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COMMENTARY

Ca^{2+} Transient, Cell Volume, and Microviscosity of the Plasma Membrane in Smooth Muscle

*Martin Ochsner**

FACULTY OF MEDICINE, BIOMEDICAL PHYSICS, UNIVERSITY OF BASEL, BASEL, SWITZERLAND

ABSTRACT. Despite pronounced differences by which membrane-depolarizing or phospholipase C-activating stimuli initiate contractile responses, a rise in $[Ca^{2+}]_i$ is considered the primary mechanism for induction of smooth muscle contractions. Subsequent to the formation of the well-characterized $Ca^{2+}{}_4$-calmodulin complex, interaction with the catalytic subunit of myosin light chain kinase triggers phosphorylation of 20 kDa myosin light chain and activates actin-dependent Mg^{2+}-ATPase activity, which ultimately leads to the development of tension. The present article reviews the fundamental mechanisms leading to an increase in $[Ca^{2+}]_i$ and discusses the biochemical processes involved in the transient and sustained phases of contraction. Moreover, the commentary summarizes current knowledge on the modulatory effect of changes in the microviscosity of the plasma membrane on the Ca^{2+} transient as well as the contractile response of smooth muscle. Evidence has accumulated that these changes in microviscosity alter the activity of membrane-bound enzymes and affect the generation of endogenous mediators responsible for the regulation of cytosolic Ca^{2+} concentrations and for the $[Ca^{2+}]_i$-sensitivity of myosin light chain phosphorylation. BIOCHEM PHARMACOL 53;12:1765–1777, 1997.

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Analogous to its function in skeletal muscle, Ca^{2+} occupies a central role in excitation-contraction coupling in smooth muscle by initiating Ca^{2+}/calmodulin-dependent processes [1]. Depending on the underlying mechanism, contractile responses may be subdivided into one of two distinct categories that account for electromechanical or pharmacomechanical coupling and imply that either depolarization-induced or receptor-driven processes are responsible for increasing cytosolic Ca^{2+} concentration and initiating smooth muscle contractions [2, 3]. Despite pronounced differences that link the initial stimulus to the contractile response, a transient rise in $[Ca^{2+}]_i$† is considered the primary mechanism for initiation of smooth muscle contractions. Ca^{2+} binds to calmodulin and the $Ca^{2+}{}_4$–calmodulin complex formed subsequently interacts with the catalytic subunit of MLCK to phosphorylate 20 kDa myosin light chain at serine 19. Phosphorylation of smooth muscle myosin is associated with an activation of its actin-stimulated Mg^{2+}-ATPase activity, thereby triggering cyclic actin–myosin interactions and, hence, contraction to occur [1]. Owing to the complicated sequence of biochemical events involved in the transmission of an excitatory stimulus at the cell membrane to the contractile machinery, there is a relatively long time delay of 0.2 to 1 sec (or even longer) between excitation and force development [4].

ELECTROMECHANICAL COUPLING

Agents such as KCl or tetraethylammonium, a K^+ channel blocker, induce smooth muscle contractions by depolarization of the plasma membrane [2, 3]. The inverted transmembrane potential increases $[Ca^{2+}]_i$ and induces smooth muscle contractions by catalyzing Ca^{2+} influx across VOCCs and by stimulating Ca^{2+} entry via Na^+/Ca^{2+} exchange carriers to restore resting Na^+ gradients across the plasmalemma lipid bilayer [5]. Whether a membrane depolarization-induced and sustained inward-directed Ca^{2+} current can trigger a Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum is still a matter of scientific discussion and remains to be elucidated.

VOCCs were classified originally into three major subtypes: L- (for long-lasting), T- (transient) and N- (neither) [6]. Both L- and, to a minor extent, T-type channels were found in smooth muscle cells, but there is no evidence to support the existence of N- or neuronal types of channels [7, 8]. In the simplest picture, Ca^{2+} channels reside in one of three distinct configurations: closed, open, or inactivated [6–8]. Under resting conditions, most channels are closed. Membrane depolarizations increase the permeability of the channels, which open for brief ($\leq$1 msec) but random

* Correspondence: Dr. Martin Ochsner, Im Kugelfang 40, CH-4102 Binningen, Switzerland. Tel. 41-61-421-11-97; FAX 41-61-421-11-97.

† *Abbreviations:* $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; cAMP and cGMP, cyclic AMP and cyclic GMP, respectively; DAG, *sn* (1,2)-diacylglycerol: $InsP_3$, D-*myo*-inositol-1,4,5-trisphosphate; LTB_4, LTC_4, LTD_4, leukotrienes (B_4, C_4, D_4); MLCK, myosin light chain kinase; PIP_2, phosphatidylinositol-4,5-biphosphate; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; VOCC, voltage-operated calcium channel; and ROCC, receptor-operated calcium channel.

periods. During maintained depolarization, Ca^{2+} channels undergo transition to an inactivated state and become nonconducting. Once inactivated by depolarization, they can reopen only when the membrane is repolarized to remove the state of inactivation. Interestingly, Ca^{2+} channel activity is also inhibited by Ca^{2+}-dependent processes [8]. The mechanism by which an elevated $[Ca^{2+}]_i$ exerts an inhibitory effect on the Ca^{2+} permeability of VOCCs is unknown, but may be linked to occupancy of a Ca^{2+} binding domain near the inner mouth of the Ca^{2+} channel [8]. Thus, depolarization and increases in $[Ca^{2+}]_i$ reduce the availability of Ca^{2+} channels and provide a negative feedback loop to prevent further Ca^{2+} influx. Whole cell patch clamp techniques have been widely used to specify the features of VOCCs in smooth muscle cells [9, 10]. Characteristically, T-type channels open at a very low threshold voltage of *ca.* −50 mV, but are inactivated rapidly at a fairly negative potential of −30 mV. In contrast, L-type channels need a higher threshold voltage of *ca.* −40 mV for activation. Their permeability is highest at a membrane potential of about 0 mV, while channel activity slowly becomes inhibited above voltages of 30 mV. Since T-type channels have a minor density and lower conductance and are more readily inactivated than channels of the L-type, the latter are thought to play a greater role in excitation–contraction coupling [8]. Surprisingly, regulation of Ca^{2+} channel activity is very poorly understood from a biochemical perspective. Cell membrane depolarization presumably exerts an electrical force on charged amino acid residues gating ionic Ca^{2+} channels [11]. In support of this hypothesis, the dependence of channel activity on membrane potential is quite satisfactorily described in terms of Boltzmann equations, which define the probability of finding a charged particle in a particular conformation [11]. Nevertheless, some scientists favor the view that Ca^{2+} channel activity is primarily regulated via mechanisms involving phosphorylation and dephosphorylation of channel proteins [12, 13]. Under physiological conditions, VOCCs are highly selective for Ca^{2+} and exhibit a low permeability for other mono- and divalent cations such as Na^+, K^+, and Mg^{2+} [13]. In should be recognized, however, that Ca^{2+} selectivity is not absolute, since the channels pass divalent Sr^{2+} and Ba^{2+} with similar efficiency [13]. Interestingly, T- and L-type channels both lose their selectivity for divalent cations and start to pass monovalent cations when Ca^{2+} concentrations in the external medium drop below 10^{-7} M [14]. Hess and co-workers therefore suggested that the presence of two neighboring, high-affinity Ca^{2+} binding sites inside the Ca^{2+} channel is a precondition for maintenance of selectivity for divalent cations [14, 15]. As long as the external medium contains enough Ca^{2+}, interactions of Ca^{2+} with specific binding sites trigger conformational changes and reduce the permeability for Na^+ and divalent cations such as Mg^{2+}. In the absence of Ca^{2+}, both Ca^{2+} binding sites are unoccupied, and VOCCs consequently lose their selectivity for divalent cations and become highly permeable for monovalent cations such as Na^+. Remarkably enough, decreases in intracellular pH shift the voltage dependence of activation (by 15 mV per pH unit) and reduce the availability of Ca^{2+} channels due to screening of negatively charged, gating amino acid residues [16]. The Ca^{2+} permeability of VOCCs consistently is down-regulated when intracellular pH is made more acidic and up-regulated when cell pH is alkalinized [17].

