Brief Review

Regulation of Contraction and Relaxation in Arterial Smooth Muscle

Christopher M. Rembold

Intracellular calcium concentration ([Ca\(^{2+}\)])-dependent activation of myosin light chain kinase and its phosphorylation of the 20-kd light chain of myosin is generally considered the primary mechanism responsible for regulation of contractile force in arterial smooth muscle. However, recent data suggest that the relation between [Ca\(^{2+}\)], and myosin light chain phosphorylation is variable and depends on the form of stimulation. The dependence of myosin phosphorylation on [Ca\(^{2+}\)], has been termed the "[Ca\(^{2+}\)], sensitivity of phosphorylation." The [Ca\(^{2+}\)], sensitivity of phosphorylation is "high" when relatively small increases in [Ca\(^{2+}\)], induce a large increase in myosin phosphorylation. Conversely, the [Ca\(^{2+}\)], sensitivity of phosphorylation is "low" when relatively large increases in [Ca\(^{2+}\)], are required to induce a small increase in myosin phosphorylation. There are two proposed mechanisms for changes in the [Ca\(^{2+}\)], sensitivity of phosphorylation: Ca\(^{2+}\)-dependent decreases in the [Ca\(^{2+}\)], sensitivity of phosphorylation induced by phosphorylation of myosin light chain kinase by Ca\(^{2+}\)-calmodulin protein kinase II and agonist-dependent increases in the [Ca\(^{2+}\)], sensitivity of phosphorylation by inhibition of a myosin light chain phosphatase. I will review the proposed mechanisms responsible for the regulation of [Ca\(^{2+}\)], and the [Ca\(^{2+}\)], sensitivity of phosphorylation in arterial smooth muscle. (Hypertension 1992;20:129–137)

KEY WORDS • aequorin • calcium • calcium sensitivity • desensitization • fura-2 • myosin light chain kinase • phosphorylation • muscle, smooth, vascular

Most contractile stimuli induce arterial smooth muscle contraction by increasing myoplasmic calcium concentration ([Ca\(^{2+}\)]) (see Figure 1 for schematic). Ca\(^{2+}\) binds to calmodulin and the Ca\(^{2+}\)-calmodulin complex removes the autoinhibition of myosin light chain kinase.1 The activated myosin light chain kinase phosphorylates the 20-kd light chain of myosin on serine 19 and activates the myosin's ATPase.2 The phosphorylated myosin cyclically binds to actin filaments producing force or shortening, or both.3 This is the most widely accepted mechanism for the primary regulation of smooth muscle contraction. However, recent data suggest that the above regulatory scheme may be incomplete. In this review, I will discuss 1) the mechanisms regulating [Ca\(^{2+}\)], 2) the mechanisms altering the dependence of myosin phosphorylation on [Ca\(^{2+}\)], (i.e., the [Ca\(^{2+}\)], sensitivity of phosphorylation), and 3) the dependence of contractile force on myosin phosphorylation.

Regulation of Myoplasmic [Ca\(^{2+}\)]

Contractile stimuli affect [Ca\(^{2+}\)] by a number of mechanisms (see Figure 1): 1) Regulation of [Ca\(^{2+}\)], by changes in membrane potential has been termed "electromechanical coupling."4 Depolarization activates L-type Ca\(^{2+}\) channels, inducing Ca\(^{2+}\) influx, elevated [Ca\(^{2+}\)], and contraction.5 Some agents (e.g., those that increase cyclic AMP (cAMP) or cyclic GMP (cGMP)) induce relaxation by activating K\(^{+}\) channels, inducing hyperpolarization and decreasing Ca\(^{2+}\) influx.6,7 Regulation of [Ca\(^{2+}\)], independent of changes in membrane potential was termed "pharmacomechanical coupling."4 There are several proposed mechanisms for pharmacomechanical coupling. One is that contractile agonist-activated G proteins8,9 increase 1,4,5-inositol trisphosphate levels (1,4,5-IP\(_3\)).10-13 The 1,4,5-IP\(_3\) releases Ca\(^{2+}\) from intracellular stores,14-16 leading to contraction. Another is that contractile agonists also increase Ca\(^{2+}\) influx,17-20 which induces a sustained contraction.21 However, the mechanism responsible is controversial. Stimulated Ca\(^{2+}\) influx may be caused either by increasing open probability of L-type Ca\(^{2+}\) channels at a constant membrane potential22,23 or by activating "receptor operated" nonspecific ion channels.24-26 Finally, 3) agonist-dependent increases in cAMP or cGMP may enhance Ca\(^{2+}\) extrusion and sequestration27-31 and therefore decrease [Ca\(^{2+}\)], and induce relaxation.

Regulation of [Ca\(^{2+}\)], Sensitivity of Phosphorylation

A fourth mechanism for pharmacomechanical coupling has been described: some contractile stimuli alter the dependence of myosin phosphorylation on [Ca\(^{2+}\)].32 The relative dependence of myosin phosphorylation on [Ca\(^{2+}\)], has been termed the "[Ca\(^{2+}\)], sensitivity of phosphorylation." The [Ca\(^{2+}\)], sensitivity of phosphorylation is "high" when relatively small increases in [Ca\(^{2+}\)],...
induce a large increase in myosin phosphorylation. Conversely, the [Ca\(^{2+}\)] sensitivity of phosphorylation is “low” when relatively large increases in [Ca\(^{2+}\)] are required to induce a small increase in myosin phosphorylation. I will extensively review the data supporting a variable [Ca\(^{2+}\)] sensitivity of phosphorylation and two mechanisms that appear to regulate changes in the [Ca\(^{2+}\)] sensitivity of phosphorylation.

