Patterns of excitation-contraction coupling in arterioles: dependence on time and concentration

J. XIA AND B. R. DULING
Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia 22906-0011

Xia, J., and B. R. Duling. Patterns of excitation-contraction coupling in arterioles: dependence on time and concentration. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H323–H330, 1998.—We sought to understand the excitation-contraction coupling process in arterioles. KCl or phenylephrine (PE) was applied via the superfusion solution or by brief pulsatile ejections from a micropipette onto unpressurized arterioles (in vitro) from either the guinea pig small intestine or hamster cheek pouch. With either mode of application, KCl caused depolarizations that were tightly and predictably correlated with subsequent constrictions (electromechanical coupling). In contrast, the relationship between membrane potential and vasoconstriction in response to phenylephrine was dependent on both stimulus duration and agonist concentration. Application of short pulses of PE (<1 s) produced mechanical responses that were dominated by pharmacomechanical coupling (i.e., they were not associated with changes in membrane potential). With longer PE stimuli, electromechanical coupling became more important and dominated microvessel responses. We conclude that adequate understanding of the signaling process in microvessels requires a consideration of both concentration and duration of application. Both the mode and duration of agonist application affect the relative degree of electromechanical or pharmacomechanical coupling in response to a vasomotor stimulus. These observations have important implications for intracellular and intercellular signaling.

THERE ARE TWO BROAD categories of signaling that link receptor occupation to contraction of arterial smooth muscle: electromechanical and pharmacomechanical coupling (22). Electromechanical coupling depends on a primary change in membrane potential that induces a vasomotor response by altering Ca\(^{2+}\) influx through L-type, voltage-sensitive Ca\(^{2+}\) channels. Pharmacomechanical coupling, in contrast, can be independent of a change in membrane potential and relies on coupling between the receptor occupation and Ca\(^{2+}\) release from intracellular stores through a mechanism such as d-myo-inositol 1,4,5-trisphosphate release or on sensitization of the contractile apparatus to Ca\(^{2+}\). Electromechanical and pharmacomechanical coupling can be activated independently by an increase in extracellular K\(^{+}\) (9) or by application of a low dose of phenylephrine (PE), respectively (6, 22). The relative participation of the two coupling mechanisms initiated by a vasomotor stimulus is also known to vary with the agonist used and the tissue tested (15, 18).

Alteration of the stimulus duration is a common variable in microvascular stimulation within both physiological and experimental situations. Under physiological conditions, blood vessels may be exposed to short bursts of neurotransmitter after nerve stimulation or to sustained stimuli as a result of altered levels of circulating vasoactive substances. In microvascular studies, brief pressure-pulse ejection of agonist from micropipettes is often used as an experimental stimulus. Despite the wide range of duration of physiological and experimental stimuli to which vessels might be exposed, the effect of stimulus duration parameters on the relative contribution of pharmacomechanical and electromechanical coupling to vascular response has received little study.

We hypothesized that the temporal patterns of application of an agonist might play an important role in determining the relative contributions of electromechanical and pharmacomechanical coupling to vasomotor responses. We tested this possibility by examining the relationship between mechanical and electrical responses to different modes of application of KCl and PE.

METHODS

All experiments were approved by the University of Virginia Animal Care and Use Committee.

Hamster Cheek Pouch Arteriole

Male golden hamsters (weight 100–180 g) were anesthetized with pentobarbital sodium (70 mg/kg ip). A segment of arteriole (~3 mm in length, 40–80 μm in diameter) was dissected from the excised cheek pouch at 4°C in 3-(N-morpholino)propanesulfonic acid (MOPS)-buffered saline (pH 7.4) containing (mM) 145 NaCl, 4.7 KCl, 2.0 CaCl\(_2\), 1.2 MgSO\(_4\), 1.2 NaH\(_2\)PO\(_4\), 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 2.8 NaOH, and 2.0 MOPS. We then attached the arteriolar segment to the silicon base of a tissue bath (volume 1 ml) by pinning through the surrounding connective tissue, and it was superfused with bicarbonate-buffered physiological saline (37°C and pH 7.4) at 6 ml/min containing (mM) 120 NaCl, 5 KCl, 2.3 CaCl\(_2\), 2 MgSO\(_4\), 25 NaHCO\(_3\), 2 NaH\(_2\)PO\(_4\), and 5 glucose equilibrated with 21% O\(_2\)-5% CO\(_2\).

