Influence of Mitochondrial Inhibition on Global and Local $[Ca^{2+}]_i$ in Rat Tail Artery

Karl Swärd, Karl Dreja, Anders Lindqvist, Erik Persson, Per Hellstrand

Abstract—Inhibition of oxidative metabolism is often found to decrease contractility of systemic vascular smooth muscle, but not to reduce global $[Ca^{2+}]$. In the present study, we probe the hypothesis that it is associated with an altered pattern of intracellular $Ca^{2+}$ oscillations (waves) influencing force development. In the rat tail artery, mitochondrial inhibitors (rotenone, antimycin A, and cyanide) reduced $\alpha_2$-adrenoceptor–stimulated force by 50% to 80%, but did not reduce global $[Ca^{2+}]$. Less relaxation (about 30%) was observed after inhibition of myosin phosphatase activity with calyculin A, suggesting that part of the metabolic sensitivity involves the regulation of myosin 20-kDa light chain phosphorylation, although no decrease in phosphorylation was found in freeze-clamped tissue. Confocal imaging revealed that the mitochondrial inhibitors increased the frequency but reduced the amplitude of asynchronous cellular $Ca^{2+}$ waves elicited by $\alpha_2$ stimulation. The altered wave pattern, in association with increased basal $[Ca^{2+}]$, accounted for the unchanged global $[Ca^{2+}]$. Inhibition of glycolytic ATP production by arsenate caused similar effects on $Ca^{2+}$ waves and global $[Ca^{2+}]$, developing gradually in parallel with decreased contractility. Inhibition of wave activity by the InsP$_3$ receptor antagonist 2-APB correlated closely with relaxation. Furthermore, abolition of waves with thapsigargin in the presence of verapamil reduced force by about 50%, despite unaltered global $[Ca^{2+}]$, suggesting that contraction may at least partly depend on $Ca^{2+}$ wave activity. This study therefore indicates that mitochondrial inhibition influences $Ca^{2+}$ wave activity, possibly due to a close spatial relationship of mitochondria and the sarcoplasmic reticulum and that this contributes to metabolic vascular relaxation. (Circ Res. 2002;90:792-799.)

Key Words: arterial smooth muscle ▪ metabolic inhibition ▪ myosin phosphorylation ▪ calcium waves ▪ confocal imaging

The distribution of blood flow in tissue depends on the sensitivity of arterial tone to the local oxygen tension, but the nature of the mediating signal is yet unclear. Proposed mechanisms include reduced $Ca^{2+}$ inflow across the cell membrane, decreased myosin phosphorylation, or inhibition of cross-bridge cycling. In some cases of vascular hypoxia or mitochondrial inhibition, the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), measured as an average from multiple cells, is unchanged or even increased at high stimulus levels while force is reduced. Thus mechanisms based on decreased global $[Ca^{2+}]$, are insufficient to explain hypoxic relaxation. It is puzzling also that in many studies myosin light chain phosphorylation does not decrease, even though exceptions are seen. Finally, insufficient MgATP for cross-bridge interaction, although clearly a possibility with severe metabolic inhibition, does not seem to be a general explanation for hypoxic relaxation. Within the vessel wall, individual cells may exhibit asynchronous $Ca^{2+}$ oscillations during agonist stimulation. These depend on the function of the sarcoplasmic reticulum (SR) and are primarily associated with InsP$_3$-induced $Ca^{2+}$ release. In addition, highly localized increases in $[Ca^{2+}]_i$ in close proximity to the cell membrane due to activation of ryanodine receptors in the SR, termed $Ca^{2+}$ sparks, can influence the activity of neighboring $K_{Ca}$ channels, modulating membrane potential and thus voltage-dependent $Ca^{2+}$ inflow. At the cellular level therefore varies both spatially and temporally as demonstrated by confocal microscopy, in contrast to the “global” $[Ca^{2+}]$, measured by conventional imaging methods. This allows for temporal and spatial coding of $Ca^{2+}$ signals, explaining why this single ion can simultaneously regulate a multitude of cellular processes. In addition to the plasma membrane and SR, mitochondria may contribute to the regulation of intracellular $Ca^{2+}$ distribution.

