Role of the Sarcolemma in Triggered Propagated Contractions in Rat Cardiac Trabeculae

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We have recently described that aftercontractions propagate through multicellular cardiac muscle preparations. These propagating contractions are triggered in damaged regions of rat right ventricular trabeculae during relaxation of electrically stimulated twitches. Propagation of triggered contractions has been attributed to calcium ions that diffuse along the preparation, causing calcium-induced calcium release from the sarcoplasmic reticulum in adjacent cells. In the present study we have investigated a possible role of the sarcolemma and delayed afterdepolarizations (DADs) in the initiation and propagation of triggered propagated contractions (TPCs) in multicellular preparations. We studied whether 1) TPCs are accompanied by delayed sarcolemmal depolarizations, 2) such depolarizations mediate local contraction, and 3) an intact sarcolemma is required for propagation of contractions. TPCs that remained stable for prolonged periods of time could be induced by trains of 15 stimuli (2 Hz, 15-second intervals) at lowered temperature (19–21°C) of the superfusing Krebs-Henseleit medium and a [Ca²⁺], of 1.0–1.5 mM. Although TPCs could be induced at 38°C and a [Ca²⁺], of 3.0–4.0 mM, they disappeared within 10 minutes. Force was measured with a silicon strain gauge; length and shortening of sarcomeres were measured at two sites of the muscle using laser diffraction techniques. Membrane potential was measured with flexible microelectrodes. Saponin was used to selectively render the sarcolemma permeable to small ions and molecules. Propagation velocity of TPCs in intact trabeculae varied from 1.7 to 13.4 mm/sec at 19–21°C. TPCs were accompanied by DADs that could reach threshold and induce triggered arrhythmias. Changes in latency, duration, and force of TPCs, induced by changing [Ca²⁺], or the number of conditioning stimuli, were closely matched by changes in latency, duration, and amplitude of DADs; DADs consistently preceded TPCs, on average by 60 msec. Local heating of the muscle, by applying a current through an insulated platinum wire (diameter 100 μm) that touched the muscle, interrupted propagation of TPCs reversibly. DADs were, in the absence of a local contraction, still recorded distal to the heated site. In muscles that were treated with saponin and exposed to solutions approximating the intracellular milieu, spontaneously occurring local contractions that propagated in both directions (at velocities of 70–200 μm/sec) were elicited at a bathing calcium concentration of ~0.6 μM. Below this threshold, propagated contractions could be triggered by pressure ejection of a calcium-containing solution from a microelectrode positioned close to the trabecula. We conclude that TPCs are accompanied by DADs that by themselves are not responsible for initiation and propagation of TPCs. Propagation of TPCs also occurs, albeit at lower velocities, in trabeculae without a functional sarcolemma that are bathed in a skinned-fiber solution. (Circulation Research 1991;68:1408–1421)

A ftercontractions in cardiac muscle preparations are accompanied by transient depolarizations of the sarcolemmal membrane that may reach activation threshold for fast sodium channels and L-type calcium channels, initiate action potentials, and underlie cardiac arrhythmias.1−3 These transient depolarizations or delayed afterdepolarizations (DADs) have been shown to result from a transient inward current4; both Na⁺-Ca²⁺ exchange and a calcium-activated nonselective cation conductance have been implied as the main charge carrying mechanisms for this current.5−7 Whereas

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earlier investigators suggested that aftercontractions are actually caused by DADs,6,9 it is now generally believed that both contraction and DAD independently result from “spontaneous” release of calcium from the sarcoplasmic reticulum.10–12

In isolated cardiac myocytes, such spontaneous calcium release may occur locally with subsequent propagation of the contraction along the cell.13–17 This propagation, at velocities ranging from 50 μm/sec to 3 mm/sec, has been attributed to diffusion of calcium ions along the cell, mediating calcium-induced calcium release from the sarcoplasmic reticulum in adjacent sarcomeres. Recently, we have described a similar phenomenon in rat cardiac trabeculae18–20: damage to end regions of the trabecula appeared to cause local aftercontractions that then propagated over several millimeters along otherwise undamaged muscle, at velocities up to 20 mm/sec. Because propagated contractions frequently induce triggered arrhythmias,18 they may provide an acceptable in vitro working model of damage-induced cardiac arrhythmias.

The observation that propagated contractions arise during the relaxation phase of the twitch19 may provide an explanation for their trigger mechanism. It is well known that the end regions of cardiac muscle preparations are stretched during the twitch by the viable cells in the central region of the muscle and are rapidly released during relaxation.19,21,22 We recently observed that elimination of such stretch, and hence the quick release, of the damaged region during the twitch prevents the induction of an aftercontraction.19 This is consistent with the hypothesis that initiation of propagated contractions results from calcium-induced calcium release from the sarcoplasmic reticulum in damaged regions of the muscle, triggered by calcium ions that dissociate from the myofilaments due to the quick release of these areas during the relaxation phase of the twitch.19 We have, therefore, denoted the phenomenon as triggered propagated contraction (TPC).

Similar to the mechanism proposed for single cardiac cells, we assumed that propagation of TPCs resulted from calcium ions that diffuse along the preparation, mediating a calcium-induced calcium release from the sarcoplasmic reticulum in adjacent sarcomeres and cells.18,19,23

However, multicellular preparations differ from isolated cells in that they are not electrically uniform. Until now, no electrophysiological studies have been performed on propagated contractions in multicellular cardiac muscle preparations. Therefore, cell-to-cell conduction of an electric event that mediates contraction cannot be ruled out as the mechanism responsible for propagation of TPCs. In fact, the differences in propagation velocity of contractions between single cardiac cells and rat trabeculae may reflect a different underlying mechanism.

The present study addresses this issue. We show that 1) TPCs in rat cardiac trabeculae are accompanied by DADs that may reach threshold membrane potential for generation of action potentials and induce triggered twitches, 2) DADs without local contraction are still present distal to a site where propagation of TPCs was blocked by local heating, and 3) propagated contractions can be elicited in saponin-treated trabeculae that lack a functional sarcolemma.