Guinea Pig Small Intestinal Arterioles

Guinea pigs (weight 180–230 g) of either sex were anesthetized with pentobarbital sodium (70 mg/kg ip). A segment of ileum (1 cm) was removed and transferred to a dissection dish filled with MOPS-buffered Ringer solution at 4°C with the same composition as that used for dissecting the hamster cheek pouch arteriole. The segment of ileum was cut open along the mesenteric line and pinned flat with the luminal...
surface up. The mucosa was removed to expose the vascularized submucosal connective tissue sheet that was then carefully separated from the underlying circular muscle layer. It was then transferred to a tissue bath where it was continuously superfused at 6 ml/min with bicarbonate-buffered Ringer solution (37°C).

Membrane Potential and Diameter Recording

Membrane potentials were recorded with an Axoclamp-2 preamplifier using glass microelectrodes filled with 2 M KCl. The tip resistances of the microelectrodes were 80–100 MΩ. To avoid tissue damage by silver ions (11), the reference Ag/AgCl electrode used to ground the organ bath was connected to the bath using a flexible, nonmetal, 3 M KCl salt bridge (WPI, New Haven, CT). The output from the Axoclamp-2 was displayed on an oscilloscope (V-650F, Hitachi) and simultaneously recorded on a strip-chart recorder and tape recorder (model 420, A. R. Vetter). The output from the Axoclamp-2 was displayed on an oscilloscope (V-650F, Hitachi) and simultaneously recorded on a strip-chart recorder and tape recorder (model 420, A. R. Vetter). To minimize capacitive coupling between the electrode tip and solution in the tissue bath, the surface level of the superfusion solution in the tissue bath was kept as low as possible. A successful recording was reflected by a sudden negative drop in voltage to -260 to -270 mV, which remained stable for over 1 min. The intracellular membrane potential recordings were typically maintained for 5–10 min. The arteriolar diameter was continuously measured on line using Diamtrak software (Monteck) (16).

Agonist Application

PE or KCl was applied either by superfusion or via pulsed ejection from a micropipette. Solution potassium concentrations were varied by replacing equal amounts of Na+ with K+. The pipette concentration of PE used for iontophoresis was 0.5 M. The KCl concentration used for pressure ejection was 225 mM in sodium-free MOPS buffer solution. Both iontophoresis and pressure pulses were used for pulsed ejection of PE, whereas only pulse pressure was used for KCl. Pipettes used for PE iontophoresis were pulled on a vertical pipette puller (model 700C, DKI) and had tip resistances of 10–20 MΩ when filled with 2 M KCl. Ejecting electrical currents of 800 nA generated by a Micro-iontophoresis programmer (model 160, WPI) were controlled by a stimulator (model SD-5, Grass Instruments). Holding currents of 20–50 nA were used to minimize leakage of PE between iontophoretic stimuli. The separation between stimulus pipette and the membrane potential recording electrode was -20 µm. Several lines of evidence suggest that only small segments of the vessel were exposed directly to agonists. The most compelling observation is based on the fact that application of agonists such as vasopressin and indolactam, which do not initiate conducted vasomotor responses, produce contractions that are confined to segments of arterioles 100–200 µm in length (data not shown). Furthermore, fluorescein, when applied by similar techniques, is typically confined to 100- to 200-µm segments of the arterioles.

Glass pipettes used for pressure application were pulled from glass stock (cat no. 1B100F-4, WPI). The tip of the pipette was ground to 5 µm in diameter, and the pressure pulse for the ejection was generated by a pneumatic PicoPump (model PV820, WPI) operating at 20 psi. Pulses between 50 and 1,000 ms are referred to as "short pulse stimulation," and pulses longer than 10 s are considered "long pulse stimulation." PE was purchased from Sigma Chemicals and dissolved in water. All other chemicals were of analytic grade.

Calculations and Statistical Analysis

The distance between the drug application pipette and the edge of the arteriole was measured at the site to which the drug was applied. The mean resting membrane potentials of the arterioles were compared using one-way analysis of variance (ANOVA). Student's t-test was used to test the difference between the regression lines of the relationship between vasoconstriction and depolarization for PE stimulations. A significance level of 0.05 was used for all the statistical tests.
RESULTS

The mean resting membrane potential of guinea pig small intestinal arterioles was $-74 \pm 3$ (SE) mV ($n = 17$). This potential was slightly more negative than those measured in hamster cheek pouch arterioles, $-67 \pm 1$ (SE) mV ($n = 30$), as determined by one-way ANOVA. Earlier observations suggested that the measurements represented a mixed cell population of smooth muscle and endothelial cells (26).

