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## A simple automated stimulator of mechanically induced arrhythmias in the isolated rat heart

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### Abstract

Transient stretching of the ventricle can trigger arrhythmias and evoke ventricular fibrillation especially when the stimulation occurs in the vulnerable period. To explore the sensitivity of small hearts we used a commercial pressure servo (HSPC-1, ALA Scientific, USA) to study the kinetic relationship of left ventricular pressure (LVP) to excitability and arrhythmias in the rat heart. Stimulation protocols were readily composed in the computer and programmed to vary the stimulus amplitude and timing relative to pacing. The pressure induced premature ventricular excitations were similar to those observed in larger hearts, but the convenience of using small hearts allows the use of inexpensive transgenic animals to explore the molecular basis of transduction.

### Keywords

mechanoelectric coupling; ventricular pressure clamp; premature ventricular excitation

### Introduction

Most ion channels are mechanosensitive (Hu & Sachs, 1997) including those called voltage dependent (Lin *et al.*, 2007; Morris & Juranka, 2007) and ligand gated (Van Wagoner, 1993; Link *et al.*, 1999) types. In a dynamically contracting organ like the heart, time dependent mechanical stresses are the normal environment and it is reasonable to suppose that there is a coupling between mechanosensitive channels and stress in the heart. To study this coupling requires a controlled mechanical stimulus, and we have found that a commercial pressure servo developed for patch clamping (Besch *et al.*, 2002; Niu & Sachs, 2003; Suchyna *et al.*, 2004a; Suchyna *et al.*, 2004b; Suchyna & Sachs, 2007) can apply controlled stimuli to small hearts. The ability to dissect the molecular mechanisms of stretch induced arrhythmias requires transgenic animals such as mice, so we needed to develop a robust measuring system that can reliably generate controlled mechanosensitive arrhythmias. Mechanical transduction, irrespective of mechanism, is commonly termed mechanoelectric coupling (MEC) (Lab, 1989) or mechanoelectric feedback (MEF) (Kohl *et al.*, 2005).

Pro-arrhythmic effects of mechanical stimulation were noticed clinically over a century ago (*Commotio cordis*) (Madias *et al.*, 2008). MEC is now commonly observed when a cardiac

catheter touches the endocardium and generates extra systoles. Mechanical stimulation induces arrhythmias in many, if not all, species including humans (Taggart *et al.*, 1992; Taggart & Lab, 2008), pigs (Madias *et al.*, 2008), dogs (Hansen *et al.*, 1990), rabbits (Zabel *et al.*, 1996; Bode *et al.*, 2006), lambs (Chen *et al.*, 2004), and rats (Salmon *et al.*, 1997). MEC has been studied in a variety of preparations including the whole organ (*in vivo or in vitro*) (Franz *et al.*, 1992; Link, 2003), bundles of muscle (papillary muscle (Nicolosi *et al.*, 2004), trabecula (Wilhelm *et al.*, 2006), etc.), isolated cells (Zeng *et al.*, 2000; Niu & Sachs, 2003; Nishimura *et al.*, 2006; Liu *et al.*, 2008) and cultured cell sheets (Kong *et al.*, 2005).

The relationship of mechanical stimulation to the generation of arrhythmias in the intact heart is best studied by stretching the chambers. In canine heart, inflation of the ventricle to approximately double the end of diastole volume raised the chance of a premature ventricular excitation (PVE) to 50% (Hansen *et al.*, 1990). In the isolated rabbit ventricle, volume pulses could successfully pace the heart (Franz *et al.*, 1992). Kinetic and pharmacologic studies have repeatedly suggested that acute mechanical stimuli act on mechanosensitive ion channels and most likely, the cation selective stretch-activated variety known as stretch-activated channels (SACs) (Kamkin *et al.*, 2001; Kelly *et al.*, 2006). We have repeated a number of these studies in the rat heart to develop a technique that we will apply to the smaller mouse heart.

## Methods

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Capital Medical University, Beijing, China, and performed in accordance with “Regulations for the Administration of Affairs Concerning Experimental Animals (the State Science and Technology Commission, China, 1988)”.

### Preparation and perfusion of the heart

We used thirty-five Sprague-Dawley rats of either sex weighing from 230 to 250 g. After the rat was anesthetized with sodium pentobarbital (50 mg/kg) and heparinized (2500 IU/kg), the heart was rapidly removed and cannulated via aorta in a Langendorff apparatus and perfused with a modified Tyrode's solution at 8 mL/min and at 37 °C. The perfusate contained (mmol/L): 137 NaCl, 5.4 KCl, 1.8 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 5 HEPES, 10 Glucose, buffered at pH 7.4 with NaOH and bubbled continuously with 100% oxygen. Gadolinium chloride (GdCl<sub>3</sub>), verapamil and streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in the perfusate to give concentrations of 50, 1 and 500 μmol/L, respectively.