Biochemical studies suggest that myosin light chain kinase activity and therefore myosin phosphorylation levels should be proportional to [Ca\(^{2+}\)]. We found a correlation between changes in aequorin-estimated myoplasmic [Ca\(^{2+}\)] and myosin phosphorylation in the KCl depolarized swine carotid artery. However, not all contractile stimuli induced a similar relation between phosphorylation and [Ca\(^{2+}\)]. Contraction agonists such as histamine or phenylephrine induced a higher [Ca\(^{2+}\)] sensitivity of phosphorylation than that observed with stimuli that activate G proteins, increase myoplasmic [Ca\(^{2+}\)] and force. A summary of the stimuli shown to induce various degrees of [Ca\(^{2+}\)] sensitivity of phosphorylation in the swine carotid artery (determined by the relation between aequorin-estimated myoplasmic [Ca\(^{2+}\)] and myosin phosphorylation levels) is qualitatively shown in Table 1. Several patterns are apparent from these data. High [Ca\(^{2+}\)] sensitivity of phosphorylation tends to be observed with stimuli that activate G proteins, increase 1,4,5-IP3, and release the intracellular Ca\(^{2+}\) store (e.g.,

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**Figure 1.** Schematic of the hypothesized [Ca\(^{2+}\)]-dependent regulatory mechanisms for contraction and relaxation present in arterial smooth muscle. EDRF, endothelium-derived relaxing factor; \(G_p\), phospholipase C activating G protein; \(G_{ac}\), adenylyl cyclase activating G protein; PLC, phospholipase C; G cyclase, guanylate cyclase; A cyclase, adenylate cyclase; SR, sarcoplasmic reticulum; MLCK, myosin light chain kinase; A kinase, cyclic AMP-dependent protein kinase; G protein kinase; CAM, calmodulin; Ca-CAMPKII, Ca\(^{2+}\)-calmodulin protein kinase II; A, actin; M, myosin; \(M_p\), phosphorylated myosin; AMp, attached phosphorylated myosin; AM, attached dephosphorylated myosin (i.e., latch bridge).

**Figure 2.** Panel A: Scatterplot shows dependence of myosin phosphorylation on the aequorin-estimated myoplasmic [Ca\(^{2+}\)] shown as log \(L/L_{max}\) change (i.e., the [Ca\(^{2+}\)] sensitivity of phosphorylation) in tissues stimulated with agents that induced high [Ca\(^{2+}\)], sensitivity of phosphorylation (open symbols, dashed line) or agents that induced low [Ca\(^{2+}\)], sensitivity of phosphorylation (filled symbols, solid line). Data are mean±SEM with \(n\geq 4,32,34\). Panel B: Scatterplot shows dependence of steady-state stress (normalized force) on myosin light chain phosphorylation. Steady state was defined as at least 30 minutes of stimulation with no recent change in force. Values are mean±SEM with \(n\geq 4,32,34\). Solid line is the latch bridge hypothesis prediction assuming that a basal phosphorylation artifact of 0.07 mol P/mol myosin light chain (MLC). Dashed line is the latch bridge model’s prediction if phosphorylase activity is decreased by 50%.34

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The dependence of force on phosphorylation is nonlinear, reported changes in the [Ca\(^{2+}\)] sensitivity of force, without measurements of myosin phosphorylation, should be interpreted with caution.

A summary of the stimuli shown to induce various degrees of [Ca\(^{2+}\)] sensitivity of phosphorylation in the swine carotid artery (determined by the relation between aequorin-estimated myoplasmic [Ca\(^{2+}\)] and myosin phosphorylation levels) is qualitatively shown in Table 1. Several patterns are apparent from these data. High [Ca\(^{2+}\)] sensitivity of phosphorylation tends to be observed with stimuli that activate G proteins, increase 1,4,5-IP3, and release the intracellular Ca\(^{2+}\) store (e.g.,

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The diagram shows the hypothesized [Ca\(^{2+}\)]-dependent regulatory mechanisms for contraction and relaxation. The schematic illustrates the roles of various components such as endothelium-derived relaxing factor (EDRF), adenylyl cyclase (G\(_{ac}\)), phospholipase C (PLC), guanylate cyclase (G cyclase), and sarcoplasmic reticulum (SR). The figure highlights the interaction between these components and their effects on myosin phosphorylation and contractile force. The text explains the significance of changes in [Ca\(^{2+}\)] on phosphorylation and force, with a detailed discussion of the mechanisms and stimuli that affect these parameters.
Table 1. Relative [Ca2+]i Sensitivities Observed With Various Stimuli in the Swine Carotid Artery