Our findings suggest that triggering and propagation of TPCs in rat cardiac trabeculae are not mediated by DADs and their subsequent passive conduction along the muscle.

Materials and Methods

Dissection and Mounting of the Preparation

Sprague-Dawley and Brown-Norway rats of either sex were anesthetized with diethyl ether. Their hearts were rapidly removed, and trabeculae running between the free wall of the right ventricle and the atrioventricular ring were dissected and mounted horizontally in a bath (which formed part of a microscope stage). The ventricular end was attached to a silicon strain-gauge force transducer (model AE 801, Senstonor, Horten, Norway), and the valve was attached to a hook. Both attaching devices were controlled by micromanipulators (Narishige, Tokyo) to change muscle and sarcomere length.

Superfusing Solutions

Standard solutions. The solutions used during dissection, mounting, and electrophysiological experiments contained (mM) Na+ 140.9, K+ 5.0, Cl− 127.5, Mg2+ 1.2, PO4 2− 2.0, SO4 2− 1.2, HCO3− 19.0, and glucose 11.0 (all obtained from Sigma Chemical Co., St. Louis); CaCl2 (analar grade, BDH Chemical Co., Toronto) was varied to induce TPCs or to change their propagation velocity (see below). The solutions were equilibrated with 95% O2-5% CO2, yielding a pH of 7.3 at both 25°C and 20°C. The presence of microelectrodes or platinum wire for local heating of the muscle precluded covering of the muscle bath with a glass slide; a diamond-shaped bath and a flow rate of 4 ml/min ensured a fluid surface smooth enough for sarcomere length measurements by laser diffraction techniques. Temperature of the superfusate was measured in the muscle bath by means of a copper/constantan thermocouple implant probe encased in Teflon and connected to a thermometer (accuracy 0.1°C, Digi-Sense 8528-20, Cole-Parmer Instrument Co., Chicago). Temperature was controlled with a cryostat (model MGW Lauda RM-T-6, Brinkmann Instruments Division, Rexdale, Ontario, Canada) and glass heat exchanger.

Saponin-treatment solutions. Since the sarcolemma contains much more cholesterol than the sarcoplasmic reticulum, it can be selectively disrupted with the cholesterol-precipitating agent saponin.24 We added saponin (Sigma Chemical) in a concentration of 50 μg/ml to a “relaxing” solution containing (mM) K+ 140, Mg2+ 5.5, Na+ 40, Cl− 111, ATP 5, creatine phosphate 15, EGTA 10 (all from Sigma Chemical), and HEPES 25 (Calbiochem Corp., La Jolla, Calif.). After saponin treatment, the muscle was superfused
with solutions of varied calcium concentration. These solutions contained 0.1 mM EGTA and 9.9 mM K$_2$-2,6-diaminohexane-N',N',N',N'-tetraacetic acid (HDTA) (Aldrich Chemical Co., Inc., Milwaukee, Wis.); their calcium concentration was increased by adding CaCl$_2$ (and, to compensate for H$^+$ ions released as EGTA binds calcium, KOH [BDH] in an amount twice that of CaCl$_2$). The calcium contamination of EGTA was determined by measurement of the pH change of an EGTA solution in response to the addition of known amounts of calcium$^{25}$; the contamination was calculated to be 10 μM (yielding a free calcium concentration in the relaxing solution of 67 nM). The method of calculating free calcium concentrations and intrinsic strength in our solutions, the choice of ion-binding constants for the various ligands, the precautions for EGTA purity, and the measurement of pH are described in detail elsewhere$^{26-28}$ experiments were performed at 22°C.

**Standard equipment.** The standard equipment used during our experiments and the principles of laser diffraction to measure sarcomere length have been described before.$^{19,29}$ The muscle was observed using an inverted microscope (model Dia-phot-TMD, Nikon Inc., Tokyo) and a video system (camera model WV 3170 and recorder model AG 2400, Panasonic). The preparation was stimulated through two punctate electrodes in the bath with rectangular pulses (5 msec, twice the threshold at 25°C) from a stimulator (model SD9, Grass Instrument Co., Quincy, Mass.) that was triggered by a computer (PC-AT, IBM). The microscope stage (with muscle bath) could be moved with respect to the laser beam (He-Ne laser, 350-μm cross section of the beam). Thus, sarcomere length could be measured at multiple sites of the muscle at distances assessed by a linear potentiometer attached to the microscope stage. Force, sarcomere length, and membrane potential were displayed on a storage oscilloscope (model V134, Hitachi), recorded with a chart recorder (model 2800S, Gould, Cleveland, Ohio), and sampled at 600 Hz via an analog-to-digital converter (model DT 2801A, Data Translation Inc., Marlboro, Mass.) installed in the computer. All recordings were stored on hard disk for later processing; the computer program for data analysis allowed display of the recordings of the last twitch of the conditioning stimulus train and a subsequent period of time on a graphics monitor (model CGA, IBM).

**Membrane potential recordings.** Manufacture and characteristics of the highly compliant microelectrodes used in this study have been described in detail elsewhere.$^{30}$ To ensure long-lasting, minimally damaging, and stable impalements in contracting trabeculae, flexible stepped electrodes were drawn from glass capillaries. The final taper of the electrode tip to <0.5 μm was confirmed by light microscopy. The electrode, filled with 3 M KCl, was held by a standard Perspex microelectrode holder (E.W. Wright, Guilford, Conn.) attached to a micromanipulator (Narashige) and connected to the head stage of a high input–impedance amplifier (Neuroprobe 1600, A-M Systems). An Ag/AgCl electrode in the bath served as the indifferent electrode.

**Local heating of the muscle.** A Teflon-coated platinum wire (diameter, 100 μm; resistance, 2 MΩ) was positioned across the muscle bath before mounting the trabecula. The wire was heated while in contact with the muscle by passing a 0.5–2.0-A current (BK Precision 1601 power supply, Dynascan Corp.) through it.

