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Cellular and molecular mechanisms regulating vascular tone. Part 1: basic mechanisms controlling cytosolic Ca^{2+} concentration and the Ca^{2+}-dependent regulation of vascular tone

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Abstract
General anesthetics cause hemodynamic instability and alter blood flow to various organs. There is mounting evidence that most general anesthetics, at clinical concentrations, influence a wide variety of cellular and molecular mechanisms regulating the contractile state of vascular smooth muscle cells (i.e., vascular tone). In addition, in current anesthetic practice, various types of vasoactive agents are often used to control vascular reactivity and to sustain tissue blood flow in high-risk surgical patients with impaired vital organ function and/or hemodynamic instability. Understanding the physiological mechanisms involved in the regulation of vascular tone thus would be beneficial for anesthesiologists. This review, in two parts, provides an overview of current knowledge about the cellular and molecular mechanisms regulating vascular tone—i.e., targets for general anesthetics, as well as for vasoactive drugs that are used in intraoperative circulatory management. This first part of the two-part review focuses on basic mechanisms regulating cytosolic Ca^{2+} concentration and the Ca^{2+}-dependent regulation of vascular tone.

Key words Vascular smooth muscle · Vascular endothelium · Intracellular Ca^{2+} concentration · Myofilament Ca^{2+} sensitivity

Introduction

During the perioperative period, anesthesiologists are appropriately concerned with blood flow, maintaining the circulation to sustain tissue viability and function [1]. The blood flow through a vascular bed is determined by the pressure gradient across it and its resistance to flow, and the overall strategy of the circulatory system is to provide all organs with a constant perfusion pressure and to allow each of them to alter its resistance to achieve the desired flow [2]. In other words, the physiological mechanisms that regulate the distribution of cardiac output and maintain tissue blood flow sufficient to fulfill metabolic requirements are intimately involved with the control of vascular tone (i.e., the contractile state of vascular smooth muscle [VSM]). Evidence is accumulating that various types of anesthetics, in clinical concentrations, threaten the functional integrity of a wide variety of cellular and molecular mechanisms regulating vascular tone (see review article [3] for details of general anesthetic actions on various vasoregulatory mechanisms). In addition, it is not uncommon, in current anesthetic management, to utilize various vasoactive agents, with the aim of controlling vascular reactivity, and thereby maintaining hemodynamic stability and organ blood flow. Understanding the physiological mechanisms regulating vascular tone thus would be important for anesthesiologists. This article reviews the basic mechanisms controlling the cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$) in vascular smooth muscle cells (VSMCs), and the Ca^{2+}-dependent regulation of vascular tone.

Role of calcium in the regulation of vascular tone

Changes in the $[Ca^{2+}]c$ are the principal mechanisms that regulate the contractile state of VSMCs. Namely, an increase and a decrease in $[Ca^{2+}]c$ result in VSM contraction and relaxation, respectively [4]. Most contractile stimuli trigger an increase in $[Ca^{2+}]c$, whereupon Ca^{2+} binds to calmodulin (CaM), inducing a conformational change in CaM. In its altered conformation, the $4Ca^{2+}$-CaM complex binds to and activates myosin light-chain kinase (MLCK). The active kinase complex, $4Ca^{2+}$-CaM-MLCK, subsequently phosphorylates myosin at 20-kD myosin regulatory light chain (MLC_{20}) on serine 19, allowing myosin adenosine triphosphatase (ATPase; ATP, adenosine triphosphate) to be activated

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by actin and the muscle to contract as a result of MgATP-dependent cyclic interactions of myosin with actin (Fig. 1) [4–6]. On the other hand, when $[Ca^{2+}]c$ decreases to less than 1 μM, as a result of extrusion from the cell or uptake into the intracellular stores, CaM dissociates from MLCK and thereby inactivates MLCK. Under these conditions, myosin phosphatase, the activity of which is independent of Ca^{2+}, dephosphorylates MLC_{20} and thereby causes relaxation by inactivating actomyosin ATPase (i.e., actin-activated, ATPase activity of myosin); (Fig. 1) [4,7,8].

Previous studies with Ca^{2+}-sensitive indicators have shown that the force-$[Ca^{2+}]c$ ratio or MLC_{20} phosphorylation-$[Ca^{2+}]c$ ratio can be variable during contractile response to receptor agonists, proposing the existence of secondary mechanisms that regulate the Ca^{2+} sensitivity of contractile myofilaments or MLC_{20} phosphorylation [4,9–13]. In addition, previous studies have reported dissociation between force and MLC_{20} phosphorylation levels during the maintenance of contraction, suggesting the existence of a regulatory mechanism(s) that maintains high contractile force at low energy (ATP) cost (i.e., low levels of MLC_{20} phosphorylation) [14,15].

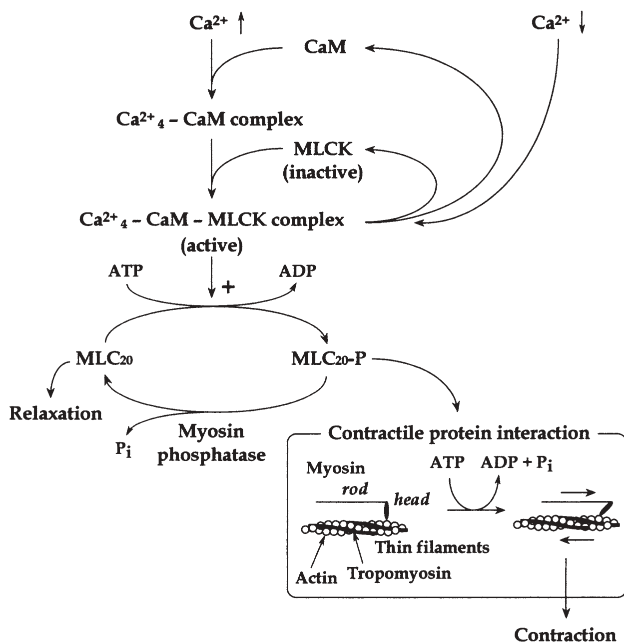

Fig. 1. Role of Ca^{2+} in the regulation of the contractile state of vascular smooth muscle cells (VSMCs). In the Ca^{2+}-calmodulin pathway, increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$) of 1 μM or more lead to contraction, while decreases in $[Ca^{2+}]c$ to 0.1 μM lead to relaxation. Contractile protein interaction: the contractile proteins are packed into thin and thick filaments; the thin filaments are composed of actin, tropomyosin, and specific thin filament-binding proteins (e.g., caldesmon, calponin), while the thick filaments are composed of myosin. When the MLC_{20} is phosphorylated, the heads of myosin molecules extend from the thick filaments to bind to actin (i.e., to form crossbridges). On association with actin, myosin converts chemical energy (i.e., adenosine 5′-triphosphate [*ATP*]) into movement of the heads (that are attached to actin), exerting force on the thin filaments and causing shortening of the VSMCs. *Upward arrows*, increases; *downward arrows*, decreases; *plus sign*, stimulation; *ADP*, adenosine 5′-diphosphate; *CaM*, calmodulin; *MLCK*, myosin light chain kinase; MLC_{20}, 20-kd regulatory light chain of myosin; MLC_{20}-*P*, phosphorylated MLC_{20}; P_i, inorganic phosphate