Various agents have been described to block Ca^{2+} entry across VOCCs [9, 18]. These so-called Ca^{2+} channel antagonists may be divided into two main groups: divalent inorganic cations (Co^{2+}, Mn^{2+}, Ni^{2+}, and Cd^{2+}) and organic compounds classified into dihydropyridines (nifedipine, nicardipine, nisoldipine), phenylalkylamines (verapamil, gallopamil), and benzothiazepines (diltiazem). In general, L-type channels are considered to be more sensitive to organic Ca^{2+} channel blockers. However, in smooth muscle the difference in susceptibility between the two types of Ca^{2+} channels is not as great as found in other excitable tissues [18]. The inhibitory effect of organic Ca^{2+} antagonists presumably results from interactions with specific binding sites that are located adjacent to the Ca^{2+} binding domains of Ca^{2+} channels and lock the channel protein in a closed conformation. Interestingly, neither nifedipine, verapamil, nor diltiazem has proven effective in asthma [19]. Results suggest that Ca^{2+} entry via VOCCs is of minor importance in airway smooth muscle contractions.

It is worth noting that stimulation of high conductance, Ca^{2+}-activated K^+ channels leads to a hyperpolarization of the plasma membrane, which exerts a dampening effect on Ca^{2+} influx across VOCCs and provides a feedback loop to terminate excitation–contraction coupling [20].

PHARMACOMECHANICAL COUPLING

In this case, activation of the contractile machinery occurs without detectable changes in membrane potential. Binding of an agonist to its surface receptor activates the phosphoinositide messenger system, triggers Ca^{2+} release from the sarcoplasmic reticulum, and enhances Ca^{2+} influx across receptor-operated Ca^{2+} channels in the plasma membrane. Agents such as angiotensin II or vasopressin cause an immediate activation of PLC via a specific, stimulatory guanine nucleotide binding protein (G_q) [21, 22]. The subsequent breakdown of PIP_2 leads to an intracellular release of $InsP_3$ and DAG. $InsP_3$ binds to a specific receptor at the sarcoplasmic reticulum with a K_d value of *ca.* 2.4 nM and mobilizes Ca^{2+} from intracellular storage sites, while the second cleavage product, DAG, activates PKC [3, 23, 24]. The initial rise in $[Ca^{2+}]_i$ from a basal level of *ca.* 100 nM to around 300 nM exerts a potentiating effect on $InsP_3$-induced Ca^{2+} release, presumably by stabilizing the $InsP_3$ receptor in a conformation that intensifies interaction of $InsP_3$ with its binding domain [25]. The $InsP_3$ receptor displays the characteristic features of a Ca^{2+}-induced Ca^{2+} release channel with a bell-shaped response curve. Low cytosolic Ca^{2+} concentrations act as coagonists

for receptor activation, reaching a maximum at about 300 nM, while higher Ca^{2+} concentrations exhibit an inhibitory effect on $InsP_3$-induced Ca^{2+} release [25]. The antagonizing effect of elevated $[Ca^{2+}]_i$ on the potency of $InsP_3$ to release stored Ca^{2+} represents a negative feedback loop and restrains mobilization of Ca^{2+} from $InsP_3$-sensitive pools as soon as cytosolic Ca^{2+} concentrations exceed a threshold value of 300 nM. The results of Mignery and co-workers [26] indicate that the $InsP_3$ receptor consists of three main sections: an amino-terminal $InsP_3$ binding domain, an intermediate coupling region, and a transmembrane-spanning, carboxyl-terminal Ca^{2+} channel. The intermediate cytoplasmic loop presumably contains a Ca^{2+} binding site(s) that strengthens the coupling of negatively charged $InsP_3$ to positively charged amino acid residues in the N-terminal region of the $InsP_3$ receptor. Nevertheless, available data suggest the existence of further regulatory Ca^{2+} binding domains, particularly in the transmembrane-spanning region of the Ca^{2+} channel.

It is well accepted today that the sarcoplasmic reticulum of smooth muscle contains a second, caffeine-releasable Ca^{2+} pool that is activated by Ca^{2+} and exhibits the properties of a Ca^{2+}-induced Ca^{2+}-release channel [24, 25, 27]. In the literature, the term ryanodine-sensitive Ca^{2+} channel is generally used to refer to this Ca^{2+} pool, since at low (nanomolar) concentrations the plant alkaloid, ryanodine, locks this Ca^{2+} channel in an open configuration, while ryanodine concentrations above 1 μM exert an inhibitory effect on Ca^{2+}-induced Ca^{2+} release [25, 28]. Nevertheless, there is considerable controversy as to the physiological relevance of this Ca^{2+} pool in excitation–contraction coupling. In smooth muscle of guinea pig taenia caeci, Ca^{2+}-induced Ca^{2+} release from ryanodine-sensitive sources was delayed until $[Ca^{2+}]_i$ exceeded 1 μM [27]. Results suggest that this Ca^{2+} channel plays a minor role in triggering physiological contractions, since in these smooth muscles tension started to develop at cytosolic Ca^{2+} concentrations just above 100 nM, and maximal contractions were reached at $[Ca^{2+}]_i$ of 1–3 μM [29]. It is possible, however, that Ca^{2+} influx across the plasma membrane into superficial compartments of the sarcoplasmic reticulum may lead to high local Ca^{2+} concentrations that activate the Ca^{2+}-induced Ca^{2+} release mechanism, while the average cytosolic Ca^{2+} concentration is only slightly elevated [30]. Recently, a computer model was developed to study $[Ca^{2+}]_i$ gradients in the neighborhood of passively buffered Ca^{2+} channels [31]. The simulation revealed that at a distance of 1 nm from the pore of L-type Ca^{2+} channels, local $[Ca^{2+}]_i$ may reach concentrations up to 100 μM, but rapidly drop to $[Ca^{2+}]_i$ below 1 μM at distances of ≥100 nm from the pore. In harmony with these considerations, recent experiments in intact smooth muscle tissues indicate that a small Ca^{2+} influx across the plasmalemma is sufficient to trigger an explosive release of stored Ca^{2+} from ryanodine-sensitive Ca^{2+} channels [32]. Moreover, even in smooth muscle of guinea pig taenia caeci, the mean cytosolic Ca^{2+} concentration required for activation of Ca^{2+}-induced Ca^{2+} release decreases drastically to values below 1 μM in the presence of high ATP or low Mg^{2+} concentrations [25, 28]. Interestingly, Ca^{2+}-sensitive ryanodine receptor channels do not possess a selectivity filter, i.e. they pass monovalent and divalent current carriers equally well and are unable to discriminate between divalent cations such as Mg^{2+} and Ca^{2+} [28].

