Hypothesis for the Initiation of Vasomotion

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Abstract—Vasomotion is the regular variation in tone of arteries. In our study, we suggest a model for the initiation of vasomotion. We suggest that intermittent release of Ca^{2+} from the sarcoplasmic reticulum (SR, cytosolic oscillator), which is initially unsynchronized between the vascular smooth muscle cells, becomes synchronized to initiate vasomotion. The synchronization is achieved by an ion current over the cell membrane, which is activated by the oscillating Ca^{2+} release. This current results in an oscillating membrane potential, which synchronizes the SR in the vessel wall and starts vasomotion. Therefore, the pacemaker of the vascular wall can be envisaged as a diffuse array of individual cytosolic oscillators that become entrained by a reciprocal interaction with the cell membrane. The model is supported by experimental data. Confocal $[Ca^{2+}]_i$ imaging and isometric force development in isolated rat resistance arteries showed that low norepinephrine concentrations induced SR-dependent unsynchronized waves of Ca^{2+} in the vascular smooth muscle. In the presence of the endothelium, the waves converted to global synchronized oscillations of $[Ca^{2+}]_i$ after some time, and vasomotion appeared. Synchronization was also seen in the absence of endothelium if 8-bromo-cGMP was added to the bath. Using the patch-clamp technique and microelectrodes, we showed that Ca^{2+} release can activate an inward current in isolated smooth muscle cells from the arteries and cause depolarization. These electrophysiological effects of Ca^{2+} release were cGMP dependent, which is consistent with the possibility that they are important for the cGMP-dependent synchronization. Further support for the model is the observation that a short-lasting current pulse can initiate vasomotion in an unsynchronized artery as expected from the model. **(*Circ Res*. 2001;88:810-815.)**

Key Words: calcium imaging ■ calcium waves ■ cGMP ■ synchronization ■ vasomotion

Synchronized oscillations in cell activity are seen in many cell types. In the vascular wall, synchronized oscillations of smooth muscle cell tension give rise to oscillations of vascular tone (vasomotion), which are of physiological and pathophysiological importance.[1,2] Vasomotion is known to be associated with slow oscillations of smooth muscle membrane potential[3] and of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).[4] It has also been demonstrated that the function of the sarcoplasmic reticulum (SR) is critically important for the activity,[5,6] whereas potassium channels play only a modulatory role.[7,8] The role of the endothelium differs between vascular beds. In some, a functional endothelium is essential for vasomotion,[3,9,10] whereas in others, vasomotion is promoted after removal of the endothelium.[6,11]

However, there is no model demonstrating how vasomotion is initiated and, in particular, which mechanisms are important for the synchronization of the membrane potential oscillations and for the regeneration of the pacing signal in the vascular wall.

In many tissues that show synchronized oscillations, designated pacemaker cells are present (eg, in the heart and the intestine), and it has also been suggested that pacemakers could play a role in the vascular wall.[2,12] However, no pacemaker cells have been demonstrated in the vascular wall, and it seems that another mechanism may be important.

In the present study, we suggest a model for determining how vasomotion is initiated (see online video, available in the data supplement at http://www.circresaha.org) in rat mesenteric resistance arteries. Repetitive transitory changes in $[Ca^{2+}]_i$, generated by the release and reuptake of Ca^{2+} from the SR, have been shown to cause Ca^{2+} waves in vascular smooth muscle cells in the present and other[13–15] studies. Our model suggests that these repetitive Ca^{2+} changes constitute the basic oscillator that paces the vasomotion. These cytosolic Ca^{2+} oscillations initially occur asynchronously in individual cells. However, the Ca^{2+} released intermittently from the SR activates a depolarizing current. This current spreads to all smooth muscle cells, presumably by means of gap junctions, and when it reaches a sufficient magnitude, it will cause simultaneous depolarization of all smooth muscle cells.[16] This leads to a synchronous influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, which enhances the likelihood of Ca^{2+} release from the SR[17] in a synchronized manner, either by promoting the release resulting from the Ca^{2+} sensitivity of either inositol 1,4,5-triphosphate receptors or ryanodine receptors or possibly by promoting the refilling of the stores

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with subsequent Ca^{2+} overflow. The individual cytosolic oscillators are now synchronized and entrained, and this initiates vasomotion.