Intracellular calcium regulation

In the resting state, $[Ca^{2+}]c$ is much lower within VSMCs (i.e., 0.1 μM) than in the extracellular fluid (1–2 mM). However, passive diffusion across the lipid bilayer of the plasmalemma is minimal because Ca^{2+} is highly water-soluble. In addition, excitable Ca^{2+} channels in the plasmalemma are closed in the resting state. Thus, in spite of the favorability of both the electrical and concentration gradients, the rate of Ca^{2+} influx across the plasmalemma is quite low in the resting state. Activation of the contractile machinery requires the $[Ca^{2+}]c$ to exceed approximately 1 μM. However, the resting-state passive Ca^{2+} entry never results in such an increase in $[Ca^{2+}]c$, because there exist several homeostatic mechanisms that maintain $[Ca^{2+}]c$ below the critical level, including: (1) active transport across the plasmalemma to the extracellular space (plasma membrane Ca^{2+}, Mg^{2+}-ATPase pump); (2) active transport across the membrane of the sarcoplasmic reticulum (SR) into its lumen (SR Ca^{2+}-ATPase pump); (3) Na^+/Ca^{2+} exchange across the plasmalemma; (4) passive transport into the mitochondrial lumen; and (5) transport into the nucleus (Fig. 2) [7,16]. However, under normal in vivo

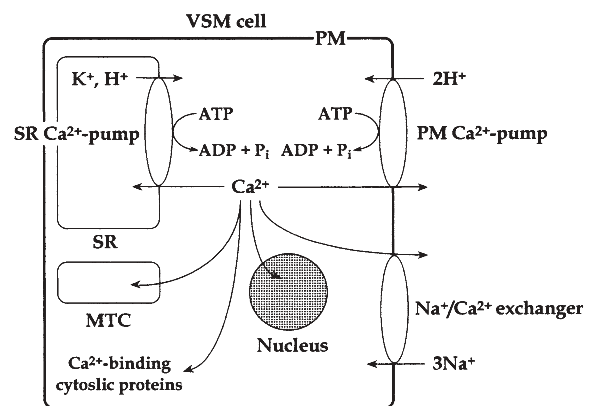

Fig. 2. Various mechanisms that reduce the cytosolic free Ca^{2+} level in VSM cells (for details see text). P_i, inorganic phosphate; *PM*, plasma membrane; *MTC*, mitochondria; *SR*, sarcoplasmic reticulum

conditions, VSMCs are not in such a resting state, but are in a partially contracted state (i.e., vasomotor tone) under the influence of various types of regulatory signal, including neural, endothelial, humoral, physicochemical, and myogenic factors, with the $[Ca^{2+}]c$ exceeding 0.1 μM [7].

Calcium-mobilization mechanisms

In response to vasoconstrictor stimuli, Ca^{2+} is mobilized from either intracellular stores (i.e., SR) or the extracellular space to increase the $[Ca^{2+}]c$ in VSMCs. The relative contribution from these two Ca^{2+} pools varies with different VSMCs and stimuli [17]. Under physiological conditions, the increases in $[Ca^{2+}]c$ are caused by either membrane depolarization (i.e., electromechanical coupling) or by the binding of contractile agonists to specific cell surface receptors without "prior" changes in the membrane potential (i.e., pharmacomechanical coupling) [4]. Note, however, that changes in membrane potential may occur secondarily in the pharmacomechanical coupling. These two mechanisms appear to operate simultaneously in a given VSMc, and excitation caused by a single agent may be the result of either electromechanical or pharmacomechanical coupling alone, or a combination of the two mechanisms [4,7].

Several mechanisms of pharmacomechanical coupling have been proposed. The most important mechanism involves the activation of the phosphatidylinositol cascade as a result of interaction between a vasoconstrictor molecule and a specific receptor coupled to phospholipase C (PLC), a Ca^{2+}-dependent phosphomonoesterase located in the cell membrane. The majority of contractile agonists (e.g., norepinephrine, angiotensin II, ATP, endothelin) provoke contraction via binding to PLC-coupled receptors [4,7]. The conformational change induced by an agonist binding to the receptor results in an interaction of the receptor-ligand complex with the heterotrimeric guanosine-5′-triphosphate (GTP)-binding protein (G-protein), which is composed of three subunits, an α, β, and γ (Fig. 3). This interaction stimulates the exchange of guanosine-5′-diphosphate (GDP) bound to the α subunit for GTP, which is subsequently hydrolyzed by the intrinsic GTPase activity of the G-protein, leading to separation of the α subunit and βγ-complex (Fig. 3). The activated α subunit then interacts with and activates PLC to catalyze the splitting of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into two messengers, i.e., inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DG; Fig. 3) [18,19]. IP_3 diffuses from the cell membrane into the cytosol, while DG remains in the cell membrane. In the cytosol, IP_3 stimulates the IP_3-sensitive SR to release Ca^{2+}, which in turn, activates contractile proteins and initiates contraction (Fig. 4).

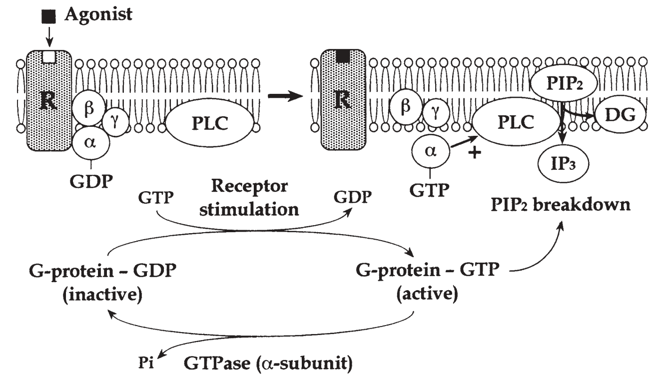

Fig. 3. Activation of the G protein-coupled receptor and resultant PLC-mediated PIP_2 breakdown (for details see text). *R*, receptor; *GDP*, guanosine-5′-diphosphate; *GTP*, guanosine-5′-triphosphate; *PLC*, phospholipase C; *PIP_2*, phospholipid phosphatidylinositol 4,5-bisphosphate; *G-protein*, guanosine-5′-triphosphate-binding protein; *IP_3*, inositol 1, 4, 5-triphosphate; *DG*, 1,2-diacylglycerol

