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RESEARCH PAPER

The negative inotropic action of canrenone is mediated by L-type calcium current blockade and reduced intracellular calcium transients

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Background and purpose: Adding spironolactone to standard therapy in heart failure reduces morbidity and mortality, but the underlying mechanisms are not fully understood. We analysed the effect of canrenone, the major active metabolite of spironolactone, on myocardial contractility and intracellular calcium homeostasis.
Experimental approach: Left ventricular papillary muscles and cardiomyocytes were isolated from male Wistar rats. Contractility of papillary muscles was assessed with force transducers, Ca^{2+} transients by fluorescence and Ca^{2+} fluxes by electrophysiological techniques.
Key results: Canrenone (300–600 μmol·L^{-1}) reduced developed tension, maximum rate of tension increase and maximum rate of tension decay of papillary muscles. In cardiomyocytes, canrenone (50 μmol·L^{-1}) reduced cell shortening and L-type Ca^{2+} channel current, whereas steady-state activation and inactivation, and reactivation curves were unchanged. Canrenone also decreased the Ca^{2+} content of the sarcoplasmic reticulum, intracellular Ca^{2+} transient amplitude and intracellular diastolic Ca^{2+} concentration. However, the time course of $[Ca^{2+}]_i$ decline during transients evoked by caffeine was not affected by canrenone.
Conclusion and implications: Canrenone reduced L-type Ca^{2+} channel current, amplitude of intracellular Ca^{2+} transients and Ca^{2+} content of sarcoplasmic reticulum in cardiomyocytes. These changes are likely to underlie the negative inotropic effect of canrenone.
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Keywords: spironolactone; canrenone; calcium; calcium channel; sarcoplasmic reticulum

Abbreviations: +dT/dt, maximum rate of tension increase; –dT/dt, maximum rate of tension decline; $B_{max\text{-}en}$, maximal Ca^{2+} binding capacity of the endogenous Ca^{2+} binding sites; $B_{max\text{-}in}$, maximal Ca^{2+} binding capacity of indo-1; $[Ca^{2+}]_i$, free intracellular Ca^{2+}; $[Ca^{2+}]_{SR}$, total Ca^{2+} content of the SR; $[Ca^{2+}]_T$, total Ca^{2+} concentration in the cytosol; $[Ca^{2+}]_{T(peak)}$, $[Ca^{2+}]_T$ at the peak of the $[Ca^{2+}]_i$ transient; $[Ca^{2+}]_{T(dia)}$, $[Ca^{2+}]_T$ immediately before application of caffeine; DT, peak developed tension; $I_{Ca\text{-}L}$, L-type Ca^{2+} channel current; k, slope factor; K_d, indo-1 apparent dissociation constant; $K_{d\text{-}en}$, K_d of the endogenous Ca^{2+} binding sites; $K_{d\text{-}in}$, K_d; L_{max}, muscle length at which DT is maximum; NCX, Na^+–Ca^{2+} exchange; RT_{50}, time from peak tension to 50% relaxation; SR, sarcoplasmic reticulum; $t_{½\text{-}rel}$, half-time for cell relaxation; $t_{½\text{-}Ca}$, half-time for calcium concentration decay at twitches; TPT, time to peak tension; V_m, membrane potential; $V_{½}$, potential of half maximum activation/inactivation

Introduction

Spironolactone has beneficial effects in heart failure of both ischaemic and non-ischaemic aetiologies such that the addition of spironolactone to the standard therapy for heart failure reduces the mortality by 30% (Pitt *et al.*, 1999). Therefore, since 2001, the American Heart Association has recommended the addition of spironolactone to the standard treatment of those patients (Hunt *et al.*, 2001). The beneficial effect was initially attributed to the blockade of aldosterone receptor, but some studies have shown a direct effect of spironolactone and its derivative metabolites on cardiac excitation–contraction coupling (Coraboeuf and Deroubaix,

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1974; Mugge *et al.*, 1984; Vassallo *et al.*, 1998; Cargnelli *et al.*, 2001). Spironolactone blocks L-type Ca^{2+} channel currents ($I_{Ca\text{-}L}$) in smooth muscle cells (Dacquet *et al.*, 1987), but this effect has not been studied in cardiomyocytes.