Guinea Pig Small Intestinal Arterioles

Responses to KCl superfusion. KCl superfusion (10, 15, 25, 55, and 105 mM) caused the expected dose-dependent depolarization and arteriolar constriction (Figs. 1 and 2A). As we have previously observed (25), the relationship between $K^+$ and membrane potential is linear rather than the more typical logarithmic relationship. The onset of the depolarization consistently preceded the constriction, and a threshold value of approximately $-50$ mV was noted for the mechanical response. Maximal contraction occurred at a $K^+$ concentration of 105 mM and a membrane potential of $-15$ mV. The amplitudes of both depolarization and constriction were stable for the duration of application of KCl. Figure 2A shows the relationship between the amplitudes of the constrictions and the membrane potential observed during steady-state KCl application.

Responses to KCl pulse application. Short pulse application (20 psi, 50- to 1,000-ms durations) of KCl also resulted in depolarization and vasoconstriction, and the relationship between the two variables was essentially the same as that seen with superfusion (Fig. 2, A and B).

Responses to PE superfusion. PE superfusion also depolarized and constricted the arteriole as illustrated by typical responses to 6 µM PE superfusion shown in Fig. 3C. The membrane potential response showed two phases: an initial depolarization followed by an abrupt transition to a steady-state and dose-dependent value. Depolarization did not, however, last during application of PE. After $-50$ s in the plateau phase, the potential declined spontaneously and rapidly back to the baseline level. The corresponding constriction also relaxed to within 7% of the basal diameter. Because of this phenomenon, dose-response curves to PE superfusion could not be constructed using cumulative drug addition. Instead, the responses to PE superfusion were studied one concentration at a time with a 30-min washout interval, as shown in Fig. 2C for PE concentrations of 0.1, 1, 3, and 6 µM.

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Fig. 2. Relationship between changes in diameter and membrane potential in guinea pig small intestinal arterioles. Stimuli were the following: A, KCl superfusion; B, application of a short pulse of KCl; C, phenylephrine (PE) superfusion; D, application of a short pulse of PE (pulse duration <1 s); and E, long pulse application of PE (pulse duration >30 s). Curves in A and B are best-fit sigmoidals, and the lines in C–E are linear regressions.
Responses to PE pulse application. Brief pulse stimulation of PE (iontophoresis, 800 nA, 50- to 1,000-ms duration) caused a constriction that was associated with a much smaller depolarization than that for pulsed application and superfusion of KCl. As shown in Fig. 2D, very large constrictions could be induced with a change of only a few millivolts in membrane potential. The relationship between constriction and membrane potential change in response to short pulses of PE was much steeper and shifted to the left compared with KCl stimulation or with observations made during PE superfusion (Student’s t-test, P < 0.01, Fig. 4).

To determine whether this striking difference between short pulse application and superfusion was due to 1) a difference in the length of the arteriole that was stimulated by PE or 2) a difference in the duration of stimulation as the pulse duration was increased to over 10 s (long pulse), we compared responses to short and long pulse stimuli. Figure 3B shows a typical record of the responses to a long-pulse application of PE (pulse duration 30 s, 800 nA). The data for several such stimuli are plotted in Fig. 2E. Note that the relationship between diameter and membrane potential is the same as the response observed during superfusion of PE (Figs. 2C and 4) but quite different from that seen with brief, pulsatile PE application (P < 0.01, Figs. 2D and 4). This demonstrates that it is the difference in the duration of stimulation rather than the length of the arteriole exposed to drug during stimulation with PE superfusion that accounts for the disparate responses to short pulse PE application or superfusion.

Relationship Between Pulse Duration and Electrical and Mechanical Responses to PE

To further explore the relationship between pulse duration and mechanical or electrical responses to PE, we stimulated the arteriole with pulse durations ranging from 0.01 to 90 s. Because depolarizations caused by PE applied with longer pulse durations tended to reach a plateau, the potential changes were related to diameter by analyzing the area of the depolarization response, a measure that we have found to be closely tied to vasoconstriction (25). The area of the depolarization response as a function of pulse duration is illustrated in Fig. 5 (dotted line). A sigmoidal relationship was observed with a steep rise as pulse duration was in-
creased beyond 10 s. The relationships among constriction, area of depolarization, and the pulse duration are shown in Fig. 6. Note that significant vasoconstriction could often be induced in the absence of detectable depolarization.

Hamster Cheek Pouch Arterioles

Responses to KCl superfusion and pulse application. The patterns of the electrical and mechanical responses of the hamster cheek pouch arteriole to either KCl superfusion or pulse application were essentially identical to those of the guinea pig small intestinal arterioles (Fig. 7, A and B).