Thus, the initial rapid phasic component observed in contractile response to these agonists reflects the PLC-mediated Ca^{2+} release from the SR [16,20–22]. The IP_3-mediated increase in $[Ca^{2+}]c$, in turn, may induce further Ca^{2+} release from ryanodine-sensitive SR via a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Fig. 4) [23]. In response to these increases in $[Ca^{2+}]c$ and in phosphoinositide levels, protein kinase C (PKC) migrates from the cytosol to the cell membrane, where it interacts with DG to become activated in the presence of phosphatidylserine [7,24]. DG can also be generated by the phospholipase D (PLD)-mediated hydrolysis of phosphatidylcholine [24]. Like other kinases, the activated PKC would act on various cytoplasmic and membrane proteins, thereby modulating a number of cellular processes, such as the regulation of transmembrane Ca^{2+} transport, myofilament Ca^{2+} sensitivity, or intracellular pH [7,24]. Following the initial increase in $[Ca^{2+}]c$ caused by PLC-mediated Ca^{2+} release, a tonic increase in $[Ca^{2+}]c$, which is dependent on extracellular Ca^{2+}, is normally observed in contractile response to receptor agonists [7,11–13]. The agonists may activate plasmalemmal Ca^{2+} channels without prior membrane depolarization, as detailed below.

Calcium release from sarcoplasmic reticulum (SR)

In VSMCs, Ca^{2+} is stored intracellularly in the SR, which is an endomembrane system consisting of a peripheral/superficial (sarcolemmal) portion and a central/deep (perinuclear) one, with radial tubular projections connecting them (Fig. 5) [25]. The peripheral SR appears to be essentially responsible for Ca^{2+} regulation in the basal state, while the central SR may be in great part responsible for Ca^{2+} removal from the cytoplasm follow-

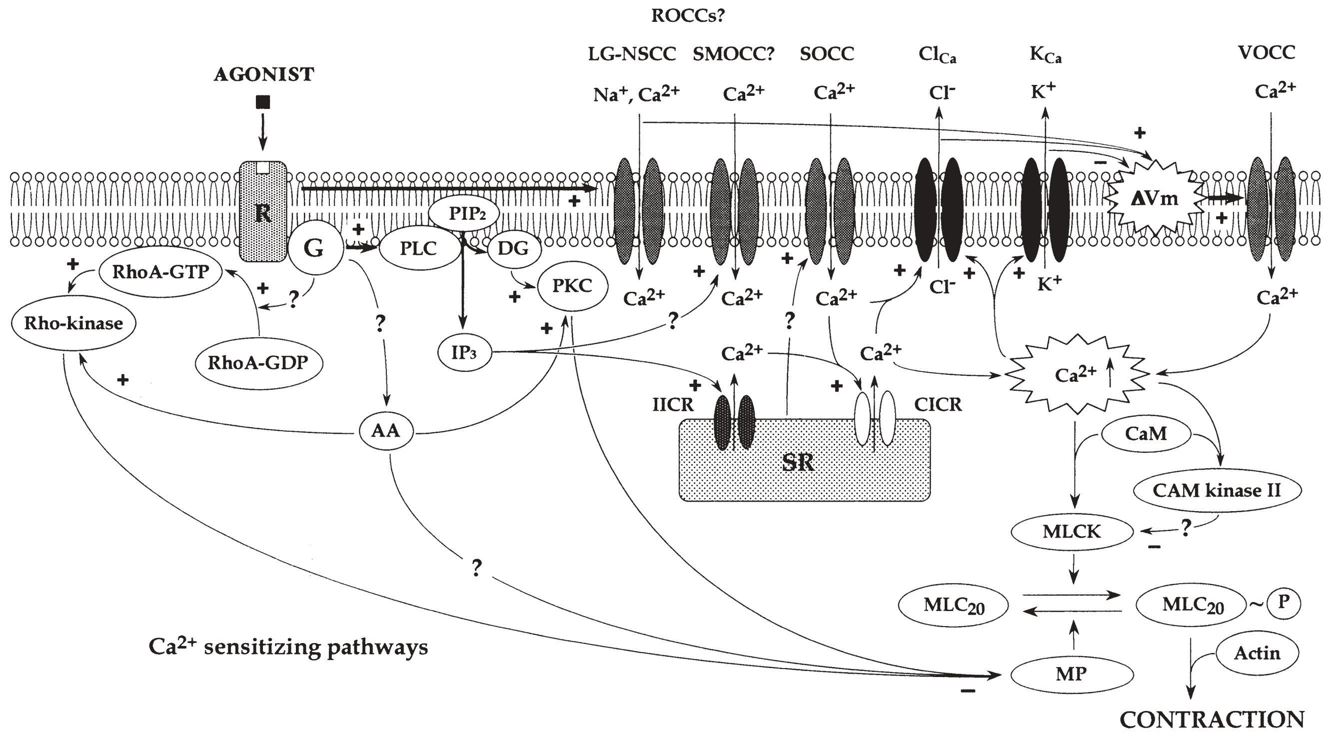

Fig. 4. Proposed mechanisms of vascular smooth muscle contraction. Rho-kinase, protein kinase C (*PKC*), and arachidonic acid (*AA*) have been proposed to increase myofilament Ca^{2+} sensitivity through the inhibition of myosin phosphatase (*MP*) during receptor stimulation (for details see part 2 of this review). The possible involvement of caldesmon, calponin, mitogen-activated protein kinases, or tyrosine kinases (for details also see part 2 of this review pp. 232–242) is not indicated for clarity of the figure. The roles of Cl^- and K^+ channels are detailed in part 2 of this review as well. *Plus sign*, stimulation; *minus sign*, inhibition; *CAM* and *CaM*, calmodulin; *CICR*, Ca^{2+}-induced Ca^{2+}-release; Cl_{Ca}, Ca^{2+}-activated Cl^- channel; *DG*, 1,2-diacyl-glycerol; *G*, guanosine-5′-triphosphate-binding protein; IP_3, inositol 1, 4, 5-triphosphate; *IICR*, IP_3-induced Ca^{2+}-release; K_{Ca}, Ca^{2+}-activated K^+ channel; *LG-NSCC*, ligand-gated nonselective cation channel; MLC_{20}, regulatory light chain of myosin (20 kDa); *MLCK*, myosin light chain kinase; PIP_2, phosphatidyl-inositol 4,5-bisphosphate; *PLC*, phospholipase C; *R*, receptor; *RhoA-GDP*, GDP-bound RhoA; *RhoA-GTP*, GTP-bound RhoA; *ROCC*, receptor-operated Ca^{2+} channel; *SMOCC*, second messenger-operated Ca^{2+} channel; *SOCC*, store-operated Ca^{2+} channel (Ca^{2+} release-activated Ca^{2+} channel [CRAC]); *SR*, sarcoplasmic reticulum; *VOCC*, voltage-operated Ca^{2+} channel; Δ*Vm*, change in membrane potential

