrane permeability to more than one ion. The fact that $V_R$ is intermediate between $E_K$ and the equilibrium potentials for all other ions suggests that $K^+$ conductances must contribute to $V_R$. Consistent with this, blockade of $K^+$ channels using nonspecific blockers such as tetraethylammonium (TEA), $Ba^{2+}$, or $Cs^+$ leads to membrane depolarisation and contraction [51]. The specific type of $K^+$ channel involved in setting $V_R$ is largely the voltage-dependent ''delayed rectifier'' subtype [52–54]. Maxi-K channels are unlikely to be active at $V_R$ and resting levels of $[Ca^{2+}]_i$. Most studies show that blockade of large-conductance $Ca^{2+}$-sensitive $K^+$ (KCa) channels (*e.g.* using charybdotoxin or iberiotoxin) has no effect on $V_R$ or resting tone [55–57], but there are exceptions [58]. In addition, STOCs are not recorded until $V_m$ exceeds -30– -40 mV in the smooth muscle cells of canine [13], guinea pig [13, 15] and porcine [59, 60] airways. Small-conductance KCa channels have been shown to be active at $V_R$ and may account for the depolarisation evoked by high concentrations of TEA; however, some find apamin has no effect on $V_R$ [61]. Adenosine triphosphate-sensitive $K^+$ channels are clearly not involved in setting $V_R$, since resting potential and tone are unaffected by glibenclamide [52, 62, 63].

With respect to the contribution to $V_R$ made by other ion conductances, recent evidence of a tonic $Cl^-$ conductance in ASM has been presented. For example, in isolated strips of canine tracheal ASM, replacement of external $Cl^-$ with the impermeant anion isethionate (which would shift $E_{Cl}$ in the positive direction) elicits membrane depolarisation and increases electrotonic potentials [64]. The current authors have described spontaneous $Cl^-$ currents at $V_m$ ranging -100– +50 mV [11, 13, 14]. Thus, $V_R$ should be found somewhere between $E_{Cl}$ (in smooth muscle, $E_{Cl}$ ranges from -33– -6 mV; [49, 50]) and $E_K$ (~ -80 mV).

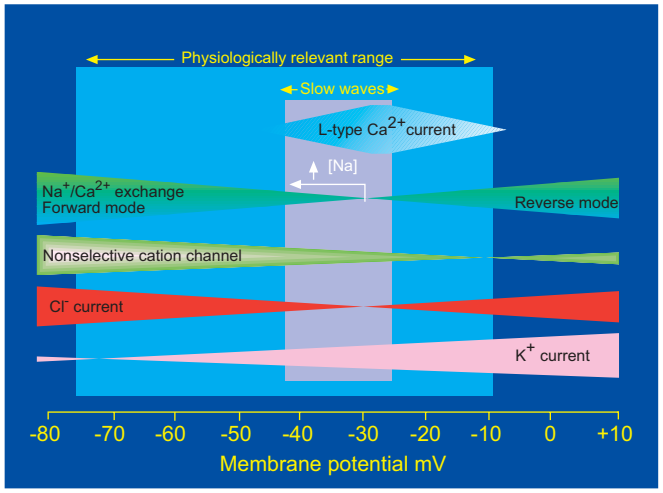


**FIGURE 2.** Ionic currents in airway smooth muscle as a function of membrane potential. The fractional magnitude of a given current at any membrane potential is reflected in the width of the bar at that potential: the reversal potential or equilibrium potential is found at the point where the bar reduces to a point. Note the physiologically relevant range of membrane potentials (bounded by the reversal potentials for nonselective cation current and $K^+$ currents), as well as the range in which the membrane tends to undergo slow wave activity (in which voltage-dependent $Ca^{2+}$ and $Cl^-$ currents are found and in which $Na^+/Ca^{2+}$ exchanger oscillates between forward and reverse modes).

With a 10,000-fold concentration gradient tending to move $Ca^{2+}$ into the cell, even a minute permeability to $Ca^{2+}$ can lead to a significant $Ca^{2+}$ influx, which will tend to depolarise the membrane. Many studies have indeed provided evidence of a persistent influx of $Ca^{2+}$ across the membrane: for example, decreasing external $[Ca^{2+}]$ causes $[Ca^{2+}]_i$ to drop [65]. The voltage-activation and -inactivation properties of VGCCs in some ASM preparations are such that there can be a persistent $Ca^{2+}$ influx or ''window current'' at membrane potentials ranging from -40–0 mV (see Voltage-dependent $Ca^{2+}$ influx section). The possible contribution of voltage-independent $Ca^{2+}$ channels will also be discussed in the Voltage-independent influx of $Ca^{2+}$ section.

### *Agonist-evoked membrane depolarisation*

Many agonists that evoke bronchoconstriction also depolarise the ASM cell membrane into the range of potentials at which VGCCs begin to activate (see Voltage-dependent $Ca^{2+}$ influx section). In human, canine, guinea-pig and equine ASM, a wide variety of spasmogens evoke inward current of up to several thousand pA at $V_R$ [9, 11, 12, 66, 67]; this current reaches a peak within a few seconds, then decays back to baseline levels in the continued presence of the agonist and is sometimes followed by a series of smaller secondary inward currents [11, 13]. A variety of pharmacological tools and electrophysiological strategies have since revealed that this net

inward current represents a mixture of conductance changes. Initially, there is a net decrease in membrane resistance [11], suggesting opening of ion channels: in all ASM preparations studied to date, these largely comprise $Ca^{2+}$-dependent $Cl^-$ channels [11, 12, 59, 60, 66–68]. In some species, this is accompanied by a more sustained activation of nonselective cation channels (NSCCs) [11, 69]. These changes are then followed by a prolonged suppression of outward $K^+$ currents [11, 56, 70, 71]. Altogether, the transient activation of $Cl^-$ and NSCC currents leads to a displacement of the $V_m$ towards the reversal potentials of those two currents (-30 and 0 mV, respectively), while the decreased $K^+$ conductance decreases the strong hyperpolarising influence of $E_K$ ($E_K$= -80 mV). During maximal excitation, the sum total of these changes is a depolarisation to ~-40 mV, often with minor oscillations or ''slow waves'' around that level (described in more detail in the Electrical slow waves section). The current authors would argue that the role of this net change in membrane conductance is not to depolarise the $V_m$ into a range that maximally stimulates VGCCs (+20 mV; see Voltage-dependent $Ca^{2+}$ influx section), but rather to effectively maintain the $V_m$ around -40 mV, which is optimal for a persistent low level of voltage-dependent $Ca^{2+}$ influx (''window current''; see Voltage-dependent $Ca^{2+}$ influx section) and for $Ca^{2+}$ entry *via* the reverse-mode NCX (as argued in subsection entitled NCX and $Ca^{2+}$ store refilling and in figures 2, 3 and 4).

$Cl^-$ current activation appears to be a direct result of elevation of $[Ca^{2+}]_i$, because it can be triggered by agonists that release internally sequestered $Ca^{2+}$ in a G-protein-independent fashion (*e.g.* caffeine) [9, 11] or by voltage-dependent $Ca^{2+}$ influx [14].

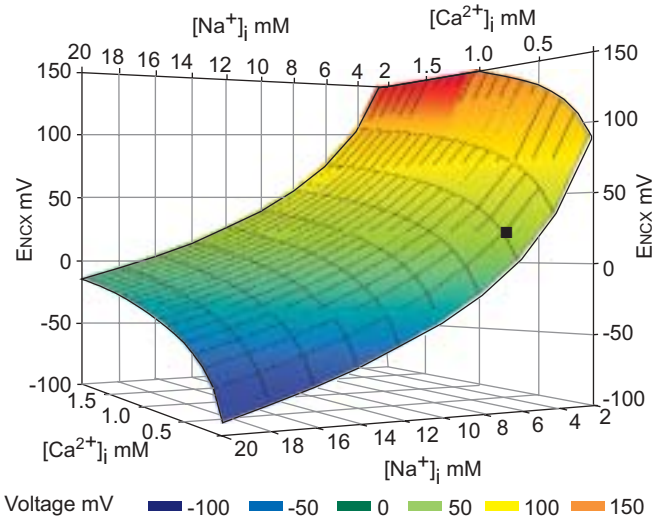


**FIGURE 3.** Reversal potentials ($E_{NCX}$) for $Na^+/Ca^{2+}$ exchanger (NCX). These were calculated over a range of intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$; 100–1,800 μM) and intracellular $Na^+$ concentrations ($[Na^+]_i$; 2–20 mM) using the equation $E_{NCX}=3E_{Na}-2E_{Ca}$ (where $E_{Na}$ and $E_{Ca}$ are the equilibrium potentials for sodium and calcium, respectively) and assuming extracellular $[Na^+]$ and $[Ca^{2+}]$ to be 140 and 1 mM, respectively (see NCX and $Ca^{2+}$ store refilling section). For example, when $[Na^+]_i$=6 mM and $[Ca^{2+}]_i$=200 nM, $E_{NCX}$ ≈+25 mV (■). Under those conditions in which $E_{NCX}$ drops below $V_m$, the NCX operates in reverse mode to bring $Ca^{2+}$ into the cell.

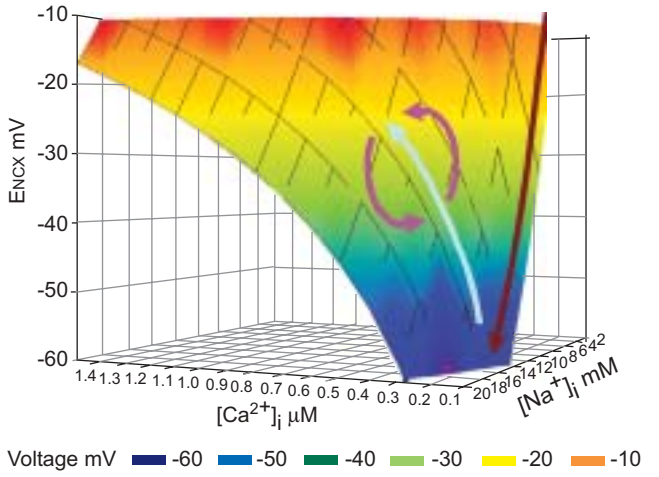


**FIGURE 4.** Reversal potentials ($E_{NCX}$) for $Na^+/Ca^{2+}$ exchanger (NCX): physiological relevance. As subplasmalemmal intracellular $Na^+$ concentration ($[Na^+]_i$) rises from a low resting value towards 15 mM (due to influx through nonselective cation channel), $E_{NCX}$ plummets below the membrane potential (burgundy arrow), causing a transition of NCX into the reverse mode. As $[Ca^{2+}]_i$ then rises from a resting value of 100 nM towards 400 nM (pale blue arrow), $E_{NCX}$ rises, thereby decreasing $Ca^{2+}$ influx through NCX and/or forcing the latter into the forward mode (depending on whether the membrane potential is more or less negative than $E_{NCX}$). During electrical slow waves, membrane potential oscillations (purple arrows) could shift NCX back and forth between forward and reverse modes.

However, these channels quickly undergo a rapid phosphorylation by calcium/calmodulin-dependent protein kinase, which uncouples their activity from $[Ca^{2+}]_i$ [72]. The channels involved in mediating these currents exhibit a very small unitary conductance (<20 pA) and voltage-dependent inactivation [13]. There have been no further characterisations of the $Cl^-$ currents in ASM since the mid-1990s, even though their molecular identity is still unknown: in other smooth muscle preparations, these may include bestrophins [73] or several different members of the $Ca^{2+}$-activated $Cl^-$ channel (CLCA) family of $Cl^-$ channels [74].

The NSCCs activated by GPCRs in ASM were first studied in detail by KOTLIKOFF and co-workers [22, 69, 75]. These are not $Ca^{2+}$-activated since they are not triggered by caffeine, but are nonetheless $Ca^{2+}$-dependent and coupled to plasmalemmal receptors *via* $G_{i/o}$ proteins. Moreover, they were determined to be $Ca^{2+}$ permeant (the fraction of current carried by $Ca^{2+}$ estimated to be 14% at -60 mV) and blocked by $Ni^{2+}$. The current authors have also recently described a NSCC which is activated by depletion of the internal $Ca^{2+}$ store (described in detail in the section entitled Voltage-independent influx of $Ca^{2+}$). It is unclear whether these channels are of a different subtype than the ones described by KOTLIKOFF and co-workers [22, 69, 75].

The $K^+$ channels suppressed by spasmogenic stimulation appear to include both the $Ca^{2+}$-dependent and voltage-dependent varieties. Suppression of the $Ca^{2+}$-dependent current has been shown to be mediated by G-proteins [70, 76], but may also involve agonist-induced depletion of the internal $Ca^{2+}$ pool with consequent decrease in $Ca^{2+}$ release

directed at the plasmalemma. Suppression of the voltage-dependent current, however, involves a shift in the voltage-dependence of activation to more positive potentials [70].

### *Voltage-dependent $Ca^{2+}$ influx*

Membrane depolarisation can result in opening of VGCCs and influx of external $Ca^{2+}$. These currents have been characterised in the trachealis of the dog [77], cow [78], horse [79] and guinea pig [80] as well as the bronchi of the human [81] and dog [82]. By and large, these currents exhibit properties typical of ''L-type'' $Ca^{2+}$ channels, including: 1) threshold potential for activation of -45– -25 mV, and maximal activation at 0–+20 mV; 2) inactivation which is relatively slow (with a time constant of 0≈5 ms) and voltage-dependent (being half maximal at -25– -30 mV); and 3) blockade by dihydropyridines (*e.g.* nifedipine, nicardipine and nitrendipine) or phenylalkylamines (*e.g.* verapamil and D-600). These $Ca^{2+}$ currents are also blocked by certain inorganic cations such as $Cd^{2+}$, $Co^{2+}$, $Mn^{2+}$ and $Ni^{2+}$, while divalents such as $Ba^{2+}$ and $Sr^{2+}$ permeate the channels. It is important to note that voltage-dependent inactivation of the channels is incomplete over the range of potentials at which the channels begin to activate (-40–0 mV); in other words, there is a range of potentials within which there is a window current, or persistent noninactivating influx of $Ca^{2+}$ [83]. Such a window current may be responsible for the $Ca^{2+}$ influx that maintains slow wave activity in intact airway tissues (see Electrical slow waves section)

While the whole-cell studies summarised above indicate that the $Ca^{2+}$ currents in tracheal smooth muscle (TSM) are predominantly of the dihydropyridine-sensitive or L-type, single-channel studies indicate the presence of two types of $Ca^{2+}$ channel, with unitary conductances of 21–26 pS and 10 pS, respectively [84]. The current authors have obtained evidence of both T- and L-type $Ca^{2+}$ currents in canine bronchial smooth muscle [82]. That is, depolarisation evokes a ''fast'' inward current (peak activation within 20 ms followed by rapid inactivation) and a ''slow'' inward current (peak activation at 50 ms and a much slower rate of inactivation); the overall $Ca^{2+}$ current is approximately a 50:50 mixture of these two types. The fast current exhibits threshold and maximal inward current at -50 and -10 mV, respectively, while these values are -40 and +10 mV for the slow current. More importantly, the two currents differ markedly with respect to the voltage-dependence of inactivation: inactivation of the fast current is first noted at -80 mV, is half-maximal at -60 mV, and is complete at -40 mV, compared with values of -60, -40 and -10 mV, respectively for the L-type $Ca^{2+}$ current. A $Ca^{2+}$ current with these voltage activation and inactivation properties has not been reported in the trachealis of any of the species in which the L-type current has been carefully characterised. T-type $Ca^{2+}$ currents are often important for pacemaking in other cell types: their role in canine bronchial smooth muscle is as yet unclear.