Responses to PE superfusion and pulsatile micropipette applications. Diminished PE stimulation was also observed in cheek pouch arterioles during continued exposure to the drug. The relationship between the amplitude of the constriction and the amplitude of the depolarization is shown in Fig. 7C. As with the guinea pig vessels, this relationship is shifted to the left compared with that seen with KCl stimulation (Fig. 8).

In response to short pulse stimulation with PE iontophoresis, brief constriction and depolarization were evoked. However, in the hamster vessels the amplitude of the depolarization was relatively greater than that observed with the same degree of constriction in the guinea pig (compare Fig. 2D with Fig. 7D). As the pulse duration was extended with the pipette application of PE, the electrical and mechanical responses approached those seen with PE superfusion (Figs. 7E and 8).

Relationship between pulse duration and electrical and mechanical responses to PE. Figure 5 (solid line) demonstrates the change in membrane potential as a function of pulse duration. The electrical response to PE with short pulse durations was much greater than its counterpart response in guinea pig small intestinal arterioles. Thus it is clear that the hamster cheek pouch arterioles behave in essentially the same manner as the submucosal arterioles, except that brief pulses of PE produced larger depolarizations.

Contribution of concentration in PE in responses to pulsed stimulation. As the agonist is ejected from a micropipette, a finite time is required for the concentration on the arteriolar surface to rise. Thus pulse length should alter either duration of exposure to the drug or the peak concentration achieved, or both. To assess the effect of changing concentration per se, we used a larger pipette tip (5 µm ID) to ensure that the concentration of PE in the immediate vicinity of the pipette tip approximated that within the pipette. We also used pressure ejection rather than iontophoresis to eject the PE. The relationship between vasoconstriction and depolarization in response to PE pulsed in this way was then tested with various PE concentrations in the pipette ($10^{-2}$–$10^{-3}$ M) and with two pulse durations, 0.5 and 10 s. We found that an electrical response was more readily initiated with pressure pulse ejection than with iontophoresis. However, as shown in Fig. 9, the diameter-membrane potential relationships resulting from stimuli using pulsed ejections of different concentrations of PE were similar to those resulting from the variations in pulse durations (Figs. 4 and 8).

DISCUSSION

This study demonstrates that the duration of application of an agonist as well as the concentration of drug applied can have a profound influence on the relative contribution of pharmacomechanical or electromechanical coupling to the vasomotor response. The relationship between vasoconstriction and depolarization during short pulse stimulation with PE is strikingly different from that observed during superfusion (Figs. 2, 4, 7, and 8). However, when stimulus duration was increased to longer than 10 s, both guinea pig and hamster arterioles showed a strong and consistent
relationship between electrical and mechanical response. Moreover, the relationship observed with PE mimicked the response seen with KCl, and this appeared to be largely the result of electromechanical coupling. This observation shows that variations in the contribution of electromechanical and pharmacomechanical coupling to the response were not due to the difference in the length of the arteriolar segment that was exposed to the PE. Rather, it appears that the duration of stimulation and/or the concentration of the agonist are the critical variables.

Although the duration of application is a determinant of the observed extent of electromechanical or pharmacomechanical coupling, phenylephrine concentration also plays a role. By varying pipette concentration rather than time, we showed that a similar relationship between diameter change and membrane potential was obtained compared with that seen when pulse duration was changed (Fig. 9). Concentration is not the sole variable, however, since a difference in the diameter-potential relationship still exists when short (0.5 s) and long pulse (10 s) stimuli are compared. These data suggest that both concentration and stimulus duration have an effect on the response to PE and on the degree to which pharmacomechanical or electromechanical coupling is invoked.

It is interesting to note that the greater role played by pharmacomechanical coupling in these isolated vessels in response to short pulses might be at least in part the result of influences from surrounding segments of the arteriole that are not exposed to PE. The membrane
potential of short segments stimulated with PE might be effectively clamped at the resting potential by the surrounding tissue. In this context, differences between hamster and guinea pig might reflect differences in gap-junctional conductance between the two vessels or a related variable. This interpretation is unlikely, however, since the electrical responses to long and short pulse KCl within each type of arteriole are not different. It is also possible that a change in gap-junctional resistance occurs during stimulation, and this might vary with pulse duration or species. These observations highlight the importance of developing a greater understanding of the relative changes in membrane resistance with respect to gap junctions under various circumstances.