Considering that adrenergic stimulation causes increased $Ca^{2+}$ wave activity in vascular tissue, it is possible that myosin phosphorylation fluctuates in a corresponding manner, whereas measurements after extraction of freeze-clamped tissue only reveal global changes. This suggests the possibility that contraction may be modulated via the spatial and temporal pattern of intracellular $Ca^{2+}$ transients as a possible site of action of metabolic inhibition. In addition to mitochondrial inhibitors, the effects of inhibited glycolytic energy supply are of interest, as there is evidence that energy...
metabolism is compartmentalized, with preferential support of membrane ion pumps via glycolysis.\textsuperscript{17}

**Materials and Methods**

**Preparation and Force Measurements**

Female Sprague-Dawley rats (Møllegaard, Copenhagen, Denmark) were killed by cervical dislocation as approved by the regional ethics committee. Segments were prepared in nominally Ca\textsuperscript{2+}-free modified Krebs solution (mmol/L) 135.5 NaCl, 5.9 KCl, 1.2 MgCl\textsubscript{2}, 11.6 glucose, and 11.6 HEPES, pH 7.35. To remove endothelium, the segment was slid over an insect needle (250 μm diameter) 20 to 30 times. Rings (1 mm wide) were mounted for isometric force registration as described.\textsuperscript{18} After equilibration in Krebs solution containing 2.5 mmol/L Ca\textsuperscript{2+} for 30 minutes at 37°C, preparations were repeatedly contracted with 0.3 μmol/L cirazoline (7 minutes) followed by relaxation for 25 minutes, until stable contractions were attained and experimentation begun.

**Global Arterial Wall [Ca\textsuperscript{2+}]\textsubscript{i} Measurements**

Arterial segments (2 mm) were turned inside out for optical access to the medial smooth muscle cells. Segments were threaded onto glass capillaries (350 μm in diameter) and loaded with 16 μmol/L fura-2 AM in Krebs solution (pH 7.4) in the dark for 3 hours. The 340:380-nm ratio of fura-2 fluorescence was determined at room temperature as an indirect measure of intracellular free Ca\textsuperscript{2+}.\textsuperscript{18} These measurements are referred to as "global [Ca\textsuperscript{2+}]\textsubscript{i}," because they represent the activity of several, although not necessarily all, medial cells.

**Confocal Imaging of Local [Ca\textsuperscript{2+}]\textsubscript{i}**

Arteries mounted on glass capillaries were loaded for 80 minutes with 10 μmol/L fluo-4 AM in Eppendorf tubes in the dark. They were subsequently mounted in a Zeiss LSM 510 laser scanning confocal microscope as described.\textsuperscript{19} Fluo-4 was excited at 488 nm, with 10% of basal fluorescence, and these were easily distinguished from movement artifacts which only occurred during development and decay of tension, not during maintained contraction (cf, online Movie found in the online data supplement available at http://www.circressa.org). Clustered events (≥10%), separated by incomplete return to baseline, were counted as one oscillation. To reduce clustering, the cirazoline concentration was kept at 0.1 μmol/L. Line-scans (20 000 scans at 3 ms each) were obtained longitudinally within the discernible contours of single cells.

**Myosin 20-kDa Light Chain Phosphorylation**

Segments of tail artery mounted on glass capillaries were frozen at 5 minutes after stimulation with cirazoline in 10% (wt/vol) trichloroacetic acid, 20 mmol/L dithiothreitol, in acetone on dry ice. Extraction, urea-glycerol gel electrophoresis, and blotting were as described by Mita and Walsh.\textsuperscript{20}

**Chemicals**

Cirazoline hydrochloride and calyculin A were from ICN; arsenate, rotenone, dinitrophenol, antimycin A, and 2-aminophenyl diphenyl borate (2-APB) were from Sigma; and fura-2 AM and fluo-4 AM from Molecular Probes. Dr Michael P. Walsh (University of Calgary, Alberta, Canada) kindly provided the polyclonal anti-LC20 antibody.