In humans, spironolactone is rapidly metabolized in the liver and does not appear in plasma or urine in measurable quantities (Sadée *et al.*, 1973). The major active metabolite of spironolactone is canrenone. Canrenone has negative inotropic action on isolated cardiac papillary muscles (Coraboeuf and Deroubaix, 1974; Mugge *et al.*, 1984). However, the mechanisms underlying the direct action of canrenone on cardiac contraction needs further clarification. Therefore, we decided to study the acute effect of canrenone in isolated preparations of cardiomyocytes and papillary muscles from the myocardium of normal rats.

Here we expanded the analysis of the effects of canrenone on isolated rat papillary muscles to both inotropic and lusitropic actions. Our results provided the first evidence of the action of canrenone on Ca^{2+} handling in cardiac muscle. We found that canrenone reduced amplitude of cardiac $I_{Ca\text{-}L}$ and the Ca^{2+} content of sarcoplasmic reticulum (SR) with consequent reduction in amplitude of intracellular Ca^{2+} transients. These changes could contribute to the negative inotropic actions of canrenone on the heart.

Methods

Animals

All animal care and the protocols of this study conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 96-23, revised, 1996) and were approved by the Ethics in Research Committee of the Federal University of São Paulo (CEP N° 640/01). A total of 65 male Wistar rats weighing 280–310 g were used. Animals were housed in light/dark cycles with food and water *ad libitum*.

Canrenone synthesis

Canrenone was obtained from potassium canrenoate (Sigma, St. Louis, MO, USA), as previously described (Megges *et al.*, 1997). Canrenone purity was determined by three methods (Megges *et al.*, 1997): thin layer chromatography, gas chromatography coupled to mass spectrometry and ^{1}H and ^{13}C-nuclear magnetic resonance, with results similar to those previously published (Megges *et al.*, 1997). Canrenone was dissolved in Tween 20 (0.001%; USB, Cleveland, OH, USA) for use in papillary muscle preparations and in dimethyl sulphoxide (DMSO; 0.0083%; Sigma) for use in cardiomyocyte preparations. DMSO at this concentration had no significant effect on $I_{Ca\text{-}L}$ (data not shown).

Papillary muscle mechanics

Myocardial contraction was evaluated in left ventricular papillary muscles, as described by others (Conrad *et al.*, 1991). Briefly, under anaesthesia (1.2 mg·g^{-1} urethane, i.p.) the hearts were quickly removed and placed in oxygenated Krebs-Henseleit solution (in mM: 118 NaCl, 4.7 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 11 glucose and 25 $NaHCO_3$). Papillary muscle was dissected free and placed in a chamber containing Krebs-Henseleit solution oxygenated by a mixture of 95% O_2 and 5% CO_2 (pH 7.4). The muscle was connected to a Grass FTO3E force transducer (Astro-Med Inc., Grass Instrument Division, West Warwick, RI, USA) that was attached to a micromanipulator to allow variation of muscle length. Developed force was recorded with Acqknowledge 3.5.7 software (Biopac Systems Inc., Santa Barbara, CA, USA). Peak developed tension (DT, g·mm^{-2}), maximum rate of tension increase (+dT/dt, g·mm^{-2}·s^{-1}) and decline (–dT/dt, g·mm^{-2}·s^{-1}), time to peak tension (TPT, ms) and time from peak tension to 50% relaxation (RT_{50}, ms) were calculated. Developed force and its time derivatives were normalized for muscle cross-sectional area. The muscle cross-sectional area was estimated from the muscle weight and length by assuming a cylindrical shape and a specific gravity of 1.0.

Preparations were stimulated (rectangular voltage pulses, 5 ms duration, 10% above threshold at 0.2 Hz) throughout the experiment. The preparations were left contracting isotonically for 60 min under a low preload and then loaded to contract isometrically for 15 min. Then the muscle was slowly stretched to the L_{max}, defined as the muscle length at which DT is maximum. The muscle was kept at this length for the rest of the experiment. Fifteen minutes later, perfusion with canrenone (150, 300 or 600 μM) or the vehicle alone (0.001% Tween 20) was started. Canrenone concentrations were chosen according to a previous study with right papillary muscles (Cargnelli *et al.*, 2001). The mechanical behaviour of the electrically stimulated papillary muscle was evaluated before and at the end of 15 min of perfusion.

