Mechanisms of Signal Transduction During α₂-Adrenergic Receptor–Mediated Contraction of Vascular Smooth Muscle

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Little is known about the signaling pathways involved in α₂-adrenergic receptor–mediated contraction of vascular smooth muscle. In the present study, we measured intracellular Ca²⁺ ([Ca²⁺]i), myosin light chain (MLC) phosphorylation, and myofilament Ca²⁺ sensitivity during stimulation with the relatively selective α₂-agonist UK 14304. These effects were compared and contrasted with corresponding changes during depolarization by elevation of the [K⁺] in the bathing medium. These studies were performed using spiral strips of the rabbit saphenous vein, a tissue with a relatively high density of postsynaptic α₂-receptors. UK 14304 (10⁻⁵ M) caused parallel changes in [Ca²⁺]i, MLC phosphorylation, and force resulting from an initial phase, followed by a sustained steady-state response. The steady-state increase in [Ca²⁺]i, MLC phosphorylation, and force caused by UK 14304 in the presence of 2.5 mM extracellular Ca²⁺ was indistinguishable from those during 51 mM K⁺ depolarization. However, when extracellular Ca²⁺ was removed in the presence of UK 14304, [Ca²⁺]i, and MLC phosphorylation fell to resting levels, but force remained significantly elevated above basal levels. UK 14304 caused no change in the steady-state [Ca²⁺]i–MLC phosphorylation relation. Thus, the [Ca²⁺]i sensitization of force was not caused by a sensitization of MLC phosphorylation. These results indicate that in a 2.5-mM Ca²⁺ bathing medium, the dominant mechanism by which α₂-adrenergic receptor stimulation causes an increase in vascular tone is through a relatively large increase in [Ca²⁺]i, and MLC phosphorylation. However, in Ca²⁺-free bathing medium, a second mechanism is unmasked which appears to involve an increased Ca²⁺ sensitivity and is independent of myosin phosphorylation. (Circulation Research 1993;72:778–785)

KEY WORDS • vascular smooth muscle • intracellular ionized calcium • myosin light chain phosphorylation • α₂-adrenergic receptor

Coupling mechanisms of α₂-adrenoceptors have been investigated in considerable detail in nonmuscle tissue, including platelets, neuroblastoma cells, renal cortex cells, and pancreatic islet cells. In these cells, inhibition of adenylate cyclase has been proposed to be the primary α₂ coupling mechanism; for review see Ruffolo et al. ¹ However, a number of studies in human as well as animal tissues have revealed that decreased cyclic AMP formation is inadequate to account for the various physiological effects elicited by α₂ stimulation.¹–³ Additionally, even from a theoretical viewpoint, it is difficult to explain the contraction caused by application of α₂-agonists to resting vascular smooth muscle preparations by a decrease in cyclic AMP to sub-basal levels. Little is known about α₂ coupling in the vasculature, but although the response has been reported to be partially coupled to intracellular release of Ca²⁺,⁴ the body of evidence demonstrates a reliance of the contraction upon the presence of extracellular Ca²⁺.⁵–⁷

It is generally well accepted that Ca²⁺-calmodulin–mediated phosphorylation of the 20-kd myosin light chain (MLC) by myosin light chain kinase (MLCK) is a major mechanism of smooth muscle contraction.⁸,⁹ A growing body of evidence, however, suggests the existence of additional mechanisms that may result in an increased sensitivity to intracellular calcium ([Ca²⁺]i) during the action of a broad range of agonists.¹⁰–¹²

Alabaster et al.¹³ and Aburto¹⁴ have shown that the α₂-adrenergic receptors in the rabbit saphenous vein (RSV) are primarily of the α₂ subtype. Therefore, in an attempt to clarify the degree of involvement of changes in [Ca²⁺]i, and MLC phosphorylation in the α₂-mediated contraction of the RSV, [Ca²⁺]i, force, and the extent of MLC phosphorylation were measured during stimulation with the relatively selective α₂-agonist UK 14304,¹⁵ and these results were compared with those in the presence of high K⁺ depolarization.

Materials and Methods

Tissue Preparation and General Methods

Adult New Zealand White rabbits of either sex weighing 2–3 kg were anesthetized with chloroform. All procedures were approved by the Institutional Commit-
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The saphenous veins were removed and placed into a physiological saline solution (PSS) bubbled with 95% O2-5% CO2. Spiral strips of RSV were isolated and any remaining endothelium was removed by gentle rubbing of the luminal surface, by a method previously shown to be effective in removing endothelium-dependent responses. One end of the muscle was clamped and the other end was attached to a Gould UC2 force transducer (Gould Inc., Cleveland, Ohio).