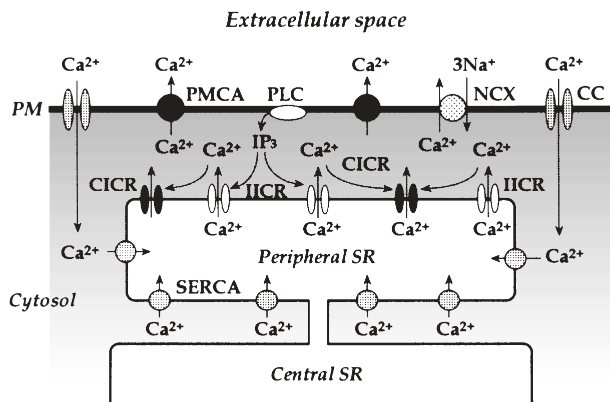

Fig. 5. Schematic representation of the superficial buffer barrier model proposed by Van Breemen and colleagues [67] (for details see text). *CC*, Ca^{2+} channel; *CICR*, Ca^{2+}-induced Ca^{2+}-release; *IICR*, IP_3-induced Ca^{2+}-release; *NCX*, Na^+/Ca^{2+} exchanger; *PM*, plasma membrane; *PMCA*, plasma membrane Ca^{2+}-ATPase (plasma membrane Ca^{2+} pump); *PLC*, phospholipase C; *SERCA*, sarcoplasmic reticulum Ca^{2+}-ATPase; *SR*, sarcoplasmic reticulum

ing the activation of myofilaments when there is rapid relaxation [16]. It is generally agreed that the SR plays a pivotal role in the regulation of $[Ca^{2+}]c$, a major determinant of vascular tone, not only as a supply of the activator Ca^{2+} but also as a buffer against VSM activation by Ca^{2+} [4,26].

The SR in VSMCs contains at least two types of Ca^{2+}-release channels, i.e., those sensitive to IP_3 (IP_3 receptor [IP_3R]/Ca^{2+}-release channels) and those sensitive to the plant alkaloid ryanodine and to caffeine (ryanodine receptor [RyR]/Ca^{2+}-release channels) [17,27]. Both of these channels are different from those of the cell membrane, and neither of them is voltage-dependent [7,24]. In recent immunohistochemical studies, both the IP_3R and RyR were present in both peripheral and central SR compartments, suggesting a structural overlap between IP_3-sensitive and ryanodine-sensitive stores [17]. Indeed, previous functional studies have suggested that there may be some overlap in the identity of IP_3-sensitive and ryanodine-sensitive stores, and that the

degree of overlap may vary among different species and different smooth muscle cell types [16,27]. Other studies have even suggested that the IP_3R and RyR are largely colocalized in the same SR membrane [28–30]. It is worth mentioning that, in smooth muscle cells where the two types of stores are discrete, high intracellular GTP levels may favor communication and Ca^{2+} transfer between them [31,32].

IP_3 receptor/Ca^{2+}-release channels (IP$_3$-induced Ca^{2+} release)

The IP_3R is coded by at least three genes, two of which have been reported to be expressed in smooth muscle, IP_3R_1 and IP_3R_3 [16,24]. The IP_3R isolated from aortic smooth muscle consists of a single tetrameric polypeptide with a molecular weight of ~224 kDa. This polypeptide has binding domains for both IP_3 and Ca^{2+}, and a central aqueous channel that permits passage of Ca^{2+} down its concentration gradient towards the cytosol [33]. The IP_3R channel is capable of releasing a large quantity of Ca^{2+} to the cytosol, and is believed to play a primary physiological role in Ca^{2+} mobilization [4,7].

It has been described that IP_3-induced Ca^{2+} release (IICR) is biphasic, with a fast-release component followed by a slow-release component, designated as "quantal Ca^{2+} release" [34]. However, its precise mechanisms and physiological significance are not fully understood. The interaction of IP_3 with its receptor appears to be regulated by $[Ca^{2+}]c$ [16,24]. Namely, $[Ca^{2+}]c$ has been shown to have a biphasic effect on the IICR; i.e., increases in $[Ca^{2+}]c$ up to 300 nM increased the effectiveness of IP_3 to release Ca^{2+} (positive feedback), while further increases in $[Ca^{2+}]c$ above 300 nM, conversely, inhibited the IICR (negative feedback) [16,24]. This bell-shaped response curve should create a "window" of $[Ca^{2+}]c$ in which IP_3 is effective in releasing stored Ca^{2+}. Outside of this window, IP_3 would be relatively ineffective [24]. This Ca^{2+} regulation is similar to that described for RyRs and, thus, IP_3R can be considered, from a functional point of view, as a CICR channel [16]. In addition, the IP_3R also seems to be modulated by cytosolic ATP; its open probability has been shown to display a bell-shaped response curve, with a maximum at around 5 mM ATP. Furthermore, the IP_3R can be phosphorylated by various protein kinases, such as cyclic adenosine 3′,5′-monophosphate (cAMP)-dependent protein kinase (protein kinase A, PKA) and PKC, and thus its function may also be modulated by these kinases [16,24].

Ryanodine receptor/Ca^{2+}-release channels (Ca^{2+}-induced Ca^{2+} release)

The RyR is coded by at least three genes, one of which is expressed in smooth muscle, RyR_3 [35]. Although the RyR shares features with the IP_3R such as a homotetrameric structure, it is not activated by IP_3. Only two physiological agonists have been identified so far, Ca^{2+} itself and cyclic adenosine diphosphoribose (cADP-ribose), a metabolite of nicotineamide adenine dinucleotide. Although cADP-ribose has been proposed as an endogenous activator of RyR, its activity in smooth muscle remains to be shown [16,24]. CICR may occur through the RyR channel, although its physiological role is not fully understood [17,27,36,37]. CICR may contribute to amplification of the agonist-induced Ca^{2+}-signal, cytosolic Ca^{2+} oscillations, and Ca^{2+} waves. In addition, it may play an important role in the superficial buffer barrier mechanism (Fig. 5), which is considered essential for cellular Ca^{2+} homeostasis in VSMCs (see below) [16,25].