### Recording of cardiac electric activities

The heart was routinely paced at 260 ms (SEN-3301, Nihon Kohden, Tokyo, Japan) using a small bipolar hooked electrodes of stainless steel thread, 0.15 mm in diameter, separated by 1.5 mm. These were placed on the surface of the right ventricle. The epicardial monophasic action potential (MAP) of the left ventricle (LV) was recorded with a suction electrode. The epicardial electrocardiogram (ECG) was recorded with two hooked electrodes (similar to the pacing electrodes, but monopolar) placed on the surfaces of the right atrium and LV, respectively. MAP, ECG and left ventricular pressure (LVP, see below) were amplified, filtered, digitized and acquired with a four-channel computer controlled data system (BL-420 System, TME Technology, Chengdu, China).

### Ventricular pressure clamping

The pressure servo or “pressure clamp” (HSPC-1, ALA Scientific Instruments, Westbury, NY, USA) has been used in patch clamp studies of stretch-activated channels in astrocytes (Suchyna *et al.*, 2004a), myotubes (Suchyna & Sachs, 2007), COS cells (Honore *et al.*,

2006) and cardiac myocytes (Niu & Sachs, 2003). The pressure clamp utilizes a piezoelectric mixing valve for pressure and vacuum with closed-loop feedback from a pressure transducer. The system rise time is about 1 ms with a closed exit tube about 20 cm in length.

The HSPC-1 was introduced as a ventricular mechanical stimulator by Wei et al (Wei *et al.*, 2006). In brief, a latex balloon filled with air was inserted into the LV via the left atrium and connected with the pressure outlet of the pressure clamp. The connecting tube was stiff and as short as possible (20 cm long and 1 mm in inside diameter). Fig.1 illustrates the signal flows. LVP was set by the pressure clamp and recorded with ECG and MAP simultaneously. A trigger from the pacing stimulator triggered pClamp7.0 software and the DigiData1200A acquisition board (Axon Instrument Inc., Foster City, CA, USA). pClamp software ran pre-edited stimulation files to control the pressure clamp.

The controlled LVP was independent of cardiac contraction creating four basic modes of mechanical stimulation (Fig. 2): 1) Zero load contraction by holding the ventricular pressure at 0 mmHg continuously (not shown). 2) Pressure pulses with adjustable intensity and timing delivered at different phases of the pacing cycle with zero load (Fig. 2Aa). 3) Mimicking an isovolumic contraction by replaying a pressure waveform from an isovolumic contraction that had been prerecorded with the ventricular balloon filled with water. The LVP during the rest period between stimuli was set at 0 mmHg (Fig. 2Ab). We termed this mode isovolumic pressure profile (IPP). 4) Variations of the IPP with additional transient mechanical stretches (Fig. 2Ac).

Fig. 2B illustrates the timing of a mechanical pulse with the left ventricular MAP (LVMAP) during a fixed pacing rhythm. The coupling interval ( $T$ ) was defined as  $T = t_m - t_e$  where  $t_e$  denotes the start of the pacing (excitation) signal and  $t_m$  the start of the pressure (mechanical) pulse. In each run, the stimulus was repeated ten times and the incidence of resultant PVEs counted to compute the probability of occurrence. To minimize adaptation (Dick & Lab, 1998), the rest interval was set to 60 s.

The pressure clamp was designed originally to meet the demands of fixed volume pipettes. Since the left ventricle has a much larger volume than a pipette, the rise time was slower and the maximum pressure was smaller, but these limits can be extended with a redesign of the valve and the use of smaller hearts. Fig. 2 shows that the rise time of a 120-mmHg pressure pulse was about 30 ms and the fall time was 10-20 ms (Fig. 2A), so a practical minimum pulse was close to 50 ms. The maximum pressure output was 150 mmHg.

The balloon pressure from the clamp is balanced by both the elasticity of the balloon and the LV, and to gain an idea contribution of the passive balloon we compared pressure-volume curves with the balloon in air and inside the LV with the balloon filled with water. The heart was perfused with Thomas solution (contains  $K^+$  16 mmol/L) at 37 °C to stop beating and Fig. 2C shows that the pressure for the balloon increased much more inside the LV than in air indicating that the inflation pressure was dominated by the LV. The balloon absorbed about 10 mmHg when LV was inflated to 150 mmHg and since the wall stiffness increases during contraction, the fractional error for the balloon elasticity will decrease.

### Statistics analysis

Data are presented as means  $\pm$  SD. A two-way analysis of variance (ANOVA) followed by multiple comparisons in ANOVA was performed to compare the effects of the coupling interval ( $T$ ) and the amplitude of the pressure pulse on PVE incidence. Student's paired  $t$  test was applied to compare the effects of gadolinium chloride and streptomycin sulfate on PVE

incidence. All data analysis was performed by means of SPSS software (SPSS, Chicago, IL, USA). Values of  $P < 0.05$  were considered statistically significant.