**Statistics**

Summarized data are presented as mean±SEM. Analysis of variance was used for multiple comparisons. Student’s t test or Wilcoxon matched pairs test was used for single comparisons. A value of \(P<0.05\) was considered significant. For \(n<6\), determinations were made in rings from separate animals. Modeling was made using MATLAB software (The Math Works Inc).

**Results**

The effect of mitochondrial inhibition with rotenone (site I, 10 μmol/L), antimycin A (site II, 5 μmol/L), cyanide (site III, 3 mmol/L), and dinitrophenol (uncoupler, 50 μmol/L) on arterial wall (global) [Ca\textsuperscript{2+}], and force was tested during stimulation with the \(\alpha_1\)-selective agonist cirazoline (0.3 μmol/L). With the exception of uncoupling, short exposures (10 minutes, Figures 1A through 1E) did not reduce stimulated [Ca\textsuperscript{2+}]. All interventions significantly inhibited contraction (Figures 1E through 1I). Longer exposure (2 hours) to...
antimycin A, but not rotenone or cyanide, reduced cirazoline-stimulated [Ca²⁺], (P<0.05, n=3, data not shown).

In Figure 1J, force is plotted versus global [Ca²⁺], for a cumulative concentration-response curve with cirazoline (control) and for the highest concentration of cirazoline (0.3 μmol/L) in the presence of mitochondrial inhibitors. The inhibitor data fall below and to the right of the control relationship, implying that a considerable fraction (50% to 70%) of force reduction is independent of global [Ca²⁺].

Myosin light chain phosphorylation (MLC-P) was determined after 25 minutes in relaxing solution and after 5 minutes stimulation with cirazoline following 5 minutes pretreatment with either vehicle or inhibitors. The protocol was thus identical to that used for force determination. As illustrated in Figures 2A and 2B, MLC-P was not significantly reduced by rotenone, antimycin A, or cyanide, whereas dinitrophenol almost totally abolished the phosphorylation response to stimulation (n=6 for all). Although we cannot exclude some reduction of MLC-P with antimycin A and cyanide (from 0.38 to ∼0.30 mol P per mol regulatory light chain), phosphorylation, being a global measurement, shows essentially the same response to mitochondrial inhibition as does global [Ca²⁺].

A straightforward interpretation of these results is that the metabolic sensitivity of vascular tone depends on mechanisms distinct from [Ca²⁺], elevation and myosin phosphorylation. However, when stable myosin phosphorylation was induced by the membrane-permeable phosphatase inhibitor calyculin A (3 μmol/L), the associated force response was reduced by only 26±10% on addition of rotenone (Figure 3A; n=4). A cirazoline-induced contraction of the same magnitude was reduced by 85±4% (n=4, P<0.001). Contractions induced by high-K⁺, which result from activation of voltage-sensitive Ca²⁺ channels, were also less sensitive to rotenone and antimycin A, although no difference was seen with cyanide (Figures 3B and 3C).

The greater effect on global [Ca²⁺], of uncoupling with dinitrophenol relative to sites I–III inhibition might be due to ATP consumption by mitochondria because reverse-mode activity of the ATP synthase is possible in the presence of dinitrophenol. When oligomycin (5 μg/mL) was used to inhibit the ATP synthase, the stimulated [Ca²⁺], after uncoupling increased by 27±9% (P<0.05, n=4; cf, online Figure A found in the online data supplement available at http://www.circresaha.org). Oligomycin had no effect by itself at 10 minutes, but long-term (2-hour) exposure inhibited the [Ca²⁺], response to cirazoline (not shown). These results suggest that mitochondrial ATP consumption may play some, although limited, role in the [Ca²⁺], response to uncoupling. Neither glibenclamide (10 μmol/L) nor tetraethyl ammonium (TEA, 1 mmol/L), which block Kᵦᵦ and Kᵥ channels, respectively, had any effect on the reduction of [Ca²⁺], by dinitrophenol (not shown).