Ca^{2+} entry across the plasmalemma

The majority of extracellular Ca^{2+} exist not as free calcium ion (Ca^{2+}), but rather, is bound to abundant anionic sites on the cell surface [38]. The carboxyl groups of sialic acid in the cell's outer glycoprotein layer would serve as the anionic sites, and the interaction between Ca^{2+} and the sialic acid residue is the first reaction in the process of plasmalemmal Ca^{2+} influx. Two kinds of Ca^{2+} binding sites are believed to exist on the surface membranes of VSMCs, related to the plasmalemmal Ca^{2+} influx—i.e., a low-affinity and a high-affinity Ca^{2+} binding site [39,40].

Plasmalemmal Ca^{2+} channels are the major routes by which Ca^{2+} enters VSMCs. Several types of plasmalemmal Ca^{2+} channels have been proposed to exist in VSMCs, including voltage-operated Ca^{2+} channels (VOCCs), receptor-operated Ca^{2+} channels (ROCCs), and store-operated Ca^{2+} channels (SOCCs; Fig. 4) [4,7,41]. ROCCs may be further subdivided into ligand-gated Ca^{2+} channels and second messenger-operated Ca^{2+} channels (SMOCCs) [7,41]. The ligand-gated Ca^{2+} channels are coupled to specific receptors and are directly activatable by receptor agonists, while SMOCCs are indirectly activated by diffusible second messengers such as IP_3 or IP_4 following receptor activation [7,41]. The open probability of VOCCs increases as the membrane potential becomes more depolarized, while the opening of ROCCs is primarily controlled by a receptor for a stimulant substance. However, this definition does not exclude the possibility that some ROCCs can be voltage-dependent [41]. SOCCs are activated in response to the emptying of intracellular Ca^{2+} stores and act to replenish these stores [42].

Passive Ca^{2+} influx

Even in the resting state, the VSM membrane is not entirely impermeable to Ca^{2+} ions, and small quantities of Ca^{2+} can move down the electrochemical gradient into the cell. The routes by which this passive Ca^{2+} influx

occurs are unidentified, although they appear to be distinct from those involved in excitatory Ca^{2+} influx [7,41].

Ca^{2+} influx via voltage-operated Ca^{2+} channels

The resting membrane potential of VSMCs, as determined in vitro using whole-cell voltage-clamp and other electrophysiological techniques, ranges from −45 to −70 mV [4,7]. However, the membrane potential of VSMCs measured in vivo is more positive than that measured in vitro (i.e., −40 to −55 mV) [43–45], and falls within the range over which VOCCs are activated, suggesting that voltage-gated Ca^{2+} influx plays an important role in maintaining vasomotor tone [7,43]. Previous studies have identified six types of VOCC (types L, T, N, R, Q, and P) to date; at least two types are present in VSM: i.e., "long-lasting" (L-type) channels and "transient" (T-type) channels [41,46].

The L-type Ca^{2+} channels are glycoproteins that span the cell membrane and consist of five subunits (α_1, α_2, β, γ, and δ) [41,43]. The α_1 subunit contains specific binding sites for Ca^{2+} channel modulators, domains sensitive to phosphorylation (e.g., PKC, Ca-calmodulin kinase II), a voltage sensor, conductance and ion selectivity filters, and activation and inactivation gates [41,43]. The L-type channels are activated by a strong depolarization (threshold ≈−40 mV, full activation at ≈0 mV) and are inactivated much less rapidly than the T-type channels (300~600 ms) [47]. The inactivation is both voltage- and Ca^{2+}-dependent, and, thus, biexponential. In most VSMCs, the L-type channels are the most numerous VOCCs and are probably the most important route of activatory Ca^{2+} influx [7]. It has been proposed that a larger proportion of Ca^{2+} entering through L-type channels directly activates contractile filaments and fills intracellular stores, and only a small proportion leads to CICR [48]. However, it still seems controversial whether Ca^{2+} entering through L-type channels replenishes intracellular stores [7]. Unlike T channels, L channels are highly sensitive to dihydropyridines and other organic and inorganic calcium antagonists [7,41,43].

The T-type Ca^{2+} channels are activated by weak depolarization (up to −30 mV) and are inactivated rapidly (within 20–60 ms) [47]. The inactivation is monoexponential and voltage-dependent [47]. The transient increase in $[Ca^{2+}]$c through T-type Ca^{2+} channels may stimulate Ca^{2+} release from intracellular stores via the CICR mechanism [32]. In addition, T-type Ca^{2+} channels may be involved in action potential generation in VSMCs with spontaneous electrical activity (i.e., pacemaker cells) and, thus, in their rhythmic and phasic contractile activity [7]. Ca^{2+} entering through T-type channels probably does not replenish the intracellular stores [48].

A "resting" (R-type) channel was previously reported to exist in aortic and renal arterial VSMCs [49]. However, its physiological role is unclear. It may contribute to the sustained slow entry of Ca^{2+} that occurs during the tonic contraction caused by high extracellular K^+ [7].

Contractile response to receptor agonists is normally associated with membrane depolarization and an increase in membrane conductance [43,50]. However, the precise mechanisms whereby the agonists cause membrane depolarization sufficient to activate VOCCs are not fully understood. The agonists may directly activate nonselective cation channels (NSCCs), and thereby cause membrane depolarization [43,51]. On the other hand, Ca^{2+} released from the SR and/or entering through NSCCs may activate Ca^{2+}-dependent cation channels and/or Cl^- channels (Cl^- equilibrium potential ~−40 mV), thereby provoking membrane depolarization [13,51]. However, the rise in $[Ca^{2+}]$c could also activate Ca^{2+}-dependent K^+ channels (K^+ equilibrium potential ~0 mV), thereby opposing the depolarizing effects of the activation of Ca^{2+}-dependent Cl^- channels [7,51].

Ca^{2+} influx via receptor-operated Ca^{2+} channels

It seems probable that the ligand-gated channels, directly activatable by receptor agonists, are NSCCs with some degree of selectivity for divalent cations [4,41]. However, it has been estimated that, under physiological conditions, most current through the channels is carried by Na^+ [4,41]. Therefore, it seems unlikely that Ca^{2+} influx through these channels itself makes significant contribution to excitation-contraction coupling. However, increased Na^+ conductance as a result of the activation of NSCCs leads to membrane depolarization and thereby activates VOCCs, causing plasmalemmal Ca^{2+} influx [4,7,41]. Several contractile agonists have been reported to activate NSCCs (or cation-selective channels) in VSMCs, including ATP, norepinephrine, vasopressin, endothelin, angiotensin II, and serotonin [41,51]. Some similarities have been found in ionic selectivity, voltage-dependence, and single-channel conductance of NSCCs opened by these agonists [41]. G-proteins and PKC may be involved in the activation of NSCCs in some VSMCs [52].