All experiments were performed at 37°C. Elevated K+ solutions were made by equimolar replacement of NaCl in the PSS with KCl. Phentolamine at 1 $\mu$M was present 20–30 minutes before and during exposure to high K+ solution. The PSS contained (mM) NaCl 137, KCl 5.9, CaCl$_2$ 2.5, MgCl$_2$ 1.2, NaHCO$_3$ 25, NaH$_2$PO$_4$ 1.2, and dextrose 11.5 at a pH of 7.4 when bubbled with 95% O2-5% CO2.

**Measurements of $[Ca^{2+}]_i$**

Aequorin was chemically loaded into the muscle strips by a previously described procedure. Briefly, the muscle was exposed to a series of four solutions of the following composition (mM) for 30 to 120 minutes: solution I: KCl 120, MgCl$_2$ 2, Tris (hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES) 20, ATP 5, and EGTA 10; solution II: KCl 120, MgCl$_2$ 2, TES 20, ATP 5, EGTA 0.1, and 0.5 mg/ml aequorin; solution III: KCl 120, MgCl$_2$ 10, TES 20, ATP 5, and EGTA 0.1; solution IV: NaCl 120, KCl 5.9, dextrose 11.5, NaHCO$_3$ 25, MgCl$_2$ 10, and NaH$_2$PO$_4$ 1.4. CaCl$_2$ (2.5 mM) was added back at the end of the incubation period.

Light emitted by aequorin was detected with a photomultiplier tube (Thorn-EMI 9635 QA or 9235 QA, Gencom Inc., Fairfield, N.J.). The muscle cells were lysed with Triton X-100 and maximal luminescence ($L_{\text{max}}$) was determined by the method of fractional

**Figure 1.** Simultaneously recorded raw tracings for force (top tracings) and aequorin luminescence (bottom tracings) during depolarization in the presence of three different concentrations of K+. NKS, normal Krebs solution. Panel A, 96 mM K+; panel B, 51 mM K+; panel C, 24 mM K+. Vertical scales represent mN for force and anode current from the photomultiplier tube for luminescence in nanoamperes. Arrows indicate the time of solution changes. The elevated K+ solutions were washed into the bath twice to assure a complete change.
luminescence ($L/L_{\text{max}}$). $L/L_{\text{max}}$ was converted to $[\text{Ca}^{2+}]_i$ by the use of an in vitro calibration curve.$^{16}$

**Myosin Light Chain Phosphorylation**

Phosphorylated and unphosphorylated forms of the 20 kd MLC (LC20 and LC20U) were determined by two-dimensional polyacrylamide gel electrophoresis as previously described.$^{17}$ The strips were frozen in liquid dichlorodifluoromethane (CCl$_2$F$_2$, or freon) precooled in liquid nitrogen. Frozen strips were put in an acetone-dry ice slurry containing 10% trichloroacetic acid and 20 mM dithiothreitol (DTT) and were allowed to thaw to room temperature, followed by three rinses with acetone containing 10 mM DTT. The strips were then homogenized in 30 vol homogenization buffer consisting of 10% glycerol, 20 mM DTT, 2% sodium dodecyl sulfate (SDS), aprotinin, and 20 mM 2-(N-morpholino)propanesulfonic acid (MOPS, pH 7.0). Following centrifugation, proteins in the supernatant were analyzed by isoelectric focusing (IEF) followed by SDS polyacrylamide gel electrophoresis. IEF gels contained (vol/vol) 20% glycerol and 5% amphotelye (80% of pH 4.5–5.5 from Pharmacia and 20% of pH 3–10 from FMC). The gels were processed for silver staining, then scanned with a Hewlett-Packard Scanjet Plus scanning densitometer (Hewlett-Packard Co., Palo Alto, Calif.). Myosin phosphorylation levels were calculated by dividing the density of LC20P by the total density of both LC20P and LC20U.

**Materials**

UK 14304 was a generous gift from Pfizer Central (Sandwich, England) or Dr. Richard Milius (NIHMH) or was purchased from Research Biochemicals, Inc. (Natick, Mass.). No difference in results was obtained with the UK 14304 from the different sources. Aequorin was purchased from Friday Harbor Photoproteins (Friday Harbor, Wash.). All other chemicals were of reagent quality or better.

**Statistics**

All values are expressed as mean±SEM. Comparisons were made using analysis of variance or Student's $t$ test. All data points were obtained from five to 25 animals. A value of $p<0.05$ was taken to indicate significance.