<table>
<thead>
<tr>
<th>[Ca2+]i Sensitivity of Phosphorylation</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Highest [Ca2+]i Sensitivity</td>
<td></td>
</tr>
<tr>
<td>High dose phorbol diesters (1 μM phorbol dibutyrate)</td>
<td>33</td>
</tr>
<tr>
<td>High [Ca2+]i Sensitivity</td>
<td></td>
</tr>
<tr>
<td>Histamine (1, 10, and 100 μM)</td>
<td>32</td>
</tr>
<tr>
<td>Phenylephrine (10 and 100 μM)</td>
<td>32</td>
</tr>
<tr>
<td>Endothelin (100 nM, sustained response)</td>
<td>34</td>
</tr>
<tr>
<td>Low dose phorbol diesters (10 nM phorbol dibutyrate)</td>
<td>33</td>
</tr>
<tr>
<td>Histamine (1 μM and 2.5 mM F- )</td>
<td>34</td>
</tr>
<tr>
<td>Histamine (10 μM and 2.5 mM F- )</td>
<td>34</td>
</tr>
<tr>
<td>Phenylephrine (10 μM) and low dose forskolin (0.3 μM)</td>
<td>89</td>
</tr>
<tr>
<td>Histamine (10 μM) and low dose forskolin (0.3 μM)</td>
<td>89</td>
</tr>
<tr>
<td>Intermediate [Ca2+]i Sensitivity</td>
<td></td>
</tr>
<tr>
<td>Histamine (1 μM) and KCl (20 mM)</td>
<td>34</td>
</tr>
<tr>
<td>Histamine (10 μM) and KCl (109 mM)</td>
<td>34</td>
</tr>
<tr>
<td>F- (2.5 mM) and KCl (20 mM)</td>
<td>34</td>
</tr>
<tr>
<td>F- (2.5 mM) and KCl (109 mM)</td>
<td>34</td>
</tr>
<tr>
<td>KCl (20 mM) and phenol dibutyrate (10 nM)</td>
<td>34</td>
</tr>
<tr>
<td>Lowest [Ca2+]i Sensitivity</td>
<td></td>
</tr>
<tr>
<td>Stretch (20 or 30% Ls)</td>
<td>111</td>
</tr>
<tr>
<td>KCl (109 mM) and high dose forskolin (30 μM)</td>
<td>89</td>
</tr>
</tbody>
</table>

[Ca2+]i sensitivity of phosphorylation is defined from the relation between [Ca2+]i and myosin light chain phosphorylation.

Histamine, phenylephrine, endothelin, and F-, a non-specific activator of G proteins, also shown in Figure 2A with a dashed line). Low [Ca2+]i sensitivity of phosphorylation is observed with agents that only promote Ca2+ influx, e.g., depolarization, Bay K-8644, ATP, stretch, and reversal of Na+-Ca2+ exchange with low extracellular sodium ([Na+]o); solid line in Figure 2A. Mixtures of contractile stimuli from these two groups induced an intermediate [Ca2+]i sensitivity of phosphorylation, indicating complex regulation for [Ca2+]i sensitivity of phosphorylation.

Ca2+-Induced Desensitization

Several investigators have hypothesized that large increases in [Ca2+]i, per se may decrease the sensitivity of the contractile apparatus to [Ca2+]i.6-9 This mechanism was termed Ca2+-induced desensitization and may act to prevent any adverse effect resulting from prolonged increases in [Ca2+]i. A molecular mechanism for Ca2+-induced desensitization has been proposed: large increases in [Ca2+]i may induce phosphorylation of myosin light chain kinase. In vitro, myosin light chain kinase activity is a function of both [Ca2+]i and calmodulin concentration.1 Myosin light chain kinase can be phosphorylated, and phosphorylated myosin light chain kinase has a lower Ca2+ sensitivity than dephosphorylated myosin light chain kinase.46 There are six phosphorylation sites on myosin light chain kinase (defined by tryptic peptide mapping). However, only phosphorylation at the peptide A site is associated with decreased Ca2+ sensitivity; phosphorylation on peptides B–F have no effect.45 Ca2+-calmodulin–dependent protein kinase II, cAMP–dependent protein kinase, and protein kinase C can phosphorylate myosin light chain kinase on peptide A.45,46

Stull et al45 hypothesized that large increases in [Ca2+]i, activate Ca2+-calmodulin–dependent protein kinase II, which phosphorylates and decreases the Ca2+ sensitivity of myosin light chain kinase. Stull's laboratory45 found that stimulation of bovine trachealis with either carbachol or KCl depolarization increased the level of myosin light chain kinase phosphorylation. Additionally, both stimuli decreased the Ca2+ sensitivity of myosin light chain kinase extracted from these stimulated tissues.45 The assay for myosin light chain kinase Ca2+ sensitivity involves incubating diluted tissue extracts with γ32PO4-ATP, calmodulin, myosin light chains, and a Ca2+-EGTA buffer.50 By varying the [Ca2+]i in the assay mixture, the Ca2+ sensitivity of 32PO4 incorporation into the myosin light chains is determined. Stull et al45 found a significant correlation between the amount of myosin light chain kinase phosphorylation on peptide A and the Ca2+ sensitivity of the extracted myosin light chain kinase in bovine trachealis. However, these investigators have not yet correlated myosin light chain kinase phosphorylation or Ca2+ sensitivity of extracted myosin light chain kinase with the [Ca2+]i sensitivity of phosphorylation observed in intact tissues.