Even though force reduction by mitochondrial inhibition could not be explained by decreased global [Ca²⁺], (Figures 1 and 4A), the possibility was considered that temporal fluctuations or spatial compartmentation might explain the results. To investigate this, laser scanning confocal microscopy was used. Frame scanning demonstrated that 9±2% (n=9) of cells in the vessel media exhibited asynchronous oscillations of [Ca²⁺], with a broad frequency spectrum. This value increased to 74±3% on stimulation with cirazoline (0.1 μmol/L, n=37; Figure 5A). During stimulation with the

**Figure 2.** Uncoupling but not site I through III inhibition reduces myosin phosphorylation. Myosin regulatory light chain phosphorylation (MLC-P) was measured under basal conditions and after stimulation with cirazoline in the presence and absence of mitochondrial inhibitors. A, Summarized data (n=6). B, Original blot with a general MLC antibody. ***P<0.001.

**Figure 3.** Effect of mitochondrial inhibition on force elicited by phosphatase inhibition or depolarization. A, Effect of rotenone (10 μmol/L) on force induced by calyculin A (3 μmol/L) or by cirazoline (0.3 μmol/L). B and C, Relative residual force after incubation with inhibitors during stimulation with 60 (B) or 140 (C) mmol/L K⁺ (open bars), or 0.3 μmol/L cirazoline (solid bars). n=4 to 5.
phosphatase inhibitor calyculin A, waves were present in 42±8% of cells (frequency 3.8±0.3 waves/min, n=10). This agent thus induces an intracellular Ca²⁺ response, but its main effect on contraction is mediated via a potent inhibitory effect on myosin phosphatase, sufficient to elicit a Ca²⁺-independent contraction.²¹

Addition of rotenone during stimulation with cirazoline increased the frequency and reduced the amplitude of the oscillations in active cells without altering time-averaged cellular [Ca²⁺]. One experiment, in which the same cells could be identified before and after addition of rotenone, is shown in Figures 4B and 4C. The majority of the focal increases in [Ca²⁺], spread along the axis of the cell and proceeded considerable distance as waves. Temporal stacks of line-scans from 2 different cells in one preparation before and after addition of rotenone clearly illustrate the altered wave pattern provoked by the mitochondrial inhibitor (Figure 4D). In 21 cells from 3 arterial rings, the frequency of oscillations increased by 71%, whereas the amplitude decreased by 46%, on addition of rotenone (Figure 5B). Spatially averaged fluorescence after addition of rotenone was 64±10 fluorescence units, versus 53±1 before addition (P=0.05), supporting the results obtained with fura-2 that rotenone does not lower global [Ca²⁺]. Cyanide likewise increased the frequency and reduced the amplitude of Ca²⁺ waves (Figure 5B). Both rotenone and cyanide slightly reduced the number of active cells (Figure 5A).

Because uncoupling, in contrast to rotenone and cyanide, lowered global [Ca²⁺], one might anticipate distinct effects on local [Ca²⁺] oscillations. At 10 minutes after the addition of dinitrophenol, the oscillatory pattern of [Ca²⁺] was completely abolished (Figure 5A). Likewise, the site II inhibitor antimycin A caused a pronounced reduction of the number of active cells. In the remaining active cells, the frequency was increased by 20% and the amplitude reduced by 61% (Figure 5B). Consistently, both global [Ca²⁺], (Figure 1) and Ca²⁺ oscillations (Figure 5) showed parallel tendencies to decrease with time of exposure to antimycin A. Similar effects do not arise with the site I and III inhibitors, because their responses are more stable in time.