In the present study, the model is supported by experimental data obtained in rat mesenteric small arteries, which when activated by norepinephrine (NE) show regular, nearly sinusoidal, oscillations in tone, which makes it likely that these could be explained by a single oscillating mechanism.

This scheme of events is radically different from the way in which synchronization and regeneration are achieved in tissues with action potentials; in such tissues, specific pacemaker cells control the frequency, and regeneration occurs through potential-sensitive ion channels. The present hypothesis does not require specific pacemaker cells in the vascular wall, and synchronization and regeneration are achieved through a reciprocal interaction between the SR and the membrane, which entrains the cytosolic oscillators present in all smooth muscle cells. We suggest that such a model might also be of relevance in groups of cells that constitute pacemakers in other tissues.

Materials and Methods

Isometric Force and Ca^{2+} Imaging of Rat Mesenteric Resistance Arteries

The intestinal mesentery was removed from 12- to 18-week-old male Wistar rats. A third-order branch of the superior mesenteric artery (internal diameter 286±5 μm, n=60) was dissected out and mounted as a ring preparation (≈2 mm long) in a custom-built myograph (Danish Myo Technology) for isometric force development. The internal circumference of the mounted artery was normalized on the basis of the passive tension-length curve to a value that gives maximal force development.[18] In experiments in which the endothelium was removed, this was performed by rubbing the inside of the artery with a 40-μm steel wire and confirmed by the disappearance of relaxation to acetylcholine. The myograph was placed on the stage of an inverted confocal laser scanning microscope (ODYSSEY XL, Noran). Confocal optical sections were acquired with a water immersion objective (×60, numerical aperture 1.2, Nikon). The arteries were loaded with calcium green-1 acetoxymethyl ester (3 μmol/L) for 3 hours at 37°C. A 77×58-μm image (640×480 8-bit pixels) was obtained every 533 ms by using a 100-ns time scan mode and 16-frame averaging. In some experiments, 77×14-μm images (640×120 8-bit pixels) were obtained every 266 ms with 32-frame averaging. The emission signals at 530 nm (excited with 488-nm laser light) were stored on a computer during the experiments, together with simultaneous force measurements.

For image analysis, the programs Intervision (Noran) and ImageSpace (Molecular Dynamics) were used. Even though we measure changes in fluorescence emission, for the ease of presentation, we use the phrase "$[Ca^{2+}]_i$ changes." The $[Ca^{2+}]_i$ changes within cells were estimated as the changes in the mean intensity of calcium green-1 fluorescence within regions of interest (ROIs; see frame 1 in Figure 1) in which all pixel values were averaged. For analysis of $[Ca^{2+}]_i$ oscillations and waves, the relative increase in fluorescence intensity was used. This was determined by dividing the fluorescence in ROIs by the baseline fluorescence in the corresponding ROI.

For the measurements of the effect of caffeine on $[Ca^{2+}]_i$, we used fura 2-AM as described previously.[19] In brief, vessels were mounted in a myograph as described above and loaded for 1 hour at 37°C with 2 μmol/L fura 2-AM. The artery was excited alternately at 340 nm and 380 nm, with generation by a 75-W xenon lamp (DeltaScan Illuminator, PTI), and the emitted light was collected by a ×40 objective (numerical aperture 0.55, Leica) and passed through a 510-nm long-pass filter to a photomultiplier tube (PMT, model 710, PTI). After completion of the experimental protocol, the signals were calibrated as described previously.[19] All the fluorescent signals and force were sampled at 1 Hz and stored on computer.

Electrophysiology

Measurements of membrane potential were made as described previously.[20] In brief, vessels were mounted in a myograph as described above. Intracellular recordings of membrane potential were obtained by using glass microelectrodes filled with 3 mol/L KCl (resistance >60 MΩ) with an Ag-AgCl electrode in the bath as a reference electrode. Potentials were measured by an Intra-767 electrometer (WPI Inc), displayed on an oscilloscope, and recorded on a chart recorder. Electrode resistance was monitored continuously by current pulse injection. Impalements were maintained between 5 and 60 minutes.