In response to the binding of excitatory agonists to specific PLC-coupled receptors, second messengers such as IP_3, IP_4, or Ca^{2+} may activate plasmalemmal Ca^{2+} channels, referred to as SMOCCs. Although SMOCCs have been proposed to exist in T-lymphocytes, mast cells, and cardiac cells, their physiological role is still poorly understood. SMOCCs are not activated by store depletion, have relatively high conductance (5–25 pS), and are not linked to a specific membrane receptor [7]. At present, there is limited evidence for their existence in VSMCs [7,41].

Ca^{2+} influx via store-operated Ca^{2+} channels (capacitative Ca^{2+} entry)

Depletion of the intracellular SR Ca^{2+} stores by diverse mechanisms (receptor agonists, CICR activators, SR-Ca^{2+} ATPase inhibitors, Ca^{2+} ionophores) has been reported to cause capacitative Ca^{2+} entry through SOCCs in a wide range of cell types, including VSMCs [42,53,54]. Because SR depletion caused by the inhibition of SR Ca^{2+}-ATPase can activate capacitative Ca^{2+} entry without continued receptor occupation or the generation of related second messengers (e.g., inositol phosphates), the Ca^{2+} entry via SOCCs is considered to be distinct from the Ca^{2+} entry via ROCCs. Namely, SR depletion acts as the primary stimulus for the activation of Ca^{2+} entry via SOCCs. In addition, electrophysiological evidence has indicated that, unlike receptor-operated NSCCs, SOCCs are highly selective for Ca^{2+} over other cations [53,55]. The cellular mechanisms linking SR depletion to the opening of SOCCs are still far from clear, and might involve the generation of a diffusible chemical messenger (i.e., calcium influx factor [CIF]) or a direct protein-protein interaction between the SR and the plasma membrane [54].

Transient receptor potential (TRP) channels, which were initially found to play a prominent role in store-operated Ca^{2+} entry in *Drosophila* photoreceptors, have recently been receiving increasing attention as excellent candidates for capacitative Ca^{2+} entry channels or SOCCs in mammalian cells [42,56]. To date, seven members of the TRP family have been cloned, and their expression has been detected within a variety of mammalian species and tissues, including VSM. In addition to contributing to capacitative Ca^{2+} entry via SOCCs, TRP channels may also be associated with receptor-operated Ca^{2+} entry via NSCCs. The topological and multimeric structures of TRP channels are similar to those of NSCCs [56]. Thus, TRP channels can be functionally organized into two groups, those that are dependent on store depletion for activation and those that are independent. The store-dependent TRP channels are more likely to be SOCCs, while the store-independent TRP channels are more likely to encode receptor-operated NSCCs [56]. Current research suggests that TRP1, TRP3, TRP6, and TRP7 encode NSCCs, and that TRP2, TRP4, and TRP5 are SOCCs. Recent evidence further suggests that TRP3, TRP4, TRP6, and TRP7 can be expressed in VSMCs [56].

In addition to its crucial role in the re-filling of the depleted SR Ca^{2+} stores, capacitative Ca^{2+} entry via SOCCs may actively contribute to the regulation of VSM tone by providing an important route of entry for activator Ca^{2+} [54,57]. The relative contribution of capacitative Ca^{2+} entry to excitation-contraction coupling probably depends on the smooth muscle type, and appears to be greatest in tonic smooth muscle [54].

Ca^{2+} influx via stretch-activated channels

VSMCs respond to an elevation in intravascular pressure (i.e., stretch) with active force development, while they dilate in response to a reduction in pressure. These myogenic responses are believed to play a key role in the fine regulation of local blood flow and basal vascular tone [58]. Previously proposed cellular mechanisms behind the myogenic responses include the activation of plasmalemmal Ca^{2+} influx [41,58,59], the release of Ca^{2+} from intracellular stores [59,60], altered myofilament Ca^{2+} sensitivity [61], and the modulation of endothelial function [58]. Stretch has been reported to induce plasmalemmal Ca^{2+} influx not only through VOCCs but also through mechanosensitive NSCCs [41,62].

Mechanisms that reduce cyotosolic calcium level

There exist several mechanisms that reduce cytosolic free Ca^{2+} level, including the plasma membrane Ca^{2+}-ATPase (PMCA) pump, the SR Ca^{2+}-ATPase (SERCA) pump, the Na^+/Ca^{2+} exchanger (NCX), and cytosolic Ca^{2+}-binding proteins (Fig. 2). Both the PMCA and the SERCA pumps are believed to play a crucial role in reducing excessive $[Ca^{2+}]c$ levels in the resting condition or in mediating the vasodilator action of various endogenous agents. Namely, under physiological conditions, decreases in the $[Ca^{2+}]c$ result mainly from either the PMCA-mediated extrusion of Ca^{2+} into the extracellular space or the SERCA-mediated uptake of Ca^{2+} into SR intracellular stores. However, recent evidence suggests that the NCX may also play a significant role in the physiological regulation of $[Ca^{2+}]c$ in VSMCs [16,25,63].

Ca^{2+}-ATPases (Ca^{2+} pump)

Two distinct Ca^{2+} transport ATPases, one localized in the plasma membrane and the other in the SR membrane, are present in VSMCs—i.e., PMCA (~130–140 kDa) and SERCA (~110 kDa) [64]. However, their amino acid sequences show a high degree of resemblance, and the structure of the PMCA pump is similar to that of the SERCA pump [65,66]. These Ca^{2+} transport ATPases are believed to play an essential role in the regulation of cytosolic Ca^{2+} homeostasis.

The PMCA pump is a multigene family consisting of at least four gene products (i.e., *PMCA 1*, *2*, *3*, and *4*). The expression of these genes is tissue-dependent; i.e., *PMCA 1* is the family found in most tissues, the rest of the PMCAs being present in a more restricted expression pattern [16]. Two isoforms of PMCA 1 are present, designated *PMCA 1a* and *PMCA 1b*. The isoform PMCA 1b, with a MW of ≈140 kDa, is the most frequently expressed in smooth muscle cells [16].

The SERCA pump consists of three genes, i.e., *SERCA 1*, *-2*, and *-3*. *SERCA 1* and *SERCA 3* show

a restricted expression pattern, while *SERCA 2* is ubiquitously expressed [16,64]. The smooth muscle 100-kDa SERCA is transcribed from the *SERCA 2* gene; however, *SERCA 3* was also shown to be expressed in smooth muscle-containing tissue [24].