Cardiomyocyte isolation

Ventricular myocytes were isolated as reported previously (Santos *et al.*, 1995). Briefly, under anaesthesia (1.2 mg·g^{-1} urethane, i.p.) the hearts were rapidly removed and attached to a modified Langendorff apparatus. The heart was perfused for 5 min at 10 mL·min^{-1} with Tyrode solution [in mM: 132 NaCl, 1 $CaCl_2$, 1.8 $MgCl_2$, 4.0 KCl, 10 HEPES (Sigma) and 5 glucose, pH 7.35] saturated with O_2, followed by Ca^{2+}-free Tyrode solution. When the heart stopped beating, collagenase II (Worthington, Lakewood, NJ, USA; 0.8 mg·mL^{-1}) and bovine albumin (0.02%; Sigma) were added. Perfusion continued until the heart became flaccid (~15–20 min). Then the enzyme was washed out with Ca^{2+}-free Tyrode solution. Next, ventricular fragments were dispersed. The cell suspension was rinsed several times with Tyrode solution in which Ca^{2+} concentration was increased up to 1 mM.

Measurement of cell shortening and calcium transients

Myocytes were plated on collagen-treated perfusion chambers. The chamber was placed on an inverted microscope (Diaphot 300, Nikon Corp., Tokyo, Japan) equipped for epifluorescence measurement (RatioMaster, Photon Technology International, Monmouth Junction, NJ, USA). Myocytes were loaded with indo-1 acetoxymethyl ester (indo-1 AM; Molecular Probes, Eugene, OR, USA) for 15 min and superfused with Tyrode solution for 20 min. Excitation wavelength was

365 nm, and fluorescence emitted by the cell was recorded at 405 and 485 nm. Fluorescence ratios were converted to free intracellular Ca^{2+} ($[Ca^{2+}]_i$) according to the equation (Grynkiewicz *et al.*, 1985):

$$[Ca^{2+}]_i = K_d \times \beta[(R - R_{min})/(R_{max} - R)]$$

where the indo-1 apparent dissociation constant (K_d) was 844 nM (Bassani *et al.*, 1995a). R_{min}, R_{max} and β were determined *in vivo* (Bassani *et al.*, 1994). Total Ca^{2+} concentration in the cytosol ($[Ca^{2+}]_T$) was calculated as:

$$[Ca^{2+}]_T = [Ca^{2+}]_i + ([Ca^{2+}]_i \times B_{max\text{-}en}/[Ca^{2+}]_i + K_{d\text{-}en}) + ([Ca^{2+}]_i \times B_{max\text{-}in}/[Ca^{2+}]_i + K_{d\text{-}in})$$

where $B_{max\text{-}en}$ and $K_{d\text{-}en}$ are the maximal Ca^{2+} binding capacity (300 μM) and K_d (0.54 μM) of the endogenous Ca^{2+} binding sites (Bassani *et al.*, 1994). $B_{max\text{-}in}$ and $K_{d\text{-}in}$ are cytosolic indo-1 concentration and apparent dissociation constant (Bassani *et al.*, 1998) assumed to be 50 μM (Bassani and Bassani, 2002) and 844 nM (Bassani *et al.*, 1995a) respectively.

To estimate the Ca^{2+} content of the SR, electrical stimulation was stopped and the perfusion medium was changed to Na^+- and Ca^{2+}-free ($0Na^+$-$0Ca^{2+}$) Tyrode solution (Li^+ replaced Na^+ and 1 mM EGTA replaced Ca^{2+}) to inhibit Na^+–Ca^{2+} exchange (NCX) and remove residual Ca^{2+}. Thirty seconds later, a 30 s perfusion with 10 mM caffeine (Sigma) in $0Na^+$-$0Ca^{2+}$ Tyrode solution was started (Bassani *et al.*, 1992; Bassani *et al.*, 1995b). The total Ca^{2+} content of the SR ($[Ca^{2+}]_{SR}$) was considered to be the difference between $[Ca^{2+}]_T$ at the peak of the $[Ca^{2+}]_i$ transient evoked by caffeine and diastolic $[Ca^{2+}]_T$ immediately before application of caffeine: $[Ca^{2+}]_{SR} = [Ca^{2+}]_{T(peak)} - [Ca^{2+}]_{T(dia)}$.