## Results

### Effects of pressure pulse stimulation from 0 mmHg

To investigate the time-dependence of mechanical stimulation we applied pulses of 120 mmHg when the LVP was held at a background of 0 mmHg. Figs. 3A and B show two records from the same heart with pulses of  $T = 60$  and 180 ms when the ventricle was paced every 260 ms. In Fig. 3A, pressure pulses induced only one PVE at  $T = 60$  ms. Fig. 3B shows five PVEs at  $T = 180$  ms, but there were eight extrasystoles in the ten repetitions. The statistical data for the timing effects on the PVE incidence is shown in Fig. 3C ( $n = 15$ ,  $P < 0.01$ ). PVEs increased with the delay of the pressure pulse and recovery of excitability.

We then tested the pressure-dependence of PVE triggering (Fig. 3). Figs. 3D and E were recorded in the same heart with  $T = 180$  ms but with different pressures. No PVEs occurred at 0 mmHg (record not shown), while sporadic PVEs could be provoked by 20 mmHg (Fig. 3D), and four PVEs were initiated by 80 mmHg (Fig. 3E) with a monotonic dependence of PVE incidence on pressure (Fig. 3F). We found it surprising that pulses as small as 20 mmHg significantly increased the occurrence of PVEs (Fig. 3F,  $n = 15$ ,  $P < 0.01$ ). In the records of Fig. 3, vertical lines mark the beginning of PVEs. They usually occurred near when the pressure had peaked (also in Figs. 5A, B and G).

### Extra pressure pulses combined with IPP

Ventricular pressure fluctuates cyclically with the cardiac rhythm. To better investigate the effects of transient pressure pulses in a loaded condition, we superimposed pressure pulses during the isovolumic contraction. The traces in Fig. 4 show the records from the same heart. The LV was alternately held at 0 mmHg and clamped with IPP from cycle to cycle. No PVEs occurred during a normal IPP (Fig. 4A). Pressure pulses of 120 mmHg with  $T = 50$  ms delivered during the rising phase of the isovolumic contraction could not induce PVEs (Fig. 4B). PVE frequency increased with the amplitude of the pressure pulse after repolarization ( $T = 150$  ms, Figs. 4C, D and E). Fig. 4E shows that every pressure pulse of 120 mmHg was followed by a PVE. Fig. 4F summarizes the data showing the PVE dependence on the intensity and timing of the pressure pulse stimulation ( $n = 9$ ,  $P < 0.01$ ). This data suggest that an abrupt increase of LVP in a range relevant to the physiological situation will induce PVEs. Pressure pulses applied during IPP caused PVEs most commonly at the beginning of the pressure pulse (Figs. 4D and E; also in Fig. 5C).

### Blocking pressure-induced PVEs with gadolinium ion and streptomycin

Cardiac MEC has been attributed to the activation of SACs. We tested whether PVEs were related to SAC activity by using pharmacologic blockers. Fig. 5 shows that  $Gd^{3+}$  and streptomycin inhibited PVEs frequency. Before application of  $Gd^{3+}$ , four PVEs were induced with four pressure pulses (Fig. 5A) while with 50  $\mu M$   $Gd^{3+}$  only one PVE occurred after 20 min of perfusion (Fig. 5B). Streptomycin (500  $\mu M$  perfused for 20 min) also blocked pressure-induced PVEs (Figs. 5C and D). The statistics emphasize that the incidence of PVEs decreased significantly (92% to 13%) after  $Gd^{3+}$  treatment and from 87% to 7% after streptomycin treatment (Figs. 5E and F,  $P < 0.01$ ,  $n = 14$ ).

Because L-type calcium channels have been shown to be stretch-sensitive (Morris *et al.* 2006) and might be also blocked by  $Gd^{3+}$  (Lacampagne *et al.*, 1994), we tested the specific L-type calcium channel blocker verapamil. After perfusion with 1  $\mu M$  verapamil for 10 min

(5 hearts), contraction was greatly reduced but PVEs could still be regularly induced by pressure pulses (Fig. 5G).

## Discussion

### Methodology of the ventricular pressure clamping: advantages and limitations

The air driven pressure clamp was introduced by Hamill and McBride (McBride & Hamill, 1992) to control the pressure on a patch. Besch et al (Besch *et al.*, 2002) improved that design and ALA produced it under the model name HSPC-1. In the small volume of pipettes the rise time is ~ 1 ms with a range of  $\pm 200$  mmHg, but the pressure range and volume flow is mostly set by the supply pumps and can be easily extended with larger pumps. The off the shelf device gave us a range of about 150 mmHg and a rise time of about 30 ms. Note that the use of a balloon is not necessary if the ventricle is filled with fluid although the HSPC should be placed above the heart to minimize the chance of fluid entry to the valve. This is particularly relevant for application to the smaller mouse heart. The present study shows that the pressure clamp is a powerful yet inexpensive tool to control the ventricular pressure of small hearts. Computer control allows arbitrary waveform stimulation emulating a variety of physiological scenarios. For example, the clamp can mimic a normal ventricular ejective contraction if we record the ventricular pressure/time curve during ejective systole *in situ*. With repeatable and controllable pressure stimuli it is now possible to explore the molecular correlates of molecular transduction with transgenic animals.