### *Electrical slow waves*

Human [85] and guinea pig [86] ASM exhibit spontaneous mechanical and electrical activities, referred to as ''basal tone'' and ''slow waves'', respectively. Electrical slow waves generally comprise oscillations in $V_m$ centred around -40– -30 mV with amplitudes of up to 25 mV and a frequency of 1 Hz. These are unaffected by neuronal blockers (*e.g.* tetrodotoxin or removal of external $Na^+$) or by antagonists of various receptors (*e.g.* antimuscarinics or antihistamines), suggesting that this activity is myogenic in origin. These spontaneous activities seem to be dependent, in part, on a constitutive metabolism of arachidonic acid, although the enzymatic pathway involved is apparently species-dependent. For example, basal tone and slow waves in guinea pig ASM are reduced by inhibitors of cyclo-oxygenase but are relatively unaffected by inhibitors of lipoxygenase and thromboxane synthase [86–88]. Spontaneous activity in human ASM, on the other hand, is not reduced by inhibition of cyclo-oxygenase but is abolished by inhibition of lipoxygenase [85, 89–91]. These observations suggest that spontaneous activity is mediated by constitutive generation of prostaglandins in guinea pig ASM, but of leukotrienes in human ASM.

The ASM of the dog [51, 92, 93], cat [94], ferret [95], cow [96] and horse [97] at rest is mechanically and electrically quiescent. However, spasmogens can evoke changes in mechanical and electrical activity that resemble the spontaneous activities described above. For example, while canine ASM is electrically and mechanically quiescent at rest, slow waves identical to those recorded from guinea pig and human ASM are evoked by cholinergic stimulation [51], the thromboxane analogue U46619 [98], leukotrienes [95, 99], or $K^+$ channel blockers [51]. Similarly, histamine-induced stimulation of bovine TSM evokes phasic contractions and slow waves [96]. In addition, indomethacin evokes contractions (apparently mediated by leukotrienes) in equine ASM tissues that were previously devoid of mechanical activity [97].

Whether recorded from tissues at rest (*e.g.* human and guinea pig) or from tissues stimulated by excitatory agonists (*e.g.* canine, bovine and equine), slow-wave activity is influenced by conditions which modulate voltage-dependent $Ca^{2+}$ currents. It is reduced by removing external $Ca^{2+}$ or by $Ca^{2+}$ channel blockers [51, 100], suggesting it is initiated and maintained by a persistent influx of $Ca^{2+}$ through those channels. In this context, it is noteworthy that the threshold potentials for slow-wave activity and for activation of L-type VGCCs in ASM are both ~-40– -30 mV (fig. 2). The repolarising phase of these oscillations in ASM seems to involve activation of $Ca^{2+}$-dependent $Cl^-$ channels [101].

Thus, ASM, which is electrically quiescent under ''basal'' conditions, is capable of exhibiting slow-wave activity identical to that recorded from spontaneously active tissues if appropriately provoked. This suggests that a myogenic oscillatory mechanism resides in all ASM tissues and is invoked by excitatory stimulation. It is tremendously interesting that these electrical slow waves oscillate up and down through the range of potentials in which the window current of VGCCs exists, as well as in the vicinity in which the NCX might transition into reverse mode under certain conditions of excitatory stimulation (see NCX and $Ca^{2+}$ store refilling section). This has enormous implications for EC coupling in ASM.

## VOLTAGE-INDEPENDENT INFLUX OF $Ca^{2+}$

### *Transient receptor potential ion channels*

Transient receptor potential (TRP) ion channels were first described in the Drosophila visual system and are named after

the role they play in phototransduction. This seminal discovery has since been followed by the identification of seven TRP homologues in mammals, namely the classical or canonical TRP (TRPC) family, the vanilloid TRP family, the melastatin TRP family, the mucolipin TRP family, the polycystin TRP family, the antigen-repeat TRP family, of which there is only one mammalian member, and no mechanoreceptor potential C channel, which does not appear to occur in the mammalian genome (for extensive reviews, see [102] and [103]). In many nonexcitable cells, including mast cells, basophils, T-cells and megakaryocytes, TRP proteins are thought to form inwardly rectifying highly $Ca^{2+}$-selective channels also referred to as $Ca^{2+}$ release-activated $Ca^{2+}$ (CRAC) channels [104]. These channels mediate $Ca^{2+}$ influx upon depletion of $IP_3$-sensitive intracellular $Ca^{2+}$ stores; a phenomenon that has been coined capacitative $Ca^{2+}$ entry (CCE) [105]. However, in excitable cells such as smooth muscle, TRP proteins form NSCCs that allow for the permeation of both mono- and divalent cations [106]. As a consequence of their ionic promiscuity, NSCCs exhibit a characteristically linear current–voltage (I–V) relationship, with a reversal potential around 0 mV, when measured in near-physiological ionic conditions. Although NSCC proteins share a structural theme reminiscent of VGCCs, they are only distantly related. Indeed, NSCCs differ from their voltage-gated cousins in that they lack an inherent voltage sensor. Consequently, NSCCs activate in a voltage-independent fashion [104].

It has been proposed that TRPC proteins are largely responsible for the formation of NSCCs expressed in mammalian smooth muscle cells including rabbit cerebral [107] and human [108] and rat [109] pulmonary arterial myocytes. In ASM, there is evidence to support the expression of TRPC1, -3, -4, -5 and -6 in human [110], rat [110], guinea pig [111] and porcine [112] airways. However, there is little knowledge of the relative levels of expression of these subunits in the native cell, or of the functional significance of their expression. Increased expression of TRPC1 [110] and TRPC3 [110] has been observed in proliferating ASM cells cultured under normal conditions or in the presence of inflammatory mediators (*e.g.* tumour necrosis factor (TNF)-α), respectively. Despite minimal biophysical and electrophysiological data regarding the properties of TRP proteins in ASM, a number of recent reviews have broadly implicated TRP channels in the pathogenesis of several airway diseases including asthma, chronic obstructive pulmonary disease, cystic fibrosis and emphysema [113–115]. Below a body of evidence is discussed that supports the present authors' current understanding of the properties and physiological implications of NSCC activation by GPCR stimulation or intracellular $Ca^{2+}$ store depletion in ASM.

### *GPCR signalling activates nonselective cation channels*

Many agonists exert their contractile effects on ASM by signalling through GPCRs (*e.g.* acetylcholine, histamine and leukotrienes). GPCR stimulation not only leads to a transient $IP_3$-dependent release of internally sequestered $Ca^{2+}$ from the SR, but also results in the recruitment of NSCCs with a low current amplitude that exhibit a significant $Ca^{2+}$ permeability under ''physiological'' conditions (representing ~14% of the inward current at -60 mV; [75]). It has been suggested that this current is responsible for the sustained elevation in $[Ca^{2+}]_i$ observed during prolonged agonist stimulation [69, 75, 116]. In contrast, patch-clamp studies reveal that $Na^+$ ions contribute the majority of the inward current conducted by NSCCs, generating speculation with respect to the role of this conductance in agonist-evoked responses. Some have proposed that the influx of $Na^+$ through NSCCs is important in effecting a substantial depolarisation, resulting in the recruitment of VGCCs to sustain or augment $Ca^{2+}$ influx, thereby contributing to contraction. Alternatively, it has been proposed that the substantial $Na^+$ permeation mediated by NSCCs may function to drive NCX in reverse mode, favouring $Ca^{2+}$ influx (see NCX and $Ca^{2+}$ store filling section). This model is supported by a recent report that TRP proteins are co-localised with NCX1 [117]. Furthermore, DAI *et al.* [17] reported that sustained contractions required $Ca^{2+}$ influx mediated by the NSCCs and the reverse mode of the NCX. Indeed, findings from the current authors' laboratory support a similar mechanism underlying sustained agonist-evoked contractions and SR $Ca^{2+}$ refilling in ASM (see NCX and $Ca^{2+}$ store filling section) [16].

In both equine [69] and porcine [116] trachealis, activation of NSCCs by histamine and/or acetylcholine relies exclusively upon the activities of $G_i/G_o$ proteins coupled to $H_1$ histamine and $M_2$ muscarinic receptors, respectively. Observations by WANG and KOTLIKOFF [69] suggest that GPCRs may signal through convergent pathways to recruit a common species of NSCCs in equine trachealis; a phenomenon evidenced by the equivalent I–V relationships measured during acetylcholine or histamine stimulation and by the lack of an additive nature of these currents upon concurrent stimulation with both agonists. However, this is most likely a species-dependent phenomenon, as acetylcholine-activated NSCC currents in porcine TSM could not be duplicated with histamine or leukotriene (LT)$D_4$ [116].

While it is apparent that an elevation in $[Ca^{2+}]_i$ alone is insufficient to activate NSCCs in several ASM preparations [69, 75, 116], both histamine- and acetylcholine-induced NSCC currents do not activate unless GPCR stimulation is accompanied by an elevation in $[Ca^{2+}]_i$. For example, acetylcholine-activated NSCC currents in porcine trachealis do not manifest at $[Ca^{2+}]_i$ <50 nM [116]. Furthermore, in equine tracheal myocytes, the inhibition of histamine-induced cation current observed in the presence of U73122 (phospholipase C inhibitor) can be restored by simultaneous application of histamine and caffeine [116].

Unfortunately, the molecular identities of the proteins responsible for NSCCs in ASM are still unclear. Work in this area has been greatly hindered by the lack of specific inhibitors for TRP channels. Nevertheless, in isolated cultured human ASM cells, $Ca^{2+}$ influx induced by acetylcholine or bradykinin is insensitive to organic $Ca^{2+}$ channel inhibitors including nifedipine, nisoldipine or diltiazem, but can be completely inhibited by several polyvalent cations including $Co^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Cd^{2+}$ or $La^{3+}$ [116, 118]. Similarly, acetylcholine-activated NSCCs in equine tracheal myocytes cannot be blocked by nisoldipine, yet are rapidly inhibited by 10 mM $Ni^{2+}$ [75]. Although nifedipine reduces $LTD_4$-induced contraction of human small bronchioles by nearly 40%, these responses are completely abolished by application of $La^{3+}$, $Gd^{3+}$ and SKF 96365, suggesting that NSCCs mediate the majority of $Ca^{2+}$

influx observed in this preparation [119, 120]. While these observations alone are insufficient to unequivocally implicate specific TRP protein(s) in forming NSCCs in ASM, the pharmacological and biophysical properties of the NSCCs examined thus far indicate that members of the TRPC family may be involved.

### *Nonselective cation channels mediate CCE*

In ASM cells, depletion of intracellular $Ca^{2+}$ stores activates a $Ca^{2+}$ influx pathway which in human [110], rat [110], guinea pig [121], porcine [65, 112] and bovine [65] ASM is mediated by a channel that is sensitive to $Ni^{2+}$, $La^{3+}$ and SKF 96365, but not to nifedipine. Although it is unclear whether this pathway is modulated by release of $Ca^{2+}$ through $IP_3$ receptors, it does appear to be modulated by release of $Ca^{2+}$ from RyRs [112]. Indeed, AY *et al.* [112] demonstrated that CCE can be induced in porcine TSM cells by depleting the internal $Ca^{2+}$ store using CPA or prolonged exposure to caffeine. While concurrent administration of acetylcholine significantly augmented CCE, this enhancement was found to be due to the recruitment of a separate group of NSCCs whose function specifically relies on release of SR $Ca^{2+}$ *via* $IP_3$ receptors (*i.e.* they are blocked by xestospongin D) but did not appear to involve diacylglycerol or activation of PKC [112]. It is still unclear which TRP proteins mediate these responses in porcine ASM; however, in cultured human ASM there is compelling evidence that a preferential increase in the expression of TRPC3 is responsible for mediating elevated resting $[Ca^{2+}]_i$ and enhanced CCE in the presence of the pro-inflammatory cytokine TNF-$\alpha$ [122]. The latter study reported that CPA-induced CCE was enhanced by TNF-$\alpha$, but inhibited by $Ni^{2+}$ and $La^{3+}$. Furthermore, these responses could be blocked using small interfering RNA directed toward TRPC3. Interestingly, in TNF-$\alpha$ treated tissue, CCE responses measured in the presence of acetylcholine were insensitive to atropine (muscarinic receptor antagonist), whereas responses in nontreated cells were reduced by blockade of the muscarinic receptors. This suggests that TNF-$\alpha$ preferentially upregulates the expression of TRPC proteins involved in the formation of ''store-operated'' NSCCs [122].