**Experimental Protocol**

**Triggered propagated contractions in intact trabeculae.** Muscle length was set at a point where passive force was 5% of active twitch force at a superfusate temperature of 25°C in the muscle bath and [Ca$^{2+}$]$_i$, of 1.0 mM. Subsequently, the temperature was lowered to 19–21°C, and the muscle was stimulated with trains of 15 stimuli at a rate of 2 Hz, interspersed with 15-second rest intervals. If this failed to induce stable TPCs after the last stimulated twitch, [Ca$^{2+}$]$_i$ was increased in steps of 0.25 mM. To vary the characteristics of TPCs, [Ca$^{2+}$], and/or the number of stimuli in the conditioning train were increased during the experiments. This has been shown to decrease the interval between the last stimulated twitch and the initiation of TPCs, as well as to increase force and propagation velocity of TPCs.$^{18,19}$

We preferred to perform our experiments at low superfusate temperatures because this has been shown to prolong the presence of stable TPCs.$^{18}$ In a separate set of experiments, we verified whether TPCs could also be induced at 38°C. After mounting of the muscle and initiation of the stimulus protocol at 25°C and a [Ca$^{2+}$], of 1.0 mM, temperature of the superfusate was rapidly (<30 seconds) raised to 38°C. If, at this temperature, no TPCs were observed, [Ca$^{2+}$], was increased in 0.5 mM steps. We did not perform electrophysiological studies at this temperature.

Once TPCs were elicited at 19–21°C, the muscle was impaled with a microelectrode at a site that showed little translation during contractions. Care was taken to avoid impalement of the damaged end region of the muscle.

In the experiments in which a platinum wire was used to locally heat the muscle, microelectrodes were inserted only after induction of TPCs and lowering of the muscle in the bath to ensure direct contact with the wire. The muscle was impaled at a site (other than the damaged area) distal to the platinum wire with respect to the direction of propagation of TPCs. Current was passed through the wire during the last five twitches of the stimulus train or, if a current of 2 A failed to interrupt propagation of TPCs, for longer periods.

**Propagated contractions in saponin-treated trabeculae.** To allow the diffusion of saponin to cells in the core of the muscle, only thin (diameter, 70±20 μm) trabeculae were used in this set of experiments. After mounting, the muscle was stimulated at 0.5 Hz for 10–15 minutes in standard superfusate. Subsequently, the muscle was exposed to the saponin-
containing solution for a varied amount of time, washed in relaxing solution (10 minutes), and superfused with a weakly calcium-buffered solution (0.1 mM EGTA). Initially, the calcium concentration of the latter solution was increased to determine the level at which propagated contractions occurred spontaneously. Subsequently, the calcium concentration was decreased so that spontaneous propagated contractions were no longer present, and a microelectrode (tip diameter, 1–2 μm) was positioned close to the muscle. This electrode was connected to a picospritzer (General Valve Corp.) that was set to eject (30-msec pulses at 30 psi) small volumes of a solution similar to the superfusate but with higher free calcium concentration (6.4 μM).

Data Analysis

Triggered propagated contractions in intact trabeculae. Measurements were made from all TPCs that followed the series of stimuli. The following parameters were measured from the stored computer waveforms of force and sarcomere length: 1) force of TPCs, 2) the duration of the force transient produced by TPCs, and 3) the interval between the last stimulus in the stimulus train and the moment of both peak TPC force and peak sarcomere shortening due to a TPC.

Time to peak TPC force was used as an index of the time of start of TPCs after the last stimulated twitch (TPC latency). Theoretically, a change in TPC propagation velocity with a constant time of start of the TPC in the end region will affect the time to peak TPC force. In experiments in which the effects of a change in [Ca\textsuperscript{2+}]\textsubscript{o} on TPCs were studied, changes in time to peak TPC force correlated closely with changes in the calculated time of start of TPCs in the end region (slope of linear regression line=1.07, r=0.95). The latter parameter of TPC latency is not influenced by changes in TPC propagation velocity and can be obtained from (t−x)/v, in which t is the time to peak sarcomere shortening due to a TPC at site X, x the distance between site X and the end region from where the TPC started, and v the propagation velocity of the TPC.

The time a TPC needs to traverse the preparation (“TPC duration”) was estimated in two ways. First, TPC duration was determined from the ratio of the length of the muscle over which the TPC propagated (as assessed by direct microscopic observation) and its propagation velocity (“calculated TPC duration”). Propagation velocity of TPCs was calculated from the interval between peak sarcomere shortening due to a TPC at two sites of the muscle (Figure 2) and the distance between these sites. This calculation was performed only on TPCs elicited during subsequent series of stimuli (with movement of the microscope stage in the 15-second rest interval) and if force tracings of these TPCs were identical. Second, TPC duration was measured as the duration of the force transient of the TPC (“measured TPC duration”). The duration of the force transient is determined by the sum of the duration of the local force transient plus the time that is required for the contractile wave to move along the muscle but does not require accurate assessment of the distance over which the propagated wave travels. Thus, duration of the TPC force transient would be expected to be inversely related to TPC propagation velocity. This was indeed found to be the case (correlation coefficient=0.89) in a separate series of experiments on six muscles in which [Ca\textsuperscript{2+}]\textsubscript{o} was varied.

Whereas latency and both calculated and measured TPC duration were expressed in absolute values (milliseconds), TPC force in a particular muscle was expressed as percentage of TPC force after 15 stimuli at a [Ca\textsuperscript{2+}]\textsubscript{o} of 1.0 mM in that muscle.

Delayed afterdepolarizations. The amplitude, the interval between last stimulus in the conditioning stimulus train and peak of DAD (DAD latency), and the duration of DADs were measured from the membrane potential recordings stored on computer. Latency and duration of DADs were expressed in milliseconds. As for the amplitude of TPCs (i.e., TPC force), DAD amplitude was expressed as percentage of the value after 15 conditioning stimuli at a [Ca\textsuperscript{2+}]\textsubscript{o} of 1.0 mM. Unlike latency and duration, the absolute amplitude of TPCs (in millinewtons) in the muscles studied did not correlate with the absolute amplitude of DADs (in millivolts) during the control phase of the experiment. This most likely reflects a variation between muscles in the (different) determinants of TPC and DAD amplitude. For example, a variation in membrane potential (which we did not control) will affect DAD amplitude but not TPC amplitude. Similarly, a difference in propagation velocity of TPCs will affect the number of force-generating cells that participate in the propagated contraction at any moment (and will thus affect TPC force) but is not expected to influence DAD amplitude. Since we did not control these variables, a normalization to control values was necessary to detect, for the group of muscles, comparable changes in the amplitudes of TPCs and DADs after an intervention.