The experimental protocol was as follows: cells were perfused with Tyrode solution and field-stimulated at 0.5 Hz (biphasic voltage pulses with amplitude 20% above threshold, 8 ms duration). After a steady state was attained: (i) $[Ca^{2+}]_i$ transients were obtained before and after 5 min perfusion with 50 μM canrenone; and (ii) $[Ca^{2+}]_{SR}$ was measured before and after 5 min perfusion with 50 μM canrenone. The concentration of canrenone for the experiments with isolated cardiomyocytes was based on previous experiments with smooth muscles cells (Dacquet *et al.*, 1987) and cardiomyocytes (Caballero *et al.*, 2003). We also analysed the time course of $[Ca^{2+}]_i$ decline during transients evoked by 10 mM caffeine in Tyrode solution.

Cell shortening was measured with a video edge detection system (Centro de Engenharia Biomédica, UNICAMP, Campinas, SP, Brazil) simultaneously with $[Ca^{2+}]_i$ measurement as previously described (Bassani *et al.*, 1994; Ricardo *et al.*, 2008).

Electrophysiological studies

Electrophysiological recordings were performed by using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). Myocytes were continuously superfused with Tyrode solution containing 2 mM 4-aminopyridine (4-AP; Sigma) and 2 mM $CaCl_2$. The glass microelectrodes had a tip resistance of 4–5 MΩ. Cells were internally dialysed with pipette solution containing in mM: 110 CsCl, 20 NaCl, 0.5 $CaCl_2$, 5 ATP-Mg (Sigma), 0.1 GTP (Sigma), 10 EGTA (Sigma), 10 HEPES and 30 TEA-Cl (Sigma), pH 7.2. Transmembrane ionic currents were recorded (low-pass filtered at 1 KHz and sampled at 5 KHz) by using Axopatch 200 amplifier, Digidata 1200 interface and pClamp 6.0.4 software from Axon Instruments, USA. Current amplitude was taken as the difference between the peak and steady-state current and normalized to the cell capacitance.

For time course analysis of the effect of canrenone, $I_{Ca\text{-}L}$ was elicited every 6 s by voltage-clamp steps from a holding potential of –70 mV to 0 mV for 250 ms. This protocol was done before, during and after (washout) 4 min of perfusion with 50 μM canrenone. For current–voltage relationship analysis, $I_{Ca\text{-}L}$ was elicited by test potentials ranging from –50 to +60 mV, in 10 mV increments, for 200 ms before and during 4 min of perfusion with 50 μM canrenone. Before each pulse, a prepulse from the holding potential to –40 mV for 25 ms was used to inactivate Na^+ and T-type Ca^{2+} channels. To investigate the effect of canrenone on the voltage dependence of the L-type calcium channel, activation and inactivation curves for $I_{Ca\text{-}L}$ were determined as described previously (Nascimento *et al.*, 2001). The activation curves were constructed from the current–voltage relationship by dividing the amplitude of $I_{Ca\text{-}L}$ at each potential by the driving force. Steady-state inactivation curves were obtained with a classical double-pulse protocol. Preconditioning steps of 1.5 s from –60 to +60 mV in 10 mV intervals from a holding potential of –50 mV were applied before a fixed 500 ms step to 0 mV. The effects of canrenone on kinetics of reactivation of the L-type Ca^{2+} channel were studied by using a standard double-pulse protocol. Two depolarizing pulses to 0 mV with a varying inter-pulse interval were applied, from a holding potential of –40 mV, every 10 s.

Steady-state activation and inactivation curves were fitted with a Boltzmann function to the data of individual experiments: $I/I_{max} = 1/[1 + \exp(V_m - V_{½})/k]$, where V_m is the membrane potential, $V_{½}$ the potential of half maximum activation/inactivation and k the slope factor (all in mV). The reactivation curve was fitted by a single exponential.

Statistical analysis

Results were expressed as mean ± SEM. Data were compared by Student's *t*-test or one-way analysis of variance followed by Student-Newman-Keuls *post hoc* analysis. In all cases, $P < 0.05$ was considered statistically significant.