### Reverse excitation-contraction coupling: stretch-induced vs. de-stretch-induced arrhythmias

Stretch-induced arrhythmias in variety of cardiac preparations, has been attributed to activation of SACs and termed MEC, a process of reverse excitation-contraction coupling (ECC). There is another type of reverse ECC that typically happens after a quick release of a cardiac myocyte during contraction (Kaufmann *et al.*, 1971; Lab *et al.*, 1984; Wakayama *et al.*, 2005; Ter Keurs *et al.*, 2008). Rapid unloading decreases the affinity of  $\text{Ca}^{2+}$  for troponin C and results in a surge of  $[\text{Ca}^{2+}]_i$  followed by delayed afterdepolarization and triggered action potentials. This type of feedback has recently been emphasized for non-uniform contractions (Ter Keurs *et al.*, 2008). The ventricle is not homogeneous in fiber orientation, mechanics and electrophysiology between different layers or regions, especially between Purkinje and working fibers. According to the reports (Wakayama *et al.*, 2005; Ter Keurs *et al.*, 2008), a border zone between the stronger and weaker myocytes might become a locus of a  $[\text{Ca}^{2+}]_i$  surge. It is possible that a de-stretch mechanism might be involved in the pressure pulse-induced PVE, especially when the pulse reached the peak, the start of the pressure fall. However, in our experiments, after treatment with verapamil that will block  $\text{Ca}^{2+}$  influx, the contraction was greatly inhibited and  $[\text{Ca}^{2+}]_i$  would be low during excitation, but the pressure pulse still regularly induced premature beats (Fig. 5G). Thus, the stretch-induced premature beat was not likely to be dependent on the  $\text{Ca}^{2+}$ -troponin C interaction.

### Electrophysiological properties of MEC revealed by pressure clamp

Our results were similar to those obtained with volume stimulation in larger hearts with respect to timing and amplitude dependence of PVE occurrence. We were surprised at the small amplitudes (20 mmHg) that were required to evoke PVEs after the refractory period. This is much below the 140 mmHg that was required in rabbit heart (Bode *et al.*, 2006). The rat PVEs were blocked by  $\text{Gd}^{3+}$  and streptomycin but not verapamil strongly indicated the involvement of cationic SACs.

The common models for SAC gating have an exponential dependency on membrane tension (Sukharev *et al.*, 1999; Markin & Sachs, 2004), thus the time required for channel activation (Suchyna *et al.*, 2004a) will strongly depend upon the applied force and the effective viscosity of the cells. We found that the contraction state was an important determinant of the latency of the stretch-induced PVE. The latency was shorter during IPP than during zero load contraction or during diastole, probably because elastic elements in parallel with the channels were preloaded. The plasmalemma of cells during zero load contraction wrinkles and was not under significant tension (except possibly in the T-tubules). Thus, the shortened myocytes are likely to respond more slowly to a pressure pulse (Fig. 3A) than in the inflated ventricle, where parallel elements are prestressed. The IPP protocol passively stretches the wall so that pulses delivered at high volume will have shorter latency (Figs. 4D and E; Fig. 5C). In diastole, the myocyte length will vary with the preload. When the preload is set to zero, the cells are shorter than in the physiological setting, and this will produce longer latency – the shorter myocyte in relaxation has to be pulled to a length necessary to activate the channels (Figs. 3B, D and E).

The similarity of the mechanically induced PVEs between the rat and the larger animals such as the rabbit and dog suggest that data learned from the small hearts can be reasonably applied to studies of the more expensive large animals, and eventually to clinical therapy in humans.