Patch-clamp recordings were made on smooth muscle cells from similar arteries isolated by overnight cold storage in papain solution as described elsewhere.[21] Whole-cell currents were recorded by using the amphotericin-perforated–patch technique with patch electrodes of 4- to 8-MΩ resistance connected to an Axopatch 200B amplifier (Axon Instruments) and analyzed by using the pClamp 7 software package (Axon). Access resistance in these experiments was in the range of 15 to 40 MΩ. Currents were recorded at a holding potential of −60 mV, and 10 mmol/L caffeine was superfused over the cell. Where indicated, 10 μmol/L ryanodine was superfused over the cell.

Solutions

PSS had the following composition (in mmol/L): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, EDTA 0.026, HEPES 5.0, and glucose 5.5. The solution was gassed with 5% CO_2/95% O_2, and pH was 7.45 to 7.5. K^+-PSS is PSS in which potassium is increased to 125 mmol/L by equimolar substitution of NaCl with KCl and in which 1 μmol/L phentolamine is added. Ca^{2+}-free K^+-PSS is K^+-PSS without Ca^{2+}. In patch-clamp experiments, the pipette contained the following (in mmol/L): KCl 102, NaCl 10, $CaCl_2$ 1, $MgCl_2$ 1, EGTA 10, and potassium HEPES 10; the extracellular solution contained the following (in mmol/L): NaCl 135, KCl 6, $CaCl_2$ 0.1, $MgCl_2$ 1, and sodium HEPES 10. Both solutions were adjusted to pH 7.4 at room temperature.

Results

Low concentrations (0.1 to 0.5 μmol/L) of NE induced $[Ca^{2+}]_i$ transients in individual smooth muscle cells within the walls of rat mesenteric small arteries (Figure 1A; see also online video, available in the data supplement at http://www.circresaha.org). These transients were not synchronized between cells and appeared to be similar to those seen in the rat tail artery,[13] in rat pressurized mesenteric arteries,[14] and in rabbit vena cava.[15] A $[Ca^{2+}]_i$ transient originated from one end of the cell and traveled as a wave along the cell (Figure 1B) at a rate of 12 to 175 $\mu m \cdot s^{-1}$ with a median of 36 $\mu m \cdot s^{-1}$ (n=225 waves). Waves started to appear even before tension began to rise (Figure 1C), consistent with the observation by Miriel et al[14] that Ca^{2+} waves were seen in pressurized arteries in the absence of tone. Two to 8 minutes after NE application, the $[Ca^{2+}]_i$ transients synchronized into regular oscillations and caused oscillating force development, ie, vasomotion (Figures 1A and 1C; see also online video, available in the data supplement at http://www.circresaha.org). During the oscillations, $[Ca^{2+}]_i$ appeared to rise simultaneously throughout the cell; ie, only global $[Ca^{2+}]_i$ changes were seen, and no waves were apparent. When the NE concentration was increased to 10 μmol/L, which gives maximal force development, vasomotion disappeared, and a tonic tension was seen. In this condition, the unsynchronized