While there is a substantial knowledge base regarding the electrophysiological properties of the NSCCs involved in mediating CCE in vascular smooth muscle preparations [106], there remains a relative dearth of information in the ASM field. Several studies have examined the effects of store depletion on $Ca^{2+}$ influx and the regulation of $[Ca^{2+}]_i$ in ASM (see above), but only three reports to date have utilised patch-clamp techniques to measure directly the resultant membrane currents [65, 110, 123]. In cultured human and rat bronchial smooth muscle cells, SWEENEY *et al*. [110] demonstrated that the current density of a $Ni^{2+}$-sensitive NSCC activated by passive store depletion was markedly increased in proliferating *versus* growth-arrested cells. This increase in membrane current density was found to be associated with enhanced CCE accompanied by a complementary increase in TRPC1 mRNA expression; unfortunately Western blots were not performed to confirm that TRPC1 protein levels were similarly increased, and other TRPC family members were not examined. The current authors' group has recently provided a detailed description of the electrophysiological properties of a CPA-evoked NSCC conductance in porcine and bovine trachealis [65]. It was described how internal $Ca^{2+}$ store depletion augments a basal NSCC conductance in TSM cells which is sensitive to micromolar concentrations of $La^{3+}$ and $Gd^{3+}$, and exhibits a marked permeability to $Na^+$ at physiologically relevant $V_m$ [65]. Indeed, the current authors demonstrated that while the bulk of the current mediated by this NSCC conductance is carried by $Na^+$, it too possesses a significant $Ca^{2+}$ permeability that is capable of sustaining elevated $[Ca^{2+}]_i$ responses in the presence of extracellular $Ca^{2+}$. Interestingly, this conductance appeared to be constitutively active under ''resting'' conditions, and was likely to be responsible for the basal, nifedipine-insensitive, $Ca^{2+}$ influx pathway observed in this preparation [65]. Others have similarly reported the existence of a basal $Ca^{2+}$ influx pathway operating in ASM [121, 122, 124, 125], speculating that this plasmalemmal $Ca^{2+}$ ''leak'' contributes to regulation of basal $[Ca^{2+}]_i$ [126].

Although it is clear that SR $Ca^{2+}$ depletion is sufficient to activate NSCC in ASM, it is not clear whether the NSCC conductance described by the present authors' group [65] and others [110] is in turn regulated by $[Ca^{2+}]_i$, as appears to be the case for GPCR-activated NSCCs in other ASM preparations [69, 75, 116]. This uncertainty stems from the fact that CPA is known to induce a biphasic change in $[Ca^{2+}]_i$ [65, 127]: this change includes an initial transient increase in $[Ca^{2+}]_i$ (due to direct antagonism of SERCA-mediated sequestration of $Ca^{2+}$ and an unmasking of SR $Ca^{2+}$ leak *via* RyRs) followed by a sustained decline in $[Ca^{2+}]_i$ (due to extrusion of $Ca^{2+}$ from the cell) [127]. To resolve this question, the current authors showed that loading the cells with BAPTA, which depletes the SR without stimulating an elevation in $[Ca^{2+}]_i$, is sufficient to activate NSCCs having pharmacological and electrophysiological properties identical to the CPA-evoked NSCC currents (unpublished observations). Although the pharmacological profile of store-depletion activated currents in ASM is similar to the GPCR-activated currents described above (see GPCR signalling activates nonselective cation channels section), only the former can be activated in the absence of an elevation in $[Ca^{2+}]_i$, suggesting that several functionally distinct species of NSCCs may be expressed in ASM.

The mechanisms responsible for conveying SR $Ca^{2+}$ status to the plasmalemmal ion channels which mediate CCE have been studied for decades but remained frustratingly elusive. However, recent work in several nonmuscle cells point to the involvement of stromal interacting molecule (STIM), initially described as a candidate tumour suppressor gene almost a decade ago [128], and its human homologue STIM1. In particular, STIM has been found to be an essential component of TG-induced CCE and CRAC currents in Drosophila S2 cells, rat basophilic leukaemia (RBL) cells, Jurkat T-cells and HEK 293 cells [129–132]. This type I transmembrane protein contains an EF-hand motif located near its amino-terminus which is thought to function as a $Ca^{2+}$ sensor of the endoplasmic reticulum (ER)/SR $Ca^{2+}$ store [133, 134]. In resting Jurkat T-cells, RBL cells, human T-cells and pheochromacytoma cells, STIM1 resides in ER-like structures, co-localised with SERCA2, but translocates to the plasmalemma upon $Ca^{2+}$ store depletion [134]. In cells made to express EF-hand mutants of STIM1 with compromised $Ca^{2+}$ binding ability, STIM1 localises to the plasmalemma but not the ER, and the cells exhibit elevated

resting $[Ca^{2+}]_i$ and persistent TG-independent $Ca^{2+}$ influx, both of which are inhibited by 2-APB, SKF 96365 and $Gd^{3+}$. Altogether, the data suggest that STIM1 translocates to the plasma membrane when it is no longer able to bind ER/SR $Ca^{2+}$ (*e.g.* when the $Ca^{2+}$ store is depleted) and directly activates CRAC channels in those cells. The question then becomes whether this mechanism operates in ASM.

Only one publication to date has addressed this question in ASM, finding both CCE and store depletion-evoked membrane currents in human bronchial smooth muscle cells to be dependent upon STIM1 but not STIM2 (a related protein) [123]. Interestingly, targeted silencing of STIM1 inhibited both CPA- and histamine-induced $Ca^{2+}$ influx and CCE, while bradykinin responses were largely unaffected. Furthermore, CPA-induced NSCC currents were almost completely abolished by targeted suppression of STIM1 (but not STIM2). Altogether these observations have several profound implications. First, STIM1 may indeed be mechanistically involved in transmitting SR $Ca^{2+}$ store status to store-operated NSCCs in ASM. Secondly, the study provides evidence of agonist-specific differences in the ability to activate store-operated NSCCs, which may reflect variations in the level of PLC activation and subsequent $IP_3$-mediated SR $Ca^{2+}$ release (*i.e.* the relative magnitude of SR depletion) associated with each agonist [123]. In addition, the study also highlights the difficulty of ascertaining the relative contribution of GPCR-activated and store-operated NSCCs in mediating agonist-evoked membrane currents and/or $Ca^{2+}$ influx.

Regardless of the mechanism by which NSCCs in ASM are activated (*i.e.* GPCR stimulation or depletion of intracellular $Ca^{2+}$ stores), they clearly contribute to the regulation of $[Ca^{2+}]_i$. In some cases, NSCCs are involved in CCE-evoked contraction of human [110, 119, 120] and rat [110] bronchial and guinea pig [121] TSM. In addition, there is mounting evidence to suggest that NSCCs may facilitate $Ca^{2+}$ entry indirectly by depolarising the membrane (thereby activating L-type $Ca^{2+}$ channels) and/or promoting reverse-mode operation of NCX. Unfortunately, it is still largely unclear whether these NSCCs actively participate in the refilling of the SR, a function that is entirely consistent with the nature of the stimulus responsible for activating these channels. Further clarification of these questions may require the use of small interfering RNA or other genomic techniques (*e.g.* gene knock-out models) in order to determine which TRP proteins are functionally involved in the regulation of ASM contraction and airway calibre.

## NOVEL MECHANISMS

### *NCX and $Ca^{2+}$ store refilling*

Following agonist-induced elevation of $[Ca^{2+}]_i$, conditions are returned to normal through the extrusion of $Ca^{2+}$ to the extracellular domain and/or re-sequestration into the SR. $Ca^{2+}$ extrusion must be carefully matched to net $Ca^{2+}$-influx: otherwise, there will be a deficit in the amount of intracellular $Ca^{2+}$ available to refill the SR. Thus, influx pathways must exist to account for the loss of extruded $Ca^{2+}$. The role of NSCCs in this function has been discussed previously in the Voltage-independent influx of $Ca^{2+}$ section, while a possible role for voltage-dependent $Ca^{2+}$ channels in this capacity will be considered later in the Transition from pharmacomechanical coupling to electromechanical coupling section. Recently, DAI *et al.* [17] introduced a third pathway contributing to $Ca^{2+}$ influx during agonist-induced contraction of porcine ASM ($Ca^{2+}$ influx mediated by the reverse mode of the NCX) as has been described in other nonairway preparations [17, 135–138]. The current authors later showed the importance of this mechanism in SR refilling in bovine ASM [16], as have others in murine ASM [139]. This $Ca^{2+}$-influx pathway appears to be dependent on the availability of extracellular $Na^+$ [16]. Interestingly, ROSKER *et al.* [140] have described an intimate relationship between the NCX and TRP proteins, building on an earlier report by MOORE *et al.* [141] that suggested that NCX and the $Na^+/K^+$ pump are co-localised in smooth muscle caveolae in close apposition to the SR. Thus, it is possible that the reverse mode of the NCX, driven by membrane depolarisation and elevated $[Na^+]_i$ in the region between the SR and the plasmalemma, may contribute to the $Ca^{2+}$ influx pathways required to replenish the smooth muscle cell of $Ca^{2+}$ and allow for maximal refilling of the SR.

The direction of ionic flux through the NCX can be predicted by calculating its reversal potential ($E_{NCX}$) under varying concentrations of $[Ca^{2+}]_i$ and $[Na^+]_i$, and comparing it to the $V_m$ under the same conditions. That is, given the stoichiometry of the NCX (three $Na^+$ ions transported for every $Ca^{2+}$), and the relative valencies of these cations, $E_{NCX}$ is defined with reference to the equilibrium potentials for sodium ($E_{Na}$) and calcium ($E_{Ca}$) thus [85]:

$$E_{NCX} = 3E_{Na} - 2E_{Ca} \quad (1)$$

Since $E_{Na}$ and $E_{Ca}$ are not static, $E_{NCX}$ is a dynamic value that will change depending on the intracellular environment (*i.e.* changes in $[Ca^{2+}]_i$ and $[Na^+]_i$; figs 3 and 4). As such, when $V_m$ is more negative than $E_{NCX}$, the NCX will act in the forward mode, extruding $Ca^{2+}$ from the cell; conversely, if $V_m$ is more positive than $E_{NCX}$, the NCX will act in the reverse mode, leading to $Ca^{2+}$ influx. During agonist-induced release of internally sequestered $Ca^{2+}$, there is membrane depolarisation and significant inward $Na^+$ current (*via* activation of NSCC and/or TRP channels) which elevates $[Na^+]_i$ in the domain immediately adjacent to the plasma membrane. Increased $[Na^+]_i$, in conjunction with low-to-moderate $[Ca^{2+}]_i$, brings $E_{NCX}$ into a range of potentials more negative than that of the depolarised membrane (*i.e.* <-30 mV; fig. 4), thereby forcing the NCX into reverse-mode operation. $Ca^{2+}$ entering the cell may be directly shuttled into the depleted SR, keeping the $[Ca^{2+}]_i$ in this subplasmalemmal region relatively low until $Ca^{2+}$ uptake slows (*i.e.* because the SR is full), at which point $Ca^{2+}$ accumulates and NCX flips back into the forward mode.

Some previous studies, including those of the current authors, have concluded a central role for L-type $Ca^{2+}$ channels in store-refilling on the basis of data obtained using techniques aimed at manipulating $V_m$ (*e.g.* KCl-induced depolarisation or cromakalim-induced $V_m$ clamping) [47, 142], even though inhibition of L-type $Ca^{2+}$ channels did not completely abolish store-refilling [47, 48]. Now recognising reverse-mode NCX as another voltage-dependent $Ca^{2+}$ influx pathway, the current authors would re-evaluate those earlier data; future studies examining $Ca^{2+}$ handling and EC coupling should not overlook the potential for $Ca^{2+}$ influx *via* the NCX.

### *$Cl^-$ and $Ca^{2+}$ handling*

As mentioned in much of the present review, $Ca^{2+}$-dependent contraction in ASM is driven primarily by the release of $Ca^{2+}$ from the SR. In addition to activating the contractile apparatus, $Ca^{2+}$ release from the SR can trigger membrane currents, including $Ca^{2+}$-dependent $Cl^-$ currents. $Cl^-$-mediated depolarisation drives $Ca^{2+}$-dependent contraction in many nonairway smooth muscle types through the activation of L-type $Ca^{2+}$ channels. However the present authors, and others, have reported that inhibition of $Cl^-$-mediated depolarisation using selective $Cl^-$ channel blockers (niflumic acid and 5-nitro-2-(3-phenylpropylamino)benzoic acid) has little effect on the cholinergic concentration–response relationship in standard organ bath experiments. This poses the question: ''what role does $Cl^-$ play in ASM?'' A growing number of reports suggest that intracellular $Cl^-$ ($Cl^-_i$) may be important in $Ca^{2+}$ handling in smooth muscle.

The movement of $Ca^{2+}$ across the SR membrane is an electrogenic processes (fig. 5). Since the efflux of $Ca^{2+}$ through RyR and $IP_3$-receptors is entirely a passive process, accumulation of charge across the SR membrane could electrostatically hinder the sustained movement of $Ca^{2+}$ into or out of the SR. However, compensatory ion fluxes across the SR membrane have been found in other nonairway preparations which are thought to neutralise any charge accumulation due to $Ca^{2+}$ movement [143, 144]. $Cl^-$ seems to be particularly important in this respect. For example, replacement of $Cl^-$ with large impermeant anions reduced the early phasic portion of agonist-induced contraction of ileal longitudinal smooth muscle in parallel with a reduction in cellular $^{45}Ca^{2+}$ uptake [145, 146]. Similarly, inhibition of SR $Cl^-$ channels reduced $Ca^{2+}$ sequestration into saponin-permeabilised gastrointestinal smooth muscle cells [144], and depletion of $Cl^-_i$ reduced angiotensin II- and norepinephrine-induced contractions of vascular smooth muscle [147]. Recently, the current authors reported that the depletion of $Cl^-_i$ through prolonged bathing in $Cl^-$-free bathing solution significantly reduced successive agonist-induced contractions in ASM [148]; additionally, the rates of contraction and relaxation (following agonist removal) were greatly reduced when $Cl^-_i$ was reduced, suggesting the release and reuptake of $Ca^{2+}$ were both hindered by depletion of $Cl^-_i$. In contrast to those effects of chronic $Cl^-_i$ depletion, acute removal of $Cl^-$ from the bathing solution accelerated contractile responses (but slowed relaxations). An interpretation of this is that sudden removal of external $Cl^-$ would augment $Cl^-$ efflux from the cell during agonist stimulation, resulting in greater reduction in $[Cl^-]_i$ in the region adjacent to the SR and allowing for more $Cl^-$ to leave the SR, which in turn would augment the rate of $Ca^{2+}$ release, as evidenced by a greater rate of contraction. Since the removal of extracellular $Cl^-$ increases $Cl^-$ efflux, the cell experiences a short-term depletion of $Cl^-_i$, resulting in a reduced ability to reuptake $Ca^{2+}$ into the SR, as seen in the decreased rate of relaxation.