We found that the Ca2+ sensitivity of myosin light chain kinase extracted from depolarized tissues was lower than the Ca2+ sensitivity of myosin light chain kinase extracted from histamine-stimulated or unstimulated tissues.51 Thus, in the case of histamine- or KCl-stimulated tissues, there was a correlation between the [Ca2+]i sensitivity of phosphorylation observed in the intact tissues and the Ca2+ sensitivity observed in the myosin light chain kinase extracted from tissues. These data suggest that phosphorylation of myosin light chain kinase is at least partially responsible for the lower [Ca2+]i sensitivity of phosphorylation observed with KCl depolarization. It is possible that the large increases in [Ca2+]i, associated with KCl depolarization activate Ca2+-calmodulin–dependent protein kinase II, which phosphorylates and decreases the Ca2+ sensitivity of myosin light chain kinase. However, the degree of myosin light chain kinase phosphorylation and the Ca2+ sensitivity of extracted myosin light chain kinase has not been determined with other stimuli that alter the [Ca2+]i sensitivity of phosphorylation (Table 1). Furthermore, we have not yet evaluated whether stimuli that induce similar levels of [Ca2+]i differentially modulate the Ca2+ sensitivity of myosin light chain kinase. Such experiments need to be performed to conclude that [Ca2+]i-dependent phosphorylation of myosin light chain kinase is primarily responsible for changes in the [Ca2+]i sensitivity of phosphorylation.

Ca2+-induced desensitization may also partially explain the phasic response of some smooth muscles. Somlyo's laboratory46,47 clamped [Ca2+]i, at high levels
with EGTA buffers in staphylococcal \( \alpha \)-toxin skinned smooth muscle. The high [Ca\( ^{2+} \)]\textsubscript{i} induced a large transient increase in force. However, force declined to significantly lower levels despite continued high [Ca\( ^{2+} \)]\textsubscript{i}, suggesting a time-dependent decrease in [Ca\( ^{2+} \)], sensitivity of force.\textsuperscript{52}

**G Protein-Induced Sensitization**

The [Ca\( ^{2+} \)]\textsubscript{i} sensitivity of phosphorylation can also be modulated at a constant [Ca\( ^{2+} \)], suggesting that another mechanism regulating the [Ca\( ^{2+} \)]\textsubscript{i} sensitivity of phosphorylation exists in smooth muscle. If histamine-stimulated swine carotid artery is treated with either diltiazem or a nominally Ca\( ^{2+} \)-free solution, aequorin-estimated myoplasmic [Ca\( ^{2+} \)] gradually decreases to resting values (i.e., log L/L\textsubscript{max} change is 0, Table 2). However, we found that myosin phosphorylation levels and contractile force remained significantly above resting values despite [Ca\( ^{2+} \)], values identical to resting.\textsuperscript{53} These data and others in skinned tissues\textsuperscript{45-47} suggest that contractile agonists such as histamine can increase the [Ca\( ^{2+} \)], sensitivity of phosphorylation at a constant [Ca\( ^{2+} \)].

Recent studies suggest that contractile agonists increase the [Ca\( ^{2+} \)], sensitivity of phosphorylation through the mediation of a G protein. [Ca\( ^{2+} \)], sensitivity of phosphorylation is increased by exogenous application of GTP analogues or contractile agonists in smooth muscle permeabilized with staphylococcal \( \alpha \)-toxin.\textsuperscript{54-56} Unfortunately, GTP analogues cannot be directly added to intact tissues. F\textsuperscript{-} (by complexing with Al\textsuperscript{3+}) can nonspecifically activate G proteins.\textsuperscript{57} Addition of either histamine or F\textsuperscript{-} to depolarized intact swine carotid artery produced similar increases in the [Ca\( ^{2+} \)], sensitivity of phosphorylation (Table 1). These data suggest that AlF\textsubscript{3}-dependent activation of G proteins can mimic contractile agonist-induced increases in the [Ca\( ^{2+} \)], sensitivity of phosphorylation.\textsuperscript{54} These data in intact tissues support the data from a-toxin-permeabilized tissues; however, the data are only suggestive because there are other actions of F\textsuperscript{-}.

Increases in the [Ca\( ^{2+} \)], sensitivity of phosphorylation may represent either activation of a [Ca\( ^{2+} \)],-independent process or an increase in the Ca\( ^{2+} \) sensitivity of myosin light chain kinase. A [Ca\( ^{2+} \)],-independent mechanism for G protein-mediated increase in [Ca\( ^{2+} \)], sensitivity of phosphorylation has been proposed: myosin light chain phosphatase may be regulated. To be consistent with the above observations, contractile agonists (e.g., histamine) should inhibit myosin light chain phosphatase activity to produce the observed changes in the [Ca\( ^{2+} \)], sensitivity of phosphorylation. Somlyo's laboratory\textsuperscript{58} found that 100 \( \mu \)M phenylephrine and 3 \( \mu \)M GTP-\gamma-S slowed dephosphorylation rates by 50\% in \( \alpha \)-toxin skinned rabbit portal vein, suggesting that myosin light chain phosphatase is inhibited by a G protein-mediated process. Similar results were observed in GTP-\gamma-S-treated tissue homogenates.\textsuperscript{59} However, these studies need to be evaluated carefully: 1) The phosphatase activity in skinned tissues was low compared with intact tissues. One potential explanation for the low phosphatase activity was the effect of low temperature (15°C) and a high Q\textsubscript{10} of myosin phosphatase. However, the skinning procedure could also have removed phosphatase activity or necessary cofactors. 2) Stimulus-dependent alterations in myosin light chain phosphatase activity have yet to be described in an intact smooth muscle. 3) Myosin light chain phosphatase has yet to be definitively identified. 4) There is no known mechanism for G protein regulation of myosin phosphatase.