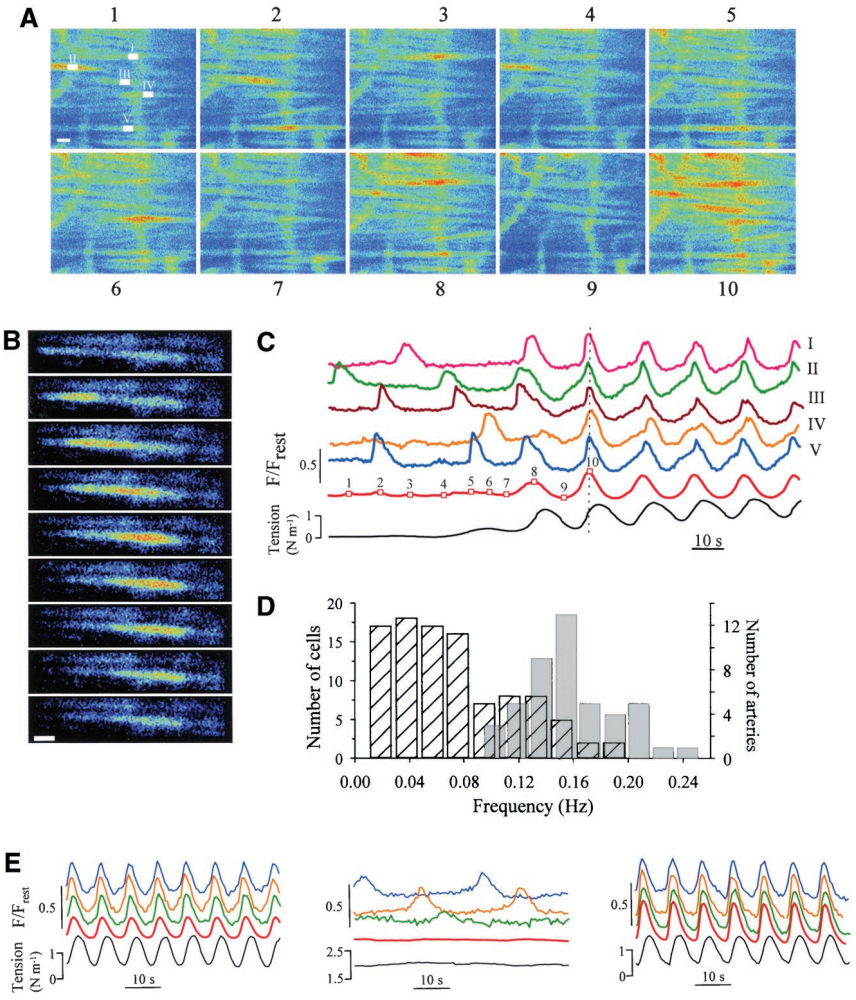

Figure 1. Ca^{2+} waves and oscillations in small arteries. Shown are $[Ca^{2+}]_i$ transients in smooth muscle cells of small mesenteric arteries activated with a low concentration of NE. A, Confocal images of $[Ca^{2+}]_i$ over time in smooth muscle cells (taken at 10 time points shown in panel C from a series of 300 sequential images recorded every 533 ms with 16-frame averaging). Intracellular changes in $[Ca^{2+}]_i$ are initially out of phase in different cells but come into phase in the last 4 frames. The first frame shows ROIs used in all frames for analysis. B, Sequential images at greater magnification of the initial $[Ca^{2+}]_i$ changes seen in images 1 to 4 in panel A, which appear as waves of $[Ca^{2+}]_i$ running along the cells. C, Plot of intensity and tension vs time of selected regions (I through V) corresponding to cells in panel A. The red curve represents the average of the entire frame, and the black trace shows tension. Numbered boxes show when images in panel A were taken. This experiment was performed at 22°C to reduce oscillation frequency. F indicates fluorescence in ROI; F_{rest}, baseline fluorescence in corresponding ROI. D, Frequency distribution of waves (hatched bars, left ordinate) and oscillations (filled bars, right ordinate). E, Traces of tension (black), average $[Ca^{2+}]_i$ (red), and $[Ca^{2+}]_i$ in 3 ROIs corresponding to 3 different cells under control conditions (stage 1), after removal of endothelium (stage 2), and after application of 300 μmol/L 8Br-cGMP to the endothelium-denuded artery (stage 3). Cellular $[Ca^{2+}]_i$ changes were initially synchronous (stage 1) and then lost synchronization after endothelium removal (stage 2) but were synchronized after application of cGMP (stage 3). This experiment was performed at 37°C. The scale bars in the images are 5 μm.

Ca^{2+} waves reappeared (n=3). The model suggests that the unsynchronized waves are pacemakers for the vasomotion. In that case, it is expected that the frequency range of the waves is overlapping with the frequency range of the vasomotion. This was indeed the case, as seen in Figure 1D. The mean frequency of oscillations was higher than that of waves, but the frequency distributions were overlapping, consistent with the model.