**Results**

Basal $[\text{Ca}^{2+}]_i$ in RSV at 37°C was 191±5 mM ($n=46$). Basal, intrinsic tone was defined as the force that could be eliminated by cooling from 37° to 2°C in the absence of any agonists. Cooling to 2°C has been found to unmask the presence of active basal tone that is resistant to extracellular Ca$^{2+}$ depletion.$^{18}$ The RSV displayed an intrinsic tone of 1.37±0.11 mN. During K$^+$ depolarization (Figure 1), $[\text{Ca}^{2+}]_i$ and force increased roughly in parallel. In response to concentrations of K$^+$ up to 51 mM (Figure 1B and 1C), the muscle reached quasi-steady-state levels of elevated $[\text{Ca}^{2+}]_i$, and force by 10 minutes. However, in response to higher concentrations of K$^+$ (96 mM K$^+$; Figure 1C), the muscle displayed continually falling $[\text{Ca}^{2+}]_i$, and force levels after an initial peak. In Figure 1B luminescence dropped below baseline after washing out the 51-mM K$^+$ solution. This was not a consistent finding and was possibly due to slight cooling of the wash solution below 37°C, which would decrease aequorin luminescence, or, alternatively, to the washout of some metabolic product. Over the equilibrium time between challenges, both luminescence and force returned to the initial baseline in all cases.

The selective $\alpha_2$-adrenergic receptor agonist UK 14304, at a maximum effective dose of 10 μM,$^{19}$ induced a rapid but transient elevation in $[\text{Ca}^{2+}]_i$, reaching its peak within 24.4±2.7 seconds (Figure 2). A lower steady-state level of $[\text{Ca}^{2+}]_i$ was reached by 5 minutes. Force generation closely paralleled the rise in $[\text{Ca}^{2+}]_i$, reaching a maximum of 2.65±0.48 mN at 1 minute, followed by a sustained but lower steady-state level of 2.1±0.48 mN by 10 minutes.
The time course of the mean (± SEM) changes in force, MLC phosphorylation, and [Ca²⁺], in response to 51 mM K⁺ PSS is illustrated in Figure 3. In most cases, the changes in aequorin luminescence during exposure to all but the lowest [K⁺] were recorded for no longer than 10 minutes in order to avoid excessive consumption of aequorin. It can be seen, however, that whereas force, on average, was fairly well maintained for the first 10 minutes of the contraction, MLC phosphorylation fell by 40% and [Ca²⁺], fell by 14% during this time. These results are similar to what has been found in many other smooth muscles, in which the “latch” phenomenon is said to occur, but they differ from what we have previously seen in ferret aorta, where [Ca²⁺], and MLC phosphorylation are maintained at high levels throughout similar [K⁺] contractions.

The control [Ca²⁺]-force relation in RSV smooth muscle was investigated by plotting the quasi–steady-state (10-minute) values for [Ca²⁺], and force against each other. A control [Ca²⁺]-force relation was generated by increasing the [K⁺] in the bathing medium or removing Ca²⁺ from the medium surrounding the depolarized muscle. The assumption that the [K⁺] curve describes the control [Ca²⁺]-force relation for the contractile apparatus is based on the concept that membrane depolarization alone would not be expected to generate second messengers that would alter myofilament Ca²⁺ sensitivity. The resulting curve is shown in Figure 5. In this curve, the level of zero force is defined as the level of basal resting tone at 37°C. As mentioned above, the RSV possesses an active intrinsic tone at rest, but even though removing the extracellular Ca²⁺ from the bathing medium surrounding the depolarized muscle was successful in decreasing [Ca²⁺], below that in resting muscles (Figure 5), it was not effective in dropping force below the resting level. Thus, it appears that the intrinsic tone in this preparation was not affected by a decrease in [Ca²⁺]. This apparent [Ca²⁺] independence of intrinsic tone is similar to what has been described for the ferret aorta. Maximal steady-state (10 minutes) force with membrane depolarization (51
Figure 5. [Ca\(^{2+}\)]\(\text{i}\)-force relations. Points represent mean±SEM except where the SEM was smaller than the size of the symbol. ○, Control curve; □, UK 14304. All points were taken at quasi-steady-state. All Ca\(^{2+}\) values represent six to 16 samples with the exception of resting [Ca\(^{2+}\)]\(\text{i}\), where n=46. All force values represent seven to 16 values. See text for further details.