In general, activation of the phosphoinositide–PLC messenger system only leads to a transient rise in cytosolic $InsP_3$ concentration ($T_{1/2} \approx 2$ min) [33, 34]. The metabolism of $InsP_3$ occurs via two separate pathways [34]. The first track covers a sequential series of dephosphorylation reactions culminating in the formation of free *myo*-inositol, whereas the second pathway includes phosphorylation of $InsP_3$ to higher phosphorylated products such as $InsP_4$, $InsP_5$, and $InsP_6$, i.e. inositol-polyphosphates implicated to possess diverse second messenger functions that remain to be elucidated [35]. Strong evidence suggests that $InsP_3$ and its phosphorylated product, Ins(1,3,4,5)P_4, act synergistically in enhancing Ca^{2+} influx across the plasma membrane, thus increasing $[Ca^{2+}]_i$ and/or refilling discharged and $InsP_3$-sensitive Ca^{2+} pools [18, 36]. Consistent with the transient elevation in intracellular $InsP_3$ concentration, the pharmacomechanically induced rapid rise in $[Ca^{2+}]_i$ is predominantly transient in nature, with Ca^{2+} levels returning to values slightly above baseline within minutes. Sequestration of Ca^{2+} back to intracellular storage sites and Ca^{2+} efflux via the membrane-bound Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers are certainly the key elements responsible for the transient character of the Ca^{2+} signal (see below) [5, 13, 22].

As mentioned above, activation of the inositol phospholipid messenger system by PLC not only leads to an intracellular release of $InsP_3$, but also generates DAG, a cleavage product known to trigger a rapid translocation of PKC from the cytoplasm to the plasmalemma and to thereby stimulate enzyme activity [37]. Although it is generally assumed that the PLC-mediated breakdown of PIP_2 results in a sustained increase in DAG content, this is not necessarily the case. Depending on the agonist, the rise in DAG concentration may be transient or sustained. In vascular smooth muscle, angiotensin II activates the phosphoinositide-PLC pathway to cause a transient increase in arachidonate-rich DAG [38]. In contrast, histamine stimulates PIP_2 turnover, but leads to a prolonged rise in DAG content [38]. The transient increase in arachidonate-rich DAG activates PKC in synergism with histamine to catalyze the phospholipase D-related hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA), which is ultimately converted to myristate-rich DAG by a specific PA phosphohydrolase [39]. Characteristically, the hydrolysis of PIP_2 yields DAG enriched in arachidonic acid, whereas PC hydrolysis generates DAG enriched in myristic acid [39]. To further complicate the situation, at least nine different isozymes of PKC have been identified, all of which possess a serine/threonine kinase as catalytic subunit [37]. The distinct pharmacological properties of these diverse

isozymes are currently a matter of intensive scientific investigation. A substantial body of information indicates that PKC acts in synergism with Ca^{2+} to potentiate smooth muscle contractions [40, 41]. Interestingly enough, activation of PKC seems to be associated primarily with the sustained phase of contraction (see below). However, there is strong evidence to support the hypothesis that PKC exerts a dual role in the regulation of smooth muscle contractions [22, 42]. Angiotensin II- and vasopressin-induced hydrolysis of polyphosphoinositides, with subsequent increases in $InsP_3$, DAG, and $[Ca^{2+}]_i$, can be attenuated by stimulation of PKC activity after short-term exposure to tumor-promoting phorbol esters [43–45]. Results suggest that PKC occupies a central function as a negative feedback regulator to terminate activation of PLC, thereby inhibiting further cleavage of PIP_2, to release $InsP_3$, DAG, and Ca^{2+}. The idea that blockage of PKC results in an increased responsiveness of the inositol signaling pathway is in excellent harmony with the observation that PKC inhibitors (H-7 [46], sphingosine [47], and staurosporine [48]) and down-regulation of PKC activity [45] potentiated the angiotensin II-stimulated breakdown of PIP_2. Since the enhanced responsiveness of smooth muscle cells was not associated with a decreased density or affinity of cellular receptors for angiotensin II, these experiments strongly suggest that feedback regulation occurs at a stage distal to receptor occupancy [45]. Most likely, PKC-mediated phosphorylations of G-proteins interrupt signal transduction by restraining dissociation of stimulatory $\alpha_s \cdot$ GTP components from affiliated $\beta\gamma$ subunits [49]. In summary, PKC plays a dual role in the regulation of cell contraction, not only mediating but also terminating cellular responses following agonist challenge [22, 42].

