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Modulation of Subcellular Ca^{2+} Signal by Fluid Pressure in Rat Atrial Myocytes

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Abstract – Atrial chambers serve as mechanosensory systems during the haemodynamic or mechanical disturbances, which initiates arrhythmia. Atrial myocytes, lacking t-tubules, have two functionally separate sarcoplasmic reticulums (SRs): those at the periphery close to the surface membrane, and those at the cell interior (center) not associated with the membrane. To explore possible role of fluid pressure (FP) in the regulation of atrial local Ca^{2+} signaling we investigated the effect of FP on subcellular Ca^{2+} signals in isolated rat atrial myocytes using confocal microscopy. FP was applied to whole area of single myocyte with pressurized automatic micro-jet (200-400 mmH_2O) positioned close to the cell. Application of FP enhanced spontaneous occurrences of peripheral and central Ca^{2+} sparks with larger effects on the peripheral release sites. Unitary properties of single sparks were not altered by FP. Exposure to higher FP often triggered longitudinal Ca^{2+} wave. These results suggest that fluid pressure may directly alter excitability of atrial myocytes by activating Ca^{2+}-dependent ionic conductance in the peripheral membrane and by enhancing spontaneous activation of central myofilaments.

Key words □ flow pressure, atrial myocytes, Ca^{2+} spark

INTRODUCTION

Contraction of mammalian cardiac myocytes is controlled by a sequence of events that include L-type Ca^{2+} current (I_{Ca})-gated opening of Ca^{2+} release channels (ryanodine receptors, RyRs) in the sarcoplasmic reticulum (SR) (Beuckelmann and Wier, 1988; Nabauer *et al.*, 1989; Niggli and Lederer, 1990; Cleemann and Morad, 1991). Most of atrial myocytes have two functionally separate SRs, junctional SR close to the peripheral membrane and non-junctional or corbular SR throughout the central regions of the cell with no associated cell membrane, as the t-tubular system is either absent (Sommer and Jennings, 1992; Carl *et al.*, 1995) or partially developed (Forssmann and Girardier, 1970; Kirk *et al.*, 2003; Woo *et al.*, 2005). It has been demonstrated that Ca^{2+} release, initiated at the peripheral junctional sites by I_{Ca}, propagates into the interior of the cell partly by local diffusion of Ca^{2+} from the peripheral sites to more centrally located sites in atrial myocytes (Berlin, 1995; Huser *et al.*, 1996; Mackenzie *et al.*, 2001; Kockskamper *et al.*, 2001; Woo *et al.*, 2002).

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Several groups have reported that diastolic Ca^{2+} release plays a role in atrial pacemaker function (Huser *et al.*, 2000; Lipsius *et al.*, 2001) and arrhythmogenesis (Mackenzie *et al.*, 2002). Junctional Ca^{2+} release in the periphery of atrial myocytes may possibly modulate membrane conductance via Ca^{2+}-dependent regulation of ion channels or transporters, e.g., K^+ channel, Cl^- channel and Na^+-Ca^{2+} exchanger (Lipsius *et al.*, 2001; Mackenzie *et al.*, 2002). Central Ca^{2+} release in atrial myocytes may play a role in activating myofilaments located throughout the cytoplasm, but control mechanisms for the central Ca^{2+} release sites are largely unknown.

Changes in the mechanical environment of the heart, caused by contractility of the heart and changes in volume and pressure, can alter cardiac excitation and contraction (Lakatta, 1993; Nazir and Lab, 1996; Kohl *et al.*, 1999). Pathological conditions, such as valve disease, hypertension or heart failure, may also lead to haemodynamic or mechanical dysfunction of the heart, causing arrhythmia (Cooper *et al.*, 1985; Komuro *et al.*, 1990; Bode *et al.*, 2001). Although accumulating evidence in atrial myocytes suggests that mechanical stress modulates intracellular Ca^{2+} signal, there is a controversy in the effects of various modes of mechanical stimuli (e.g., stretch and shear stress). For example, in atrial tissue from rat, electrically stimulated Ca^{2+} transients and contraction were enhanced by stretch

with no change in the diastolic Ca^{2+} level (Tavi *et al.*, 1998). However, direct application of shear stress to single rat atrial myocytes has transiently increased fura-2 fluorescence in resting condition (Morad *et al.*, 2005).