As previously reported,[13–15] $[Ca^{2+}]_i$ waves required a functional SR, inasmuch as they were always abolished after pretreatment with 10 mmol/L caffeine (releasing Ca^{2+} from the SR), 10 μmol/L ryanodine (in this concentration, inhibiting the release of Ca^{2+} from the SR), or 10 μmol/L thapsigargin (inhibiting the uptake of Ca^{2+} into the SR) (Table). All experiments with thapsigargin were made after removal of the endothelium, because thapsigargin has an endothelium-dependent hyperpolarizing effect in rat mesenteric small arteries.[22]

In contrast, Ca^{2+} influx was not necessary for waves to appear, inasmuch as they were seen in the presence of 2 nmol/L of the L-type Ca^{2+} channel blocker felodipine (preincubation for 25 to 60 minutes), after hyperpolarization of the smooth muscle cells with 10 μmol/L pinacidil[23] (preincubation for 20 to 30 minutes), or after the omission of Ca^{2+} from the bathing medium with the membrane either at resting potential or fully depolarized (5 or 125 mmol/L potassium, respectively, in the bath; preincubation for 7 to 15 minutes). Thus, the $[Ca^{2+}]_i$ waves are evoked by a cytosolic oscillator,[24] consistent with previous observations,[13–15] and provide the basis for the proposed model.

If the waves are the pacemakers for the vasomotion, vasomotion should depend on an intact SR. This has been demonstrated previously[5,6] and is confirmed in the present study. Also, the oscillations of $[Ca^{2+}]_i$ and tension were always prevented by inhibitors of the SR function (Table). In addition, not only did thapsigargin at 1 μmol/L inhibit the oscillations, but in 10 experiments, we found that thapsigargin at a concentration of 0.03 μmol/L reduced the frequency of the oscillations to 85±5%, and with 0.1 μmol/L thapsigargin, a more complex oscillatory pattern emerged, as described by Griffith and Edwards.[6] Thus, a thapsigargin-sensitive uptake of Ca^{2+} into the SR also seems to play a central role in the process that leads to synchronized oscillations.

However, the model predicted that the vasomotion and synchronized Ca^{2+} oscillation are dependent on extracellular Ca^{2+}. This was confirmed by the observation that in contrast to the situation with waves, felodipine, pinacidil, or the omission of extracellular Ca^{2+} always inhibited oscillations (Table) and caused asynchronous waves to reappear.

Role of Membrane Potential and SR Function for $[Ca^{2+}]_i$ Transients

Treatment	Asynchronous $[Ca^{2+}]_i$ Waves	Synchronous $[Ca^{2+}]_i$ Oscillations	Experiments, n
Membrane potential			
NE+pinacidil	Yes	No	6
NE K^+/Ca^{2+} free	Yes	No	3
NE+felodipine	Yes	No	9
SR function			
NE+thapsigargin	No	No	3
NE+ryanodine	No	No	3
NE+caffeine	No	No	3

It has previously been shown that removal of the endothelium prevents vasomotion in these arteries but that vasomotion can be restored by application of the membrane-permeable analogue of cGMP, 8-bromo-cGMP (8Br-cGMP).[3] In 3 experiments in which the endothelium was removed, NE induced only asynchronous waves. However, these always became synchronized into oscillations after the addition of 300 μmol/L 8Br-cGMP (Figure 1E). This finding strongly suggests that the endothelium and cGMP are essential for synchronization.

An important prediction from the model is that the Ca^{2+} release from the SR can induce a depolarization and that this depolarization is cGMP dependent. To test this prediction, we investigated the effect on the membrane potential of releasing Ca^{2+} from the SR in these arteries. Release of Ca^{2+} by 10 mmol/L caffeine was associated with a transient force development and a depolarization of 11.0±1.5 mV (n=10 cells in 7 arteries, Figure 2A). Ca^{2+} release (not shown), force, and depolarization were all abolished by ryanodine (Figure 2B), indicating that they were due to a specific effect of caffeine on the SR. In contrast, we found in 3 experiments that 1 mmol/L caffeine caused a hyperpolarization of 7.3±1.3 mV (n=3 cells in 3 arteries). This suggests that both inward and outward currents can be activated by Ca^{2+} release in these arteries.