The effect of inhibited glycolysis on Ca²⁺ waves was investigated using arsenate, which uncouples ATP formation during the conversion of 1,3-diphosphoglycerate to phosphoglycerate. Although it does not inhibit glycolysis, there is no net glycolytic generation of ATP.²² Treatment with arsenate (2 mmol/L) progressively reduced the cirazoline-induced global [Ca²⁺] response (n=4, P<0.001; cf, online Figure C). Basal [Ca²⁺], increased in parallel (n=4, P<0.01). Similar
but more dramatic effects were obtained using iodoacetic acid, which halts glycolysis (online Figure B).

Arsenate treatment for 1 hour (n=4) greatly reduced local Ca\(^{2+}\) oscillations in the presence of cirazoline (0.1 µmol/L, black bar) with addition of arsenate (2 mmol/L, gray bar). B, Mean force (black line)±SEM (dotted line) after addition of 2 mmol/L arsenate at 0 minutes (n=4). Filled symbols indicate mean force at the times when confocal Ca\(^{2+}\) images were obtained. Open symbols show the product of percent active cells (C), normalized frequency (D), and normalized amplitude (E) of Ca\(^{2+}\) waves.

Figure 6. Global [Ca\(^{2+}\)], force, and wave activity after inhibition of glycolysis. A, Representative trace (n=3) of global [Ca\(^{2+}\)] during stimulation with cirazoline (0.1 µmol/L, black bar) with addition of arsenate (2 mmol/L, gray bar). B, Mean force (black line)±SEM (dotted line) after addition of 2 mmol/L arsenate at 0 minutes (n=4). Filled symbols indicate mean force at the times when confocal Ca\(^{2+}\) images were obtained. Open symbols show the product of percent active cells (C), normalized frequency (D), and normalized amplitude (E) of Ca\(^{2+}\) waves.

Figure 7. Inhibition of Ca\(^{2+}\) waves by thapsigargin or 2-APB reduces adrenergically stimulated force. A, Effects of cirazoline (0.3 µmol/L, black bar) and verapamil (1 µmol/L, gray bar) on global [Ca\(^{2+}\)]. After thapsigargin treatment (10 µmol/L, 10 minutes), stimulation was repeated. First and second breaks are 25 and 60 minutes. B, Force recorded in another ring taken through the same protocol. C, Typical traces of Ca\(^{2+}\) wave activity under conditions as in A and B, except that 0.1 µmol/L cirazoline was used. Da, Concentration-dependent (IC\(_{50}=11\) µmol/L) inhibition of cirazoline (0.1 µmol/L)-induced force by 2-APB. Db through Dd, Percent active cells, normalized frequency, and normalized amplitude of Ca\(^{2+}\) waves, together with logistic fit to force data (dotted line). De, Product of data from Db through Dd.

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waves/min) and amplitude (66±6 versus 67±5 fluorescence units, 39 and 35 cells). After thapsigargin treatment, global [Ca\(^{2+}\)]\(_{i}\), in the stimulated preparations was maintained (85±7.4%. Figure 7Ac, versus 78±6%. Figure 7Ab; NS), whereas waves were completely abolished (Figure 7Cc) and contraction reduced by an additional 54±3% (Figure 7Bc; P<0.001).

To further establish a role of waves in force maintenance, we used the InsP\(_3\) receptor antagonist 2-APB, which dose-dependently inhibited cirazoline (0.1 \(\mu\)mol/L)-induced force (Figure 7Da) with an IC\(_{50}\) value of 11 \(\mu\)mol/L. Force inhibition by 2-APB was associated with dose-dependent reductions of the percentage of active cells (Figure 7Db), Ca\(^{2+}\) wave frequency (Figure 7De), and amplitude (Figure 7Dd). The product of the number of active cells, wave frequency, and amplitude correlated well with tissue-averaged force (Figure 7De).