The finding that a G protein appears to inhibit myosin phosphatase does not diminish the importance of Ca\( ^{2+} \)-induced desensitization by phosphorylation of myosin light chain kinase. A decrease in phosphatase activity of 50\% can explain only half of the difference in the [Ca\( ^{2+} \)], sensitivity of phosphorylation observed in Figure 1A. Supporting this hypothesis was the finding that stimulation of swine carotid artery with histamine and high KCl induced an intermediate [Ca\( ^{2+} \)], sensitivity of phosphorylation (Table 1). If inhibition of myosin phosphatase was the only mechanism responsible for changes in [Ca\( ^{2+} \)], sensitivity of phosphorylation, then stimulation with histamine and high KCl should have induced a high [Ca\( ^{2+} \)], sensitivity of phosphorylation similar to that induced by histamine alone.

Alternatively, G protein–dependent increases in the [Ca\( ^{2+} \)], sensitivity of phosphorylation could result from dephosphorylation of myosin light chain kinase by either agonist-dependent activation of the phosphatase that dephosphorylates myosin light chain kinase or agonist-dependent inhibition of the enzyme that phosphorylates myosin light chain kinase. Maximal phosphorylation rates were unaffected by GTP-\gamma-S in \( \alpha \)-toxin skinned rabbit portal vein,\textsuperscript{58} suggesting that phosphorylation of myosin light chain kinase was not affected directly by G proteins. However, the effect of GTP-\gamma-S on myosin light chain kinase activity was investigated only at one relatively high [Ca\( ^{2+} \)] (300 nM); there may be effects of G proteins on myosin light chain kinase at lower [Ca\( ^{2+} \)]

### Table 2. Agonist-Induced Sensitization in Swine Carotid Artery

<table>
<thead>
<tr>
<th>Condition</th>
<th>Aequorin-estimated [Ca( ^{2+} )] (log L/L\textsubscript{max} change)</th>
<th>Myosin phosphorylation (mol Pi/mol MLC)</th>
<th>Stress (( \times 10^3 ) N/m\textsuperscript{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine (10 ( \mu )M 30 min)</td>
<td>0.34±0.04*</td>
<td>0.44±0.07*</td>
<td>1.87±0.19*</td>
</tr>
<tr>
<td>Histamine (10 ( \mu )M 10 min+Ca( ^{2+} ) free 13 min)</td>
<td>0.00±0.02</td>
<td>0.21±0.02*</td>
<td>0.97±0.12*</td>
</tr>
<tr>
<td>Histamine (10 ( \mu )M 10 min+10 ( \mu )M diltiazem 12 min)</td>
<td>0.00±0.01</td>
<td>0.19±0.01*</td>
<td>1.17±0.09*</td>
</tr>
<tr>
<td>Unstimulated control</td>
<td>0.00±0.00</td>
<td>0.09±0.01*</td>
<td>0.11±0.02</td>
</tr>
</tbody>
</table>

\*Significantly (p<0.05) different from unstimulated control. MLC, myosin light chain. Data adapted from Reference 53.
It should be noted that the latch bridge hypothesis (see below) makes a specific prediction regarding myosin-phosphatase activity. Decreased phosphatase activity should change the dependence of steady-state stress on phosphorylation. A twofold decrease in phosphatase activity induces a small rightward and downward shift in the model-predicted dependence of steady-state stress on phosphorylation (Figure 2B). However, we could not detect a measurable difference in the dependence of steady-state stress on phosphorylation with stimuli inducing high \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation (including agents that activate G proteins such as histamine or F; see Figure 2B) or low \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation (see Figure 2B). Unfortunately, the small shift in the predicted dependence of stress on phosphorylation would not be detected within the error of our measurements. These data suggest that modest inhibition of myosin phosphatase is not in disagreement with the latch bridge hypothesis. It is possible that minor degrees of phosphatase inhibition could induce relatively large changes in the \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation that would not be detected by analysis of dependence of stress on phosphorylation.

**Protein Kinase C and \([\text{Ca}^{2+}]_i\), Sensitivity of Phosphorylation**

Phorbol diesters, activators of protein kinase C, contract smooth muscle. In several types of smooth muscle, phorbol diester–induced contractions are associated with an increase in the force without changes in \([\text{Ca}^{2+}]_i\). In the swine carotid, we found that low dose (10 nM) phorbol dibutyrate, a water soluble phorbol diester, produced very small but significant increases in \([\text{Ca}^{2+}]_i\), and phosphorylation, yielding a \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation similar to that induced by histamine.