We compared the latency, duration, and amplitude of DADs at varied levels of [Ca\textsuperscript{2+}]\textsubscript{o}, and numbers of conditioning stimuli with the latency, duration, and amplitude of TPCs. The apparently linear relations were further described using linear regression analysis.

Propagated contractions in saponin-treated trabeculae. The propagation velocities of spontaneously occurring propagated contractions and of TPCs elicited by a jet of calcium-containing solution from the microelectrode were measured by frame-to-frame analysis of video recordings. Propagation velocity was related to time of exposure to saponin and to calcium concentration of the superfusate.

Results

Triggered Propagated Contractions at 38°C

In five muscles tested at 38°C, TPCs could be induced by rapid stimulation and an increase in [Ca\textsuperscript{2+}]\textsubscript{o} to 3.0 mM (in two muscles) or 4.0 mM (three
Triggered Propagated Contractions at 19–21°C

TPCs could be induced in all 15 intact trabeculae tested by lowering the temperature of the superfusate from 25°C to 19–21°C at a [Ca^{2+}]_o between 1.0 and 1.5 mM. Nine of the 15 preparations were used for quantitative studies on DADs; the remaining six were subjected to local heating. Length of the preparations varied from 1.34 to 5.59 mm (mean, 2.13 mm); width, from 82 to 257 μm; and thickness, from 20 to 90 μm. The length constants of our preparations were calculated according to linear cable theory, in which the length constant of an infinite cable is given by

\[ \text{length constant} = \left( \frac{r_m}{r_a} \right)^{1/2} \]

where \( r_m \) is the membrane resistance and \( r_a \) is the axial resistance of the fiber. Using a cytosolic resistivity of 100 Ωcm and a sarcolemmal resistivity of 10 kΩcm², the length constant for a cylindrical muscle composed of longitudinally oriented cells with a diameter of 100 μm is 5.0 mm. As the ends of the preparation are likely to heal over and be of high resistance, the value of 5.0 mm is a lower limit for the length constant in our preparations.

Resting membrane potential at the low (19–21°C) temperature for all muscles was −58±5 mV (range, −40 to −72 mV). During rapid stimulation, the membrane potential hyperpolarized by 8.0±1.4 mV in seven muscles. In three muscles (with resting membrane potentials of −40, −48, and −52 mV), temperature of the superfusate was increased to 25°C at the end of the experimental protocol; at this temperature, resting membrane potentials were −73, −76, and −70 mV, respectively.

Electrophysiological Measurements During Triggered Propagated Contractions in Intact Trabeculae

In all but two muscles, TPCs were accompanied by transient sarcolemmal depolarizations that occurred after the preceding action potential (DADs). The two muscles in which we failed to record DADs during TPCs did not differ from other trabeculae studied with respect to resting membrane potential (−51 and −64 mV at lowered temperature) or size. The present analysis thus reflects complete measurements in seven trabeculae.

Figure 2 shows the combined presence of a TPC and DAD (measured at two sites) in one muscle and their response to an increase in number of conditioning stimuli. The DADs were nearly synchronous at both measuring sites and occurred superimposed on a depolarizing drift, as was noted in all muscles that hyperpolarized during rapid stimulation. With an increase in the number of stimuli, TPC force, TPC propagation velocity, and DAD amplitude increased,

Figure 1. Recordings showing induction of a triggered propagated contraction and triggered arrhythmia at 38°C. Panel A: Force (F) and sarcomere length (SL) recordings of the last stimulated twitch of a series of 15 and a subsequent triggered contraction. Sarcomere length was measured at two sites of the muscle (X and Y). The interval between the peak sarcomere shortening transients at sites X and Y during a triggered contraction reflects the propagating character of the contraction (directed from site X toward site Y). [Ca^{2+}]_o was 3.0 mM. Initial sarcomere length was 2.15 μm at both sites, X and Y. Propagation velocity of triggered propagated contraction was 12 mm/sec. Panel B: Force and sarcomere length recordings at one site of the muscle. A series of stimulated twitches (ending at the vertical line) at 38°C and a [Ca^{2+}]_o of 5.0 mM was followed by a triggered arrhythmia. Note that the interval between the triggered twitches progressively prolonged before a triggered propagated contraction ended the series of twitches.

muscles). Figure 1A shows the force and sarcomere length tracings during the last stimulated twitch and a subsequent TPC obtained in one of these muscles. In agreement with previous descriptions, TPCs invariably started in one of the end regions of the muscle and propagated as a localized “wave” of contraction along the trabecula. Propagation velocity of TPCs at this temperature ranged from 7.0 to 18.1 mm/sec. In contrast to the stability of TPCs at 19–21°C, TPCs at 38°C disappeared within 3–10 minutes in all muscles. However, they could be reinduced when [Ca^{2+}]_o was further increased. At a [Ca^{2+}]_o of 5.0 mM, three of the muscles exhibited a series of triggered twitches (i.e., unstimulated contractions that occurred synchronously throughout the muscle and of which the rapid upstroke of force production was comparable with that of electrically stimulated twitches) after termination of rapid stimulation (Figure 1B). These arrhythmias ended spon-

taneously with an increase in their cycle length and a TPC. We did not perform electrophysiological studies on these preparations.
Sarcolemma and Triggered Propagated Contractions