**Discussion**

This study demonstrates a novel mechanism for an influence of metabolism on excitation-contraction coupling in vascular smooth muscle. The temporal and spatial pattern of intracellular Ca\(^{2+}\) fluctuations (waves) was shown to be affected by inhibition of oxidative or glycolytic metabolism, revealing correlations with force development that are not shown by conventional (global) measurements of either [Ca\(^{2+}\)]\(_{i}\), or MLC-P. Potentially, this explains earlier puzzling observations, but it should be emphasized that the evidence for a causal relationship between altered Ca\(^{2+}\) wave activity and relaxation during metabolic inhibition so far is indirect, as discussed later. A further aspect is that Ca\(^{2+}\) waves may influence other cellular processes, such as signaling pathways for gene expression and apoptosis, and that the effects shown in this study might reveal mechanisms for long-term effects of hypoxia on cellular function. Further work to explore these possibilities should prove fruitful.

In the rat tail artery, normal wave activity was found in preparations where the ryanodine receptors had been functionally inactivated, and thus InsP\(_3\) receptor activity seems to be the major cause of waves in this vessel. A crucial factor in the generation of waves is the Ca\(^{2+}\) sensitivity of the InsP\(_3\) receptor, implying a positive feedback control to increase the rate of Ca\(^{2+}\) release. High Ca\(^{2+}\) concentrations exert a negative effect on InsP\(_3\) receptor activity, of possible importance in a negative feedback loop to limit emptying of Ca\(^{2+}\) stores. A close spatial contact between mitochondria and the endoplasmic reticulum has been demonstrated in HeLa cells, and it is possible that a similar arrangement exists with the sarcoplasmic reticulum in smooth muscle. Indirect evidence for this are the functional results showing that Ca\(^{2+}\) released from the SR is rapidly taken up into mitochondria, and that mitochondrial inhibition reduces the amount of Ca\(^{2+}\) released by InsP\(_3\). These findings suggest that mitochondria effectively reduce the Ca\(^{2+}\) concentration in a restricted space near the SR, as it would otherwise be high enough to inhibit further release. Of direct relevance to the present findings, Jouaville et al showed that addition of mitochondrial substrates decreases the frequency and increases the amplitude of Ca\(^{2+}\) waves in Xenopus laevis oocytes, effects that were blocked by rotenone.

Ca\(^{2+}\) waves in Xenopus laevis oocytes, effects that were blocked by rotenone.