Plasma membrane Ca^{2+}-ATPases (PMCA)

PMCA utilize energy from ATP hydrolysis to produce Ca^{2+} efflux in exchange for $2H^+$ influx against a high electrochemical gradient across the plasmalemma (Fig. 2), suggesting that this electroneutral pump may also contribute to intracellular pH regulation [16,64]. In addition, the exchange is coupled to Na^+/K^+-ATPase or the Na^+ pump, inducing active extrusion of $3Na^+$ and accumulation of $2K^+$, thereby maintaining transmembrane Na^+ and K^+ gradients [16].

The C-terminal of the cytosolic domain of the PMCA pump contains both CaM-binding sites and substrates for several protein kinases such as PKA, PKC, and cyclic guanosine 3′,5′-monophosphate (cGMP)-dependent protein kinase (protein kinase G; PKG). The activation of the PMCA pump by CaM increases its affinity for Ca^{2+} and the velocity of Ca^{2+} transport, due to conformational changes produced in its CaM binding domain [16]. Conversely, when CaM is absent, the CaM binding domain interacts with cytosolic regions of the enzyme, inhibiting its activity (i.e., autoinhibition). Phosphorylation of some sites in the CaM binding regions by several protein kinases—such as PKA, PKC, PKG, and Ca^{2+}/CaM-dependent protein kinase (CaMK)—results in activation of the PMCA pump [16,64]. The PMCA pump is embedded in membrane lipids and its activity is critically dependent on the presence of surrounding phospholipids. Namely, membrane phospholipids such as PIP_2, PIP, and PI ($PIP_2 > PIP > PI$) increase the affinity for Ca^{2+} and activate the PMCA pump. Thus, changes in the amounts of these phospholipids in the plasmalemma may be important factors in the regulation of PMCA activity. The inhibitory action of agonists on plasmalemmal Ca^{2+} extrusion could be due to a decrease in PIP_2 in the plasmalemma [16,64].

Sarcoplasmic reticulum Ca^{2+}-ATPases (SERCA)

The SERCA pump is generally considered to be electroneutral, as, for each Ca^{2+} influx into the SR lumen, one K^+ and one H^+ are extruded from the lumen to the cytosol [66], although a different stoichiometry has also been described (Fig. 2) [65]. The SERCA pump also utilizes the energy supplied by the hydrolysis of ATP to accumulate Ca^{2+} inside the SR. The hydrolysis of 1 mol of ATP is normally coupled to the transfer of two Ca^{2+} atoms across the membrane ($ATP + 2Ca^{2+}_{cyt} \longleftrightarrow 2Ca^{2+}_{luminal} + ADP + Pi$) [16]. Once Ca^{2+} is taken up into the SR lumen, it is bound by several small hydrophilic proteins, such as calreticulin or calsequestrin, that are capable of binding large amounts of Ca^{2+}. Binding to these Ca^{2+} buffering proteins reduces the luminal free Ca^{2+} concentration, decreasing the energy necessary to pump up Ca^{2+} from the cytoplasma against a concentration gradient. Thus, the SR can store large amounts of Ca^{2+}, with luminal Ca^{2+} concentrations reaching up to 10–15 mM [7,16].

Because the SERCA pump, unlike the PMCA pump, lacks the CaM-binding domain, its activity is not regulated by CaM. Instead, phospholamban, a small transmembrane protein of the SR formed by 52 amino acids assembled in a stable homopentamer of 6 kD, plays a central role in the regulation of SERCA activity [16,64]. The role of phospholamban in regulating SERCA activity, however, is similar to that of the CaM-binding domain in regulating PMCA activity; i.e., in both cases, the respective enzymes are inhibited when they are in a dephosphorylated state. Indeed, phospholamban shows ~50% sequence identity with the CaM-binding domain [16]. Phospholamban physically interacts with the SERCA pump, and is thought to inhibit its activity by promoting the aggregation of pump molecules into catalytically unfavorable conformations or by interfering with the catalytic site [7]. The phosphorylation of phospholamban releases SERCA from this kinetically unfavorable associated state, increasing its Ca^{2+} affinity and the rate of its Ca^{2+} transport [16,24]. Phospholamban can be phosphorylated by PKA and PKG at the same amino acid, but it can also be phosphorylated by CaMK at other amino acids, and also by PKC, with some differences in the efficacy of the phosphorylated compound [16,64]. The PKG-mediated activation of the SERCA pump probably contributes to the vasodilator actions of various agents that increase the cytosolic cGMP level, such as β-adrenergic agonists, NO, or atrial natriuretic factor (ANF) [64]. Despite these properties of phospholamban, its physiological roles are not fully understood [16].

Na^+/Ca^{2+} exchanger (NCX)

VSMCs possess a high capacity (i.e., large maximal velocity), cardiac-type NCX in their plasmalemma [16,63]. The exchanger molecules are distributed in organized patterns across the plasmalemma in VSMCs; however, the density of their distribution in VSMCs is much lower than that in cardiac myocytes or neurons [63]. The NCX is driven by the transmembrane Na^+ gradient and is sustained by the Na^+ pump, normally transporting 1 Ca^{2+} out of the cell in exchange for 3 Na^+. The NCX may contribute to either Ca^{2+} extrusion or even Ca^{2+} influx, depending on the conditions. Because even large changes in the Na^+ electrochemical gradient across the plasmalemma do not significantly alter the resting $[Ca^{2+}]c$ level in VSMCs, the physiological significance of the NCX in VSMCs has been questioned for many years

[63]. However, the NCX has recently been proposed to play a key role in the superficial buffer barrier mechanism, which seems essential for the fine regulation of $[Ca^{2+}]c$ in VSM (see below) [25,67].

Myoplasmic Ca^{2+} buffering molecules

Smooth-muscle myoplasma contains many Ca^{2+}-binding proteins that could bind a significant fraction of myoplasmic Ca^{2+} [24]. According to similarities in the Ca^{2+} binding domain, they are classified into two groups, i.e., (i) the EF-hand (i.e., common Ca^{2+} binding motif) family, such as CaM, troponin C, saponin, calbindin, calretinin, and parvalbumin; and (ii) the annexin family [68–70]. The myoplasma also contains many inorganic compounds or other small organic molecules (amino acids, nucleotides), and organic acids (pyruvic, acetic and citric acids), which have the ability to bind to Ca^{2+} with a relatively low affinity [69]. These Ca^{2+} buffering molecules may provide a mechanism for the rapid removal of Ca^{2+}, limiting its diffusion and thus making Ca^{2+} a local second messenger within a restricted subcellular space. However, their precise roles in $[Ca^{2+}]c$ regulation remain to be investigated [24].