Higher doses of phorbol dibutyrate (1 μM) induced a large contraction with resting levels of \([\text{Ca}^{2+}]_i\), (i.e., the highest \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation observed in the swine carotid). These data suggest that phorbol diesters either increase the \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation or activate a \([\text{Ca}^{2+}]_i\),-independent process. Recently, Nishimura et al. showed that 12-O-tetradecanoylphorbol 13-acetate increased the \([\text{Ca}^{2+}]_i\), sensitivity of force in e-tongue skinned rat mesenteric artery to a degree similar to that observed with GTPγS. In the canine trachealis, 12-deoxyphorbol-13-isobutyrate increased the \([\text{Ca}^{2+}]_i\), sensitivity of force in depolarized tissues, but decreased both \([\text{Ca}^{2+}]_i\), and force in carbachol-stimulated tissues. A relatively specific inhibitor of protein kinase C (H7) decreased the \([\text{Ca}^{2+}]_i\), sensitivity of force without much effect on changes in \([\text{Ca}^{2+}]_i\). These studies suggest that protein kinase C may regulate \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation. However, the physiological significance of this response is unclear because large doses of phorbol diesters were necessary to obtain these responses. There are studies suggesting that protein kinase C can be activated by contractile agonists in smooth muscle cells and intact tissues. However, the question remains whether protein kinase C is activated during physiological smooth muscle contraction. Phorbol diester stimulation (even 10 nM phorbol dibutyrate) may induce more protein kinase C activation than that induced by contractile agonists. We found no measurable sustained histamine- or endothelin-induced increase in diacylglycerol mass, although others found an increased diacylglycerol mass.

**Accuracy of \([\text{Ca}^{2+}]_i\), Estimates**

We have assumed that aequorin is a true estimate of mean myoplasmic \([\text{Ca}^{2+}]_i\) because myosin phosphorylation values were proportional to aequorin-estimated \([\text{Ca}^{2+}]_i\) with agonist activation. However, this assumption may not be valid. It is possible that aequorin may overestimate mean myoplasmic \([\text{Ca}^{2+}]_i\) when tissues are depolarized because high KCl may induce focal elevations in \([\text{Ca}^{2+}]_i\). KCl-induced \([\text{Ca}^{2+}]_i\) entry could potentially induce these focal high elevations in \([\text{Ca}^{2+}]_i\), due to the dynamics of a point source (the \([\text{Ca}^{2+}]_i\) channel) filling a large sink (cellular \([\text{Ca}^{2+}]_i\) binding proteins and \([\text{Ca}^{2+}]_i\) pumps). In one study, which assumed that \([\text{Ca}^{2+}]_i\) influx was only passively buffered, a computer model indicated that steady-state \([\text{Ca}^{2+}]_i\), 1 nm from the pore of an L-type \([\text{Ca}^{2+}]_i\) channel was \(\sim 100\) μM, the \([\text{Ca}^{2+}]_i\), 10 nm from the channel was \(\sim 10\) μM, and the \([\text{Ca}^{2+}]_i\), 100 nm from the channel was \(\sim 1\) μM. These values will be smaller when active processes such as \([\text{Ca}^{2+}]_i\) pumps are considered; however, this model does suggest that significant intracellular \([\text{Ca}^{2+}]_i\) gradients can exist near the pore of an activated \([\text{Ca}^{2+}]_i\) channel.

If KCl depolarization produces focal high elevations in \([\text{Ca}^{2+}]_i\), near the pore of the \([\text{Ca}^{2+}]_i\) channel, then the aequorin signal would predominantly reflect the \([\text{Ca}^{2+}]_i\), in those regions with high \([\text{Ca}^{2+}]_i\), aequorin overestimates \([\text{Ca}^{2+}]_i\) because there is a steep relation between light and \([\text{Ca}^{2+}]_i\); aequorin light is proportional to \([\text{Ca}^{2+}]_i\), raised to the 2.5 power. Thus, aequorin light production will be large from hot spots with high \([\text{Ca}^{2+}]_i\). Therefore, in the presence of focal increases in \([\text{Ca}^{2+}]_i\), \([\text{Ca}^{2+}]_i\), estimates using aequorin may overestimate the \([\text{Ca}^{2+}]_i\), in the region of the myofilaments. Potentially, apparent changes in the \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation (Figure 2A) could be an artifact of the \([\text{Ca}^{2+}]_i\), measurement with aequorin. Fura-2 has a less steep fluorescence/[\text{Ca}^{2+}]_i relation and therefore should not overestimate \([\text{Ca}^{2+}]_i\) as much as aequorin in the presence of focal increases in \([\text{Ca}^{2+}]_i\). We investigated this possibility by measuring myoplasmic \([\text{Ca}^{2+}]_i\) with both fura-2 and aequorin in intact swine carotid media. Although there were some differences in the \([\text{Ca}^{2+}]_i\), signals, both indicators revealed that the histamine-induced \([\text{Ca}^{2+}]_i\), signal was lower than the KCl-induced \([\text{Ca}^{2+}]_i\), signal indicating that histamine induced a higher \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation than that observed with KCl depolarization. It should be noted that inhomogeneities in fura-2 loading and other artifacts can also alter fura-2 \([\text{Ca}^{2+}]_i\), estimates. However, these data using two mechanistically different \([\text{Ca}^{2+}]_i\), indicators suggest that changes in the \([\text{Ca}^{2+}]_i\), sensitivity of phosphorylation are not an artifact of the \([\text{Ca}^{2+}]_i\), indicator.
phosphorylate myosin light chain kinase, and therefore decrease the \([\text{Ca}^{2+}]_j\) sensitivity of myosin light chain kinase.