The effect of UK 14304 on the [Ca\(^{2+}\)]\(\text{i}\)-force relation (Figure 5) was investigated by using a constant concentration (10 μM) of UK 14304 in the presence of varying extracellular [Ca\(^{2+}\)]. The steady-state increases in [Ca\(^{2+}\)] and force caused by UK 14304 in the aequorin studies in the presence of normal extracellular [Ca\(^{2+}\)] were 258±20 nM and 2.1±0.48 mN, respectively, and were both indistinguishable statistically from the [Ca\(^{2+}\)] and force in the presence of 51 mM K\(^+\) depolarization. However, when the extracellular Ca\(^{2+}\) was removed (without the addition of EGTA), [Ca\(^{2+}\)] fell to levels that were below resting levels, but force was statistically significantly elevated above basal levels. When EGTA was added to the Ca\(^{2+}\)-free bathing medium, [Ca\(^{2+}\)], was driven to levels far below resting [Ca\(^{2+}\)]\(\text{i}\), and no significant increase in force above basal levels was caused by UK 14304. Thus, at the higher range of [Ca\(^{2+}\)], there was no evidence that UK 14304 caused any deviation from the "control" [Ca\(^{2+}\)]-force relation, as defined by the [K\(^+\)]-depolarization curve. The contraction appears to be solely due to an increase in [Ca\(^{2+}\)]. In contrast, at lower [Ca\(^{2+}\)] levels (between 150 and 200 nM) a second effect, to increase the [Ca\(^{2+}\)] sensitivity of the contractile apparatus, appears to be unmasked.

The question arises as to whether the steady-state forces plotted in Figure 5 are affected by the initial...
[Ca$^{2+}$], transients that occurred in some cases at the onset of the contraction (e.g., Figure 2 and also Figures 3 and 4). A history dependence of the [Ca$^{2+}$]$_i$–force relation in smooth muscle has been previously suggested by the formation of latch bridges.\textsuperscript{20} We cannot completely rule out this possibility, but in past experiments performed under conditions where initial [Ca$^{2+}$]$_i$, transients in response to $\alpha$-agonists were either allowed to occur or were prevented, steady-state forces were found to be unaffected.\textsuperscript{22} Specifically, with respect to whether the leftward shift in the [Ca$^{2+}$]$_i$–force relation in the presence of UK 14304 at low [Ca$^{2+}$]$_i$ could be due to the occurrence of an initial [Ca$^{2+}$] transient, we believe that this possibility can be ruled out. Even though $\alpha_2$-agonists can release Ca$^{2+}$ from intracellular stores,\textsuperscript{4} this was prevented in the present study by prolonged exposure to Ca$^{2+}$-free solutions (designed to assure that [Ca$^{2+}$]$_i$, reached a definable steady-state level) and initial [Ca$^{2+}$]$_i$, transients were not observed in Ca$^{2+}$-free solutions.

The relation between [Ca$^{2+}$]$_i$ and MLC phosphorylation levels is shown in Figure 6. It is of interest that the resting saphenous vein has a relatively low basal level of MLC phosphorylation (7%), but that when [Ca$^{2+}$]$_i$ was driven to sub-basal levels with the removal of extracellular Ca$^{2+}$ and the addition of EGTA, phosphorylation levels decreased even further to 3%. This decrease in MLC phosphorylation had no apparent effect to decrease the level of intrinsic tone. A comparison of the [Ca$^{2+}$]$_i$–MLC phosphorylation relation during the action of UK 14304 reveals that there is no detectable shift from the “control” [Ca$^{2+}$]$_i$–MLC phosphorylation relation even in the range of basal [Ca$^{2+}$]$_i$, values, where force is significantly elevated above basal values (see Figure 5). Thus, the apparent increased [Ca$^{2+}$]$_i$ sensitivity of the contractile apparatus at basal [Ca$^{2+}$]$_i$, in the presence of UK 14304 does not appear to involve a decrease in the [Ca$^{2+}$]$_i$, requirement of MLC phosphorylation.

The steady-state relation between MLC phosphorylation and force is shown in Figure 7. Again, the level of zero force is defined as the resting force at 37°C before the addition of any agonist, since the true level of intrinsic active tone was not determined in all of these experiments. Consistently higher forces were obtained in the experiments summarized in Figure 7 compared with the aequorin experiments summarized in Figure 5. In general, this was due to the fact that larger strips were used for the phosphorylation–force studies. The MLC phosphorylation–force relation is very steep, with maximal force occurring with only about 15% phosphorylation. Similarly steep relations between MLC phosphorylation and force have been described for several other tissues.\textsuperscript{17,23} At higher force and MLC phosphorylation values, the presence of UK 14304 did not appear
to alter the relation, but at basal MLC phosphorylation values, there was a statistically significant increased level of force in the presence of UK 14304 compared with that of the control.