The release of arachidonic acid from arachidonate-rich DAG appears to be a secondary reaction after stimulation of G-protein-coupled PLC activity [50, 51]. The mechanisms responsible for triggering the release of arachidonic acid from phosphoinositides either result from the combined action of PLC and diacylglycerol lipase or from activation of phospholipase A_2 in response to increases in $[Ca^{2+}]_i$. The availability of free arachidonic acid is considered the rate-limiting step for the synthesis of eicosanoids, i.e. prostaglandins, thromboxanes, and leukotrienes. While cyclooxygenase, a multienzyme complex, catalyzes the conversion to thromboxanes (A_2, B) and prostaglandins (PGD_2, PGE_2, PGI_2, 6-keto-$PGF_{1\alpha}$), 5-lipoxygenase converts arachidonic acid to leukotrienes (B_4, C_4, D_4) and hydroxy acids [50–52]. The binding of eicosanoids to specific receptors at the surface of the plasma membrane initiates a wide variety of physiological responses. Current evidence suggests that, depending on the activated receptor subtype, eicosanoids may induce either contractile or relaxant effects [51]. Nevertheless, in smooth muscle tissue the relaxing action of generated prostaglandins (PGD_2, PGE_2, PGI_2) generally predominates and leads to a vasodilatation of terminal arterioles. In contrast, binding of thromboxanes and/or leukotrienes to their associated receptor sites induces smooth muscle contractions, causing arteriolar and venular vasoconstrictions. It is noteworthy that in contrast to the 11- and 7-fold enhancement of PGE_2 synthesis observed after stimulation of mesangial cells, a specialized subtype of vascular smooth muscle cell, with angiotensin II (100 nM) or platelet-activating factor (PAF; 1 μM), neither LTC_4 nor LTD_4, in concentrations up to 1 μM, affected PGE_2 production and/or generation of other prostanoids [53]. This result is of major importance, since the constrictor effects of angiotensin II and PAF are attenuated by concomitant increases in generation of relaxant prostaglandins such as PGE_2 or PGI_2 [53]. The mechanism by which PGE_2 and PGI_2 induce the relaxation of smooth muscle cells has been clearly identified. Both prostanoids dose-dependently stimulate adenylate cyclase and/or guanylate cyclase activity, thereby increasing intracellular cAMP and/or cGMP concentrations and inducing a relaxation of the cells [51, 54]. As outlined by Mené and co-workers [54], PGE_2 and PGI_2 help to maintain the physiological function of mesangial cells by counteracting the contractile effects of vasoconstrictive agents such as angiotensin II and PAF. The inability of leukotrienes to stimulate synthesis of relaxant prostaglandins drastically increases their potency to contract mesangial cells and explains their severe pathological actions on kidney function.

Ca^{2+} EXTRUSION MECHANISMS

In this section, we shall review in more detail the major Ca^{2+} removal mechanisms responsible for maintaining cytosolic Ca^{2+} concentrations at low steady-state levels in resting cells and for returning agonist-induced increases in $[Ca^{2+}]_i$ to basal values during smooth muscle relaxation [13, 55–57]. Diverse enzyme systems have been implicated in contributing to the elimination of Ca^{2+} from the cytoplasm, promoting extrusion of Ca^{2+} into extracellular fluids and/or sequestration back to intracellular organelles such as the sarcoplasmic reticulum and the mitochondria.

Two ATP-driven Ca^{2+} pumps in the plasma membrane (calmodulin-dependent) and the sarcoplasmic reticulum (calmodulin-independent) have been identified to be of particular importance for the maintenance of Ca^{2+} homeostasis in resting smooth muscle cells, thus compensating for passive Ca^{2+} influx across the plasma membrane along a steep electrochemical Ca^{2+} gradient. Both Ca^{2+} pumps possess a high affinity for Ca^{2+} ($K_m \approx 0.2$ to 0.3 μM), and their Ca^{2+} turnover is sufficient to maintain basal $[Ca^{2+}]_i$ at low steady-state levels of 100–200 nM. Since translocations of Ca^{2+} across lipid bilayers are coupled to a counter-transport of protons in a stoichiometric ratio of 1:2, these ATP-driven Ca^{2+} transport systems operate in an electroneutral mode [13, 55–57].

The Ca^{2+}-ATPase of the plasmalemma membrane has a molecular mass of *ca.* 130 kDa, and its activity is strongly stimulated by interaction with calmodulin, which presumably stabilizes the Ca^{2+} pump protein in an active confor-

mation. Nevertheless, available data suggest that regulation of the plasmalemmal Ca^{2+} transport ATPase is very complex. The Ca^{2+} pump is not only activated by calmodulin, but may also be stimulated by cAMP- and cGMP-dependent protein kinases [13, 55–57], by proteolytic cleavage [58], by self-association [59], and by a decrease in the microviscosity of the cell membrane [60]. Moreover, acidic phospholipids, which immediately surround the Ca^{2+}-ATPase and act to embed the membrane protein into the lipid bilayer, have been shown to exert a direct stimulatory action on Ca^{2+} pump activity [61].

The Ca^{2+}-ATPase of the sarcoplasmic reticulum has a molecular mass of *ca.* 105 kDa, and its cytoplasmic loop contains a specific binding domain for phospholamban, a regulatory protein known to exert an inhibitory effect on Ca^{2+} transport activity. In the dephosphorylated state, phospholamban remains permanently attached to the Ca^{2+}-ATPase, but upon phosphorylation dissociates from its binding site, thereby relieving its damping action on the Ca^{2+} pump and enhancing Ca^{2+} turnover [13, 55]. Recent *in vivo* experiments demonstrate that phospholamban is an endogenous substrate of various protein kinases, including Ca^{2+}/calmodulin-dependent protein kinase II, PKC, and cAMP- and cGMP-dependent protein kinases [62, 63].

It has been estimated that in bovine pulmonary artery 43% of the Ca^{2+} pumping activity was due to the Ca^{2+}-ATPase of the sarcoplasmic reticulum and 57% to that of the plasma membrane [64]. Nevertheless, the maximum rate at which these Ca^{2+} pumps can sequester and extrude Ca^{2+} from the cytoplasm is too low ($\leq$1 nmol per mg protein per sec) to account for the rapid rate of Ca^{2+} removal after a challenge with membrane-depolarizing and/or PLC-activating stimuli. As a consequence, additional regulatory mechanisms must be involved in returning elevated $[Ca^{2+}]_i$ to basal values. It is well accepted today that the plasmalemmal Ca^{2+}-ATPase operates in parallel with an Na^+/Ca^{2+} exchanger [5, 56, 65]. Since its Ca^{2+} affinity is rather low ($K_m \approx 2$–5 μM), the Na^+/Ca^{2+} exchanger is particularly active when the cytosolic Ca^{2+} concentration exceeds a specific threshold value, with any fall below this critical level shutting off the exchange mechanism. However, compared with the ATP-driven Ca^{2+} pumps of the plasmalemma and the sarcoplasmic reticulum, the bidirectional Na^+/Ca^{2+} exchange carrier has a much larger transport capacity (or maximum transport rate), reaching values of 30 nmol per mg protein per sec. Since the electrochemical Na^+ gradient across the plasma membrane is the driving force behind Na^+-coupled Ca^{2+} movement, the performance of the Na^+/Ca^{2+} exchanger intimately depends on the activities of membrane-bound Na^+/K^+-ATPases [56, 65–67] and Na^+/H^+ antiporters [68, 69]. A reduction in the Na^+ gradient stimulates both enzyme systems to restore equilibrium Na^+ gradients across the plasmalemma, thereby counteracting membrane depolarization, lowering intracellular pH, catalyzing Ca^{2+} extrusion via the Na^+/Ca^{2+} exchanger, and promoting a relaxation of contracted smooth muscle [69]. Interestingly, the Na^+/H^+ antiporter [68] and the Ca^{2+} transport ATPases of the plasmalemma and sarcoplasmic reticulum all operate in an electrically silent mode, while the Na^+/Ca^{2+} exchanger and Na^+/K^+-ATPase are both electrogenic, exchanging Na^+ for Ca^{2+} and Na^+ for K^+ in a stoichiometric ratio of 3:1 and 3:2, respectively [5, 13, 55–57].