Mitochondria, nucleus, and cellular Ca^{2+} homeostasis

Mitochondria were previously thought to play an important role in the regulation of cytosolic Ca^{2+} homeostasis in VSMCs. However, it is now generally believed that their role as an intracellular Ca^{2+} store is relatively unimportant in comparison with that of the SR [71]. The major route for Ca^{2+} transport into the mitochondria is an electrophoretic uniporter with low Ca^{2+} affinity [7]. The uniporter uses the electropotential gradient across the inner mitochondrial membrane, producing Ca^{2+} influx by exchange with H^+ [71]. This mitochondrial Ca^{2+} accumulation may be activated only in pathological conditions associated with elevated $[Ca^{2+}]c$ [16].

The nucleus is directly interconnected with the SR and, thus, it is reasonable to suspect that this organelle acts as an intracellular Ca^{2+} store and participates in Ca^{2+} homeostasis [7]. Indeed, the Ca^{2+} level in the nucleus, as is that in the SR, seems to be greater than that in the myoplasma. However, the precise role of the nucleus in cellular Ca^{2+} homeostasis, as well as the mechanisms of regulation of nuclear Ca^{2+} homeostasis, are currently unclear [16].

Superficial buffer barrier model

The superficial buffer barrier model, originally proposed by van Breemen and colleagues [67], has been highly appreciated in recent review articles [16,24,25]. In this model a portion of Ca^{2+} entering the cells is sequestered by the peripheral SR via the SERCA before it accumulates in the central myoplasma to initiate contraction (Fig. 5). In other words, the model proposes that, in addition to the function of the SR as a sink and a source of Ca^{2+}, the SR also functions as an effective barrier against Ca^{2+} influx across the plasmalemma, preventing the great amounts of Ca^{2+} entering the cells from reaching the myofilaments to evoke contraction. Thus, areas of heterogeneity in $[Ca^{2+}]c$ (i.e., Ca^{2+} gradients) are postulated, with higher $[Ca^{2+}]c$ in the subsarcolemmal space near SR vesicles. However, one prerequisite for the peripheral SR in assuming this function is its ability to maintain a Ca^{2+} storage reserve under resting conditions. It has thus been proposed that the peripheral SR releases Ca^{2+} essentially towards the junctional/subsarcolemmal space between the SR and the sarcolemma—designated vectorial Ca^{2+} release—to maintain the storage reserve (Fig. 5). Both IP_3R/Ca^{2+}-release and RyR/Ca^{2+}-release channels are thought to contribute to vectorial Ca^{2+} release. Namely, it has been proposed that IP_3, synthesized by basal PLC activity, continually activates IP_3Rs facing the sarcolemma, producing a localized increase in Ca^{2+} level in the junctional space through circumscribed IICR. The IP_3-mediated increase in Ca^{2+} level in the junctional space, in turn, stimulates CICR via the RyR channels [67]. The Ca^{2+} unloaded into the junctional space via the regulated vectorial release is subsequently extruded into the extracellular space by the NCX and the PMCA pump. The superficial buffer barrier mechanism, presumably, plays an important role in preventing excess $[Ca^{2+}]c$ and maintaining $[Ca^{2+}]c$ at lower levels under physiological conditions [16,24,25,67].

Cytosolic calcium oscillations

Rhythmic oscillations have been observed in contractile responses to a variety of agonists in isolated arterial and venous preparations [72–77], and this phenomenon, referred to as vasomotion, is especially evident in small arteries or arterioles [75,78,79]. Correspondingly, rhythmic oscillatory changes in vessel diameter, blood flow, or even oxygen tension have been observed in the in vivo vascular beds of intact subjects, including healthy human volunteers [80–84]. However, the frequency, amplitude, and regularity of the oscillations observed both in vitro and in vivo are known to vary among vessels, and the functional importance of the oscillations are still a matter of discussion. In small arteries or arterioles, the oscillations may play important physiological roles in regulating vascular resistance without disturbing tissue perfusion or oxygen delivery to tissue, minimizing fluid filtration into the extravascular space by reducing hydrostatic pressure, and enhancing lymphatic drainage through the pumping action of closely adjacent arterioles [85–87].

The cellular mechanisms behind the generation of oscillatory contractile responses have not been fully

clarified. However, they could correlate with cytosolic Ca^{2+} oscillations in VSMCs [88,89]. A variety of different mechanisms have been proposed to explain the Ca^{2+} oscillations, including a periodic release of Ca^{2+} from the SR [89,90], and oscillatory changes in membrane potential due to an interplay between VOCCs and K^+ channels [89–92]. In addition, endothelium, Na^+/K^+ pump, and Ca^{2+}-dependent Cl^- channels may play a role in the generation of cytosolic Ca^{2+} oscillations [73,75,77,78,89]. In any case, the rhythmic contractions of multicellular preparations would require considerable coordination of activity among VSMCs. Because VSMCs are electrically coupled via gap junctions [89], the cell-to-cell coupling via gap junctions among VSMCs could be essential in the generation of oscillatory contractile responses.

Summary

Changes in $[Ca^{2+}]c$ are the principal mechanisms that regulate the contractile state of VSMCs. In response to vasoconstrictor stimuli, Ca^{2+} is mobilized from intracellular stores (i.e., SR) and/or the extracellular space through various types of Ca^{2+} channels to increase the $[Ca^{2+}]c$ in VSMCs. The increase in $[Ca^{2+}]c$, in turn, activates the Ca^{2+}-CaM-MLCK pathway and stimulates MLC_{20} phosphorylation, leading to myosin-actin interactions and, hence, contraction—i.e., the primary mechanism for the initial development of contractile force in VSMCs. On the other hand, in response to vasodilator stimuli or the removal of vasoconstrictor stimuli, the $[Ca^{2+}]c$ decreases, mainly as a result of plasmalemmal Ca^{2+} extrusion and/or Ca^{2+} uptake into the SR, leading to the inactivation of MLCK and, hence, relaxation.

Besides the above-described basic mechanisms controlling the $[Ca^{2+}]c$ and Ca^{2+}-dependent activation of contractile myofilaments, there exist numerous regulatory mechanisms that modulate myofilament Ca^{2+} sensitivity and/or Ca^{2+} mobilization in VSMCs—such as Rho/Rho kinase, PKC, arachidonic acid, Ca^{2+}/CaM-dependent protein kinase II, caldesmon, calponin, mitogen-activated protein kinases, tyrosine kinases, cyclic nucleotides, K^+ channels, and Cl^- channels. These regulatory mechanisms, which are possibly involved in the physiological regulation of vascular tone, are reviewed in part 2 of this review (pp. 232–242).

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