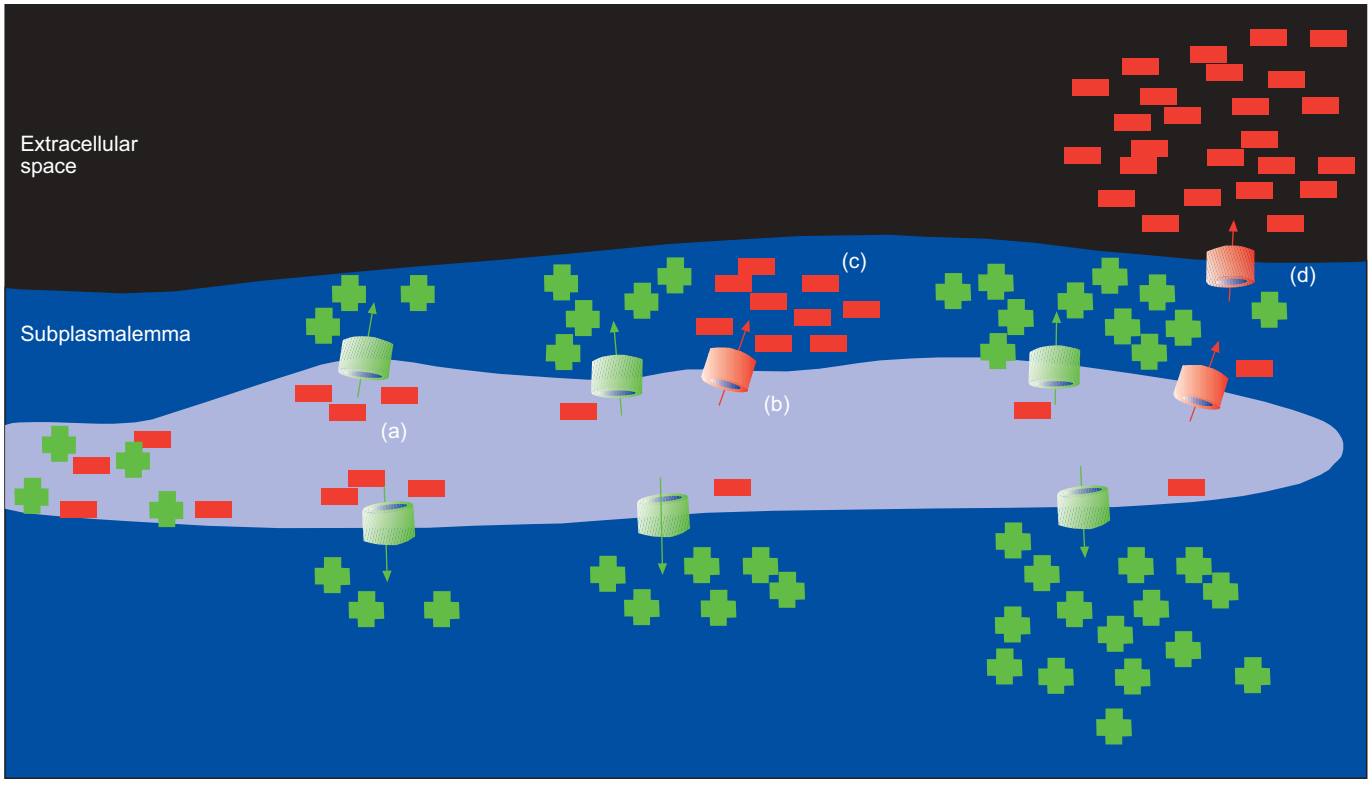


**FIGURE 5.** $Cl^-$ channels serve to neutralise charge build-up on the sarcoplasmic reticulum (SR) membrane. a) As internal $Ca^{2+}$ (green) is released, charge builds up on the SR membrane, decreasing the driving force on $Ca^{2+}$. b) Channels for $Cl^-$ (red) on the SR dissipate the charge build-up on the SR membrane, removing this impediment to $Ca^{2+}$ efflux from the SR. c) However, accumulation of $Cl^-$ in the restricted subplasmalemmal space decreases the driving force on $Cl^-$. d) Plasmalemmal $Cl^-$ channels disperse cytosolic $Cl^-$, thereby increasing the driving force on $Cl^-$ from the SR (and thus enhancing $Ca^{2+}$ release).

Altogether, then, there is a growing body of evidence to suggest that the burst of $Ca^{2+}$-release leads to a rapid activation of $Cl^-$ currents which in turn boosts further $Ca^{2+}$ release (by promoting efflux of $Cl^-$ from the SR to compensate for the charge build-up). The rapid inactivation of those $Cl^-$ channels by calcium/calmodulin-dependent protein kinase (described in the Agonist-evoked membrane depolarisation section) may represent an important timing mechanism, allowing for ''resetting'' of the $Cl^-$ and $Ca^{2+}$ gradients by SR and plasmalemmal pumps, and thus setting the stage for subsequent bursts of $Ca^{2+}$ (*i.e.* repetitive $Ca^{2+}$ waves).

### *Regulation of Rho/ROCK by $Ca^{2+}$, $Cl^-$ and $V_m$*

The current authors were the first to show in ASM that RhoA is activated by 60 mM KCl [149], contrary to initial expectations. Follow-up work showed that this is directly related to elevated $[Ca^{2+}]_i$, although membrane depolarisation *per se* may also be involved [150]. Changes in Rho-associated kinase (ROCK) activity paralleled those in RhoA, suggesting KCl does not exert an additional effect on ROCK (*i.e.* is only stimulating RhoA). It will be important, then, to ascertain how $Ca^{2+}$ and $V_m$ stimulate RhoA activity.

Rho activation itself may ultimately prove to be $Ca^{2+}$ dependent: the current authors have already ruled out roles for $Ca^{2+}$/calmodulin-dependent kinase II and for PKC (which is also $Ca^{2+}$ dependent) in activation of Rho [150]; others have shown focal adhesion kinase (FAK) phosphorylation following GPCR stimulation to be $Ca^{2+}$ dependent [151, 152]. Another possibility is that $Ca^{2+}$, being a divalent cation, influences the interactions between $G_{12,13}$, RhoGDI, RhoGEF, RhoGAP and/or RhoA by interfering with $Mg^{2+}$-dependent activation of RhoA ($Mg^{2+}$ influences guanosine triphosphate/guanosine diphosphate exchange activity [3, 153–156]).

Conversely, $V_m$ *per se* could influence Rho activity, both directly and indirectly. Directly, Rho is a charged molecule and would be expected to be influenced by the transmembrane voltage gradient as it approaches and inserts into the membrane (both steps are prerequisite for its activation). Indirectly, voltage-sensitive channels may signal to Rho upon membrane depolarisation: there is a growing literature describing direct physical interactions between various enzymes and ion channels, including L-type $Ca^{2+}$ channels [157, 158]. As such, the voltage-dependence of Rho-activation could be conferred by the $Ca^{2+}$ channels. Such a direct channel–enzyme interaction does not necessarily imply that $Ca^{2+}$-channel blockers should prevent Rho activation, because these only prevent ion conduction through the channels and do not prevent the depolarisation-induced conformational changes that might stimulate Rho activity.

$Ca^{2+}$-dependent $Cl^-$ channels may also transduce the signal between the plasmalemma and Rho. There are two pieces of evidence that suggest that $Cl^-_i$ modulates RhoA/ROCK signalling in ASM. First, while characterising the agonist-evoked $Cl^-$-currents in ASM [11, 12, 66, 67], the current authors noted anecdotally that cells perfused internally with a $Cl^-$-deficient electrode solution quickly lost the ability to contract to acetylcholine, even though the membrane current responses persisted. In contrast, control cells perfused internally with a 140 mM $Cl^-$ electrode solution could contract repeatedly for a number of hours [9]; more interestingly, those contractions could be evoked during voltage-clamp at -60 mV and in the presence of CPA, indicating they are independent of both voltage-dependent $Ca^{2+}$ influx and release of internal $Ca^{2+}$. At that time, the reason for the discrepancy between high and low $Cl^-$ conditions was unknown. Additionally, the current authors have more recently found that the $Cl^-$ channel blocker niflumic acid markedly suppresses cholinergically-induced RhoA-activation (unpublished data). These observations lead to a suggestion that $Cl^-_i$ may modulate Rho/ROCK activity. Changes in subplasmalemmal $[Cl^-]$ might facilitate translocation of RhoA to the membrane, or enhance interactions between the different components of this signalling cascade: others have shown G-protein activity to be modulated by $Cl^-_i$ [159].

### *$Ca^{2+}$ oscillation frequency determines contractile function*

The traditional approach to $Ca^{2+}$-dependent contraction in smooth muscle considers only global and sustained elevations of $[Ca^{2+}]_i$. However, there is increasing evidence for considerable spatial and temporal heterogeneities in $[Ca^{2+}]_i$ elevation [17, 21, 39, 160, 161]. In particular, agonists evoke a large $Ca^{2+}$ wave which sweeps across the length of the cell, followed by a series of subsequent $Ca^{2+}$ waves at periodic intervals with a frequency which seems to correlate with the degree of tone [39, 161]. $Ca^{2+}$ waves in neighbouring cells are generally asynchronous. In some cases, unitary $Ca^{2+}$ events referred to as $Ca^{2+}$ sparks have been described [15].

The mechanisms underlying $Ca^{2+}$-wave generation and propagation have been studied for over a decade, yet are still poorly understood. Agonist-induced oscillations in $[Ca^{2+}]_i$ have been proposed to be triggered by $IP_3$. However, DAI *et al.* [17] reported the generation of oscillations in the presence of the $IP_3$-receptor antagonists xestospongin C and 2-APB. Continuous $Ca^{2+}$ oscillations can be inhibited by caffeine and ryanodine [17, 39, 162], reinforcing the central role of the SR and possibly suggesting that CICR is involved. The importance of SR $Ca^{2+}$ is also highlighted by the sensitivity of $Ca^{2+}$ oscillations to SERCA inhibitors or chelation of intracellular $Ca^{2+}$ [17, 39]. In addition to $Ca^{2+}$ released from the SR, $Ca^{2+}$ influx pathways play a crucial role in the continuous generation of agonist-induced asynchronous $Ca^{2+}$ oscillations. Removal of extracellular $Ca^{2+}$ from bathing solutions results in a gradual run-down of oscillations. Early studies revealed that agonist-induced oscillations were sensitive to verapamil and enhanced by BAY K-8644, suggesting a role for L-type $Ca^{2+}$ channels [60]. It is unlikely that the $Ca^{2+}$ waves are triggered by electrical slow waves, given that the frequency of the former is roughly 1–2 orders of magnitude lower than that of the latter. Conversely, inhibitors of NSCCs (*e.g.* SKF 96365 and $Ni^{2+}$) and of reverse-mode NCX inhibit oscillations and the corresponding force generation in responses to cholinergic stimulation [17], suggesting involvement of those other pathways as well. The contributions of many other molecular and cellular entities and signalling events have also been examined, including: mitochondria; cytosolic $Ca^{2+}$-buffering proteins; $Ca^{2+}$uptake and extrusion rates; and the rates of activation and inactivation of $IP_3$-receptors and RyR, *etc*. The integration of these various parameters into a mathematical model is beyond the scope of the present manuscript, but has already been carried out elsewhere [163–165].

Very little is known about how agonist-induced $Ca^{2+}$ oscillations in ASM determine the level of sustained force generation: how are these $Ca^{2+}$ signals integrated and transduced at the level of the contractile apparatus? It is evident from the study of genetically hyper- and hyporesponsive mice that $Ca^{2+}$ waves of similar frequency can result in very different contractile responses [160], and comparisons of agonist-evoked constriction of airways *versus* pulmonary vasculature reveal very different frequency-response relationships [21, 166–168]. Thus, it appears that the $Ca^{2+}$-integrative ability of the contractile apparatus in smooth muscle can vary considerably. However, the details of this frequency-sensitive integration in $Ca^{2+}$-dependent contraction is lacking. One hypothesis relates this integration to the activity of MLCP. MLCK activity is directly proportional to $[Ca^{2+}]_i$, whereas MLCP activity is increased by accumulation of its substrate: phosphorylated myosin. As such, sustained contraction would require continuous stimulation of MLCK activity, and the net level of tone would be determined by the relative levels of MLCK and MLCP activities: in other words, the stimulus to contract would be encoded within the absolute magnitude of a tonic change in $[Ca^{2+}]_i$ (fig. 6a). Such a mechanism requiring continuous myosin phosphorylation/dephosphorylation and $Ca^{2+}$ release/uptake would be very expensive energetically. Instead, if MLCP were briefly but reversibly suppressed by $Ca^{2+}$, a $Ca^{2+}$ wave would be sufficient to stimulate MLCK and evoke a contraction that would eventually resolve (be reversed by MLCP) unless/until another $Ca^{2+}$ wave were triggered to reinvigorate MLCK and keep MLCP in check: in other words, net myosin phosphorylation and excitation could be encoded within the frequency of $Ca^{2+}$ spiking (fig. 6c) in a much more economical fashion. Other possible targets that might be regulated by $Ca^{2+}$ wave frequency include MLCK, RhoA or PKC, all of which can regulate the activity of the contractile apparatus.