The present study was designed to explore whether fluid pressure affects local and focal Ca^{2+} signaling in the peripheral and central regions of atrial myocytes. The fluid pressure was applied as puffing of pressurized fluid to whole area of single cell using electronically controlled micro-jet. This method may reproduce mechanical stress to the atrial chamber arising from strong haemodynamic turbulence and excess pressure. Our data demonstrate that fluid pressure generates longitudinal Ca^{2+} wave by increasing spark occurrences in the peripheral and central region.

MATERIALS AND METHODS

Application of fluid pressure to the single atrial myocytes

Rat atrial myocytes were enzymatically isolated from male Wistar WKY rats (200~300 g) as described previously (Woo *et al.*, 2002). The surgical procedure was carried out in accordance with university ethical guidelines. Fluid pressure was applied to the whole area of single myocyte using an automatic fluid-jet system. Tip of a microbarrel (outer diameter of 500 ìm), that was connected to a reservoir with 200- (25 dynes/cm^2) or 400-mm height (50 dynes/cm^2) and filled with fluid, was positioned at ≈150 μm away from the myocyte. Electronically controllable solenoid valve was installed in the middle of tube connecting the fluid reservoir and the microbarrel. Bottom of the tip, touching at the chamber bottom, was tilted to one side with an angle of 45°. The positioning of the microbarrel was performed under microscope (Nikon TS2000, Nikon, Japan) using micromanipulator (Prior England 48260).

Two-dimensional confocal Ca^{2+} imaging

Cells were loaded with the Ca^{2+} indicator dye fluo-4 AM (2 μM, 30 min) at room temperature and were imaged using a Noran Odyssey XL 2-D laser scanning confocal microscopy system (Noran Instruments, Madison, WI) attached to a Zeiss Axiovert TV135 inverted microscope fitted with a ×40 water-immersion objective lens (Zeiss 440052 C-Apochromat, NA 1.2). The excitation wavelength of the argon ion laser was set to 488 nm (Omnichrome), and fluorescence emission (>510 nm) was detected by a high-efficiency PMT (Hamamatsu, Middlesex, NJ). The *y* direction was scanned at 30 Hz. The confocal slit, stretching in the *x* direction, was set to values corresponding to a width of 0.6 mm in confocal plane of the objective. The measured point-spread-function of the confocal microscope was approximately a truncated cylinder 0.3 mm in radius and 0.8 mm in length. Data were acquired by the Intervision program in a workstation computer (IRIX-operating system, Indy, Silicon Graphics).

Image analysis

Images were analyzed using a custom-written PC computer program in Visual Basic 6.0 (Microsoft). Focal Ca^{2+} releases were identified by a computerized algorithm as previously described (Cleemann *et al.*, 1998; Woo *et al.*, 2003). The focal Ca^{2+} releases that had one stationary center for their growth and decay were then subjected to 2-D Gaussian approximations in a restricted area (30 pixels × 30 pixels), which allowed routine measurements of the amplitude, width and equivalent area of sparks originating from the peripheral and central regions (Woo *et al.* 2003). We have routinely measured the quality of the fit by calculating the standard deviation (SD, root-mean-square) between the raw data and the fitted Gaussian distribution. We compared these SD values to an estimate of the noise (individual pixels as compared to the mean their 8 nearest neighbors) and found that they agree within ~20% in ~85% of analyzed sparks.

We assumed that the major part of cell image excluding both ends of the cell, recorded at 30 Hz (see Figure 1B), to be a square. Then, the area up to 1.5 μm immediately underneath the cell membrane was denoted as the peripheral domain (peripheral area = 2 × 1.5 μm × image length, μm). The remaining area, measured as a difference between the whole image area and the peripheral area was considered as center.

Statistical analysis

Statistical comparisons were carried out using Student's *t* test. Differences were considered to be statistically significant to a level of $P < 0.05$. Numerical results are given as means ± SEM. All experiments were carried out at room temperature (22-24°C).