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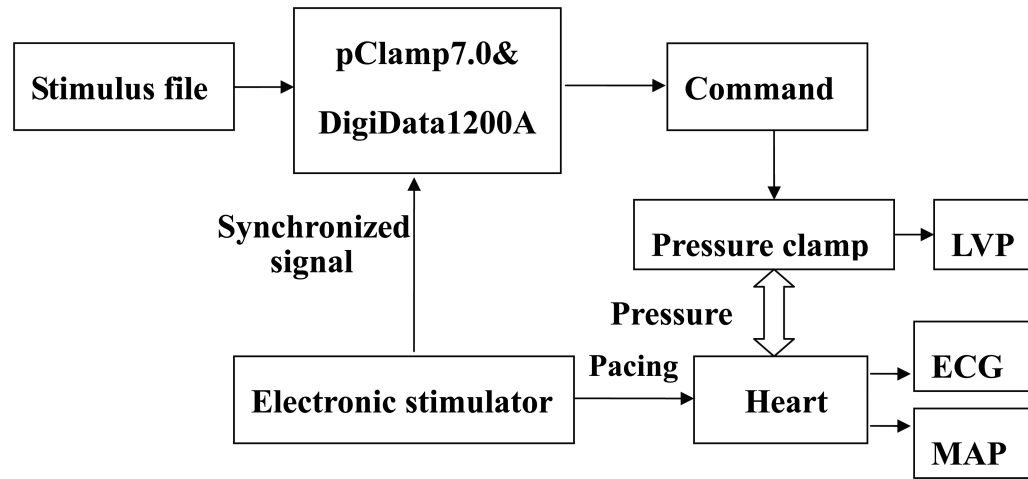
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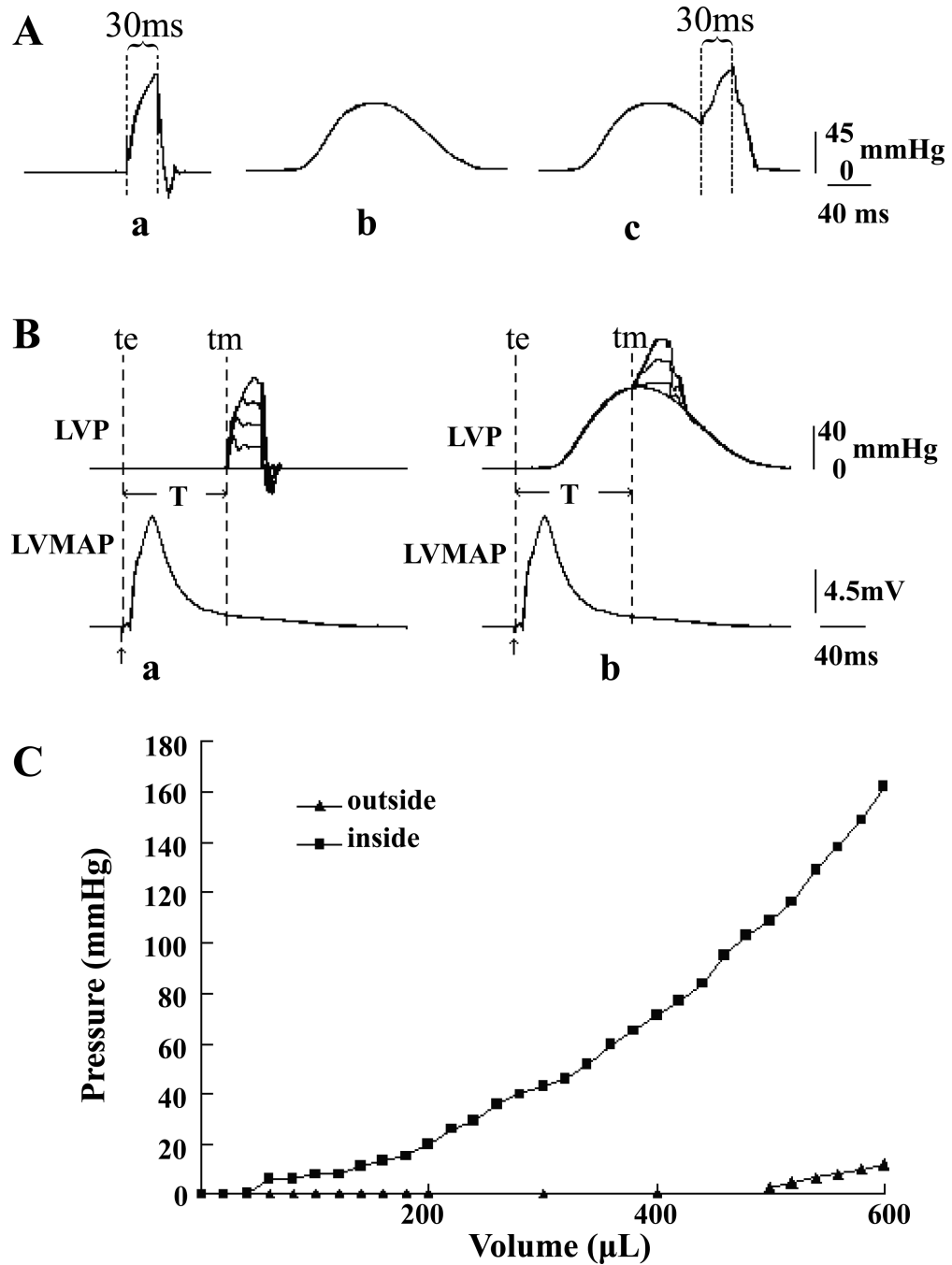