### *Superficial buffer barrier*

Electron microscopy of vascular smooth muscle cells shows the SR to form sheets around the internal periphery of the cell [169], thereby dividing the cytosol into two spaces in which $[Ca^{2+}]_i$ could be regulated independently. Ion channels and plasmalemmal-associated enzymes would sense changes in $[Ca^{2+}]_i$ in the peripheral space immediately underneath the plasmalemma, while the contractile apparatus would sense $[Ca^{2+}]_i$ in the deep cytosolic space underneath the SR. Another recent study suggests that the same anatomical arrangement can be found in ASM [170]. Such an arrangement has important physiological consequences, as it may allow the cell to effectively dissociate the influences of $[Ca^{2+}]_i$ on mechanical and electrical activities. The current authors have also hypothesised that RyR may be preferentially located on the subplasmalemmal face of the SR and play an important role in directing $Ca^{2+}$ from an overloaded SR towards $Ca^{2+}$-extrusion mechanisms on the plasmalemma (*e.g.* PMCA; NCX), whereas $IP_3$ receptors play the primary role in EC coupling. Others have provided functional evidence for this. One group described the differential distribution of RyR subtypes at the periphery and central regions of ASM cells [28]. An earlier study showed the β-agonist isoproterenol to simultaneously decrease $[Ca^{2+}]_i$ in the deep cytosol while elevating $[Ca^{2+}]_i$ in the periphery of the cell [171]: presumably, the goal of these changes is to move

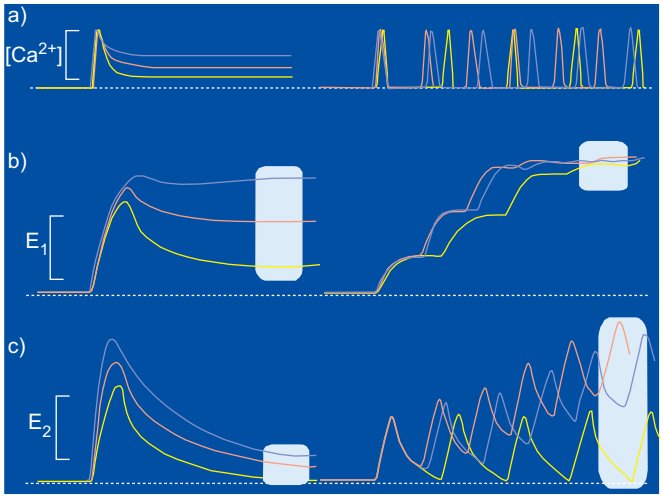


**FIGURE 6.** The amplitude and frequency modulation (AM and FM, respectively) of $Ca^{2+}$-signalling. a) Repetitive $Ca^{2+}$ spikes can trigger enzymes (or ion channels) with different $Ca^{2+}$-sensing properties. b) One hypothetical enzyme ($E_1$) may increase its activity with each elevation of $[Ca^{2+}]$ and not deactivate quickly (left): as such, its activity will progressively increase with each spike and become maximal after a few waves have passed, irrespective of frequency (right). c) Another enzyme ($E_2$) might be more strictly dependent upon $[Ca^{2+}]$, activating quickly but then falling back to baseline as soon as the $Ca^{2+}$-wave passes (left); as such, summation is seen with increasing $Ca^{2+}$-wave frequency (right). Enzymes such as $E_1$ best decode information within the magnitudes of the $Ca^{2+}$ transients (AM-signalling), while those such as $E_2$ best decode spike frequency (FM-signalling).

$Ca^{2+}$ away from the contractile apparatus toward the membrane for subsequent extrusion out of the cell. This close apposition of the SR and plasmalemmal membranes, with juxtaposition of RyR on the SR and various $Ca^{2+}$-handling entities on the plasmalemma (PMCA; NCX), is a *sine qua non* for a peculiar phenomenon described in the next section in which $Ca^{2+}$ flows in the opposite direction, from the extracellular space directly into the SR, bypassing SERCA.

The division of the cytosol into two compartments may not only be relevant to $Ca^{2+}$ signalling. A recent study of Rho/ROCK-signalling in ASM found two distinct pools of Rho, one near the membrane and one in the deep cytosol, with different time-courses of activation following agonist stimulation and different roles in regulation of myosin light chain phosphorylation [172].

### *Transition from pharmacomechanical coupling to electromechanical coupling*

Under normal physiological conditions, agonist-evoked contractions in ASM are sustained and largely insensitive to classical $Ca^{2+}$-channel blockers. However, many groups have shown that under conditions in which handling of $Ca^{2+}$ by the SR is disrupted, those responses become acutely dependent upon voltage-dependent $Ca^{2+}$-influx. For example, following functional depletion of the SR using CPA, cholinergic stimulation of ASM leads to substantial phasic contractile responses which are precipitously abrogated by subsequent exposure to dihydropyridine blockers of L-type $Ca^{2+}$ channels [46, 173–176]; in this respect ASM acts much like vascular and gastrointestinal smooth muscle preparations. Similar observations are

made in ASM tissues challenged with cholinergic agonists in the presence of ryanodine [177–179]. Although the conditions surrounding these responses are totally artificial, such experiments may reveal something important and fundamental about EC coupling in ASM.

Data from several groups, including that of the current authors, suggest a direct coupling between L-type $Ca^{2+}$ channels and the SR, allowing $Ca^{2+}$ to move directly from the extracellular space into the SR without the involvement of SERCA activity. This mechanism was first proposed on the basis of mechanical data (contractions evoked by repeated cholinergic stimulation) as an indirect measure of $Ca^{2+}$ handling [45–48]. More compelling, though, was the monitoring of $Ca^{2+}$ handling using membrane $Cl^-$ currents, which are directly $Ca^{2+}$ dependent (in contrast to mechanical responses, which are quite heterogeneous with respect to $Ca^{2+}$ dependence): using this approach, the present authors demonstrated SR depletion (reflected in disappearance of the $Cl^-$ currents) and then refilling using voltage pulses (reflected in reappearance of those currents) in voltage-clamped TSM pretreated with CPA [9]. There have been innumerable studies of $Ca^{2+}$ uptake in a wide variety of cell types: all the evidence suggests SERCA is the only type of $Ca^{2+}$ pump on the SR, and no study has identified a SERCA that is insensitive to CPA. As such, refilling in the presence of CPA implies that voltage-dependent $Ca^{2+}$ influx is somehow directed into the SR, but not necessarily *via* SERCA. Elsewhere, a model has been proposed which describes one such alternative refilling pathway [180–185]. Briefly, agonist-induced depletion of the internal store triggers activation of protein tyrosine kinases (PTK), Ras and reorganisation of the cytoskeleton in such a way as to directly couple $IP_3$ receptors on the SR with $Ca^{2+}$ channels on the plasmalemma. Several observations made in ASM are consistent with such a mechanism: 1) spasmogenic stimulation of ASM is accompanied by activation of PTKs [186, 187] and Ras/Rho [188–191], as well as cytoskeletal rearrangement [189, 191, 192]; 2) inhibition of PTK compromises SR refilling [193]; 3) ASM depleted of FAK, which regulates cytoskeleton stability, shows marked suppression of cholinergic $Ca^{2+}$ transients and contractions as well as changes in voltage-dependent $Ca^{2+}$ channel function, without any disruptive changes in the contractile apparatus *per se* when assessed by addition of $Ca^{2+}$ to permeabilised muscle strips [194]; 4) in the rat ASM, RyR1 on the SR co-localise with voltage-dependent $Ca^{2+}$ channels on the plasmalemma [27]. However, the possible role for this novel SR refilling pathway has not yet been tested in ASM: the use of inhibitors of cytoskeletal organisation (*e.g.* cytochalasin D, jasplakinolide, colchicine and vinblastine) would be invaluable in this respect.

It is still quite unclear how $Ca^{2+}$ enters the SR, if not *via* SERCA. Although SERCA appears to be the only pathway for active uptake into the SR, this does not mean it is the only possible route for $Ca^{2+}$ entry. Under normal physiological conditions, $IP_3$ receptors and RyRs allow $Ca^{2+}$ to leave the SR: however, this movement is entirely passive, with $Ca^{2+}$ moving down its concentration gradient. It is conceivable, then, that if the driving force on $Ca^{2+}$ were reversed, these channels could allow $Ca^{2+}$ back into the SR: detailed electrophysiological studies show these channels do not exhibit current rectification [195, 196]. $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels, TRP channels and/or reverse-mode NCX could allow $[Ca^{2+}]_i$ to accumulate within the ''superficial buffer barrier'' formed by the close apposition of the plasmalemmal and SR membranes: as such, under conditions in which the SR were depleted (*e.g.* by agonist stimulation or by CPA), opening of those $Ca^{2+}$-permeable channels on the SR would allow refilling. Given that $Ca^{2+}$ release through RyR and $IP_3$ receptors is inhibited by $Ca^{2+}$ at micromolar concentrations *via* a negative feedback mechanism [24, 25] it might be reasonable to assume that retrograde flux back into the SR might likewise be susceptible to higher $[Ca^{2+}]$ in the subplasmalemmal space. However, fluorimetric measurements of $[Ca^{2+}]$ during agonist stimulation show that, while the initial $Ca^{2+}$ spike may rise into the micromolar range, the sustained (or plateau) portion is well below that level. Also, it is possible that the cytoskeletal rearrangements which bring the plasmalemmal and SR-associated ion channels into close proximity are accompanied by (or cause) changes in the properties of the RyR and/or $IP_3$-receptors (*e.g.* obstruction of the $Ca^{2+}$ binding site which mediates channel inactivation).

$Ca^{2+}$ which enters the depleted SR in this way could continue down its electrochemical gradient out of the other side of the SR and contribute to contraction. The contractions evoked under these conditions would necessarily be completely sensitive to L-type $Ca^{2+}$-channel blockers, consistent with the otherwise paradoxical observations summarised at the beginning of this section.

## UNRESOLVED QUESTIONS: DIRECTIONS FOR FUTURE RESEARCH

The exact distribution of the various ionic channels, pumps and exchangers needs to be ascertained with a high degree of precision. For example, are RyR and $IP_3$ receptors uniformly distributed across the SR, or are they clustered heterogeneously in ways which have important functional implications? That is, are these SR-associated entities brought into close proximity with others on the plasmalemma, such as $Ca^{2+}$-channels, NCX and PMCA (as demanded by the models proposed in the Superficial buffer barrier and Transition from pharmacomechanical coupling to electromechanical coupling sections)? Are NSCCs co-localised with NCX (as implied in the NCX and $Ca^{2+}$ store refilling section)? Do VGCCs or $Ca^{2+}$-dependent $Cl^-$ channels interact with Rho (see Regulation of Rho/ROCK by $Ca^{2+}$, $Cl^-$ and $V_m$ section)?

The mechanisms underlying discharge and refilling of the internal $Ca^{2+}$ pool need to be better understood. What molecular entities are involved in producing the periodicity of the $Ca^{2+}$ waves, and how are these waves transduced by the contractile apparatus? What might be the relationship between electrical slow waves and $Ca^{2+}$ waves? The former would be expected to influence the driving forces on the cations which permeate NSCCs and the ''window current'' of the VGCCs, which in turn would influence the driving forces on NCX (as would the slow waves themselves); is there then crosstalk between the electrical slow waves and $Ca^{2+}$ waves? Likewise, how might $Cl^-$ currents contribute to emptying/refilling of the $Ca^{2+}$ store? What refilling pathways are operative other than SERCA: can there be reverse flow through RyR and/or $IP_3$-receptors?

Another important area for further investigation is the regulation of Rho/ROCK by $Ca^{2+}$, $Cl^-$ or Vm. Does that enzyme interact with certain ion channels at the membrane? Is there crosstalk between Rho/ROCK activities and the $Ca^{2+}$ waves?

The molecular identities of the plasmalemmal and SR $Cl^-$ and NSCC channels remain unknown. This is in stark contrast to our relatively specific knowledge of the subtypes of voltage-dependent $K^+$ and $Ca^{2+}$ channels in ASM [52, 53]. Is the $Cl^-$ conductance a member of the CLCA family [74, 197] or a bestrophin [73]? Which of the many TRP proteins make up the NSCCs in ASM?

Much of the work summarised in the present review was obtained using tracheal preparations, even though there is a growing body of literature attesting to the important differences between tracheal and bronchial smooth muscle. These express different ion channels [82, 198] and appear to rely differently upon various EC coupling mechanisms [175]. This is an urgent matter, since the bronchi play a far more important role than the trachea in determining resistance to airflow, and are the locus of inflammation and other functional/structural changes associated with asthma. Thus, it will be crucial to examine these mechanisms in bronchial preparations, and to rely less heavily on tracheal ones. Likewise, there are far too many examples of species-related differences to list here: this fact demands that there be a greater emphasis on human-derived preparations rather than those from other animals. Cultured human cells are not the best answer to this problem, given the many examples of phenotype transitions which occur in those preparations.

## CONCLUSION

Perhaps the most fundamental conclusion that can be drawn is that a simplistic view of airway smooth muscle can no longer be held, *i.e.* that airway smooth muscle has: relatively uniform cytosolic ionic concentrations; homogeneous distributions of ion channels and pumps; simple fluxes of $Ca^{2+}$; and smooth transitions from the resting state to the excited state. The more that is known about ionic mechanisms in airway smooth muscle, the more these are found to be exceedingly complex: nothing is static, very little is homogeneous. There are temporal and spatial heterogeneities in the intracellular concentrations of $Ca^{2+}$, $Na^+$ and $Cl^-$, and perhaps also for other ions; even enzyme activities (*e.g.* Rho, Rho-associated kinase, protein kinase C, protein kinase A, adenylate and guanylate cyclases) are now found to exhibit marked spatial and temporal heterogeneities. There are oscillations in membrane potential and in the intracellular $Ca^{2+}$ concentration. Not only can the $Na^+/Ca^{2+}$ exchanger operate normally to reduce the intracellular $Ca^{2+}$ concentration, but it can also operate in the reverse mode to bring $Ca^{2+}$ into the cell. Relaxants can simultaneously stimulate uptake of $Ca^{2+}$ from the deep cytosol (*via* sarco-endoplasmic $Ca^{2+}$ adenosine triphosphatase) and $Ca^{2+}$ release into the subplasmalemmal space (*via* ryanodine receptor) for subsequent extrusion out of the cell (*via* plasma membrane $Ca^{2+}$ adenosine triphosphatase). Perhaps more surprisingly, $Ca^{2+}$ may also flow backwards through that same pathway, entering through various inward conductances (voltage-dependent $Ca^{2+}$ channels, nonselective cation channels, $Na^+/Ca^{2+}$ exchanger) to accumulate in the subplasmalemmal space and then enter the sarcoplasmic reticulum (through ryanodine receptor), only to be re-released towards the contractile apparatus. Electrophysiologists are often focusing on the ''wrong'' end of the range of membrane potential: the important physiological events are not occurring at the positive potentials at which $Ca^{2+}$ channels are maximally activated, nor at 0 mV at which we typically artificially set the equilibrium potential of $Cl^-$ and at which nonselective cation channels are obviated, but instead at $\sim$ -40 mV at which the physiological equilibrium potential of $Cl^-$, inward nonselective cation channels, slow waves, window current and reverse-mode $Na^+/Ca^{2+}$ exchanger are found (fig. 2).