Even though mitochondria have a large capacity to accumulate Ca^{2+}, mitochondrial Ca^{2+} uptake does not play an important role under physiological conditions, because their Ca^{2+} transport affinity ($K_m \approx 10$ μM) is very low. Nevertheless, when $[Ca^{2+}]_i$ exceeds *ca.* 5 μM, mitochondria are capable of sequestering a substantial amount of Ca^{2+} with a high uptake rate ($\leq$10 nmol per mg protein per sec), thereby protecting cells against Ca^{2+} overload under pathological conditions [13, 57]. Mitochondria contain an electrophoretic Ca^{2+} uniporter driven by the large electrochemical potential across mitochondrial membranes, taking up Ca^{2+} without compensation of charge. Notably, the inner leaflets of mitochondrial membranes carry cardiolipin, a negatively charged phospholipid that has a high affinity for Ca^{2+} and is able to bind considerable amounts of it. Moreover, available data suggest that mitochondrial Ca^{2+} uptake may also be coupled to a proton ejection mechanism using an electroneutral Ca^{2+}/H^+ antiporter [13, 57].

SMOOTH MUSCLE CONTRACTION

Transient Phase of Contraction

Changes in cytosolic Ca^{2+} concentration are considered the major determinant in initiation of smooth muscle contractions. Following a challenge with membrane-depolarizing or PLC-stimulating agents, activation of the contractile machinery primarily results from an increase in $[Ca^{2+}]_i$ and correlates with phosphorylation of 30 kDa myosin light chain at serine 19 [1]. Depending on the stimulus, the agonist-induced rise in $[Ca^{2+}]_i$ may be primarily attributed to an enhanced Ca^{2+} influx across voltage- and/or receptor-operated membrane channels and/or to a Ca^{2+} release from the sarcoplasmic reticulum [2].

However, in contrast to the data obtained during the initial phase of contraction, the mechanisms leading to sustained smooth muscle contractions are remarkably different for agents acting via the electromechanical or pharmacomechanical pathway [70].

Sustained Phase of Contraction

In tracheal smooth muscle, tonic contractions could be induced by a challenge with either carbachol or KCl [71]. However, major differences between the action of these agents were apparent. Membrane-depolarizing stimuli such as KCl required higher intracellular Ca^{2+} concentrations to procure the same degree of isometric force, and long-lasting membrane depolarizations and bursts of action potentials were necessary for induction of tetanic contractions. In KCl

contracted smooth muscles, myosin light chain phosphorylation decreased to a minor extent (from 59 to 40%) after 2 hr of sustained contraction [71] and closely correlated with a persistent increase in cytosolic Ca^{2+} concentration [72]. In contrast, after a challenge with carbachol, myosin light chain phosphorylations [71] and $[Ca^{2+}]_i$ time courses [40] were both essentially transient, although a constant level of isometric force was maintained [71]. Obviously, additional regulatory mechanisms must be involved for induction and maintenance of pharmacomechanically induced sustained smooth muscle contractions. The state of force maintenance, with reduced levels of myosin phosphorylation, has been termed the "*latch bridge*" state, implying that phosphorylated actin-myosin cross-bridges are dephosphorylated, while the myosin head remains attached to the actin component and merely forms slowly cycling cross-bridges [73]. The latch bridge hypothesis is in reasonable harmony with the low rates of oxygen and ATP consumption during the maintained phase of contraction [40, 73]. In tracheal smooth muscle, carbachol-induced PIP_2 turnover was associated with a sustained activation of PKC that resulted from a prolonged increase in DAG content induced by a transient rise in arachidonate-rich DAG and persistent elevation in myristoylated DAG [74]. Results suggest that, in the pharmacomechanical case, activation of PKC plays a critical role in maintenance of sustained smooth muscle contractions [40, 70, 75]. In agreement with this hypothesis, PKC-stimulating phorbol esters caused slow, but tonic contractions of tracheal and vascular smooth muscle cells [76, 77]. Strikingly, the generated pattern of phosphoproteins closely matched that observed after agonist challenge with carbachol, but was markedly different from the pattern found after stimulation with KCl [40, 70]. The involvement of PKC received further support from the observation that PKC inhibitors strongly antagonized the tonic phase of carbachol-induced tracheal smooth muscle contractions, but had no effect on contractile responses triggered by a challenge with KCl [70]. Recent experiments in endothelium-denuded porcine pulmonary arteries support these data and demonstrate that preincubation with the PKC inhibitor staurosporine selectively blocked the tonic component of phorbol ester and/or thrombin-stimulated vessel constrictions without having any effect on the phasic component of contraction [33]. However, staurosporine failed to decrease tension in concentrations up to 0.1 μM when added to KCl-precontracted arterial segments [33].

Strikingly, carbachol- and phorbol ester-induced sustained tracheal smooth muscle contractions could be abolished completely by complexation of extracellular Ca^{2+}, and under these conditions no increase in the phosphorylation of any of the late-phase proteins was observed [78]. Obviously, the calcium ions leading to sustained smooth muscle contractions come from the extracellular fluid rather than from internal reservoirs. These results support Murray's hypothesis that the tonic phase of contraction is maintained by a small influx of Ca^{2+} across ROCCs [79]. Even though PKC activation by DAG and/or DAG analogs (12-O-tetradecanoylphorbol-13-acetate) requires Ca^{2+} and phosphatidylserine as cofactors for maximal enzyme activity [42], strong evidence exists that a small Ca^{2+} influx across ROCCs leads to local $[Ca^{2+}]_i$ just at and/or beneath the plasma membrane, which is sufficient to keep membrane-associated PKC enzymes in an activated state [31, 40, 70, 80]. Recent results demonstrate that cAMP may relieve sustained smooth muscle contractions by impairing enzymes of the PKC branch of the Ca^{2+} messenger system and by activating phosphatases that catalyze dephosphorylation of a number of late-phase phosphoproteins [41]. The physiological functions of some of these regulatory phosphoproteins, including those of desmin, caldesmon, synemin, and calponin, are well understood today [70]. Nevertheless, the majority of low-molecular-weight phosphoproteins remains to be identified and functionally characterized [41, 70]. In smooth muscle, caldesmon, a component of the actin-myosin-tropomyosin domain, is considered to play a key role in the maintenance of sustained contractions [78, 81]. Diverse protein kinases including PKC cause a rapid phosphorylation of caldesmon, thereby triggering dissociation of this regulatory peptide from its binding site at the myosin moiety, enabling slow cyclic actin-myosin interactions and hence contraction to occur. Strikingly, the extent of the phosphorylation of caldesmon not only follows the time course of PKC activation, but this key enzyme becomes dephosphorylated as soon as smooth muscle relaxes [78]. Nevertheless, a wide variety of other phosphoproteins has also been implicated in playing an important role during the tonic phase of contraction. The results of Rasmussen *et al.* [40] indicate that various structural components of the filamin-actin-desmin fibrillar domain are phosphorylated rapidly by PKC-dependent processes and exhibit a modulatory effect on induction and maintenance of sustained smooth muscle contractions.