Discussion

The results reported in this paper demonstrate that the dominant mechanism by which α2-adrenergic receptor stimulation causes contraction of the RSV is through a relatively large increase in [Ca2+] and MLC phosphorylation. However, the removal of extracellular Ca2+ unMASKS an additional component of force generation caused by α2-adrenergic receptor stimulation, in which significantly elevated force levels can be maintained in the presence of resting [Ca2+] levels. This suggests that α2-receptor stimulation also evokes a second mechanism of action involving an increase in the apparent Ca2+ sensitivity of the contractile apparatus.

The ability of α2-adrenergic receptor stimulation to cause relatively large sustained increases in [Ca2+] and MLC phosphorylation is in contrast to previous reports of signalling mechanisms during α2-adrenergic receptor stimulation of a variety of vascular smooth muscles. During α2-adrenergic receptor stimulation, only very low levels of [Ca2+] and MLC phosphorylation are present during the maintained stress levels equal to or greater than that produced by α2-adrenergic receptor stimulation in this preparation. For comparison, in the present study, the [Ca2+], MLC phosphorylation and force levels were not statistically significantly different during sustained α2-adrenergic receptor stimulation and maximal (51 mM) K+ depolarization. In contrast, using the same methods, we have found that during α2-adrenergic receptor stimulation, both [Ca2+], and MLC phosphorylation are dramatically less than those produced by maximal (51 mM) K+ stimulation.17,21,22 Similar results have been found by other investigators using α2-adrenergic receptor stimulation.24,25 We are not aware of any other reports of the measurement of [Ca2+] or MLC phosphorylation with α2-receptor stimulation in smooth muscle.

In studies performed with the use of Ca2+-free bathing medium, it became apparent that α2-receptor-mediated contraction of the RSV also involved a second signalling pathway. This part of the contraction occurred without an increase in MLC phosphorylation, suggesting that a different kinase other than MLCK might be involved. This part of the contraction also occurred at resting [Ca2+] levels, indicating that this second signalling pathway involved an apparent increase in [Ca2+] sensitivity of the contractile apparatus. In other smooth muscle tissues, activation of protein kinase C has been associated with an apparent increase in the [Ca2+] sensitivity of the contractile apparatus.16,26 Since the relatively selective protein kinase C antagonist staurosporine can also inhibit α2-receptor-mediated contractions of RSV, it seems likely that this second signalling pathway involves protein kinase C.

To our knowledge, this is the first report of [Ca2+] measurements or [Ca2+]–force relations in the RSV. The resting [Ca2+] for saphenous vein is similar to that which we previously reported, using the same methods for the ferret portal vein,27 another phasic tissue, but lower than that previously reported for ferret aorta, a tonic tissue.27 In both cases, aequorin was used as the [Ca2+] indicator. Because of complications in calibrating signals in terms of absolute [Ca2+], especially when results from different laboratories are compared, it is difficult to compare these aequorin values to those obtained with fura-2 in other laboratories. However, it is of interest that when fura-2 was used in ileum and pulmonary artery, another pair of phasic and tonic muscles, respectively, [Ca2+] was reported to be higher for the tonic muscle compared with the phasic.28 The relatively low pK and high steepness of the [Ca2+]–force relation in the RSV is similar to that reported for other vascular smooth muscles.27,29,30

The phenomenon of sag in maintained force appears to be a general property of phasic muscles. As can be seen in Figure 1A, however, the sag in force does not appear to involve a decrease in the apparent [Ca2+] sensitivity of the contractile apparatus since it is accompanied by a parallel decrease in [Ca2+]. It is of interest that Himpen et al.30 have reported different results for guinea pig ileum, another phasic smooth muscle, in that a desensitization of the contractile apparatus to [Ca2+] was seen in that study. Thus it appears that the sag in force that is characteristic of phasic tissues can occur by more than one mechanism and that these mechanisms are tissue specific.

In summary, in RSV, α2-adrenoceptor-mediated contraction involves a dual mechanism of action. Part of the contraction is clearly explained by [Ca2+]–dependent MLC phosphorylation, but a second mechanism is also involved. The second mechanism appears to involve a MLC phosphorylation–independent pathway and results in an apparent increase in the [Ca2+] sensitivity of the contractile apparatus.

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