**Could Myosin Be Phosphorylated By Other Kinases?**

During physiological stimulation of intact tissues, myosin appears to be phosphorylated only at the myosin light chain kinase sites (serine 19 and threonine 18, the latter occurring after the former only with very high myosin light chain kinase activity)\(^{83,84}\). Myosin kinase activity in smooth muscle homogenates is dependent on the presence of \([\text{Ca}^{2+}]_j\). Thus, if another kinase is phosphorylating myosin in tissues, it should phosphorylate the light chains at myosin light chain kinase sites and be \([\text{Ca}^{2+}]_j\)-dependent. A potential candidate is \([\text{Ca}^{2+}]_j\)-calmodulin protein kinase II, which can phosphorylate myosin at serine 19 and increase myosin's ATPase activity\(^{85}\). However, the activity and \([\text{Ca}^{2+}]_j\) sensitivity of \([\text{Ca}^{2+}]_j\)-calmodulin protein kinase II is substantially lower than that of myosin light chain kinase.\(^1\)

**Cyclic Nucleotide-Induced Relaxation**

Elevations in cyclic nucleotide concentration may relax smooth muscle by several different mechanisms: 1) by decreasing \([\text{Ca}^{2+}]_j\), 2) by decreasing the \([\text{Ca}^{2+}]_j\) sensitivity of phosphorylation, or 3) by uncoupling force from myosin phosphorylation. Before analyzing the data available in smooth muscle, I will introduce the proposed mechanism for action of cAMP and cGMP in smooth muscle.

It was always assumed that cAMP activated cAMP-dependent protein kinase and that cGMP activated cGMP-dependent protein kinase. However, cGMP-dependent protein kinase can also be activated by cAMP, although it requires nearly a 10-fold higher cAMP concentration (\([\text{cAMP}]_j\)) than cGMP concentration (\([\text{cGMP}]_j\)) to activate the kinase.\(^{29,60,87}\) Similarly, cAMP-dependent protein kinase can also be activated by cGMP, although it requires nearly a 10-fold higher \([\text{cGMP}]_j\) than \([\text{cAMP}]_j\) to activate the kinase. In smooth muscle \([\text{cAMP}]_j\) is typically nearly 10-fold greater than \([\text{cGMP}]_j\).\(^{88}\) Therefore, elevations in \([\text{cAMP}]_j\) potentially could activate both cAMP-dependent protein kinase and cGMP-dependent protein kinase, whereas cGMP should only activate cGMP-dependent protein kinase (Figure 1). Data supporting the hypothesis that cAMP can activate cGMP-dependent protein kinase in vivo came from the laboratory of Tom Lincoln: in a smooth muscle cell line deficient in cGMP-dependent protein kinase, cAMP does not decrease \([\text{Ca}^{2+}]_j\), unless cGMP-dependent protein kinase is reintroduced by electroporation.\(^{31}\)

CAMP-dependent protein kinase phosphorylates myosin light chain kinase on peptide A in vitro;\(^{45}\) therefore, activation of this kinase could potentially decrease the \([\text{Ca}^{2+}]_j\) sensitivity of phosphorylation in vivo. It should be noted that activation of cAMP-dependent protein kinase is not responsible for the changes in the \([\text{Ca}^{2+}]_j\) sensitivity of phosphorylation observed with agonist stimulation or KCl depolarization because neither stimulus altered \([\text{cAMP}]_j\) or \([\text{cGMP}]_j\) in the swine carotid artery.\(^{89}\) CAMP-dependent protein kinase has also been shown to increase \([\text{Ca}^{2+}]_j\) influx by phosphorylating L-type \([\text{Ca}^{2+}]_j\) channels, a mechanism that could increase \([\text{Ca}^{2+}]_j\).\(^{90}\)

Activation of cGMP-dependent protein kinase reduces \([\text{Ca}^{2+}]_j\); however, the mechanism (or mechanisms) for the reduction in \([\text{Ca}^{2+}]_j\) is controversial (Figure 1): 1) cGMP-dependent protein kinase can phosphorylate phospholamban, which removes the inhibition of smooth muscle sarcoplasmic reticulum \([\text{Ca}^{2+}]_j\) ATPase.\(^{91,92}\) 2) cGMP-dependent protein kinase may also activate a smooth muscle plasma membrane \([\text{Ca}^{2+}]_j\) ATPase.\(^{93,94}\) 3) cGMP-dependent protein kinase may activate K+ channels in smooth muscle, inducing hyperpolarization and decreasing \([\text{Ca}^{2+}]_j\) influx.\(^7\) Some studies have shown a correlation between cGMP-mediated relaxation and changes in membrane potential; however, there are other studies that dissociate hyperpolarization and relaxation (possibly due to "endothelial-dependent hyperpolarizing factor").\(^6\)