Results

Effects of canrenone on contractility of papillary muscle

A clear negative inotropic effect was observed after 15 min of canrenone exposure as shown by changes in DT and +dT/dt. Indeed, DT decreased significantly at the end of perfusion with 150 μM (3.9 ± 0.5 to 3.6 ± 0.4 g·mm^{-2}; $P < 0.05$; $n = 7$), 300 μM (4.4 ± 0.4 to 3.5 ± 0.3 g·mm^{-2}; $P < 0.01$; $n = 10$) and 600 μM canrenone (4.0 ± 0.4 to 2.8 ± 0.3 g·mm^{-2}; $P < 0.001$; $n = 8$), amounting to a relative reduction of DT values compared with those observed with vehicle perfusion (Table 1), when compared with basal levels. In addition, +dT/dt

Table 1 Effects of canrenone on contractile and relaxation properties of left ventricular papillary muscle from rats

	Tween (n = 9)	*150 μM CRN* (n = 7)	*300 μM CRN* (n = 10)	*600 μM CRN* (n = 8)
DT (%)	96 ± 1	92 ± 1	80 ± 2†	71 ± 2†
+dT/dt (%)	99 ± 2	98 ± 5	82 ± 2†	73 ± 2†
TPT (%)	98 ± 2	102 ± 3	98 ± 1	96 ± 1
–dT/dt (%)	105 ± 6	93 ± 6	91 ± 4	80 ± 2*
RT_{50} (%)	94 ± 4	100 ± 8	87 ± 3	87 ± 2

Contractile variables (mean ± SEM) were measured after 15 min exposure to vehicle (0.001% Tween 20) or canrenone (CRN) at the concentrations shown. Basal values in control, untreated papillary muscles were set to 100%.
+dT/dt, maximum rate of tension increase; –dT/dt, maximum rate of tension decline; DT, peak developed tension; RT_{50}, time from peak tension to 50% relaxation; TPT, time to peak tension.
*$P < 0.01$ compared with vehicle.
†$P < 0.001$ compared with vehicle.

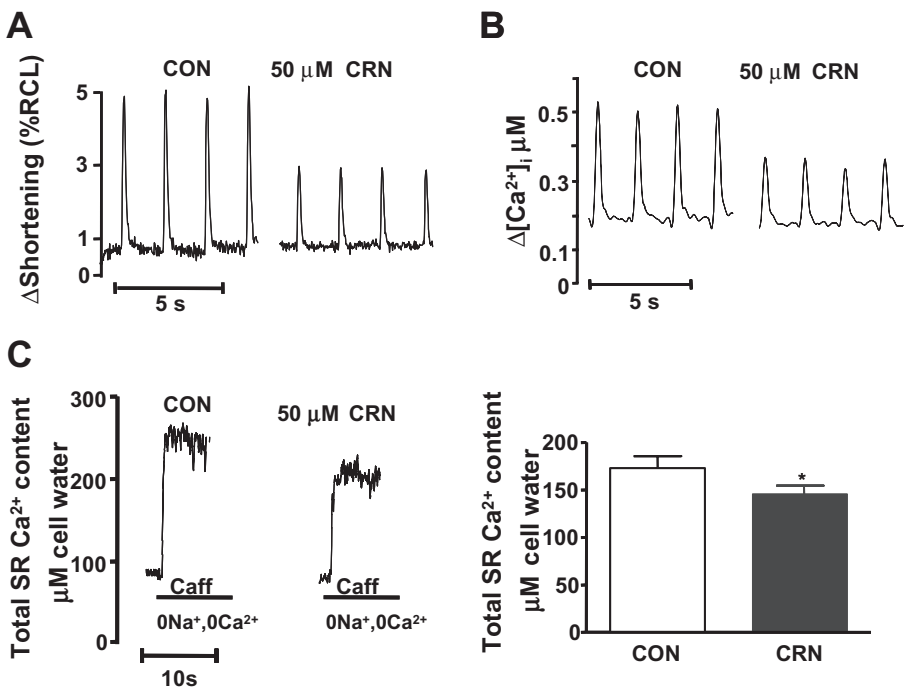

Figure 1 Effect of CRN on cardiomyocyte shortening and intracellular calcium transients. (A) Representative experimental record of changes in cardiomyocyte shortening (ΔShortening) as percentage of RCL during electrically induced twitches before and after 5 min of superfusion with 50 μM CRN. (B) Intracellular calcium transients ($\Delta[Ca^{2+}]_i$) of cardiomyocytes during electrically induced twitches before and after 5 min of superfusion with 50 μM CRN. (C) Left: representative experimental record showing total SR Ca^{2+} content, estimated from caffeine-induced calcium release in a Na^+- and Ca^{2+}-free medium before and after 5 min of superfusion with 50 μM CRN. Right: total SR Ca^{2+} content, estimated from caffeine-induced calcium release, before and after 5 min of superfusion with 50 μM CRN ($n = 6$). *$P < 0.05$. $0Na^+$, Na^+-free; $0Ca^{2+}$, Ca^{2+}-free; $[Ca^{2+}]_i$, free intracellular Ca^{2+}; Caff, caffeine; CON, control; CRN, canrenone; RCL, resting cell length; SR, sarcoplasmic reticulum.