RESULTS

Effect of fluid pressure on subcellular Ca^{2+} signal in rat atrial myocytes

We first examined if FP induces change in Ca^{2+} differentially at the central (nonjunctional) and peripheral (junctional) domains of quiescent atrial myocytes. Imaging in extended periods at 30

Hz was used to monitor the major part of the cell and compensate for the sparsity of spontaneously occurring sparks. Application of FP (200 mmH_2O) to atrial myocytes elicited transient increase of Ca^{2+} at local sites both in the periphery and center (Fig. 1A). The FP-induced Ca^{2+} transients were recorded more often in the cell periphery than the center (Fig. 1A, compare *green* and *red* traces). In addition immediately after the exposure to FP the events of local Ca^{2+} transients were observed at distinct focal sites (Fig. 1Bb-c). FP significantly increased background basal Ca^{2+} level in the center of myocytes with no change in the magnitudes of transient Ca^{2+} rises (Fig. 1Ac) while the focal Ca^{2+} signals in the cell periphery became more diffused and larger (Fig. 1Ad and Bd). The FP-induced focal and local Ca^{2+} transients were often detected at specific local spots repetitively (Fig. 1C, traces 1 and 3). Such highly active sites were observed in the periphery more frequently than in the center of cells.

To determine the effect of FP on focal Ca^{2+} signals in more detail we attempted to identify focal release sites in the 2-D confocal Ca^{2+} images using center-minus-surround detection kernel (see *Materials and Methods*; Woo *et al.*, 2003). Figure 2A compares 2-D confocal Ca^{2+} fluorescence images recorded in the absence and presence of FP (200 mmH_2O; duration: 1.5 s) in a representative rat atrial myocyte. Single Ca^{2+} sparks were normally seen at 2-3 sequential frames. The average frequency of sparks (events/[10^3 $\mu m^2 s$]) in the control condition was significantly higher in the cell periphery (12.5 ± 2.3) compared to the center (2.61 ± 1.52, $P < 0.01$, n = 12), which is consistent with our previous report on the spark frequency in fluo-3 dialyzed voltage-clamped rat atrial myocytes (Woo *et al.*, 2003). The occurrence of spontaneous Ca^{2+} sparks was significantly increased by the application of FP (Fig. 2), which was observed both in the center and periphery of the myocytes (compare panel B and C in Fig. 2). The mean frequency of peripheral sparks was increased to ~6 fold by FP, whereas that of central sparks was increased to ~3 fold (Fig. 2D), such that the effects of FP was 2-fold larger in the cell periphery.

Individual 2-D spark images were quantified by a spatial Gaussian approximation (Woo *et al.*, 2003). Figure 3 compares the unitary properties of spontaneous central and peripheral Ca^{2+} sparks measured in the presence and absence of FP. Intensity (F_1/F_0), full-width at half maximal amplitude (FWHA) and area (mm^2) of Ca^{2+} sparks in the periphery and center were not significantly changed by FP (Fig. 3).