Figure 2. Schematic representation of rat trabecular preparation (top panel) and recordings showing parallel changes in the characteristics of triggered propagated contraction (TPC) and delayed afterdepolarization after two (middle panel) and 15 (bottom panel) conditioning stimuli. The top panel represents a trabecula with its ventricular end positioned in the cradle that was attached to a force transducer and its valvular side attached to a hook. Sarcomere length and membrane potential (V_m in the top panel) were monitored at two sites along the preparation (X and Y). The recordings in the middle and bottom panels show force (F), sarcomere length (SL), and membrane potential (V) of the last stimulated twitch and a subsequent TPC and delayed afterdepolarization. The preparation hyperpolarized slightly during stimulation, accounting for the depolarizing drift at which delayed afterdepolarizations occurred. At the two measuring sites, membrane potential tracings were very similar. SL was measured during consecutive series of stimuli at a proximal (X) and distal (Y) site with respect to the direction of propagation of the TPC; the tracings have been artifically shifted. Propagation velocity of the TPC was calculated from the interval 1–2 and the distance between sites X and Y (630 μm in this muscle). With an increase in the number of conditioning stimuli from two to 15, TPC propagation velocity increased from 2.4 to 9.5 mm/sec. Furthermore, TPC force and delayed afterdepolarization amplitude increased, whereas TPC latency, delayed afterdepolarization latency, and duration of both TPC force and delayed afterdepolarizations decreased. After 15 stimuli, a second TPC and delayed afterdepolarization were induced. [Ca^{2+}]_o was 1.0 mM, temperature was 20.8°C, and resting membrane potential was −68 and −64 mV at sites X and Y, respectively. Muscle length was 2.23 mm.

whereas the latency of both TPC and DAD, as well as the duration of both the TPC force transient and DAD, decreased. Similar changes were observed in the other muscles tested, both for an increase in the number of stimuli and an increase in [Ca^{2+}]_o. These interventions also increased TPC propagation velocity, which, in the present study, varied from 1.7 to 13.4 mm/sec. In three additional muscles, multiple impalements revealed that the shape, duration, latency, and amplitude of DADs were nearly identical at different locations along the preparation (Figure 2 and Table 1). Quantification of any differences in DAD characteristics along the muscles was, therefore, not attempted in the remaining experiments.

The relation between characteristics of TPCs and DADs for all muscles in this study are quantified in Figure 3. Figure 3A and Figure 3B show that the duration of DADs was closely correlated with both the measured and calculated TPC duration. The relation between measured and calculated TPC duration was given by the line: measured TPC duration (msec)=0.88 calculated TPC duration (msec)+265 msec, where r=0.88.

Figure 3C demonstrates that, with an increase in latency of TPCs, DAD latency increased linearly. The analysis suggests a difference in latency for DADs and TPCs with DADs preceding TPCs by, on average, 60 msec. Indeed, time to peak DAD was shorter than time to peak TPC force in virtually all records in all muscles studied.

Figure 3D shows the matching changes in the amplitude of DADs and in TPC force after a change in [Ca^{2+}]_o or in the number of conditioning stimuli.

A large number of conditioning stimuli or a sufficient increase in [Ca^{2+}]_o sometimes induced action potentials and triggered twitches. Termination of

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Values are mean±1 SEM. X and Y, muscle sites; DAD, delayed afterdepolarization. Triggered contractions propagated from site X toward site Y. A minimum of five measurements was made at each site after 15 conditioning stimuli at a [Ca^{2+}]_o of 1.0 mM. None of the X–Y pairs was significantly different (differences were considered significant at p<0.05 by analysis of variance).
such triggered arrhythmias either occurred abruptly or, more often, was preceded by an increase in cycle length for both triggered contractions and triggered action potentials; in either case, the triggered arrhythmia was always followed by a TPC (compare with Figure 1B) and a subthreshold DAD.

**Effect of Local Heating on Triggered Propagated Contractions and Delayed Afterdepolarizations**

The high-resistance Teflon coating of the platinum wire was intended to prevent current flow across the muscle membrane. Although the flow of current through the platinum wire sometimes caused a large artifact on the membrane potential recording, the diastolic membrane potential immediately after termination of current flow in these muscles was within 5 mV of its value before local heating. Furthermore, the membrane potential hyperpolarized maximally by 4 mV in muscles that did not show such an artifact on the membrane potential recording during current flow through the platinum wire (e.g., see Figure 4). It is unlikely that this small modification of the membrane potential due to local heating of the muscle might have affected propagation of the TPC.

Local heating completely blocked propagation of the contraction at the heated site in two of six muscles. This is demonstrated for one of the two muscles in Figure 4: the TPC originated in the remnant of the ventricular free wall and subsequently propagated toward the valvular end, opposite the direction of the superfusing flow. Before passing current through the platinum wire, a clear local contraction due to a TPC was seen at both measuring sites along the trabecula (X and Y in Figure 4), as well as a DAD distal to the wire (Figure 4, middle panel). Local heating did not prevent the triggering of a TPC, as was witnessed by the proximally occurring local contraction (site X, Figure 4, bottom panel). It did, however, block TPC propagation at the wire; consequently, no contraction was seen distal to the wire (site Y, Figure 4, bottom panel). In contrast, local heating did not abolish the DAD at the distal location. The dissociation between contraction and the DAD distal to the heating wire was reversible when current flow through the platinum wire was terminated. Note also that during heating DAD duration decreased. Although only minor in this example, TPC force usually decreased to a similar extent.

Triggering of propagated contractions was prevented by local heating in two other muscles. Triggered twitches occurred in the remaining two muscles that were locally heated. No relation was found between length or diameter of the muscle and its
**Propagated Contractions in Saponin-Treated Trabeculae**

Length of the saponin-treated muscles ranged from 1.3 to 3.5 mm, and diameter ranged from 41 to 88 μm. As expected from the effects of saponin and the high-potassium-containing bathing media, the muscles failed to contract after electrical stimulation during and after saponin treatment. As a verification of the action of saponin, we bathed one muscle in a solution containing 10 mM EGTA and no ATP. In accordance with previous observations, the muscle immediately went into rigor. Also, the free calcium threshold concentration for steady-state activation under the conditions of our experiments was comparable to that found in previous experiments with skinned trabeculae (i.e., ~1 μM).