decreased significantly at the end of 15 min perfusion with 300 μM (53.8 ± 5.2 to 44.0 ± 4.2 $g{\cdot}mm^{-2}{\cdot}s^{-1}$; $P < 0.001$; $n = 10$) and 600 μM (50.5 ± 5.4 to 36.8 ± 4.0 $g{\cdot}mm^{-2}{\cdot}s^{-1}$; $P < 0.001$; $n = 8$) with significant reduction, compared with values obtained with vehicle (Table 1). TPT decreased with 600 μM canrenone (143 ± 2.5 to 135 ± 3.8 ms; $P < 0.05$; $n = 8$), but this reduction did not differ from that after vehicle perfusion (Table 1). Less influence of canrenone was noted in papillary muscle relaxation. After 600 μM canrenone –dT/dt decreased in absolute (27.1 ± 3.9 to 21.6 ± 3.0 $g{\cdot}mm^{-2}{\cdot}s^{-1}$; $P < 0.05$; $n = 8$) and relative values (Table 1), whereas the values for RT_{50} after canrenone did not differ from those obtained after vehicle perfusion (Table 1).

Effect of canrenone on cardiomyocyte shortening, $[Ca^{2+}]_i$ transients and sarcoplasmic reticulum Ca^{2+} load

Canrenone (50 μM) reduced cardiomyocyte shortening amplitude from 5.2 ± 0.6% to 3.3 ± 0.5% ($n = 14$; $P < 0.01$; Figure 1A) of resting length. Reduction of shortening was accompanied by a reduction in the amplitude of the $[Ca^{2+}]_i$ transient from 0.51 ± 0.04 to 0.33 ± 0.03 μM ($n = 13$; $P < 0.001$, Figure 1B). Canrenone also reduced diastolic Ca^{2+} concentration from 0.20 ± 0.01 to 0.18 ± 0.01 μM ($n = 13$; $P < 0.01$, Figure 1B). Canrenone did not affect the half-time for cell relaxation ($t_{½\text{-rel}}$ from 83 ± 4.8 to 88 ± 5.8 ms; $n = 12$) and calcium concentration decay at twitches ($t_{½\text{-Ca}}$ from 122 ± 6.5 to 115 ± 7.7 ms; $n = 12$). The time course of $[Ca^{2+}]_i$ decline