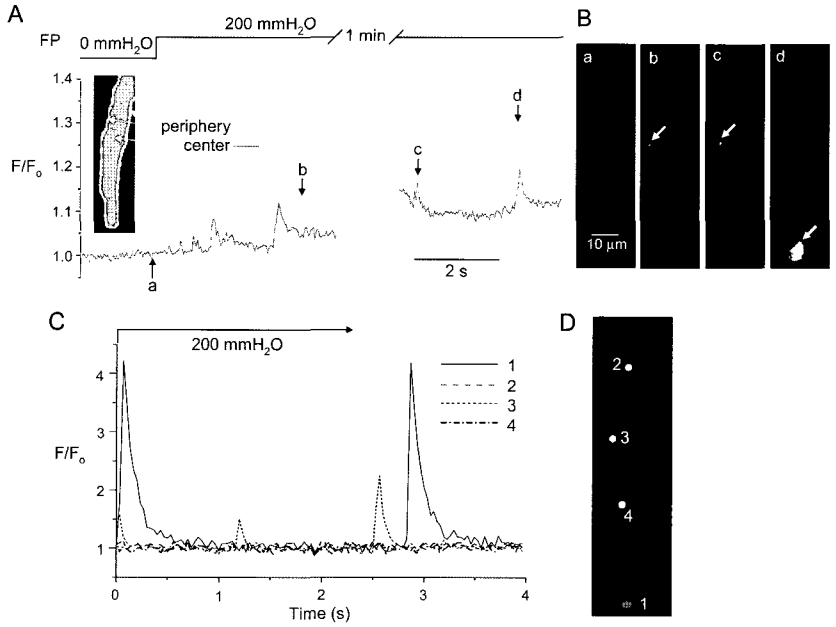

Fig. 1. Fluid pressure-induced local and focal Ca^{2+} release in rat atrial myocytes. (A) Effect of fluid pressure (FP; 200 mmH_2O) on time courses of local Ca^{2+} signals (30 Hz) measured in the periphery and center of rat atrial myocytes loaded with fluo-4 AM. Same extracellular solution was applied continuously for 2 min using pressurized microjet. Inset image show pixel masks to measure Ca^{2+} signal from the cell periphery (gray) and center (black). (B) 2-D confocal Ca^{2+} images, measured at the time points indicated by *a, b, c* and *d* in the panel A. Color scale for fluorescence intensity is shown in the Fig. 4A. (C) Repetitive focal Ca^{2+} releases from the same local sites during FP (see traces *1* and *3*). The numbered Ca^{2+} signals (*1-4*) were measured from the subcellular sites indicated by the corresponding numbers in panel D. (D) Map of the locations of focal sites, where the Ca^{2+} signals in panel C were measured.

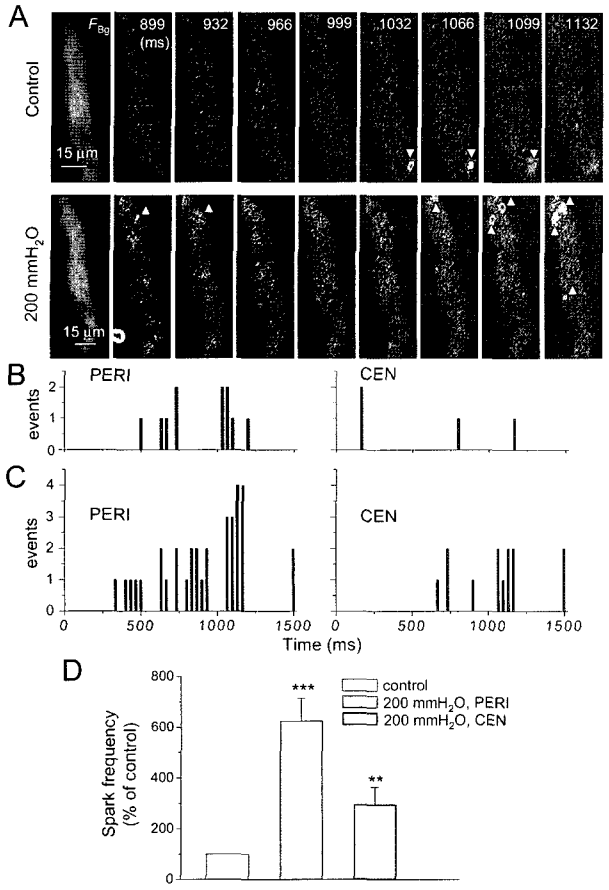

Fig. 2. Enhancement of spontaneous Ca^{2+} sparks by FP. (A) Sequential 2-D confocal Ca^{2+} images measured at 30 Hz during periods indicated by the numbers on top of each image. Upper and lower images were obtained before (control) and after applying FP (200 mmH_2O). Image F_{Bg} shows average fluorescence, revealing the clear outline of the myocytes. Numbered images, 899 to 1132 ms, are sequential frames measured differentially as the increase in fluorescence (ΔF) relative to the average fluorescence. In the differential measurements of fluorescence, the outline of the cell is seen as only variations in the noise, but Ca^{2+} sparks appear clearly (arrowheads). (B) and (C) Right and left panels show time course of the occurrences of peripheral (PERI) and central (CEN) sparks in cell shown partly in panel A before (B) and after (C) application of FP. (D) Mean increase in the frequency of central and peripheral Ca^{2+} sparks by FP (200 mmH_2O) compared to the control condition. ***P<0.001 *vs.* peripheral spark frequency in the control condition. **P<0.01 vs. central spark frequency in the control condition (n=12).