The effect of stimulus duration and concentration of PE on the pattern of the response might also be explained by the presence of two populations of α-receptors with distinct pharmacomechanical and electromechanical coupling patterns and/or different affinities. α1A- and α1B-receptor subtypes have been identified (8, 9, 12), and the two subtypes are postulated to be coupled to different Ca2+ signaling mechanisms (8, 23). α1A-receptors appear to stimulate inositol phosphate formation and/or intracellular Ca2+ mobilization and cause functional responses that are largely independent of extracellular Ca2+, whereas α1B-receptors cause a physiological response that is predominantly dependent on the influx of extracellular Ca2+. The distribution of α1A- and α1B-receptors is also species dependent and varies from vessel to vessel (5, 10, 23).

The dependence of the response to PE on the stimulus duration might also reflect differences in the characteristics of the receptor-operated channels (ROC) that are activated by PE and cause the resultant depolarization. Depolarization after activation of ROC by PE can be caused either by a voltage-dependent channel (e.g., K+ or Ca2+ channel) or by an indirect effect on such pathways that are activated by a rise in intracellular Ca2+. There are several potential ionic mechanisms that may be responsible for agonist-induced depolarization, including opening of nonspecific cation channels (1, 4, 7, 24) or inhibition of K+ channel activity (13, 20). Furthermore, there exists whole cell patch-clamp evidence that the duration of receptor occupancy might influence the open probability of each of these channels and thus the pattern of electrical response. For example, Miyoshi et al. (14) showed that depolarization of porcine coronary artery smooth muscle cells by endothelin was gradual and increased dramatically after 3 min of endothelin application. Similarly, blockade of K+ channel currents induced by endothelin required >10 s to occur. Dependence of voltage-gated Ca2+ channel behavior on stimulation time has also been reported (21).

The process active in our experiments has yet to be established. However, the fact that the relationship between constriction and depolarization was independent of pulse duration for KCl in both types of arterioles suggests that the effects of pulse duration are not consistent with the kinetics of L-type Ca2+ channels.

It is conceivable that differences between responses to short and long pulses arise as a result of stimulation of different cell types. However, in hamster cheek pouch arterioles, the smooth muscle cells and endothelial cells appear to be electrically coupled and exhibit the same resting membrane potential as well as local and conducted electrical responses. Thus cell-dependent responses that do arise must reflect different calcium dependencies in the two cells or differences in second messenger systems.

Delashaw and Duling (3) found that the ability to initiate conducted vasomotor response in hamster cheek pouch arterioles by different agonists was very heterogeneous from vessel to vessel and was dependent on the agonist applied. They hypothesized that the heterogeneity in vasomotor conduction originated in the signaling process at some point between receptor occupation and alteration of the membrane potential. The present study supports their hypothesis by demonstrating that the degree of electromechanical coupling involved in a response is dependent on the drug used as well as the duration of application.

The dependence of the electrical response on stimulus duration will contribute to variability in the conducted response, since membrane depolarization appears to be required to initiate conduction (25). The present report clearly demonstrates that a robust mechanical response has little predictive value for the presence or absence of the electrical response and thus for the conducted response.

The escape phenomenon (Fig. 3C), initially defined by Ross (19), is striking and consistent with the observa-
tion made by Neild and Kotecha (17) in the guinea pig small intestinal arterioles. They hypothesized that this desensitization might be due to a combined effect of a rise in intracellular adenosine 3',5'-cyclic monophosphate and an increased rate of intracellular Ca²⁺ sequestration. It is noteworthy, however, that no such behavior is observed in in vivo hamster cheek pouch arterioles (our unpublished observation), suggesting a major difference in signaling between in vitro and in vivo situations.

Such differences could reflect the change in vascular environment or absence of intravascular pressure or flow. The greater membrane potentials seen in these vessels must be considered in trying to establish the physiological significance of our findings, but the unpressurized vessels are likely to exist in the pathological situation of shock associated with hemorrhage.

In summary, we have shown that stimulus duration, drug concentration, duration of application, and species all have a profound effect on patterns of electromechanical versus pharmacomechanical coupling in vaso-motor response. We hypothesize that the effect of stimulus duration might be a result of activation of two subtypes of α-receptor on the smooth muscle cells in these arterioles. Alternatively, it may be due to activation of a ROC with a probability of opening following agonist stimulation that changes with the duration of stimulation.

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Present address for J. Xia: Dept. of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce St., Philadelphia, PA 19104-6008.

Address for reprint requests: B. R. Duling, Dept. of Molecular Physiology and Biological Physics, Univ. of Virginia Health Sciences Center, PO Box 10011, Charlottesville, VA 22906-0011.

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