## REFERENCES


1 Janssen LJ. Ionic mechanisms and $Ca^{2+}$ regulation in airway smooth muscle contraction: do the data contradict dogma? *Am J Physiol Lung Cell Mol Physiol* 2002; 282: L1161–L1178.
2 Huxley AF. Cross-bridge action: present views, prospects, and unknowns. *J Biomech* 2000; 33: 1189–1195.
3 Somlyo AP, Somlyo AV. $Ca^{2+}$ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 2003; 83: 1325–1358.
4 Tang DD, Gunst SJ. Selected contribution: roles of focal adhesion kinase and paxillin in the mechanosensitive regulation of myosin phosphorylation in smooth muscle. *J Appl Physiol* 2001; 91: 1452–1459.
5 Gunst SJ, Wu MF. Selected contribution: plasticity of airway smooth muscle stiffness and extensibility: role of length-adaptive mechanisms. *J Appl Physiol* 2001; 90: 741–749.
6 Roux E, Marhl M. Role of sarcoplasmic reticulum and mitochondria in $Ca^{2+}$ removal in airway myocytes. *Biophys J* 2004; 86: 2583–2595.
7 Sanders KM. Invited review: mechanisms of calcium handling in smooth muscles. *J Appl Physiol* 2001; 91: 1438–1449.
8 Dai J, Kuo KH, Leo JM, van Breemen C, Lee CH. Rearrangement of the close contact between the mitochondria and the sarcoplasmic reticulum in airway smooth muscle. *Cell Calcium* 2005; 37: 333–340.
9 Janssen LJ, Sims SM. Emptying and refilling of Ca2+ store in tracheal myocytes as indicated by ACh-evoked currents and contraction. *Am J Physiol* 1993; 265: C877–C886.
10 Janssen LJ, Betti PA, Netherton SJ, Walters DK. Superficial buffer barrier and preferentially directed release of $Ca^{2+}$ in canine airway smooth muscle. *Am J Physiol* 1999; 276: L744–L753.
11 Janssen LJ, Sims SM. Acetylcholine activates non-selective cation and chloride conductances in canine and guinea-pig tracheal myocytes. *J Physiol* 1992; 453: 197–218.
12 Janssen LJ, Sims SM. Histamine activates Cl- and K+ currents in guinea-pig tracheal myocytes: convergence with muscarinic signalling pathway. *J Physiol* 1993; 465: 661–677.
13 Janssen LJ, Sims SM. Spontaneous transient inward currents and rhythmicity in canine and guinea-pig tracheal smooth muscle cells. *Pflugers Arch* 1994; 427: 473–480.

14 Janssen LJ, Sims SM. $Ca^{2+}$-dependent $Cl^-$ current in canine tracheal smooth muscle cells. *Am J Physiol* 1995; 269: C163–C169.
15 ZhuGe R, Sims SM, Tuft RA, Fogarty KE, Walsh JV Jr. $Ca^{2+}$ sparks activate $K^+$ and $Cl^-$ channels, resulting in spontaneous transient currents in guinea-pig tracheal myocytes. *J Physiol* 1998; 513: 711–718.
16 Hirota S, Pertens E, Janssen LJ. The reverse mode of the $Na^+/Ca^{2+}$ exchanger provides a source of $Ca^{2+}$ for store refilling following agonist-induced $Ca^{2+}$ mobilization. *Am J Physiol Lung Cell Mol Physiol* 2007; 292: L438–L447.
17 Dai JM, Kuo KH, Leo JM, van Breemen C, Lee CH. Mechanism of ACh-induced asynchronous calcium waves and tonic contraction in porcine tracheal muscle bundle. *Am J Physiol Lung Cell Mol Physiol* 2006; 290: L459–L469.
18 Kawanishi M, Baba K, Tomita T. Effects of Na removal and readmission on the mechanical response in the guinea-pig tracheal smooth muscle. *Jpn J Physiol* 1984; 34: 127–139.
19 Janssen LJ, Walters DK, Wattie J. Regulation of $[Ca^{2+}]_i$ in canine airway smooth muscle by $Ca^{2+}$-ATPase and $Na^+/Ca^{2+}$ exchange mechanisms. *Am J Physiol* 1997; 273: L322–L330.
20 Yang C, Tsao HL, Chiu CT, Fan LW, Yu SM. Regulation of 5-hydroxytryptamine-induced calcium mobilization by CAMP-elevating agents in cultured canine tracheal smooth muscle cells. *Pflugers Arch* 1996; 432: 708–716.
21 Bai Y, Sanderson MJ. Airway smooth muscle relaxation results from a reduction in the frequency of $Ca^{2+}$ oscillations induced by a CAMP-mediated inhibition of the IP3 receptor. *Respir Res* 2006; 7: 34.
22 Wang YX, Fleischmann BK, Kotlikoff MI. M2 receptor activation of nonselective cation channels in smooth muscle cells: calcium and $Gi/G_o$ requirements. *Am J Physiol* 1997; 273: C500–C508.
23 Howard S, Chan-Yeung M, Martin L, Phaneuf S, Salari H. Polyphosphoinositide hydrolysis and protein kinase C activation in guinea pig tracheal smooth muscle cells in culture by leukotriene D4 involve a pertussis toxin sensitive G-protein. *Eur J Pharmacol* 1992; 227: 123–129.
24 Parker I, Ivorra I. Confocal microfluorimetry of $Ca^{2+}$ signals evoked in *Xenopus* oocytes by photoreleased inositol trisphosphate. *J Physiol* 1993; 461: 133–165.
25 Parker I, Ivorra I. Inhibition by $Ca^{2+}$ of inositol trisphosphate-mediated $Ca^{2+}$ liberation: a possible mechanism for oscillatory release of $Ca^{2+}$. *Proc Natl Acad Sci USA* 1990; 87: 260–264.
26 Hyvelin JM, Martin C, Roux E, Marthan R, Savineau JP. Human isolated bronchial smooth muscle contains functional ryanodine/caffeine-sensitive Ca-release channels. *Am J Respir Crit Care Med* 2000; 162: 687–694.
27 Du W, McMahon TJ, Zhang ZS, Stiber JA, Meissner G, Eu JP. Excitation-contraction coupling in airway smooth muscle. *J Biol Chem* 2006; 281: 30143–30151.
28 Du W, Stiber JA, Rosenberg PB, Meissner G, Eu JP. Ryanodine receptors in muscarinic receptor-mediated bronchoconstriction. *J Biol Chem* 2005; 280: 26287–26294.
29 Kuemmerle JF, Makhlouf GM. Agonist-stimulated cyclic ADP ribose. Endogenous modulator of $Ca^{2+}$-induced $Ca^{2+}$ release in intestinal longitudinal muscle. *J Biol Chem* 1995; 270: 25488–25494.
30 Sitsapesan R, McGarry SJ, Williams AJ. Cyclic ADP-ribose, the ryanodine receptor and $Ca^{2+}$ release. *Trends Pharmacol Sci* 1995; 16: 386–391.
31 Wang YX, Zheng YM, Mei QB, *et al.* FKBP12.6 and CADPR regulation of $Ca^{2+}$ release in smooth muscle cells. *Am J Physiol Cell Physiol* 2004; 286: C538–C546.
32 Deshpande DA, Walseth TF, Panettieri RA, Kannan MS. CD38/cyclic ADP-ribose-mediated Ca2+ signaling contributes to airway smooth muscle hyper-responsiveness. *FASEB J* 2003; 17: 452–454.
33 Prakash YS, Kannan MS, Walseth TF, Sieck GC. Role of cyclic ADP-ribose in the regulation of $[Ca^{2+}]_i$ in porcine tracheal smooth muscle. *Am J Physiol* 1998; 274: C1653–C1660.
34 Coronado R, Morrissette J, Sukhareva M, Vaughan DM. Structure and function of ryanodine receptors. *Am J Physiol* 1994; 266: C1485–C1504.
35 Meissner G. Molecular regulation of cardiac ryanodine receptor ion channel. *Cell Calcium* 2004; 35: 621–628.
36 Xu L, Tripathy A, Pasek DA, Meissner G. Potential for pharmacology of ryanodine receptor/calcium release channels. *Ann N Y Acad Sci* 1998; 853: 130–148.
37 Nelson MT, Patlak JB, Worley JF, Standen NB. Calcium channels, potassium channels, and voltage dependence of arterial smooth muscle tone. *Am J Physiol* 1990; 259: C3–C18.
38 Bai Y, Sanderson MJ. Modulation of the $Ca^{2+}$ sensitivity of airway smooth muscle cells in murine lung slices. *Am J Physiol Lung Cell Mol Physiol* 2006; 291: L208–L221.
39 Bergner A, Sanderson MJ. Acetylcholine-induced calcium signaling and contraction of airway smooth muscle cells in lung slices. *J Gen Physiol* 2002; 119: 187–198.
40 Gerthoffer WT, Murphey KA, Khoyi MA. Inhibition of tracheal smooth muscle contraction and myosin phosphorylation by ryanodine. *J Pharmacol Exp Ther* 1988; 246: 585–590.
41 Ethier MF, Madison JM. IL-4 inhibits calcium transients in bovine trachealis cells by a ryanodine receptor-dependent mechanism. *FASEB J* 2006; 20: 154–156.
42 Farley JM, Miles PR. Role of depolarization in acetylcholine-induced contractions of dog trachealis muscle. *J Pharmacol Exp Ther* 1977; 201: 199–205.
43 Farley JM, Miles PR. The sources of calcium for acetylcholine-induced contractions of dog tracheal smooth muscle. *J Pharmacol Exp Ther* 1978; 207: 340–346.
44 Coburn RF, Yamaguchi T. Membrane potential-dependent and -independent tension in the canine tracheal muscle. *J Pharmacol Exp Ther* 1977; 201: 276–284.
45 Bourreau JP, Abela AP, Kwan CY, Daniel EE. Acetylcholine $Ca^{2+}$ stores refilling directly involves a dihydropyridine-sensitive channel in dog trachea. *Am J Physiol* 1991; 261: C497–C505.
46 Bourreau JP. Cross talk between plasma membrane and sarcoplasmic reticulum in canine airway smooth muscle. *Biol Signals* 1993; 2: 272–283.
47 Bourreau JP, Kwan CY, Daniel EE. Distinct pathways to refill ACh-sensitive internal $Ca^{2+}$ stores in canine airway smooth muscle. *Am J Physiol* 1993; 265: C28–C35.

48 Qian Y, Bourreau JP. Two distinct pathways for refilling $Ca^{2+}$ stores in permeabilized bovine trachealis muscle. *Life Sci* 1999; 64: 2049–2059.
49 Aickin CC. Chloride transport across the sarcolemma of vertebrate smooth and skeletal muscle. *In*: Alvarez-Leefmans FJ, Russell JM, eds. Chloride Channels and Carriers in Nerve, Muscle, and Glial Cells. New York, Plenum Press, 1990; pp. 209–249.
50 Aickin CC, Vermue NA. Microelectrode measurement of intracellular chloride activity in smooth muscle cells of guinea-pig ureter. *Pflugers Arch* 1983; 397: 25–28.
51 Janssen LJ, Daniel EE. Depolarizing agents induce oscillations in canine bronchial smooth muscle membrane potential: possible mechanisms. *J Pharmacol Exp Ther* 1991; 259: 110–117.
52 Adda S, Fleischmann BK, Freedman BD, Yu M, Hay DW, Kotlikoff MI. Expression and function of voltage-dependent potassium channel genes in human airway smooth muscle. *J Biol Chem* 1996; 271: 13239–13243.
53 Boyle JP, Tomasic M, Kotlikoff MI. Delayed rectifier potassium channels in canine and porcine airway smooth muscle cells. *J Physiol* 1992; 447: 329–350.
54 Fleischmann BK, Washabau RJ, Kotlikoff MI. Control of resting membrane potential by delayed rectifier potassium currents in ferret airway smooth muscle cells. *J Physiol* 1993; 469: 625–638.
55 Savaria D, Lanoue C, Cadieux A, Rousseau E. Large conducting potassium channel reconstituted from airway smooth muscle. *Am J Physiol* 1992; 262: L327–L336.
56 Kume H, Graziano MP, Kotlikoff MI. Stimulatory and inhibitory regulation of calcium-activated potassium channels by guanine nucleotide-binding proteins. *Proc Natl Acad Sci USA* 1992; 89: 11051–11055.
57 Miura M, Belvisi MG, Stretton CD, Yacoub MH, Barnes PJ. Role of potassium channels in bronchodilator responses in human airways. *Am Rev Respir Dis* 1992; 146: 132–136.
58 Murray MA, Berry JL, Cook SJ, Foster RW, Green KA, Small RC. Guinea-pig isolated trachealis: the effects of charybdotoxin on mechanical activity, membrane potential changes and the activity of plasmalemmal $K^+$-channels. *Br J Pharmacol* 1991; 103: 1814–1818.
59 Liu X, Farley JM. Acetylcholine-induced $Ca^{2+}$-dependent chloride current oscillations are mediated by inositol 1,4,5-trisphosphate in tracheal myocytes. *J Pharmacol Exp Ther* 1996; 277: 796–804.
60 Liu X, Farley JM. Acetylcholine-induced chloride current oscillations in swine tracheal smooth muscle cells. *J Pharmacol Exp Ther* 1996; 276: 178–186.
61 Allen SL, Beech DJ, Foster RW, Morgan GP, Small RC. Electrophysiological and other aspects of the relaxant action of isoprenaline in guinea-pig isolated trachealis. *Br J Pharmacol* 1985; 86: 843–854.
62 Hiramatsu T, Kume H, Kotlikoff MI, Takagi K. Role of calcium-activated potassium channels in the relaxation of tracheal smooth muscles by forskolin. *Clin Exp Pharmacol Physiol* 1994; 21: 367–375.
63 Jones TR, Charette L, Garcia ML, Kaczorowski GJ. Selective inhibition of relaxation of guinea-pig trachea by charybdotoxin, a potent $Ca^{2+}$-activated $K^+$ channel inhibitor. *J Pharmacol Exp Ther* 1990; 255: 697–706.
64 Daniel EE, Jury J, Bourreau JP, Jager L. Chloride and depolarization by acetylcholine in canine airway smooth muscle. *Can J Physiol Pharmacol* 1993; 71: 284–292.
65 Helli PB, Pertens E, Janssen LJ. Cyclopiazonic acid activates a $Ca^{2+}$-permeable, nonselective cation conductance in porcine and bovine tracheal smooth muscle. *J Appl Physiol* 2005; 99: 1759–1768.
66 Janssen LJ, Sims SM. Substance P activates $Cl^-$ and $K^+$ conductances in guinea-pig tracheal smooth muscle cells. *Can J Physiol Pharmacol* 1994; 72: 705–710.
67 Janssen LJ. Acetylcholine and caffeine activate $Cl^-$ and suppress $K^+$ conductances in human bronchial smooth muscle. *Am J Physiol* 1996; 270: L772–L781.
68 Janssen LJ, Wattie J. Electrophysiological effects of erythromycin, but lack of mechanical effects, in airway smooth muscle. *Eur J Pharmacol* 2003; 475: 93–97.
69 Wang YX, Kotlikoff MI. Signalling pathway for histamine activation of non-selective cation channels in equine tracheal myocytes. *J Physiol* 2000; 523: 131–138.
70 Kotlikoff MI, Kume H, Tomasic M. Muscarinic regulation of membrane ion channels in airway smooth muscle cells. *Biochem Pharmacol* 1992; 43: 5–10.
71 Kume H, Kotlikoff MI. Muscarinic inhibition of single KCa channels in smooth muscle cells by a pertussis-sensitive G protein. *Am J Physiol* 1991; 261: C1204–C1209.
72 Kotlikoff MI, Wang YX. Calcium release and calcium-activated chloride channels in airway smooth muscle cells. *Am J Respir Crit Care Med* 1998; 158: S109–S114.
73 Qu Z, Wei RW, Mann W, Hartzell HC. Two bestrophins cloned from *Xenopus laevis* oocytes express $Ca^{2+}$-activated $Cl^-$ currents. *J Biol Chem* 2003; 278: 49563–49572.
74 Pauli BU, Abdel-Ghany M, Cheng HC, Gruber AD, Archibald HA, Elble RC. Molecular characteristics and functional diversity of CLCA family members. *Clin Exp Pharmacol Physiol* 2000; 27: 901–905.
75 Fleischmann BK, Wang YX, Kotlikoff MI. Muscarinic activation and calcium permeation of nonselective cation currents in airway myocytes. *Am J Physiol* 1997; 272: C341–C349.
76 Wang YX, Fleischmann BK, Kotlikoff MI. Modulation of maxi-$K^+$ channels by voltage-dependent $Ca^{2+}$ channels and methacholine in single airway myocytes. *Am J Physiol* 1997; 272: C1151–C1159.
77 Kotlikoff MI. Calcium currents in isolated canine airway smooth muscle cells. *Am J Physiol* 1988; 254: C793–C801.
78 Welling A, Felbel J, Peper K, Hofmann F. Hormonal regulation of calcium current in freshly isolated airway smooth muscle cells. *Am J Physiol* 1992; 262: L351–L359.
79 Fleischmann BK, Wang YX, Pring M, Kotlikoff MI. Voltage-dependent calcium currents and cytosolic calcium in equine airway myocytes. *J Physiol* 1996; 492: 347–358.
80 Hisada T, Kurachi Y, Sugimoto T. Properties of membrane currents in isolated smooth muscle cells from guinea-pig trachea. *Pflugers Arch* 1990; 416: 151–161.
81 Marthan R, Martin C, Amedee T, Mironneau J. Calcium channel currents in isolated smooth muscle cells from human bronchus. *J Appl Physiol* 1989; 66: 1706–1714.
82 Janssen LJ. T-type and L-type $Ca^{2+}$ currents in canine bronchial smooth muscle: characterization and physiological roles. *Am J Physiol* 1997; 272: C1757–C1765.