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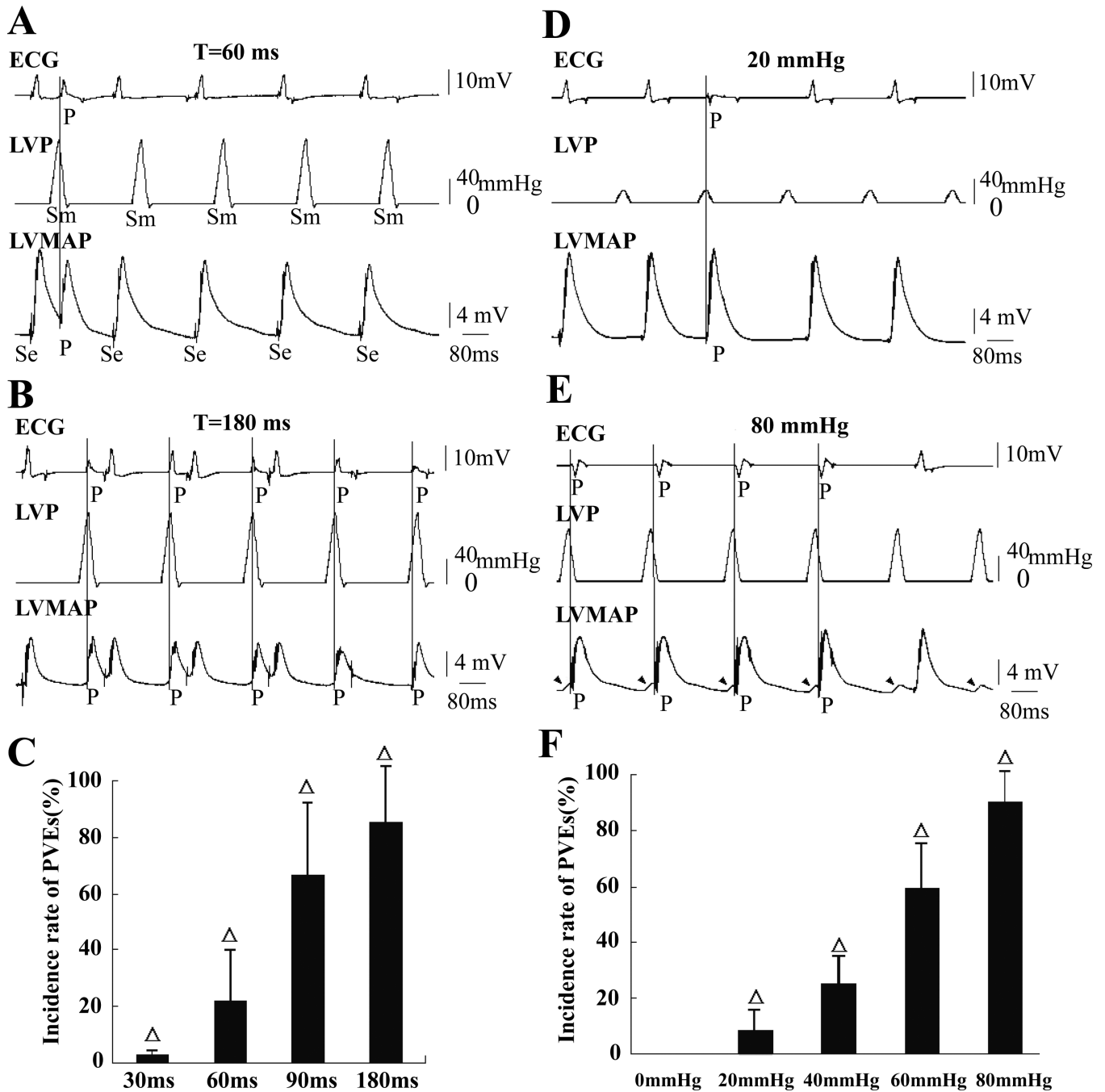
**Figure 1. Flow chart of ventricular pressure clamping**