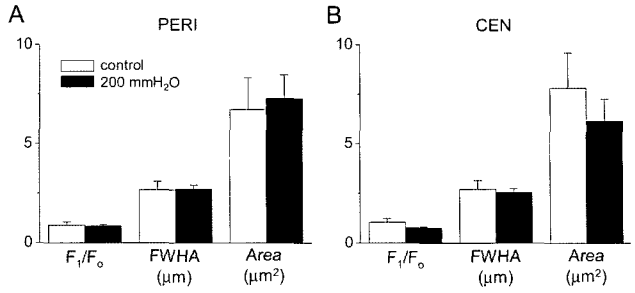

Fig. 3. Effect of FP on the unitary properties of Ca^{2+} sparks. Comparison of means of amplitude (F_1/F_0), full-width at half maximal amplitude (FWHA) and area of single peripheral (PERI: control, n=41; FP, n=210) and central (CEN: control, n=73, FP, n=196) sparks in the presence and absence of FP (200 mmH_2O; 12 cells). P values were >0.05 in the comparisons of all three parameters between control and FP. For detail see *Materials and Methods*.

Development of longitudinal Ca^{2+} wave by high FP

When a higher fluid pressure was applied to atrial myocytes, global Ca^{2+} waves were developed. Figure 4A shows a representative Ca^{2+} wave developed by application of higher FP (400 mmH_2O). The global Ca^{2+} wave started from the focal Ca^{2+} release site in the periphery (Fig. 4A, third image), and it always propagated in a longitudinal direction from its focal origin (Fig. 4A). The location of the focal origin of Ca^{2+} waves was found at end or perinucleous of the cell, or at a site close to the cell membrane. The velocity of FP-induced longitudinal Ca^{2+} waves was calculated as a distance between two sites, starting point and around ending point (1 and 2 in the image of Fig. 4B), divided by difference (delay) of time-to-peaks of Ca^{2+} releases from the two sites. Mean velocity of FP-induced Ca^{2+} waves measured in 7 cells was 78.4 ± 14.1 μm/s.

DISCUSSION

In the present study we showed that fluid pressure significantly increased the spontaneous Ca^{2+} spark frequency in the periphery and center of atrial myocytes without changing the unitary properties of single sparks. The effect of fluid pressure on the spark occurrences was two times larger in the cell periphery than the center (Fig. 2D), suggesting that a junctional component in the cell periphery may be involved in the signaling of flow pressure. Relatively high flow pressure generated longitudinal Ca^{2+} waves which were normally derived from the Ca^{2+} sparks. How atrial myocytes accelerate the frequency of spark occurrences remains unclear. Since unitary properties of individual sparks were not changed by flow pressure, the flow pressure may not directly affect gating kinetics of RyRs but rather lowers a threshold of the RyR openings. In this regards, it has been recently suggested that flow pressure-induced increase in global Ca^{2+} concentration in quiescent rat atrial myocytes, loaded with fura-2 AM, is not affected by the selec-