If the 10 mmol/L caffeine–induced depolarization is important for synchronization, this effect should be absent after endothelium removal and should reappear after the addition of 8Br-cGMP. Figures 2C and 2D show that this is so. Removal of the endothelium inhibited the depolarizing effect of caffeine (2.4±1.2 mV, n=8 cells in 6 arteries), and 8Br-cGMP restored the caffeine-induced depolarization in arteries without endothelium (12.0±0.6 mV, n=3 cells in 3 arteries).

On the basis of these findings and the proposed model, we tested whether 10 mmol/L caffeine could induce an inward current in isolated voltage-clamped smooth muscle cells in the presence of 8Br-cGMP. This was indeed found (Figure 2E). Thus, caffeine in the presence of 8Br-cGMP induced an inward current of 168±28 pA (n=20); in the presence of 10 μmol/L ryanodine, the response was abolished, suggesting that it was a consequence of Ca^{2+} release (Figure 2F). Furthermore, in the absence of 8Br-cGMP, the current was markedly reduced (15±4 pA, n=6) (Figure 2G), indicating that it is cGMP dependent.

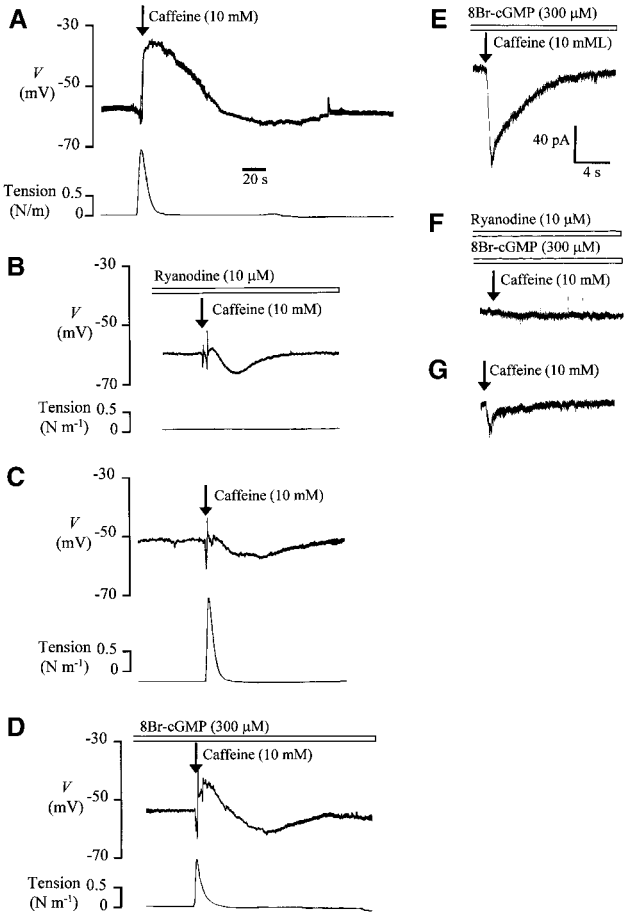

Figure 2. Effect of caffeine on membrane potential and current. A, Application of caffeine under control conditions caused a transient depolarization (top) and contraction (bottom). V indicates voltage. B, Inhibiting intracellular release of Ca^{2+} by ryanodine inhibited contraction and depolarization. C, Removal of endothelium eliminated the depolarization but did not inhibit the contraction to caffeine application. D, Applying a membrane-permeable analogue of cGMP (8Br-cGMP) to the endothelium-denuded artery caused depolarization to reappear. E, Patch-clamp experiments revealed an inward current activated by 10 mmol/L caffeine application in the presence of extracellular 8Br-cGMP. F and G, This current was absent in the presence of ryanodine (F) and was much reduced in both amplitude and duration in the absence of 8Br-cGMP (G).

Thus, we have demonstrated a current and a corresponding depolarization, which because of their cGMP dependence are likely to correspond to the membrane events predicted by the model. These events are predicted to synchronize the cells, which subsequently remain entrained to generate vasomotion. Therefore, it was important to show that a transient depolarization can initiate vasomotion. This is, in effect, "defibrillating" the arterial wall. As shown in Figure 3, applying a single 1-ms electrical impulse across the blood vessel or applying a short pulse of PSS containing 62 mmol/L potassium (KCl substituted for NaCl) in the presence of a low concentration of NE triggered sustained oscillatory activity that was consistent with the prediction.