The pressure clamp was controlled by analog voltages from the interface generated in response to pre-edited stimulus protocols. The double ended arrow denotes the pressure transfer with a connecting tube. The left ventricular pressure (LVP), epicardial electrocardiogram (ECG) and left ventricular monophasic action potential (LVMAP) were recorded synchronously.



**Figure 2. Pressure-clamping modes and the pressure-volume curves of the balloon inside and outside the LV**

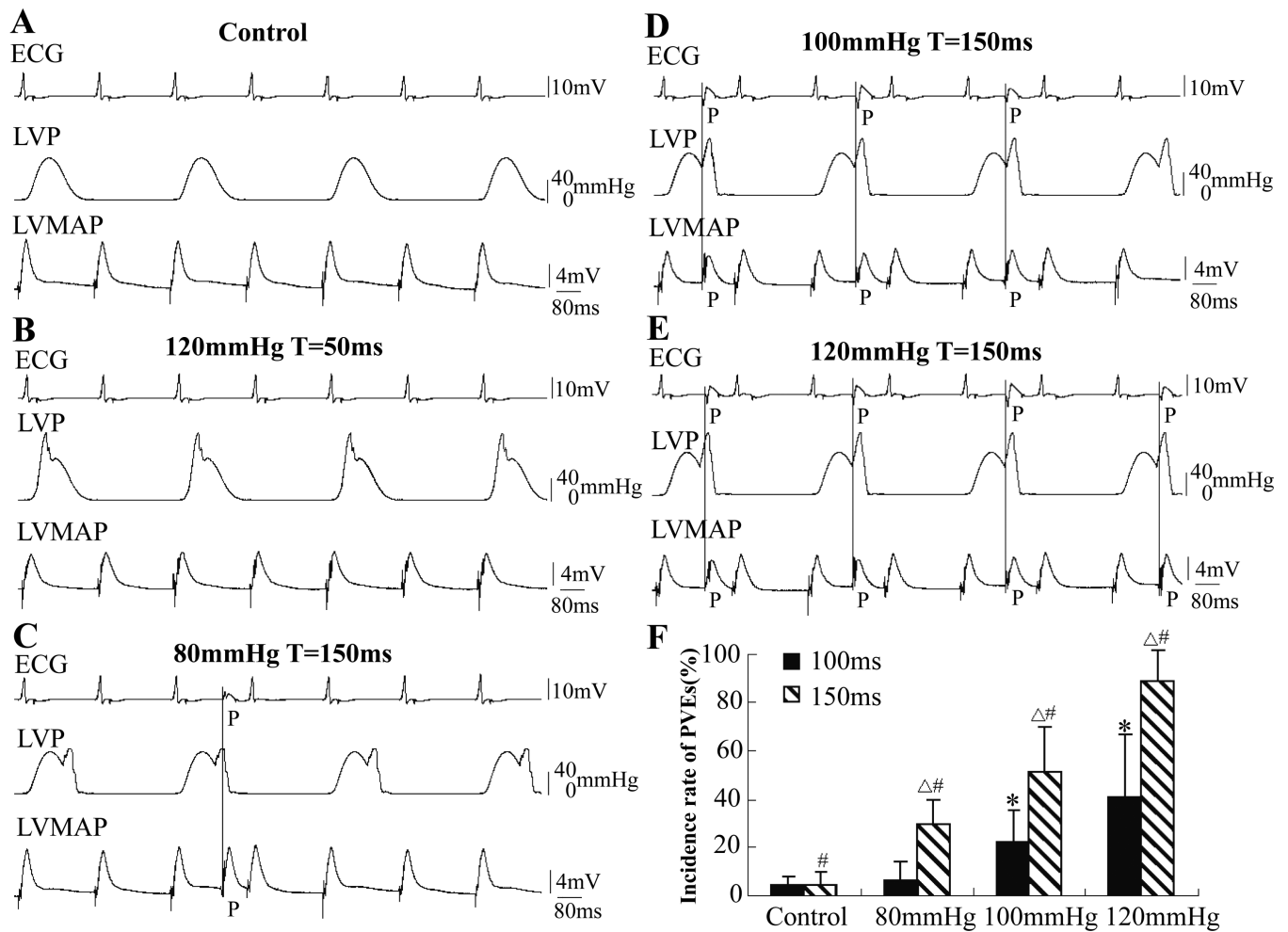
The LVP could be clamped at 0 mmHg continuously (not shown) and there were three other modes. Fig. Aa: clamping with a short pressure pulse from a background pressure of 0 mmHg. Fig. Ab: clamping with an isovolumic pressure profile (IPP). Fig. Ac: clamping with a short pressure pulse superimposed on an IPP. Panel B shows an example of the timing of LVP clamping relative to LVMAP.  $T$  is the coupling time defined as the time from the beginning of a pacing signal ( $t_e$ ) to pressure pulse ( $t_m$ ). Panel C shows pressure-volume relations when the LV balloon was outside ( $\blacktriangle$ ) and inside ( $\blacksquare$ ) the LV. See text for more detail.