In all 12 muscles studied, propagated contractions occurred spontaneously at a bathing free calcium concentration of nominally 0.57 μM. Contractions started at, and propagated away from, several sites along the muscle, the positions of which appeared to be constant throughout the observation period. Lowering the calcium concentration of the superfusate prevented the spontaneous occurrence of propagated contractions; under these conditions, exposure of any site long the muscle to a jet of calcium-containing fluid that was pressure-ejected from a microelectrode triggered a local contraction that subsequently propagated in both directions at identical velocities, as is visualized in Figure 5. In four muscles, propagation velocity of contractions was 117±18 and 98±11 μm/sec (mean±SD) at bathing free calcium concentrations of 0.39 and 0.57 μM, respectively, and was similar for both spontaneous and triggered contractions.

In three muscles, propagation velocity of contractions was studied at varied times of exposure to saponin. A minimal exposure of 20 minutes was required to induce propagated contractions. Additional exposures to saponin, to a total of 30–85 minutes, did not change the propagation velocity of contractions at constant bathing calcium concentration. Propagation velocity of both spontaneous and triggered contractions in all experiments ranged from 70 to 200 μm/sec.

**Discussion**

Since triggered activity is considered to be one of the mechanisms underlying clinically important cardiac arrhythmias, it is important to identify the cellular events responsible for triggering and propagation of unstimulated (spontaneous) contractions. We have previously postulated that propagation of triggered contractions in rat cardiac trabeculae results from calcium diffusion along the preparation, mediating calcium-induced calcium release from the sarcoplasmic reticulum in adjacent cells. DADs occur in conjunction with aftercontractions in single cells as well as in multicellular preparations. These afterdepolarizations and their underlying transient inward current are believed to result from intra-
Figure 5. Photomicrographs showing triggered contractions propagating in both directions in a saponin-treated trabecula after pressure ejection of calcium-containing solution on the muscle. Photographs were taken directly through the microscope (final magnification, ×35) at random moments. From top to bottom: Calcium ejection from the microelectrode (visible below the muscle) induced a local contraction (persisting into record 3) that subsequently propagated in both directions at similar propagation velocities (85 μm/sec). The contraction reached the left end of the preparation in record 4; in record 5, only the rightward propagating contraction remained present. Direction of superfusing flow was from right (valvular attachment site) to left. Length of the muscle was 2.61 mm, and width was 88 μm.
cellular calcium release rather than to cause such release and a subsequent contraction.\textsuperscript{10–12} However, the role of passive conduction of DADs along the sarcolemma as possible mediators of calcium-induced calcium release from the sarcoplasmic reticulum, and thus as mediators of propagation of TPCs in multicellular preparations, has not been investigated. Since conduction of an electric event along the sarcolemma may be expected to be faster than diffusion of calcium, the presence of such a mechanism may perhaps account for the higher propagation velocities of triggered contractions in intact multicellular preparations as compared with the magnitude of propagation velocity in single cells. Therefore, we undertook the present study, which was aimed at resolving three questions: 1) Do DADs accompany TPCs in rat cardiac trabeculae? 2) Do DADs, as possible mediators of calcium-induced calcium release from the sarcoplasmic reticulum, induce local contractions? 3) Is an intact sarcolemma required as an electrical transmission cable for propagation of triggered contractions in multicellular cardiac muscle preparations?

We preferred to study TPCs at 19–21°C, a temperature at which TPCs remain stable for prolonged periods of time\textsuperscript{18} and can easily be visualized. To exclude the possibility that TPCs only occur at these temperatures and can therefore not be involved in the pathogenesis of cardiac arrhythmias, we first studied rat cardiac trabeculae at 38°C. Figure 1 shows that both TPCs and triggered arrhythmias can be induced at this temperature if [Ca\textsuperscript{2+}]\textsubscript{o} is sufficiently elevated. Recently, we studied whether TPCs can also be induced in human cardiac muscle preparations (M.C.G. Daniels, T. Kieser, and H.E.D.J. ter Keurs, unpublished data). In all 10 right atrial trabeculae, TPCs occurred after rapid stimulation at a [Ca\textsuperscript{2+}]\textsubscript{o} of 6.0 mM. The changes in characteristics of TPCs in human atrial trabeculae with varied [Ca\textsuperscript{2+}]\textsubscript{o} or varied number of conditioning stimuli were qualitatively similar to the changes observed in rat ventricular trabeculae. This further suggests that TPCs in rat cardiac trabeculae provide a model of (patho)physiological important phenomena that may be involved in arrhythmogenesis in the human heart.

**Triggered Propagated Contractions Are Accompanied by Synchronously Occurring Delayed Afterdepolarizations**

DADs have been found to accompany aftercontractions in a variety of multicellular cardiac muscle preparations.\textsuperscript{1–3} The present study demonstrates that this is also the case for triggered contractions that propagate along the muscle (Figure 2).

To correlate changes in latency, amplitude, and duration of TPCs with changes in the same characteristics of DADs, a valid measurement of the parameters is crucial (see also “Materials and Methods”).

**Mechanical aspects.** Changes in latency (i.e., the interval between the last stimulated twitch and triggering of TPCs) were assessed by measurement of time to peak TPC force. Theoretically, a change in propagation velocity of the TPC with a constant moment of its initiation in the end region of the muscle will affect time to peak TPC force. However, the close correlation between changes in time to peak TPC force and changes in the calculated time of start of a TPC in the end region (which is not influenced by changes in TPC propagation velocity) suggests that the time to peak TPC force reliably reflects changes in latency.

**Electrical aspects.** The resting membrane potential of −58±5 mV at the temperature at which the experiments were performed (19–21°C) requires further consideration. These depolarized values might be related to decreased activity of the Na\textsuperscript{+}-K\textsuperscript{+} pump that is due to absence of stimulation\textsuperscript{34,35} at low temperature.\textsuperscript{36,37} The small hyperpolarization that we sometimes observed during rapid stimulation may have resulted from activation of the Na\textsuperscript{+}-K\textsuperscript{+} pump.\textsuperscript{38–40} The repolarization to virtually normal resting membrane potentials at 25°C in the three most depolarized muscles at low temperature indicates that this depolarization was not the result of cellular damage.