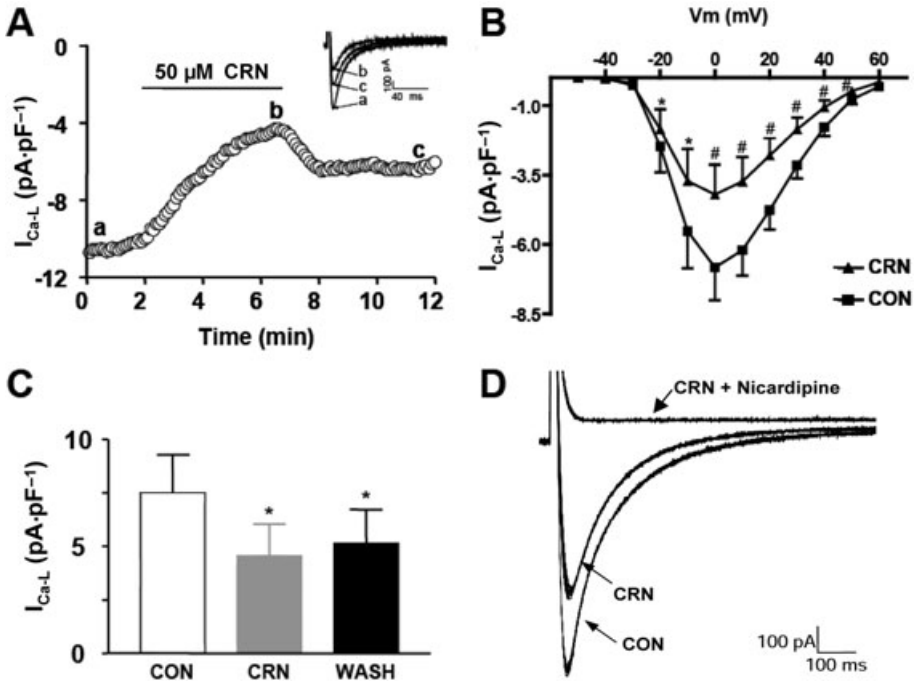

Figure 2 Effect of CRN on L-type Ca^{2+} channel current (I_{Ca-L}). (A) Representative experimental record showing time course changes in I_{Ca-L} current amplitude before (a), during (b) and after superfusion with CRN (c – washout). Inset shows current traces recorded at the indicated times (a, b and c). The current was elicited from a holding potential of –70 to 0 mV with a prepulse to –40 mV for 25 ms, activated every 6 s, in rat ventricular myocytes. (B) Current–voltage relationship of the peak I_{Ca-L} elicited by test potentials ranging from –50 to +60 mV, before and after 4 min of perfusion with CRN ($n = 12$). (C) Mean ± SEM values of maximal I_{Ca-L} amplitude before, during and after CRN perfusion ($n = 5$). (D) Representative experimental record showing I_{Ca-L} amplitude in control conditions and after sequential addition of CRN (50 μM) and nicardipine (1 μM). *$P < 0.05$ and #$P < 0.001$ CRN versus CON. CON, control; CRN, canrenone.

during transients evoked by caffeine in the presence of extracellular Na^+, which relies mainly on Ca^{2+} extrusion via the NCX (Bassani *et al*., 1992; 1994), was similar in the absence and presence of canrenone ($t_{½\text{-}Ca} = 1.5 \pm 0.4$ s, $n = 5$, and 1.9 ± 0.3 s, $n = 5$ respectively).

Additionally, we studied the effect of canrenone on the $[Ca^{2+}]_{SR}$ by measuring Ca^{2+} release induced by caffeine. We observed that canrenone decreased $[Ca^{2+}]_{SR}$ by about 16% ($n = 6$; $P < 0.01$; Figure 1C).

Effect of canrenone on I_{Ca-L}

The amplitude of I_{Ca-L} decreased progressively during superfusion with canrenone (50 μM) over the 4 min superfusion period (Figure 2A,C; $n = 5$; $P < 0.05$) but did not recover significantly during the subsequent 4 min of canrenone washout (Figure 2A,C). In Figure 2B is shown the current–voltage relationship of I_{Ca-L} before and after 4 min of perfusion with canrenone. The current we measured was completely blocked by 1 μM nicardipine (Figure 2D) suggesting that it consisted of I_{Ca-L} without significant contamination by other currents.

Figure 3 shows the normalized steady-state activation and inactivation curves in control conditions and in the presence of canrenone. Under control conditions, the analysis of steady-state activation curves revealed that $V_{½}$ was –8.5 ± 1.7 mV and k was 5.0 ± 0.2 mV. In the presence of canrenone, $V_{½}$ was –3.6 ± 1.7 mV and k was 7.0 ± 1.0 mV ($n = 7$, $P > 0.05$; Figure 3A). Analysis of steady-state inactivation curves also revealed similar parameters under control conditions ($V_{½}$ was –32.2 ± 0.3 mV and k was 5.8 ± 0.2 mV) and in the presence of canrenone ($V_{½}$ was –35.1 ± 1.2 mV and k was 7.3 ± 0.6 mV ($n = 5$, $P > 0.05$; Figure 3B).

The canrenone effect on the time constant of reactivation of L-type Ca^{2+} channels was also studied. The time constant in control conditions was 229 ± 26.8 ms and 182 ± 11 ms ($n = 5$, $P > 0.05$; Figure 3C) in the presence of 50 μM canrenone. In all experiments reactivation was complete about 1 s. Thus, canrenone did not affect the steady-state activation and inactivation, and reactivation curves of the L-type Ca^{2+} channel.