In summary, after stimulation with KCl, the extent of myosin light chain phosphorylation closely correlates with the contractile response even during the sustained phase of contraction, while the mechanism by which pharmacomechanical agonists induce tonic smooth muscle contractions depends on the induction of biochemical events that activate the PKC branch and lead to a dissociation of maintained force and myosin light chain phosphorylation.

MODULATION OF EXCITATION–CONTRACTION COUPLING

Despite the fact that increases in $[Ca^{2+}]_i$ are the main factor in determining the strength of smooth muscle contractions, various biochemical agents have been implicated in modulating the efficacy of the excitation–contraction coupling, primarily by activating enzymes that trigger removal of Ca^{2+} from the cytoplasm or that decrease the responsiveness of the contractile machinery toward Ca^{2+}.

MODULATION OF Ca^{2+} TRANSIENTS BY CYCLIC NUCLEOTIDES

In smooth muscle, regulation of cytosolic Ca^{2+} concentrations and of the $[Ca^{2+}]_i$-sensitivity of contraction are under potent feedback control by cyclic nucleotides. Agonist-induced increases in $[cAMP]_i$ and/or $[cGMP]_i$ consequently play an important role in relaxation of contracted smooth muscle [82]. As the intracellular concentrations of cyclic nucleotides are regulated by their rates of synthesis and degradation, these concentrations crucially depend on the activity ratios of adenylate cyclase (or guanylate cyclase) and their corresponding phosphodiesterases [83, 84]. Diverse endo- and exogenous mediators have been identified to exert a stimulatory or inhibitory effect on synthesis and/or degradation rates and thereby to modify intracellular cAMP and cGMP levels.

As far as cAMP is concerned, increases in $[cAMP]_i$ primarily result from a stimulation of adenylate cyclase activity by agents such as PGE_2, forskolin, or β_2-adrenoceptor agonists or from the restraining action of phosphodiesterase inhibitors such as rolipram or theophylline [83, 84]. The dominant mechanism by which cAMP impairs excitation–contraction coupling in smooth muscle depends on its down-regulating effect on cytosolic Ca^{2+} concentration and includes activation of cAMP-dependent protein kinases (PKAs) [82, 85]. Contrasting with earlier conclusions, the inhibitory effect of PKA on myosin light chain kinase and on force development could be observed only at very low, and not at moderately high $[Ca^{2+}]_i$ [86, 87]. This suggests that activation of the cAMP branch usually does not lead to a decreased sensitivity of contractile structures toward Ca^{2+} (see below). The Ca^{2+} transport ATPases of the plasma membrane and the sarcoplasmic reticulum are both stimulated by cAMP-dependent protein kinases. Phosphorylation of the plasmalemmal Ca^{2+} pump at a serine residue near the calmodulin-binding domain has been shown to increase its affinity for Ca^{2+} and enhance Ca^{2+} turnover approximately 2-fold [88]. In contrast, regulation of the Ca^{2+}-ATPase of the sarcoplasmic reticulum occurs indirectly by PKA-dependent phosphorylation of phospholamban, a protein unit that in its dephosphorylated state exerts a damping action on the Ca^{2+} pump, but upon phosphorylation dissociates from its binding site at the Ca^{2+}-ATPase, thus relieving its inhibitory effect on Ca^{2+} turnover [13, 55, 62, 63]. Further sites of action of cAMP-dependent protein kinases include activation of Na^+/K^+-ATPases [66, 67] and stimulation of Na^+/Ca^{2+} exchangers [89] and of Ca^{2+}-activated K^+ channels, thereby diminishing Ca^{2+} influx across VOCCs [90, 91]. In addition, PKAs have been shown to lower Ca^{2+} influx across the plasma membrane [92, 93] and to inhibit the phospholipase C-mediated phosphoinositide hydrolysis so that agonist-induced formation of $InsP_3$ and DAG is suppressed, and the mobilization of Ca^{2+} from internal stores consequently lowered [41].

In smooth muscle, the conversion of GTP to cGMP is catalyzed by both soluble and membrane-bound forms of guanylate cyclase. Endothelium-derived relaxing factor or other nitric oxide-releasing compounds strongly stimulate soluble guanylate cyclase activity [94]. In contrast, the particulate enzyme is activated by atrial natriuretic peptides and *Escherichia coli* heat-stable enterotoxin [94]. In arterial smooth muscle, activation of the cGMP messenger system resulted in a significant decrease in cytosolic Ca^{2+} concentration [95] and antagonized the mobilization of Ca^{2+} after a challenge with vasopressin [96]. Various sites of action have been suggested for cGMP-dependent protein kinases, including the target enzymes involved in the regulation of $[Ca^{2+}]_i$ by protein kinases of the cAMP branch. Nevertheless, the relative importance of these different sites of action still awaits to be established. Recent *in vitro* and *in vivo* experiments demonstrate that cGMP-dependent protein kinases are at least as effective as PKA in phosphorylating phospholamban and lead to a rapid activation of the Ca^{2+}-ATPase of the sarcoplasmic reticulum [63, 97]. However, most results support the view that the transmembrane-spanning Ca^{2+}-ATPase of the plasmalemma is itself not a substrate of cGMP-dependent protein kinases, but is activated indirectly via phosphorylation of associated proteins [55, 98]. In vascular smooth muscle, cGMP-mediated phosphorylations have been clearly implicated in inhibiting the pharmacomechanically induced breakdown of phosphoinositides and, thereby, in antagonizing the generation of $InsP_3$, DAG, and Ca^{2+} [94]. Interestingly enough, cGMP-dependent protein kinases may also be activated by increases in cAMP [82, 94]. However, compared with cGMP, *ca.* 10-fold higher cAMP concentrations are required to stimulate protein kinases of the cGMP branch [99]. The possibility that part of the biochemical action of cAMP may be transduced by activation of the cGMP messenger system should not be excluded. As shown by Rembold, basal and histamine-stimulated intracellular levels of cAMP are typically 10-fold higher than those of cGMP, so that sufficient cAMP molecules should be generated after a challenge with pharmacomechanical agonists to activate both messenger systems, i.e. cAMP- and cGMP-dependent protein kinases [22, 100]. Such cross-activation of the cGMP branch by cAMP was observed recently and resulted in a relaxation of arterial smooth muscle [101]. Notably, there exists an alternative pathway by which cAMP may enhance the activity of cGMP-dependent protein kinases. Experiments using analogs of cAMP and/or forskolin demonstrated that in smooth muscle increases in $[cAMP]_i$ amplify the transcription and expression of the inducible nitric oxide synthase gene and consequently the synthesis of nitric oxide through the L-arginine/L-citrulline pathway [102]. Nitric oxide is a potent activator of soluble guanylate cyclase and substantially elevates the intracellular level of cGMP, which subsequently stimulates cGMP-dependent protein kinases [103].