In intact smooth muscle, some studies\(^{25,36,95}\) but not all\(^{96,97}\) found that elevations in \([\text{cAMP}]_j\) induced by forskolin or \(\beta\)-adrenergic stimulation of smooth muscle were associated with decreases in \([\text{Ca}^{2+}]_j\). High dose forskolin treatment induced myosin light chain kinase phosphorylation in intact tissues.\(^98\) However, the \([\text{Ca}^{2+}]_j\) sensitivity of myosin light chain kinase extracted from bovine trachealis treated with modest doses of isoproterenol was not significantly different from the \([\text{Ca}^{2+}]_j\) sensitivity of myosin light chain kinase extracted from unstimulated tissues, suggesting that isoproterenol did not alter the \([\text{Ca}^{2+}]_j\) sensitivity of phosphorylation (the \([\text{Ca}^{2+}]_j\) sensitivity of myosin light chain kinase was much lower with carbachol or KCl stimulation than with isoproterenol).\(^{95,96}\) We evaluated low dose (\(\leq 0.3 \mu M\)) forskolin-induced relaxations of histamine- or phenylephrine-precontracted swine carotid media tissues. Low dose forskolin treatment increased \([\text{cAMP}]_j\) two-fold and significantly decreased \([\text{Ca}^{2+}]_j\), phosphorylation, and stress without changing the \([\text{Ca}^{2+}]_j\) sensitivity of phosphorylation (Table 1),\(^99\) suggesting that small elevations in \([\text{cAMP}]_j\) relax arterial smooth muscle primarily by decreasing \([\text{Ca}^{2+}]_j\). Similar results were observed in isolated tracheal smooth muscle cells.\(^{97}\) However, much higher concentrations of forskolin were required to induce relaxation of KCl-depolarized swine carotid artery (addition of 30 \(\mu M\) forskolin was associated with a 30-fold increase in \([\text{cAMP}]_j\), a significant decrease in the \([\text{Ca}^{2+}]_j\) sensitivity of phosphorylation, and no change in \([\text{Ca}^{2+}]_j\); Table 1).\(^99\) These data suggest that large elevations in \([\text{cAMP}]_j\) relax arterial smooth muscle primarily by decreasing \([\text{Ca}^{2+}]_j\) sensitivity of phosphorylation (potentially through myosin light chain kinase phosphorylation).

Results with elevations in \([\text{cGMP}]_j\) are more controversial. One investigator suggested that nitroprusside primarily decreased the \([\text{Ca}^{2+}]_j\) sensitivity of force (phosphorylation levels were not measured).\(^3\) Preliminary data suggest that low concentrations of nitroprusside relax submaximally stimulated swine carotid media by decreasing \([\text{Ca}^{2+}]_j\). Larger concentrations of nitroprusside transiently decreased the \([\text{Ca}^{2+}]_j\) sensitivity of phosphorylation. Intriguingly, nitroprusside also appeared to uncouple force from myosin phosphorylation.\(^98\)

**Regulation of Contractile Force by Myosin Light Chain Phosphorylation (Latch Phenomenon)**

Activated myosin light chain kinase phosphorylates the 20-kd light chain of myosin on serine 19. This phosphorylation is associated with an increase in the
actin-activated myosin ATPase activity. This finding suggested the phosphorylation “switch” hypothesis in which phosphorylation-dependent activation of myosin is equivalent to Ca$^{2+}$-dependent troponin C activation of thin filaments in skeletal muscle. A prediction of a switch model is that stress would be linearly proportional to phosphorylation; however, this was not the case (Figure 2B). Additionally, sustained stimulation of intact smooth muscle was associated with decreasing yet suprabasal levels of [Ca$^{2+}$],93,97 phosphorylation,99 unloaded shortening velocity (V$_{o}$),100 and energy consumption,101 whereas stress (normalized force) remained high. This has been termed the “latch” phenomenon (high stress with lower activation). The latch bridge hypothesis of Murphy et al.102 is the most accepted explanation of the above observations. Detailed explanation of the latch bridge model is beyond the scope of this review. It proposes the existence of a latch bridge, which is an attached dephosphorylated cross bridge formed by dephosphorylation of an attached phosphorylated cross bridge (Figure 1). The latch bridge is proposed to be force generating like a phosphorylated cross bridge, and therefore, force can be maintained at higher levels than would be expected based on the number of phosphorylated cross bridges. Latch bridges are assumed to be identical to phosphorylated cross bridges except that they have a fivefold slower detachment rate (this accounts for the reduction in V$_{o}$ and ATP consumption).

However, there are several examples of uncoupling force from myosin phosphorylation: nitrovasodilators,80 high extracellular [Mg$^{2+}$],103 high intracellular [Mg$^{2+}$],104 and Ca$^{2+}$-repletion protocols.105 These data do not invalidate the latch bridge hypothesis. However, these data do suggest that regulatory systems other than myosin light chain phosphorylation may modulate contractile force, potentially by a thin filament mechanism.72,106,107

In this review I concentrated on data in arterial smooth muscle. Contraction and relaxation of arterial smooth muscle in response to physiological neurotransmitters and hormones are regulated primarily by changes in [Ca$^{2+}$],108-111 Changes in [Ca$^{2+}$] sensitivity of phosphorylation are observed experimentally and may have physiological relevance (Table 1). There are several conditions in which force is uncoupled from myosin phosphorylation.

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