**83** Fleischmann BK, Murray RK, Kotlikoff MI. Voltage window for sustained elevation of cytosolic calcium in smooth muscle cells. *Proc Natl Acad Sci USA* 1994; 91: 11914–11918.

**84** Tomasic M, Boyle JP, Worley JF, Kotlikoff MI. Contractile agonists activate voltage-dependent calcium channels in airway smooth muscle cells. *Am J Physiol* 1992; 263: C106–C113.

**85** Honda K, Tomita T. Electrical activity in isolated human tracheal muscle. *Jpn J Physiol* 1987; 37: 333–336.

**86** Honda K, Satake T, Takagi K, Tomita T. Effects of relaxants on electrical and mechanical activities in the guinea-pig tracheal muscle. *Br J Pharmacol* 1986; 87: 665–671.

**87** Boyle JP, Davies JM, Foster RW, Good DM, Kennedy I, Small RC. Spasmogen action in guinea-pig isolated trachealis: involvement of membrane $K^+$-channels and the consequences of $K^+$-channel blockade. *Br J Pharmacol* 1988; 93: 319–330.

**88** Small RC, Foster RW, Boyle JP, Davies JM. The relationship between electrophysiological and mechanical events in airways smooth muscle. *Prog Clin Biol Res* 1988; 263: 205–217.

**89** Davis C, Kannan MS, Jones TR, Daniel EE. Control of human airway smooth muscle: *in vitro* studies. *J Appl Physiol* 1982; 53: 1080–1087.

**90** Brink C, Grimaud C, Guillot C, Orehek J. The interaction between indomethacin and contractile agents on human isolated airway muscle. *Br J Pharmacol* 1980; 69: 383–388.

**91** Ito M, Baba K, Takagi K, Satake T, Tomita T. Some properties of calcium-induced contraction in the isolated human and guinea-pig tracheal smooth muscle. *Respir Physiol* 1985; 59: 143–153.

**92** Janssen LJ, Daniel EE. Pre- and postjunctional muscarinic receptors in canine bronchi. *Am J Physiol* 1990; 259: L304–L314.

**93** Janssen LJ, Daniel EE. Characterization of the prejunctional beta adrenoceptors in canine bronchial smooth muscle. *J Pharmacol Exp Ther* 1990; 254: 741–749.

**94** Ito Y, Takeda K. Non-adrenergic inhibitory nerves and putative transmitters in the smooth muscle of cat trachea. *J Physiol* 1982; 330: 497–511.

**95** Murlas CG, Doupnik CA. Electromechanical coupling of ferret airway smooth muscle in response to leukotriene C4. *J Appl Physiol* 1989; 66: 2533–2538.

**96** Kirkpatrick CT. Excitation and contraction in bovine tracheal smooth muscle. *J Physiol* 1975; 244: 263–281.

**97** Gill KK, Kroeger EA. Effects of indomethacin on neural and myogenic components in equine airway smooth muscle. *J Pharmacol Exp Ther* 1990; 252: 358–364.

**98** Janssen LJ, Daniel EE. Pre- and postjunctional effects of a thromboxane mimetic in canine bronchi. *Am J Physiol* 1991; 261: L271–L276.

**99** Inoue T, Ito Y. Pre- and post-junctional actions of prostaglandin I2, carbocyclic thromboxane A2 and leukotriene C4 in dog tracheal tissue. *Br J Pharmacol* 1985; 84: 289–298.

**100** Allen SL, Foster RW, Small RC, Towart R. The effects of the dihydropyridine Bay K 8644 in guinea-pig isolated trachealis. *Br J Pharmacol* 1985; 86: 171–180.

**101** Janssen LJ, Hague C, Nana R. Ionic mechanisms underlying electrical slow waves in canine airway smooth muscle. *Am J Physiol* 1998; 275: L516–L523.

**102** Clapham DE, Julius D, Montell C, Schultz G. International Union of Pharmacology. XLIX. Nomenclature and structure-function relationships of transient receptor potential channels. *Pharmacol Rev* 2005; 57: 427–450.

**103** Pedersen SF, Owsianik G, Nilius B. TRP channels: an overview. *Cell Calcium* 2005; 38: 233–252.

**104** Parekh AB, Penner R. Store depletion and calcium influx. *Physiol Rev* 1997; 77: 901–930.

**105** Putney JW Jr. A model for receptor-regulated calcium entry. *Cell Calcium* 1986; 7: 1–12.

**106** Albert AP, Large WA. Store-operated $Ca^{2+}$-permeable non-selective cation channels in smooth muscle cells. *Cell Calcium* 2003; 33: 345–356.

**107** Flemming R, Xu SZ, Beech DJ. Pharmacological profile of store-operated channels in cerebral arteriolar smooth muscle cells. *Br J Pharmacol* 2003; 139: 955–965.

**108** Golovina VA, Platoshyn O, Bailey CL, *et al.* Upregulated TRP and enhanced capacitative $Ca^{2+}$ entry in human pulmonary artery myocytes during proliferation. *Am J Physiol Heart Circ Physiol* 2001; 280: H746–H755.

**109** Ng LC, Gurney AM. Store-operated channels mediate $Ca^{2+}$ influx and contraction in rat pulmonary artery. *Circ Res* 2001; 89: 923–929.

**110** Sweeney M, McDaniel SS, Platoshyn O, *et al.* Role of capacitative $Ca^{2+}$ entry in bronchial contraction and remodeling. *J Appl Physiol* 2002; 92: 1594–1602.

**111** Ong HL, Brereton HM, Harland ML, Barritt GJ. Evidence for the expression of transient receptor potential proteins in guinea pig airway smooth muscle cells. *Respirology* 2003; 8: 23–32.

**112** Ay B, Prakash YS, Pabelick CM, Sieck GC. Store-operated $Ca^{2+}$ entry in porcine airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2004; 286: L909–L917.

**113** Gosling M, Poll C, Li S. TRP channels in airway smooth muscle as therapeutic targets. *Naunyn Schmiedebergs Arch Pharmacol* 2005; 371: 277–284.

**114** Li S, Westwick J, Poll C. Transient receptor potential (TRP) channels as potential drug targets in respiratory disease. *Cell Calcium* 2003; 33: 551–558.

**115** Ong HL, Barritt GJ. Transient receptor potential and other ion channels as pharmaceutical targets in airway smooth muscle cells. *Respirology* 2004; 9: 448–457.

**116** Yamashita T, Kokubun S. Nonselective cationic currents activated by acetylcholine in swine tracheal smooth muscle cells. *Can J Physiol Pharmacol* 1999; 77: 796–805.

**117** Rosker C, Graziani A, Lukas M, *et al.* $Ca^{2+}$ signaling by TRPC3 involves $Na^+$ entry and local coupling to the $Na^+/Ca^{2+}$ exchanger. *J Biol Chem* 2004; 279: 13696–13704.

**118** Murray RK, Kotlikoff MI. Receptor-activated calcium influx in human airway smooth muscle cells. *J Physiol* 1991; 435: 123–144.

**119** Snetkov VA, Hapgood KJ, McVicker CG, Lee TH, Ward JP. Mechanisms of leukotriene $D_4$-induced constriction in human small bronchioles. *Br J Pharmacol* 2001; 133: 243–252.

**120** Gorenne I, Labat C, Gascard JP, Norel X, Nashashibi N, Brink C. Leukotriene $D_4$ contractions in human airways are blocked by SK&F 96365, an inhibitor of