A central consideration in interpreting the present results is whether an altered wave pattern toward increased frequency and decreased amplitude is able to explain a decrease in force production in spite of maintained (or at least nearly so) levels of global [Ca\(^{2+}\)]\(_{i}\), and MLC-P. Relevant factors are that the relationships between [Ca\(^{2+}\)]\(_{i}\), and MLC-P, and between MLC-P and force, are both nonlinear and time-dependent. A simple model may be used to illustrate the impact of frequency and amplitude variations of Ca\(^{2+}\) waves on cell force to allow comparison with the observed effects. A sigmoidal [Ca\(^{2+}\)]\(_{i}\),-force relationship with an EC\(_{50}\) of 7·10\(^{-7}\) mol/L, obtained from experiments on skinned preparations (unpublished data) was used for conversion of fluorescence traces to force before and after treatment with rotenone (Figures 8A to 8B). Calculation of mean values from the individual traces shows that the inhibitor has a relatively greater effect on force than on [Ca\(^{2+}\)]\(_{i}\). Because the fluo-4 recording does not allow an absolute measure of [Ca\(^{2+}\)]\(_{i}\), the data in Figures 8A and 8B were generated using an assumed calibration factor giving physiologically reasonable [Ca\(^{2+}\)]\(_{i}\), values. To investigate the effect of calibration, a range of [Ca\(^{2+}\)]\(_{i}\), values was assigned to the means calculated for the 3 cells in Figure 4 were obtained for a range of physiologically reasonable calibrations by varying the calibration factor (k) between fluorescence and [Ca\(^{2+}\)]. Equation 1: [Ca\(^{2+}\)]\(_{i}\) = Fluorescence (AU) · k + m; Equation 2: Force (%); Equation 3: [Ca\(^{2+}\)]\(_{i}\) / EC\(_{50}\) - a; where k is the calibration factor (AU), chosen to give peak [Ca\(^{2+}\)] - 750 nmol/L in A, varied in C: m = 30 nmol/L (not critical <100 nmol/L); EC\(_{50}\) = 700 nmol/L (unpublished data from permeabilized tail artery); a = -4.7 (from Gomez and Swärd\(^{39}\); and a = 0.98 (not critical).
adrenergic stimulation in the tail artery, force was considerably reduced for similar \( \text{Ca}^{2+} \) levels during mitochondrial inhibition. A further prediction of the model is that force can be reduced as a consequence of reduced interwave \( [\text{Ca}^{2+}] \), because this would affect the position of the waves peaks vis à vis the threshold for contraction. The partial relaxation seen during L-type channel blockade (cf, Figure 7), which did not abolish wave activity, could thus be due to a decrease in \( \text{Ca}^{2+} \) influx across the cell membrane, affecting interwave \( [\text{Ca}^{2+}] \) and apparent in the measurements of global \( [\text{Ca}^{2+}] \). Different contributions to activator \( \text{Ca}^{2+} \) by influx via L-type channels may also explain discordant results in the literature, as in pressurized mesenteric resistance arteries addition of nifedipine was found to totally relax force, but not to eliminate wave activity. The model allows for both \([\text{Ca}^{2+}]\) versus MLC-P and for MLC-P versus force being nonlinear. On the other hand, it does not incorporate temporal latencies inherent in activation and force development, which will cause phase shifts between \([\text{Ca}^{2+}]\), and force. Effects of such phase shifts, if significant, would be expected to decrease the amplitude of force when oscillation frequency increases, as the system will function as a low-pass filter.

\( \text{Ca}^{2+} \) waves engaging only a section of a cell at a time might be expected to cause a segmental force development influenced by cell and tissue elasticity. However, we never observed length oscillations in individual cells during agonist-stimulated asynchronous wave activity. An interesting comparison is the study by Peng et al in rat mesenteric resistance arteries. During the initial phase of low-level adrenergic stimulation, asynchronous \( \text{Ca}^{2+} \) waves and slowly developing force were observed, whereas after prolonged stimulation, intracellular \( \text{Ca}^{2+} \) oscillations became synchronous between cells and force started to oscillate. This suggests that although cells are able to contract and relax in phase with the \( \text{Ca}^{2+} \) transients, segmental movements are only observed when tissue stress varies synchronously over sufficiently large areas to allow measurable changes in elastic extension.

Some level of metabolic sensitivity is seen with all modes of activation tested in this study, i.e., in addition to adrenergic stimulation, depolarization by high-K⁺ solution, and inhibition of myosin phosphatase activity using calyculin A. It is not likely that inhibited wave activity accounts for the relaxation with these latter types of stimulation. In addition, uncoupling of oxidative phosphorylation using 2,3-dinitrophenol caused a relaxation that was more pronounced than that produced by site I through site III inhibitors and was accompanied by decreased MLC-P. Thus, both stimulus conditions and modes of metabolic inhibition can be found that do not fit the pattern considered here with respect to the role of wave activity. This is to be expected because excitation-contraction coupling, and contraction itself, are energy-dependent at several steps. In a recent comprehensive investigation of the energetics of hypoxic relaxation in porcine coronary artery, Shimizu et al were able to rule out changes in either intracellular pH, ATP, or inorganic phosphate concentrations as causes of the response. Yet, given sufficiently potent metabolic inhibition, any of these factors may change sufficiently to cause loss of contractile force. In addition, as pointed out by Shimizu et al, the \( \text{Ca}^{2+} \) sensitivity of contraction is by definition decreased when force is reduced in the presence of elevated \( [\text{Ca}^{2+}] \), and in addition to effects mediated by altered myosin phosphorylation, this may involve a putative thin filament-mediated regulatory system. Whatever the nature of such energy-limited steps, they do not produce additive effects, i.e., even if inhibition of a later step in the sequence produces (nearly) as much relaxation as that of an earlier step, this would be revealed only when the early step is bypassed.