DADs can only be measured locally, in contrast to force of the TPC, which reflects characteristics of the whole muscle. As part of a propagating event, DAD latency may not be the same at different sites of the muscle. Indeed, a small difference in DAD latency has been observed during triggered contractions in dog ventricular muscle at sites >5 mm apart.\textsuperscript{41} Unfortunately, this report did not mention whether the contractions did propagate. On the other hand, spontaneous voltage and current oscillations in multicellular preparations showed synchronous fluctuations of the same amplitude at different sites.\textsuperscript{11,42,43} In the present study, we never observed consistent changes in latency, duration, amplitude, and shape of DADs with location of measurement (Figure 2 and Table 1). This is consistent with the view that virtually all trabeculae we studied were shorter than their length constants. Although we did not directly measure the decrement of injected current pulses along the length of trabeculae, the calculated length constant for the muscles was minimally 5.0 mm.\textsuperscript{31} Therefore, we are confident that our results are not confounded by selection of DAD measurement sites.

This study demonstrates that changes in TPC latency, duration of the presence of a TPC in the muscle, and TPC force, induced by changes in the number of conditioning stimuli or [Ca\textsuperscript{2+}]\textsubscript{o}, were closely matched by changes in the characteristics of DADs. A similar close relation between amplitude and temporal characteristics of DADs or their underlying transient inward current on the one hand and triggered contractions on the other hand have been recognized before in Purkinje fibers of several mammalian species\textsuperscript{10,11,42,44,45} and in guinea pig ventricular myocytes.\textsuperscript{6} In these tissues, a transient inward current was shown to precede triggered contractions by 50–140 msec.\textsuperscript{6,10,11,46} In our study, peak DAD occurred, on average, nearly 60 msec
earlier than peak TPC force (Figure 3C). Such a delay between DAD and TPC may suggest a causal relation between the two (the DAD mediating the TPC) but is no proof of such a relation. In fact, a mechanism in which one event, such as an intracellular calcium transient, causes both DAD and TPC explains the data equally well if it is assumed that the change in membrane conductance occurs faster than activation of the myofilaments. A time lag in the response of the contractile filaments to calcium is well known from studies of intracellular calcium transients and mechanical properties of cardiac muscle. In addition, even the slow repolarization phase of the action potential in the rat heart, which is presumably generated by Na+/Ca2+ exchange, occurs before peak twitch force.

Delayed Afterdepolarizations Do Not Mediate Local Contractions

A novel observation in this study is that the duration of the DAD is closely correlated with the time that is required for a localized TPC to travel along the muscle (Figures 3A and 3B). The correlation is so strict that the hypothesis that both the electrical and the mechanical events share the same causal mechanism is obvious. The observed correlation between the spatially nonuniform mechanical event and the concurrent virtually synchronous depolarization of several millimeters of muscle (Figure 2) clearly rules the depolarization out as a causal factor for the relatively slow propagation of the contraction. For the same reason, it is also highly unlikely that an electrically propagated transient inward current can be held responsible for propagation of the contraction. Because no mechanism of chemical propagation of a transient inward current is known to us, we conclude that the only plausible mechanism that explains the data consists of propagation of a calcium release from an intracellular store; this process then leads to the secondary phenomena of contraction and depolarization. In this model, the synchronicity of DADs over rather long distances can be explained by the long length constants of our preparations: we assume that a DAD at one particular site consists of the combination of a local inward current and the passively conducted DAD from other sites.

We sought further ways to dissociate the electrical from the mechanical event during TPCs. If successful, this would provide definite proof that DADs are not the cause of TPCs. In two muscles, we observed TPCs without identifiable DADs. The failure to detect DADs in representative muscles may be due to technical imperfections; hence, this observation alone is not enough to discard a causal relation between DAD and TPC. However, other investigators have also observed triggered contractions without concomitant afterdepolarizations. Therefore, these findings need not be due to technical limitations and may again suggest that DADs do not cause TPCs. The observation itself may be explained if a threshold level of intracellular calcium must be reached to induce a DAD.

In a study on the effects of temperature on the characteristics of TPCs (M.C.G. Daniels and H.E.D.J. ter Keurs, unpublished data), we noticed that TPCs can be abolished in most muscles by increasing the temperature of the superfusate at >23°C at a constant [Ca2+]o in some muscles, such an increase in temperature induced triggered twitches. In the present study, we heated the preparation over a length of 100 μm in the expectation that this might halt propagation of TPCs at the heated site; such intervention would be expected not to affect passive conduction of electrical phenomena. It is important to point out that some variability of the effect of passing current through the platinum wire may be expected for several reasons; first, the geometry of the muscles was not constant. Second, the extent of the damaged end region and therefore the distance of that region to the heating wire may have been variable. Also, the local temperature must be varied with the current, thermal contact between the wire and the muscle, and heat lost from the muscle into the bath. An estimate of the temperature increase in a 100-μm-thick muscle, based on the resistivity of the wire (2 mΩmm), the rate of heat transfer through the Teflon insulation, and heat loss, yields a temperature increase of 2°C/A (at an efficiency of overall heat transfer of 2%). Although it is not surprising that the superfusate temperature in the muscle bath did not change measurably, this estimate allows for the possibility that passing current through the platinum wire caused some generalized heating of the muscle. That such generalized heating occurred is suggested mostly by the accelerated relaxation of electrically stimulated twitches during local heating. In addition, we observed that in some muscles local heating abolished triggered contractions altogether or induced triggered twitches. Finally, even in the muscles in which propagation of TPCs was interrupted after heating, TPC latency decreased, and force of stimulated twitches increased (Figure 4). However, neither the presence of a slight generalized heating of the trabecula nor the fact that we do not know the exact local temperature in the heated region will affect the qualitative interpretation of our results.

Figure 4 shows that, when propagation of TPCs was blocked completely by the locally applied heat, a DAD persisted distal to the platinum wire. If DADs mediate calcium-induced calcium release from the sarcoplasmic reticulum and underlie triggered contractions, a local contraction should have occurred distal to the heated site. The absence of such contraction in the presence of a DAD proves that DADs themselves do not induce local contractions and therefore are neither responsible for triggering of TPCs nor for their propagation. In addition, the side distal to the platinum wire (with respect to the direction of the TPC) in one of the muscles in which local heating blocked
Propagation (Figure 4) was actually the “upstream” side of the superfusing flow, excluding the possibility that the TPC was abolished in the distal portion of the muscle because it was warmed up by heat carried by the flowing superfusate.