In analogy to the effect of cAMP on the cGMP branch, cAMP-dependent protein kinases can also be activated by

increases in cGMP. However, compared with cAMP, approximately 10-fold higher cGMP concentrations are required to activate the protein kinases of the cAMP branch, as a consequence of which intracellular levels of cGMP are generally too low to activate PKA [82, 100].

In summary, cAMP- and/or cGMP-dependent protein kinases contribute synergistically to decrease Ca^{2+} influx across the plasma membrane, to catalyze sequestration of Ca^{2+} back to intracellular storage sites, to stimulate extrusion of Ca^{2+} into the extracellular space, and to downregulate the generation of Ca^{2+}-releasing $InsP_3$. The net effect of all these processes results in a reduced cytosolic Ca^{2+} concentration and causes a relaxation of smooth muscle [82, 85, 104]. Nevertheless, the results of Rasmussen and Barrett [105] indicate that interactions between the calcium and cyclic nucleotide messenger systems are somewhat more complicated than previously explained. Cyclic nucleotides not only reduce cytosolic Ca^{2+} concentrations, but the latter, in turn, modulate the intracellular concentrations of cAMP and cGMP by regulating the activities of adenylate cyclase, guanylate cyclase, and certain phosphodiesterases.

MODULATION OF THE CONTRACTILE RESPONSE TOWARD Ca^{2+}

Early investigation demonstrated that stimulation of β_2-adrenoceptors may result in a dissociation between $[Ca^{2+}]_i$ and force [106]. Since these changes were associated with a phosphorylation of MLCK, it was concluded that the sensitivity of the contractile system to $[Ca^{2+}]_i$ is variable and drastically lowered by phosphorylation of MLCK. In support of this hypothesis, the results of Goldie and co-workers [92] indicated that phosphorylated MLCK exhibits a lower affinity for the $Ca^{2+}{}_4$–calmodulin complex, causing a decrease in the phosphorylation of 20 kDa myosin light chains and a concomitant reduction in actin–myosin coupling. The term $[Ca^{2+}]_i$-sensitivity of phosphorylation was therefore coined to specify the dependence of myosin light chain phosphorylation on $[Ca^{2+}]_i$. When the $[Ca^{2+}]_i$-sensitivity of the contractile system is high, small increases in $[Ca^{2+}]_i$ result in extensive phosphorylations of the myosin light chain. Conversely, the $[Ca^{2+}]_i$-sensitivity of phosphorylation is low when large increases in $[Ca^{2+}]_i$ are required to induce minor changes in myosin light chain phosphorylation.

Recent phosphopeptide mapping studies have revealed that there exist at least six distinct phosphorylation sites (A–F) on MLCK [86]. The primary site of phosphorylation depends on various conditions and varies with the agonist used. Strikingly, only phosphorylation at peptide site A was associated with a decreased $[Ca^{2+}]_i$-sensitivity, while phosphorylation on peptides B–F did not result in a reduced activity of MLCK. In other words, only phosphorylation at peptide site A impairs the ability of MLCK to phosphorylate myosin light chain and decreases the $[Ca^{2+}]_i$-sensitivity of the contractile system. In harmony with these results, phosphorylation at site A specifically incorporated phosphate at a serine residue near the calmodulin-binding domain of MLCK, i.e. at a position implicated in playing an important physiological function in the delicate regulation of MLCK activity. Remarkably enough, the extent of phosphorylation at site A strictly correlated with increases in the concentration of Ca^{2+}/calmodulin complex required for activation of MLCK.

It is well accepted today that cAMP-dependent protein kinases are capable of phosphorylating MLCK. The results of Stull and co-workers [86] clearly indicate that the sites of phosphorylation strongly depend on the microenvironment within the cell. In the absence of Ca^{2+}/calmodulin, cAMP-dependent protein kinases phosphorylated purified MLCK from gizzard smooth muscle at two amino acid residues (sites A and B), and the concentration of the Ca^{2+}/calmodulin complex required for half-maximal activation of MLCK increased by a factor of *ca.* 10 [86, 87]. Conversely, in the presence of Ca^{2+}/calmodulin, phosphate was incorporated exclusively into site B by cAMP-dependent phosphorylations and had no effect on MLCK activity [86, 87].

Analogous to the results obtained with PKA, MLCK may also be phosphorylated and inhibited by cGMP-dependent protein kinases [107]. However, the physiological relevance of cGMP-dependent phosphorylations in the regulation of smooth muscle contractions is still a matter of scientific discussion. Recently, considerable amounts of evidence have accumulated supporting the notion that the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II is one of the key elements in inducing desensitization of the contractile system toward Ca^{2+} by phosphorylation of MLCK at its regulatory site A [86, 87]. Published data suggest that MLCK phosphorylations by Ca^{2+}/calmodulin-dependent protein kinase II are at least partially responsible for the lower $[Ca^{2+}]_i$-sensitivity observed after a challenge with KCl [108]. The large increases in $[Ca^{2+}]_i$ triggered by KCl-induced membrane depolarizations supposedly activate the Ca^{2+}/calmodulin-dependent protein kinase II and thus decrease the sensitivity of the contractile machinery toward Ca^{2+}.

Of course, the extent of myosin light chain phosphorylation not only depends on the activities of protein kinases to phosphorylate MLCK, but also on the efficiencies of phosphatases to catalyze dephosphorylation of MLCK and/or of the myosin light chain. It is well documented that various metabolites of the phosphoinositide pathway possess a sensitizing effect on the contractile response through inhibition of myosin light chain phosphatase (MLCPase). For example, the potentiating action of arachidonic acid on force development in rat femoral artery strips at constant $[Ca^{2+}]_i$ resulted from its inhibitory effect on myosin light chain phosphatase and was accompanied by an increased phosphorylation of the myosin light chain [109]. Moreover, unhydrolyzable GTP analogs lead to an increase in the $[Ca^{2+}]_i$-sensitivity of contraction due to inhibition of MLCK phosphorylation and impairment of myosin light

chain phosphatase [82, 110]. Thus, desensitization of the contractile system toward Ca^{2+} is caused primarily by an altered balance between the activities of MLCK and MLCPase and results in a decreased phosphorylation/dephosphorylation ratio.