The fact that InsP₃, receptor blockade by 2-APB caused relaxation that correlated with decreased wave activity indicates that waves in fact support force development. Furthermore, several observations suggest that wave activity is a better correlate to force than global \([\text{Ca}^{2+}]\), as inhibition of glycolytic ATP production by arsenate caused a time-dependent relaxation correlating with decreased wave activity (active cells×frequency×amplitude), even though global \( \text{Ca}^{2+} \), as well as wave frequency, increased during the process. Similarly, force development in the presence of verapamil demonstrates that inflow of \( \text{Ca}^{2+} \) via L-type membrane channels is not necessary to support force; however, the subsequent elimination of waves by exposure to thapsigargin caused relaxation despite maintained elevation of global \([\text{Ca}^{2+}]\). Both thapsigargin and arsenate are expected to decrease extrusion of \( \text{Ca}^{2+} \) from the cell because thapsigargin inhibits \( \text{Ca}^{2+} \) uptake into the SR and arsenate inhibits glycolytic energy production, and thus, e.g., \( \text{Na}^{+}-\text{K}^{+} \) pump activity, which is of importance for \( \text{Na}^{+}-\text{Ca}^{2+} \) exchange over the plasma membrane.

Even though the different inhibitors of oxidative phosphorylation all produced similar results, there was a weaker relaxing effect of cyanide than of rotenone and antimycin A. This may have been due to the fact that cyanide has a low solubility at neutral pH and thus is difficult to administer in defined concentration. Metabolic inhibition may in some cases produce effects similar to hypoxia, so in addition to elucidating mechanisms due to metabolic inhibition or dysfunction, the present results may be relevant to hypoxic vasorelaxation. In summary, this study shows that inhibition of oxidative or glycolytic metabolism leads to relaxation of the vascular wall that may occur through an altered pattern of \( \text{Ca}^{2+} \) wave activity, which affects contractile activity of individual cells in the tissue but is not reflected in measurements of global \([\text{Ca}^{2+}]\), or MLC-P.

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References


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**Supplement 1**: During sustained contraction individual muscle cells are isometrically suspended and do not contract when Ca$^{2+}$ waves traverse the cells. An area of the tail artery, after stimulation with 0.1 µmol/L cirazoline is shown. The film runs at four times normal speed. Loading and mounting of the tissue was as described in Material and Methods.

**Supplement 2**: Effect of uncoupling and glycolytic inhibition on basal and stimulated [Ca$^{2+}$].

* A: the mitochondrial ATPase inhibitor oligomycin (5 µg/ml) enhances the response to cirazoline (black horizontal bars) after mitochondrial uncoupling with dinitrophenol (50 µmol/L). *B, C*: the glycolytic inhibitors iodoacetic acid (0.5 mmol/L) and arsenate (2 mmol/L) increase basal [Ca$^{2+}$] and reduce cirazoline-induced responses. Traces are representative of 4 independent experiments.
A

![Graph A](image)

**Ratio**

0.45 0.50 0.55 0.60 0.65

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cirazoline

dinitrophenol

B

![Graph B](image)

**Ratio**

0.5 0.6 0.7 0.8 0.9

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oligomycin

C

![Graph C](image)

**Ratio**

0.50 0.55 0.60 0.65

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2 mmol/L arsenate

5 min

0.5 mmol/L iodoacetic acid

Online supplementary material 2