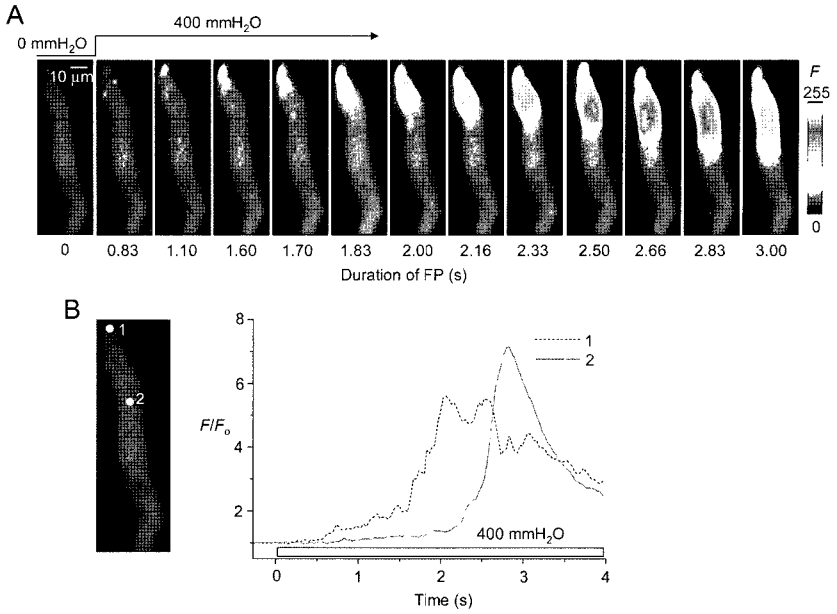

Fig. 4. High FP-induced longitudinal Ca^{2+} wave. (A) 2-D confocal Ca^{2+} images, recorded at the indicated times (see below each image) following application of FP of 400 mmH_2O, show development of a longitudinal Ca^{2+} wave by FP. (B) Ca^{2+} fluorescence signals (1 and 2) measured from the corresponding local sites, "*1* (dash)" and "*2* (solid)", in the left confocal image. Imaging was performed at 30 Hz.

tive blockers for the L-type Ca^{2+} channel, Na^+-Ca^{2+} exchanger, and stretch activated channel, but it is sensitive to FCCP that disrupts mitochondrial potential (Morad *et al.*, 2005). Since mitochondria are located both at the center and periphery in atrial myocytes (Forssmann and Girardier, 1970; Carl *et al.*, 1995), the question on the mechanism for larger effects of flow pressure on peripheral Ca^{2+} concentration still remains. In addition novel effectors to explain the fluid pressure-induced increase in the spark frequency should not be excluded.

Our finding on the effects of flow pressure on the frequency of spontaneous sparks and basal Ca^{2+} is somewhat disparate from the previous report on the responses of rat atrial tissue to membrane stretch. In fact, the stretch of atrial muscle by increasing intra-atrial pressure did not affect diastolic indo-1 fluorescence ratio (Tavi *et al.*, 1998). Although the different observations may be, in part, due to the distinct mode of stimuli (i.e., fluid pressure vs. stretch) it should be also noted that when epifluorescence of the heart muscle is used in estimation of the intracellular calcium of the mycoytes, part of the fluorescence signal may originate from cells other than myocytes, forming a possible source of error and low spatial resolution (Shinozaki *et al.*, 1993). Interestingly stretch of the rat ventricular myocytes enhanced spontaneous spark frequency but did not change diastolic Ca^{2+} level (Vila Petroff *et al.*, 2001). This report is somewhat comparable to our observation on the increased spark frequency in atrial myocytes during fluid pressure. No Ca^{2+} removal mechanism through the t-tubules in atrial myocytes may cause frequent development of central Ca^{2+} waves from the Ca^{2+} sparks, which may explain the increase in resting Ca^{2+} only in the atrial myocytes.

In the intact hearts it has been previously reported that an increase in the intra-atrial pressure with pressurized flow into the atrial chambers induces atrial fibrillation, a sustained arrhythmias (Bode *et al.*, 2001). The production of Ca^{2+} spark-derived global Ca^{2+} wave by fluid pressure, demonstrated in the present study, may provide an insight into a cellular mechanism for the previously observed atrial fibrillation during the excessive intra-atrial pressure. We suggest that fluid pressure-induced Ca^{2+} releases may directly alter excitability of atrial myocytes by activating Ca^{2+}-dependent ionic conductance in the peripheral membrane and by enhancing spontaneous activation of central myofilaments. How flow pressure are sensed and transmitted to RyRs in the junctional and nonjunctional SR remains to be worked out.

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