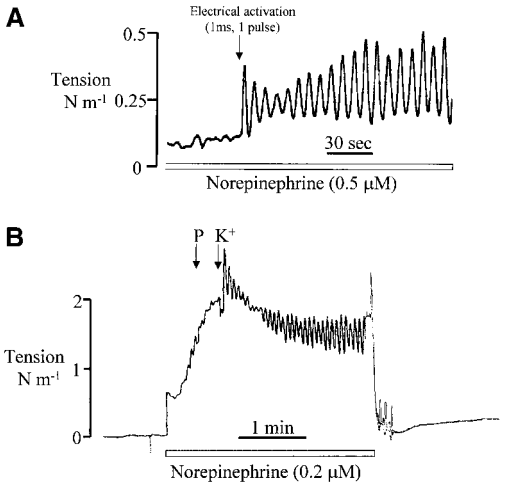

Figure 3. Synchronization triggered by a short-lasting electrical stimulus. Under conditions in which we had shown asynchronous changes in $[Ca^{2+}]_i$ (in the presence of a low concentration of NE, which caused a slight increase in force), applying a brief electrical pulse (1 ms, 45 mA, ≈5 V) between 2 electrodes in the bath at either side of the artery (A) or a bolus injection of 5 μL PSS with 62 mmol/L potassium near the vessel (B) triggered the appearance of coordinated oscillations in tone. The experiment was performed in the presence of 10 μmol/L guanethidine, to prevent NE release from intramural nerves. Similar results were obtained for electrical field stimulation in 7 of 9 trials in 3 experiments and for potassium stimulation in 10 of 18 trials in 4 experiments. In 9 of 9 trials in 3 experiments, a bolus injection of 5 μL PSS used as a control for the potassium activation did not elicit oscillations.

Discussion

The hypothesis tested in the present study is as follows: During agonist stimulation, Ca^{2+} is released intermittently from the SR. Released Ca^{2+} can activate a membrane conductance carrying inward (depolarizing) current. When a sufficient number of cells become active at the same moment, the current will overcome the current sink in the preparation and depolarize all cells (coupled via gap junctions). Depolarization causes Ca^{2+} influx that activates Ca^{2+} release in all parts of all cells (also in inactive cells). This converts Ca^{2+} waves into global Ca^{2+} oscillations, synchronizes the cells, and causes contraction. All cells now have released Ca^{2+} and start refilling their SR simultaneously, which leads to a new synchronous release, thus reiterating the cycle.

This hypothesis was found to be consistent with the experimental data obtained. Vasomotion was correlated with the appearance of synchronous Ca^{2+} changes in the smooth muscle cells, which required a functional SR. Vasomotion does not appear if the membrane potential is clamped and is only present when cGMP levels are above basal. Ca^{2+} release was shown to cause depolarization, but this occurred only when cGMP levels were elevated. Similarly, Ca^{2+} release evoked an inward current, but this occurred only in the presence of cGMP. A current pulse or other brief depolarization was shown to be sufficient to synchronize and entrain the cells in the preparation. We believe that these observations match the predictions so closely that they strongly support the suggested model.

Several questions remain to be resolved in further work. One is the question of the nature of the ion channel. Ca^{2+}-activated inward currents have been demonstrated in vascular smooth muscle to be either cation or chloride currents[25–29]; to our knowledge, none of these has been characterized as being cGMP dependent. There are other questions regarding the action of cGMP: Does cGMP act directly on the channels, is this action mediated via protein kinase G on the channel, or does the action of cGMP possibly influence the magnitude or direction of Ca^{2+} release instead? The events occurring during the delay between the application of NE and the initiation of vasomotion also need to be studied in greater detail.

In summary, the present model explains how a large group of electrically coupled cells can initiate and maintain synchronous activity even without dedicated pacemaker cells. Although the details of the model may be specific for the vascular bed investigated, the general concept of synchronization of intracellular oscillators via a reciprocal interaction with the membrane may be applicable to a broader range of electrically syncytial tissues.

Acknowledgments

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