The recording of a DAD distal to the heated site is consistent with the view that this DAD was generated at the proximal side of the platinum wire and was conducted passively across the heated site. An increase in the temperature of the cells overlying the platinum wire may be expected to accelerate calcium binding and uptake into the sarcoplasmic reticulum and/or reduce the open probability of the calcium release channel at this site. Theoretically, this should interrupt propagation of the contraction if propagation is mediated by calcium release from the sarcoplasmic reticulum coupled by calcium diffusion. Thus, the observed effects of local heating are consistent with the hypothesis that both electrical and mechanical events during triggered contractions independently result from intracellular release of calcium. A reduction in diffusion distance of calcium ions would also explain the observed decrease in duration of DAD and TPC during local heating.

**Propagation of Contractions Does Not Require an Intact Sarcolemma**

In the previous section, we have shown that DADs can occur without concomitant local contraction. From this, we concluded that the DADs do not mediate triggering and propagation of TPCs. If this is true, it should also be possible to demonstrate the reverse: the presence of TPCs in the absence of DADs. To achieve this, we studied propagated contractions in saponin-treated trabeculae.

Saponin is assumed to render the sarcolemma permeable to ions and small molecules. This assumption is based on several lines of evidence. First, the pCa–tension relation in highly calcium-buffered saponin-treated preparations appeared to be within the same general range as that of muscle fibers “skinned” by other techniques. In our preparations, the threshold bathing calcium concentration for direct myofilament activation was 1 mM. This is similar to values reported previously and well below the [Ca$^{2+}$], required to activate intact cardiac muscle preparations, indicating that saponin rendered the sarcolemma permeable to calcium ions. Second, saponin-treated as well as mechanically skinned preparations exhibit a strong contractile response in the absence of ATP, a response that does not occur if the sarcolemma is intact. In our preparations, removal of ATP from the bathing solution resulted in a contracture. Third, ions and molecules that do not normally penetrate through an intact sarcolemma do so in saponin-treated preparations. Finally, the myofilament lattice spacing after saponin treatment shows an expansion similar to that caused by other chemical skinning procedures.

Thus, it is reasonable to assume that saponin effectively disrupted the sarcolemma of our preparations. This effect, together with the presence of 140 mM potassium in the superfusate, eliminates 1) the occurrence of electrical phenomena over the sarcolemma, 2) events that depend on the presence of transsarcolemmal voltage differences or transsarcolemmal currents, 3) chemical messenger pathways that depend on an intact sarcolemma, and 4) the barrier to the extracellular space for many molecules involved in intracellular regulation processes.

Sufficient elevation of the free calcium concentration of the weakly calcium-buffered superfusate triggered local contractions in saponin-treated trabeculae, in agreement with the observations in a preliminary study. The local contractions subsequently propagated both with and against the direction of the superfusing flow. Propagated contractions appeared to start from sites at a constant location along the otherwise quiescent preparations. We speculate that damage to the sarcoplasmic reticulum at these sites (perhaps as a result of saponin exposure) lowered the threshold for overload-induced calcium release, thus promoting the occurrence of spontaneous local contractions. Diffusion of calcium from these areas along the preparation, mediating calcium release from the sarcoplasmic reticulum, might explain the propagating character of the contraction.

At bathing calcium levels low enough to prevent the spontaneous occurrence of propagated contractions, such contractions could be triggered by the ejection of a jet of calcium-containing solution from a microelectrode on the muscle (Figure 5). This presumably induced both a local contraction and calcium-induced calcium release from the sarcoplasmic reticulum. Since the contraction subsequently propagated at identical velocities in both directions (Figure 5), propagation could not have been due to ejected calcium that was carried along the muscle by the superfusing flow. Apparently, propagation of triggered contractions does not require an intact sarcolemma. Thus, propagation does not critically depend on sarcolemmal depolarizations or ionic fluxes nor on activation of sarcolemma-dependent chemical messengers.

A limitation in the comparison between saponin-treated and intact trabeculae is the difference of one order of magnitude in propagation velocity of triggered contractions. This difference was not related to the time of exposure to saponin and therefore probably not to the extent of damage to the sarcolemma. Neither can it be explained by insufficient calcium loading of the sarcoplasmic reticulum of saponin-treated muscles: propagation velocity of contractions at free bathing calcium concentrations high enough to induce spontaneously occurring contractions (0.57 µM) were not higher than triggered ones at lower (0.39 µM) calcium concentrations (98±11 versus 117±18 µm/sec). Interestingly, the propagation velocities that we observed in saponin-treated muscles are comparable to the velocities reported in studies on unstimulated single cardiac myocytes, although higher velocities have been measured after...
stimulation of isolated myocytes. At present, we cannot explain the difference in propagation velocities of contractions in saponin-treated and intact trabeculae. Whether it is due to 1) a loss of normally present intracellular constituents from saponin-treated muscles, 2) a rapid loss of calcium through the permeable sarcolemma after its release from the sarcoplasmic reticulum (thus limiting calcium diffusion to adjacent cells), or 3) another modulating effect of the sarcolemma on propagation velocity of contractions is speculative and requires further study.

Conclusion

TPCs in intact rat cardiac trabeculae are accompanied by near-synchronous DADs. This excludes the possibility that propagation of the contraction is mediated by depolarization of the sarcolemma. Furthermore, propagation of triggered contractions occurs in the absence of an intact sarcolemma, which is consistent with the hypothesis that propagation of triggered contractions results from calcium diffusion along the trabecula, mediating calcium-induced calcium release from the sarcoplasmic reticulum. Calcium release that elicits the propagating contraction may also activate a depolarizing current across the sarcolemma that leads to DADs, which then may reach threshold and initiate action potentials and triggered twitches. A modulating role of the sarcolemma on propagation velocity can not be excluded in view of the slow propagation of contractions in saponin-treated trabeculae as compared with the propagation velocity of TPCs in muscles with an intact sarcolemma.

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Role of the sarcolemma in triggered propagated contractions in rat cardiac trabeculae.

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