receptor-mediated calcium entry. *J Pharmacol Exp Ther* 1998; 284: 549–552.
**121** Ito S, Kume H, Yamaki K, *et al.* Regulation of capacitative and noncapacitative receptor-operated $Ca^{2+}$ entry by Rho-kinase in tracheal smooth muscle. *Am J Respir Cell Mol Biol* 2002; 26: 491–498.
**122** White TA, Xue A, Chini EN, Thompson M, Sieck GC, Wylam ME. Role of transient receptor potential C3 in TNF-α-enhanced calcium influx in human airway myocytes. *Am J Respir Cell Mol Biol* 2006; 35: 243–251.
**123** Peel SE, Liu B, Hall IP. A key role for STIM1 in store operated calcium channel activation in airway smooth muscle. *Respir Res* 2006; 7: 119.
**124** Bazan-Perkins B, Sanchez-Guerrero E, Carbajal V, Barajas-Lopez C, Montano LM. Sarcoplasmic reticulum $Ca^{2+}$ depletion by caffeine and changes of $[Ca^{2+}]_i$ during refilling in bovine airway smooth muscle cells. *Arch Med Res* 2000; 31: 558–563.
**125** Bazan-Perkins B, Flores-Soto E, Barajas-Lopez C, Montano LM. Role of sarcoplasmic reticulum $Ca^{2+}$ content in $Ca^{2+}$ entry of bovine airway smooth muscle cells. *Naunyn Schmiedebergs Arch Pharmacol* 2003; 368: 277–283.
**126** Montano LM, Bazan-Perkins B. Resting calcium influx in airway smooth muscle. *Can J Physiol Pharmacol* 2005; 83: 717–723.
**127** Ethier MF, Yamaguchi H, Madison JM. Effects of cyclopiazonic acid on cytosolic calcium in bovine airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2001; 281: L126–L133.
**128** Sabbioni S, Barbanti-Brodano G, Croce CM, Negrini M. GOK: a gene at 11p15 involved in rhabdomyosarcoma and rhabdoid tumor development. *Cancer Res* 1997; 57: 4493–4497.
**129** Putney JW Jr. Capacitative calcium entry: sensing the calcium stores. *J Cell Biol* 2005; 169: 381–382.
**130** Soboloff J, Spassova MA, Dziadek MA, Gill DL. Calcium signals mediated by STIM and Orai proteins–a new paradigm in inter-organelle communication. *Biochim Biophys Acta* 2006; 1763: 1161–1168.
**131** Soboloff J, Spassova MA, Tang XD, Hewavitharana T, Xu W, Gill DL. Orai1 and STIM reconstitute store-operated calcium channel function. *J Biol Chem* 2006; 281: 20661–20665.
**132** Roos J, DiGregorio PJ, Yeromin AV, *et al.* STIM1, an essential and conserved component of store-operated $Ca^{2+}$ channel function. *J Cell Biol* 2005; 169: 435–445.
**133** Zhang SL, Yeromin AV, Zhang XH, *et al.* Genome-wide RNAi screen of $Ca^{2+}$ influx identifies genes that regulate $Ca^{2+}$ release-activated $Ca^{2+}$ channel activity. *Proc Natl Acad Sci USA* 2006; 103: 9357–9362.
**134** Zhang SL, Yu Y, Roos J, *et al.* STIM1 Is a $Ca^{2+}$ sensor that activates CRAC channels and migrates from the $Ca^{2+}$ store to the plasma membrane. *Nature* 2005; 437: 902–905.
**135** Takai N, Yamada A, Muraki K, Watanabe M, Imaizumi Y. KB-R7943 reveals possible involvement of $Na^+$-$Ca^{2+}$ exchanger in elevation of intracellular $Ca^{2+}$ in rat carotid arterial myocytes. *J Smooth Muscle Res* 2004; 40: 35–42.
**136** Bradley E, Hollywood MA, Johnston L, *et al.* Contribution of reverse $Na^+$-$Ca^{2+}$ exchange to spontaneous activity in interstitial cells of Cajal in the rabbit urethra. *J Physiol* 2006; 574: 651–661.
**137** Leblanc N, Leung PM. Indirect stimulation of $Ca^{2+}$-activated $Cl^-$ current by $Na^+/Ca^{2+}$ exchange in rabbit portal vein smooth muscle. *Am J Physiol* 1995; 268: H1906–H1917.
**138** Motley ED, Paul RJ, Matlib MA. Role of $Na^+$-$Ca^{2+}$ exchange in the regulation of vascular smooth muscle tension. *Am J Physiol* 1993; 264: H1028–H1040.
**139** Parvez O, Voss AM, de Kok M, Roth-Kleiner M, Belik J. Bronchial muscle peristaltic activity in the fetal rat. *Pediatr Res* 2006; 59: 756–761.
**140** Rosker C, Graziani A, Lukas M, *et al.* $Ca^{2+}$ signaling by TRPC3 involves $Na^+$ entry and local coupling to the $Na^+/Ca^{2+}$ exchanger. *J Biol Chem* 2004; 279: 13696–13704.
**141** Moore ED, Etter EF, Philipson KD, *et al.* Coupling of the $Na^+/Ca^{2+}$ exchanger, $Na^+/K^+$ pump and sarcoplasmic reticulum in smooth muscle. *Nature* 1993; 365: 657–660.
**142** Liu X, Farley JM. Depletion and refilling of acetylcholine- and caffeine-sensitive $Ca^{2+}$ stores in tracheal myocytes. *J Pharmacol Exp Ther* 1996; 277: 789–795.
**143** Fink RH, Stephenson DG. $Ca^{2+}$-movements in muscle modulated by the state of $K^+$-channels in the sarcoplasmic reticulum membranes. *Pflugers Arch* 1987; 409: 374–380.
**144** Pollock NS, Kargacin ME, Kargacin GJ. Chloride channel blockers inhibit $Ca^{2+}$ uptake by the smooth muscle sarcoplasmic reticulum. *Biophys J* 1998; 75: 1759–1766.
**145** Rangachari PK, Triggle CR. Chloride removal and excitation-contraction coupling in guinea pig ileal smooth muscle. *Can J Physiol Pharmacol* 1986; 64: 1521–1527.
**146** Rangachari PK, Triggle CR, Daniel EE. Chloride removal selectively inhibits phasic contractions of guinea pig ileal longitudinal smooth muscle. *Can J Physiol Pharmacol* 1982; 60: 1741–1744.
**147** Jensen BL, Ellekvist P, Skott O. Chloride is essential for contraction of afferent arterioles after agonists and potassium. *Am J Physiol* 1997; 272: F389–F396.
**148** Hirota S, Trimble N, Pertens E, Janssen LJ. Intracellular $Cl^-$ fluxes play a novel role in $Ca^{2+}$ handling in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2006; 290: L1146–L1153.
**149** Janssen LJ, Tazzeo T, Zuo J, Pertens E, Keshavjee S. KCl evokes contraction of airway smooth muscle *via* activation of RhoA and Rho-kinase. *Am J Physiol Lung Cell Mol Physiol* 2004; 287: L852–L858.
**150** Liu C, Zuo J, Pertens E, Helli PB, Janssen LJ. Regulation of Rho/ROCK signaling in airway smooth muscle by membrane potential and $[Ca^{2+}]_i$. *Am J Physiol Lung Cell Mol Physiol* 2005; 289: L574–L582.
**151** Fan RS, Jacamo RO, Jiang X, Sinnett-Smith J, Rozengurt E. G protein-coupled receptor activation rapidly stimulates focal adhesion kinase phosphorylation at Ser-843. Mediation by $Ca^{2+}$, calmodulin, and $Ca^{2+}$/calmodulin-dependent kinase II. *J Biol Chem* 2005; 280: 24212–24220.
**152** Calandrella SO, Barrett KE, Keely SJ. Transactivation of the epidermal growth factor receptor mediates muscarinic stimulation of focal adhesion kinase in intestinal epithelial cells. *J Cell Physiol* 2005; 203: 103–110.
**153** Weber TP, Widger WR, Kohn H. Metal dependency for transcription factor Rho activation. *Biochemistry* 2003; 42: 1652–1659.
**154** Rossman KL, Worthylake DK, Snyder JT, Cheng L, Whitehead IP, Sondek J. Functional analysis of cdc42

residues required for guanine nucleotide exchange. *J Biol Chem* 2002; 277: 50893–50898.
**155** Weber TP, Widger WR, Kohn H. The $Mg^{2+}$ requirements for Rho transcription termination factor: catalysis and bicyclomycin inhibition. *Biochemistry* 2002; 41: 12377–12383.
**156** Zhang B, Zhang Y, Wang Z, Zheng Y. The role of $Mg^{2+}$ cofactor in the guanine nucleotide exchange and GTP hydrolysis reactions of Rho family GTP-binding proteins. *J Biol Chem* 2000; 275: 25299–25307.
**157** Kobrinsky E, Schwartz E, Abernethy DR, Soldatov NM. Voltage-gated mobility of the $Ca^{2+}$ channel cytoplasmic tails and its regulatory role. *J Biol Chem* 2003; 278: 5021–5028.
**158** Soldatov NM. $Ca^{2+}$ channel moving tail: link between $Ca^{2+}$-induced inactivation and $Ca^{2+}$ signal transduction. *Trends Pharmacol Sci* 2003; 24: 167–171.
**159** Higashijima T, Ferguson KM, Sternweis PC. Regulation of hormone-sensitive GTP-dependent regulatory proteins by chloride. *J Biol Chem* 1987; 262: 3597–3602.
**160** Bergner A, Sanderson MJ. Airway contractility and smooth muscle $Ca^{2+}$ signaling in lung slices from different mouse strains. *J Appl Physiol* 2003; 95: 1325–1332.
**161** Kuo KH, Dai J, Seow CY, Lee CH, van Breemen C. Relationship between asynchronous $Ca^{2+}$ waves and force development in intact smooth muscle bundles of the porcine trachea. *Am J Physiol Lung Cell Mol Physiol* 2003; 285: L1345–L1353.
**162** Prakash YS, Kannan MS, Sieck GC. Regulation of intracellular calcium oscillations in porcine tracheal smooth muscle cells. *Am J Physiol* 1997; 272: C966–C975.
**163** Roux E, Noble PJ, Noble D, Marhl M. Modelling of calcium handling in airway myocytes. *Prog Biophys Mol Biol* 2006; 90: 64–87.
**164** Sneyd J, Tsaneva-Atanasova K, Reznikov V, Bai Y, Sanderson MJ, Yule DI. A method for determining the dependence of calcium oscillations on inositol trisphosphate oscillations. *Proc Natl Acad Sci USA* 2006; 103: 1675–1680.
**165** Haberichter T, Roux E, Marhl M, Mazat JP. The influence of different $InsP_3$ receptor isoforms on $Ca^{2+}$ signaling in tracheal smooth muscle cells. *Bioelectrochemistry* 2002; 57: 129–138.
**166** Bai Y, Zhang M, Sanderson MJ. Contractility and $Ca^{2+}$ signaling of smooth muscle cells in different generations of mouse airways. *Am J Respir Cell Mol Biol* 2007; 36: 122–130.
**167** Perez JF, Sanderson MJ. The contraction of smooth muscle cells of intrapulmonary arterioles is determined by the frequency of $Ca^{2+}$ oscillations induced by 5-HT and KCl. *J Gen Physiol* 2005; 125: 555–567.
**168** Perez JF, Sanderson MJ. The frequency of calcium oscillations induced by 5-HT, ACH, and KCl determine the contraction of smooth muscle cells of intrapulmonary bronchioles. *J Gen Physiol* 2005; 125: 535–553.
**169** Nixon GF, Mignery GA, Somlyo AV. Immunogold localization of inositol 1,4,5-trisphosphate receptors and characterization of ultrastructural features of the sarcoplasmic reticulum in phasic and tonic smooth muscle. *J Muscle Res Cell Motil* 1994; 15: 682–700.
**170** Darby PJ, Kwan CY, Daniel EE. Caveolae from canine airway smooth muscle contain the necessary components for a role in $Ca^{2+}$ handling. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L1226–L1235.
**171** Yamaguchi H, Kajita J, Madison JM. Isoproterenol increases peripheral $[Ca^{2+}]_i$ and decreases inner $[Ca^{2+}]_i$ in single airway smooth muscle cells. *Am J Physiol* 1995; 268: C771–C779.
**172** Miyazaki K, Yano T, Schmidt DJ, *et al.* Rho-dependent agonist-induced spatio-temporal change in myosin phosphorylation in smooth muscle cells. *J Biol Chem* 2002; 277: 725–734.
**173** Amoako D, Qian Y, Kwan CY, Bourreau JP. Probing excitation-contraction coupling in trachealis smooth muscle with the mycotoxin cyclopiazonic acid. *Clin Exp Pharmacol Physiol* 1996; 23: 733–737.
**174** Tao L, Huang Y, Bourreau JP. Control of the mode of excitation-contraction coupling by $Ca^{2+}$ stores in bovine trachealis muscle. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L722–L732.
**175** Janssen LJ, Wattie J, Lu-Chao H, Tazzeo T. Muscarinic excitation-contraction coupling mechanisms in tracheal and bronchial smooth muscles. *J Appl Physiol* 2001; 91: 1142–1151.
**176** Montano LM, Barajas-Lopez C, Daniel EE. Canine bronchial sustained contraction in $Ca^{2+}$-free medium: role of intracellular $Ca^{2+}$. *Can J Physiol Pharmacol* 1996; 74: 1236–1248.
**177** Tao L, Huang Y, Bourreau JP. Control of the mode of excitation-contraction coupling by $Ca^{2+}$ stores in bovine trachealis muscle. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L722–L732.
**178** Cortijo J, Sanz CM, Villagrasa V, Morcillo EJ, Small RC. The effects of phorbol 12,13-diacetate on responses of guinea-pig isolated trachea to methylxanthines, isoprenaline and ryanodine. *Br J Pharmacol* 1994; 111: 769–776.
**179** Tao L, Huang Y, Bourreau JP. Control of the mode of excitation-contraction coupling by $Ca^{2+}$ stores in bovine trachealis muscle. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L722–L732.
**180** Rosado JA, Sage SO. The actin cytoskeleton in store-mediated calcium entry. *J Physiol* 2000; 526: 221–229.
**181** Patterson RL, van Rossum DB, Gill DL. Store-operated $Ca^{2+}$ entry: evidence for a secretion-like coupling model. *Cell* 1999; 98: 487–499.
**182** Yao Y, Ferrer-Montiel AV, Montal M, Tsien RY. Activation of store-operated $Ca^{2+}$ current in *Xenopus* oocytes requires SNAP-25 but not a diffusible messenger. *Cell* 1999; 98: 475–485.
**183** Berridge MJ. Capacitative calcium entry. *Biochem J* 1995; 312: 1–11.
**184** Kobayashi S, Gong MC, Somlyo AV, Somlyo AP. $Ca^{2+}$ channel blockers distinguish between G protein-coupled pharmacomechanical $Ca^{2+}$ release and $Ca^{2+}$ sensitization. *Am J Physiol* 1991; 260: C364–C370.
**185** Rosales C, Brown EJ. Calcium channel blockers nifedipine and diltiazem inhibit $Ca^{2+}$ release from intracellular stores in neutrophils. *J Biol Chem* 1992; 267: 1443–1448.
**186** Smith PG, Garcia R, Kogerman L. Mechanical strain increases protein tyrosine phosphorylation in airway smooth muscle cells. *Exp Cell Res* 1998; 239: 353–360.
**187** Tolloczko B, Tao FC, Zacour ME, Martin JG. Tyrosine kinase-dependent calcium signaling in airway smooth

muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2000; 278: L1138–L1145.

**188** Hakonarson H, Grunstein MM. Regulation of second messengers associated with airway smooth muscle contraction and relaxation. *Am J Respir Crit Care Med* 1998; 158: S115–S122.

**189** Hirshman CA, Emala CW. Actin reorganization in airway smooth muscle cells involves $G_q$ and $G_{i-2}$ activation of Rho. *Am J Physiol* 1999; 277: L653–L661.

**190** Emala CW, Liu F, Hirshman CA. $G_i\alpha$ but not $G_q\alpha$ is linked to activation of $p21^{Ras}$ in human airway smooth muscle cells. *Am J Physiol* 1999; 276: L564–L570.

**191** Togashi H, Emala CW, Hall IP, Hirshman CA. Carbachol-induced actin reorganization involves $G_i$ activation of Rho in human airway smooth muscle cells. *Am J Physiol* 1998; 274: L803–L809.

**192** Gunst SJ, Tang DD. The contractile apparatus and mechanical properties of airway smooth muscle. *Eur Respir J* 2000; 15: 600–616.

**193** Madison JM, Ethier MF, Yamaguchi H. Refilling of caffeine-sensitive intracellular calcium stores in bovine airway smooth muscle Cells. *Am J Physiol* 1998; 275: L852–L860.

**194** Tang DD, Gunst SJ. Depletion of focal adhesion kinase by antisense depresses contractile activation of smooth muscle. *Am J Physiol Cell Physiol* 2001; 280: C874–C883.

**195** Gillespie D, Xu L, Wang Y, Meissner G. (De)constructing the ryanodine receptor: modeling ion permeation and selectivity of the calcium release channel. *J Phys Chem B Condens Matter Mater Surf Interfaces Biophys* 2005; 109: 15598–15610.

**196** Uehara A, Fill M, Velez P, Yasukochi M, Imanaga I. Rectification of rabbit cardiac ryanodine receptor current by endogenous polyamines. *Biophys J* 1996; 71: 769–777.

**197** Gandhi R, Elble RC, Gruber AD, *et al.* Molecular and functional characterization of a calcium-sensitive chloride channel from mouse lung. *J Biol Chem* 1998; 273: 32096–32101.

**198** Snetkov VA, Ward JP. Ion currents in smooth muscle cells from human small bronchioles: presence of an inward rectifier $K^+$ current and three types of large conductance $K^+$ channel. *Exp Physiol* 1999; 84: 835–846.