**Figure 3. Effects of pressure pulses when holding ventricular pressure at 0 mmHg**

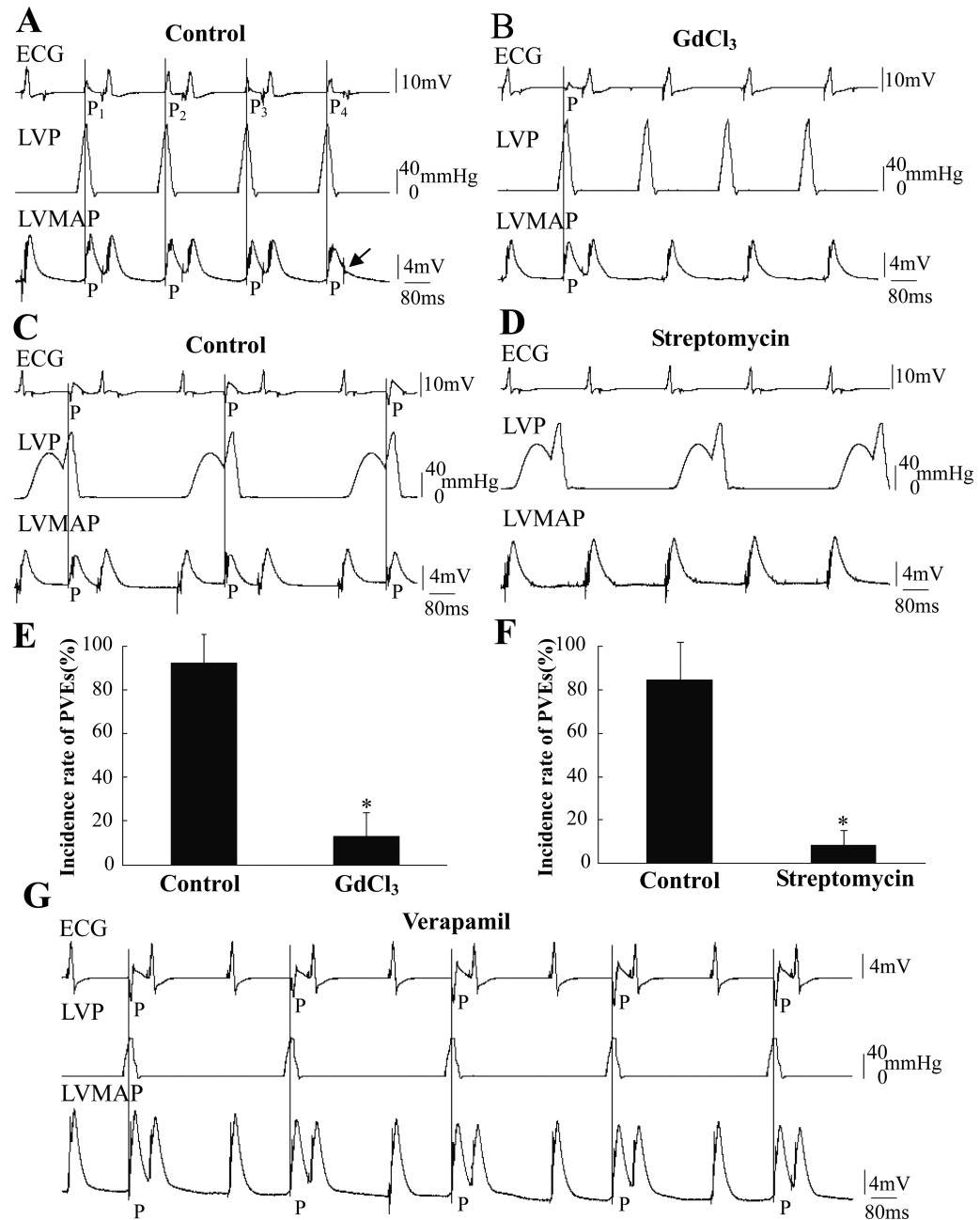
The ventricle was electrically paced at a cycle length of 260 ms. P denotes a PVE caused by pressure stimulation and the vertical line marks the time of PVE occurrence. In this figure LVP was held at a background 0 mmHg. The electrical pacing artifact (Se) and the mechanical stimulus (Sm) are marked only in A. Pressure pulses of 120 mmHg were delivered with coupling intervals ( $T$ ) of 30, 60, 90 and 180 ms. Only records for  $T=60$  ms and  $T=180$  ms are shown in A and B, respectively. PVE incidence increased as  $T$  lengthened from 30 to 180 ms (C,  $n=15$ ,  $\Delta: P<0.01$  vs each other). In D and E, the left ventricle was exposed to pressure pulses of 20 and 80 mmHg delivered at  $T=180$  ms, respectively. More PVEs were induced by the larger pressures (F,  $n=15$ ,  $\Delta: P<0.01$  vs 0 mmHg group and each

other). In E, stretch activated depolarizations (SADs) marked with arrowheads and four were followed by PVEs.



**Figure 4. Effects of pressure pulses during IPP (the same heart)**

No PVEs occurred when the LV was alternately held at 0 mmHg and clamped with an isovolumic contraction beat by beat (A). Pulses of 120 mmHg with short  $T$  (50 ms) could not induce a PVE (B). In C, D and E, transient pulses of 80, 100 and 120 mmHg with  $T=150$  ms were superimposed on the IPP, respectively. F shows the data summary, focusing on the effects of intensity and timing of the pressure pulse.  $\Delta$  signifies  $P < 0.01$  compared to the paired 100 ms group; # signifies  $P < 0.01$  compared to each other in the 150 ms group; \* signifies  $P < 0.01$  compared to each other, the control, and 80 mmHg in the 100 ms group;  $n=9$ .



**Figure 5. Effects of GdCl<sub>3</sub> and streptomycin on pressure-induced PVEs**

A and B were recorded in the same heart before and after application of GdCl<sub>3</sub>. The LV was regularly inflated by pulses of 120 mmHg at  $T=180$  ms from a resting pressure of 0 mmHg. In A, four numbered PVEs with four different shapes might indicate multiple PVE loci. The arrow in A indicated a pacing signal failed to pace the heart that occurred during the effective refractory period of the previous PVE. C and D were recorded before and after application of 500  $\mu$ M streptomycin. The LV was regularly inflated by pulses of 120 mmHg with  $T=150$  ms superimposed on the IPP during every other pacing cycle. The incidence of PVEs decreased after treatment with Gd<sup>3+</sup> and streptomycin (E and F,  $n=14$ , \*:  $P<0.01$  vs. control). G shows a record when the LVP was held at 0 mmHg and stimulated with 60-

mmHg pulse for  $T=180$  ms after application of  $1 \mu\text{M}$  verapamil. The PVEs caused by pressure pulses could not be blocked by verapamil in five hearts.