INFLUENCE OF MEMBRANE MICROVISCOSITY ON $[Ca^{2+}]_i$ AND EXCITATION–CONTRACTION COUPLING

The physical state, or microviscosity, of the plasma membrane has been implicated in altering the conformation of transmembrane-spanning ion channels and/or in changing the activities of enzymes embedded in the phospholipid matrix [60, 111]. As shown by Stubbs and Smith [60], a decrease in membrane microviscosity may lead to substantial modifications in the local organization within the plasmalemma (e.g. lateral phase separations) and facilitate conformational transitions coupled to the catalytic functions of enzymes. Since the ring of lipids that immediately surround a specific enzyme acts to solvate the membrane protein into the lipid bilayer, it is not surprising that the microviscosity of the cell membrane not only influences the lateral migration of enzymes, but also affects internal motions of groups and peptide chains connected to their catalytic function [60, 111]. This explains the high sensitivity of enzyme activities to minor changes in the microenvironment.

The physical properties of membranes, in particular their fluidity or rigidity, closely correlate with the composition and state of phospholipid bilayers. Polyunsaturated fatty acids, phosphatidylcholine, and phosphatidylethanolamine have been shown to increase membrane fluidities, whereas proteins, cholesterol, and sphingomyelin act as rigidifiers [60, 112]. In addition, the results of Storch and Schachter [113] indicate that high extracellular Ca^{2+} concentrations decrease the fluidity of phospholipid bilayers by promoting direct binding of Ca^{2+} to phospholipid head groups and by stimulating the activity of membrane-bound enzymes that alter the acyl chain composition of the plasmalemma, i.e. by decreasing the double-bond index in the fatty acid region. Consistently, most membrane-associated and negatively charged phospholipids possess high affinity constants for Ca^{2+} and are able to bind considerable amounts of Ca^{2+} [13].

Based on the considerations of Stubbs and Smith [60], enzyme activities are expected to be enhanced as the membrane microviscosity is lowered. However, it should be kept in mind that the lipids found in the intimate neighborhood of an enzyme may also exert a direct stimulatory or inhibitory effect on enzymatic functions. As mentioned above, acidic phospholipids exhibit a direct stimulatory action on the plasmalemmal Ca^{2+}-ATPase, presumably by interference with the calmodulin-binding site [61]. The mechanism of activation of Ca^{2+} pump proteins by phosphatidylethanolamine is even more complex, since ATP-driven Ca^{2+} turnover is stimulated directly as well as indirectly by reduction of bilayer fluidity [114]. Strikingly, the activities of the Ca^{2+} transport ATPases of the plasmalemma and the sarcoplasmic reticulum are extremely sensitive to minor changes in membrane fluidity [55, 60]. Compared with the control group, Ca^{2+} pump turnover was actually reduced in intact erythrocyte membranes of spontaneously hypertensive rats due to a higher membrane microviscosity [115]. Dominiczak and Bohr [116] therefore suggested that in hypertensive patients hypercholesterolemia leads to an increase in cholesterol content of membranes of vascular smooth muscle, causing phospholipid bilayers to become more rigid and enhancing their permeability to Ca^{2+}. In harmony with these considerations, excess membrane cholesterol reduced bilayer fluidity in artery smooth muscle [117], increased basal and norepinephrine-activated Ca^{2+} influx across the plasma membrane, and enhanced the $[Ca^{2+}]_i$-sensitivity of the contractile system [118]. Moreover, cholesterol enrichment in synthetic bilayers and reconstituted membranes depressed the activities of plasmalemmal Na^+/K^+-ATPase [119] and of Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum [120]. Interestingly, cultured vascular smooth muscle cells isolated from stroke-prone spontaneously hypertensive rats showed all the pathological features found in cholesterol-enriched artery smooth muscle [117, 118]. In contrast with the results of Storch and Schachter [113], a 5 mM increase in extracellular Ca^{2+} concentration significantly reduced elevated membrane microviscosities in vascular smooth muscle of spontaneously hypertensive rats toward the values found in normotensive animals, depressed Ca^{2+} influx across the plasmalemma lipid bilayer, and antagonized all other pathophysiological effects observed in cholesterol-enriched artery smooth muscle [117, 118, 121]. Remarkably enough, it has been recognized for more than three decades that elevated extracellular Ca^{2+} concentrations exhibit a membrane-stabilizing effect, depress membrane excitability, and lower vascular reactivity toward pharmacomechanical and/or electromechanical agonists [111, 121]. In summary, whatever the precise mechanism (altered cholesterol/phospholipid ratio or unsaturated fatty acid content), the cell membranes of patients with essential hypertension are stiffer, resulting in a reduced capacity of vascular smooth muscles to extrude Ca^{2+} from the cytoplasm [116].

In harmony with these data, it has been shown recently that pretreatment of vascular smooth muscle cells with formoterol, a β_2-adrenoceptor agonist, decreases steady-state microviscosity of the plasmalemma lipid bilayer and concurrently leads to a faster elimination of Ca^{2+} from the cytoplasm after a challenge with angiotensin II [122]. Characteristically, the β_2-adrenergic receptor is linked to the ultimate cellular response (i.e. muscle relaxation) by a transduction mechanism that consists of a stimulatory G protein (G_s) and the catalytic subunit of adenylate cyclase. The interaction has been termed "collision coupling" and has been found to be enhanced when the microviscosity of the cell membrane is lowered [60, 111, 123]. The results of Houslay and Gordon [123] show that the activity of adenylate cyclase is regulated by the nature of its lipid

environment and that a decrease in membrane microviscosity has a potentiating effect on its catalytic function. Notably, increases in the content of unsaturated fatty acids enhance adenylate cyclase activity directly as well as indirectly by increasing bilayer fluidity [123]. Whether long-term exposure of cultivated smooth muscle cells to formoterol alters the double bond index in the acyl chain region as expected from a higher Ca^{2+} turnover is currently a matter of investigation [113]. Nevertheless, our data demonstrate that intercalation of formoterol into cell membranes lowers their microviscosity and consequently leads to a stronger coupling between the β_2-adrenoceptor glycoprotein and adenylate cyclase [60, 123]. Moreover, published data suggest that the activities of phospholipase A_2, phospholipase C, PKA, and myosin light chain kinase are also enhanced due to the higher fluidity of the hydrophobic environment in muscle fibers following a pretreatment with formoterol [60].

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