Discussion and conclusions

We have demonstrated for the first time that the acute negative cardiac inotropic effect of canrenone is related to a decreased amplitude of I_{Ca-L} and of $[Ca^{2+}]_I$ transients, together with a decreased $[Ca^{2+}]_{SR}$. We also observed that canrenone reduced diastolic $[Ca^{2+}]_i$. The observation that canrenone modulated cardiac contractility is in agreement with many earlier findings that aldosterone antagonists produce cardiac effects that may not depend on mineralocorticoid receptor antagonism (Tanz and Kerby, 1961; Baskin *et al*., 1973; Coraboeuf and Deroubaix, 1974; Sorrentino *et al*., 1996; 2000; Vassallo *et al*., 1998; Cargnelli *et al*., 2001; Barbato *et al*., 2002; Sugiyama *et al*., 2004).

The negative inotropic effect of canrenone, indicated by the reduction in DT and +dT/dt, was previously observed in

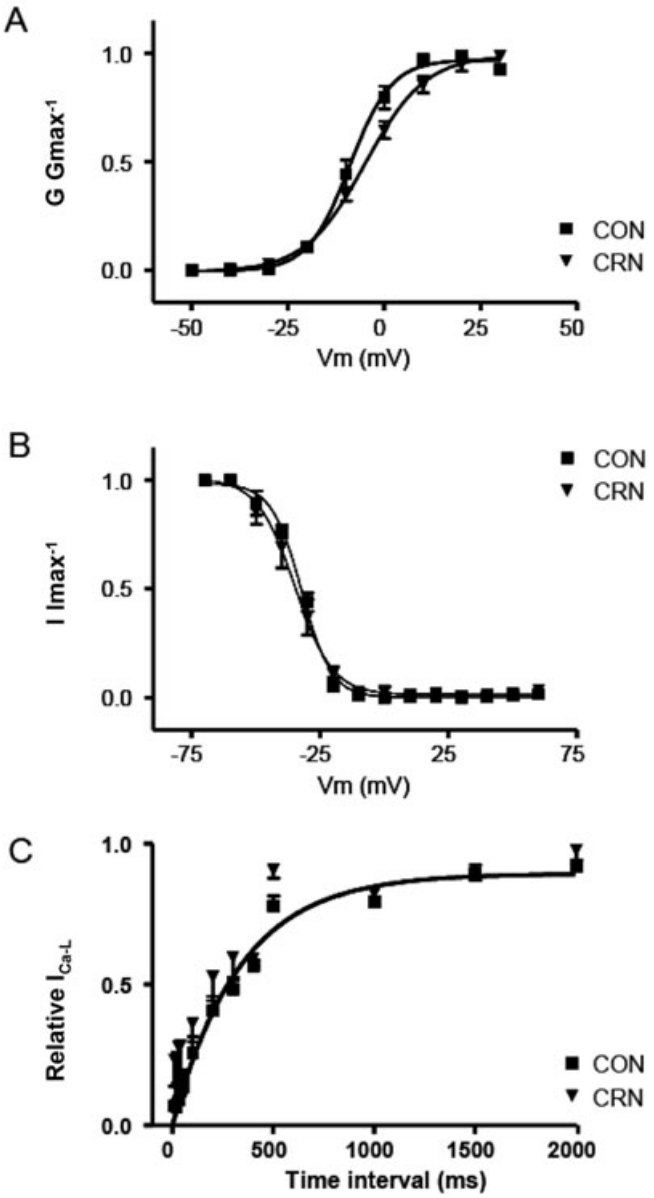

Figure 3 Lack of effect of CRN on voltage-dependent characteristics of L-type Ca^{2+} channel current ($I_{Ca\text{-}L}$) in rat cardiomyocytes. (A) The steady-state activation curves were calculated as normalized conductance values from the current-voltage curves before and 2 min after exposure to CRN (50 μM; $n = 7$). (B) Steady-state inactivation curve, $I_{Ca\text{-}L}$ at the test step to 0 mV was normalized to maximum current and plotted against the potential of a 1 s inactivating conditioning prepulse between –60 and +60 mV before and 2 min after exposure to CRN ($n = 5$). (C) Time constant of recovery from inactivation of $I_{Ca\text{-}L}$. Depolarizing pulses were applied every 6 s at a holding potential of –40 mV. The recovery from inactivation was fitted by a single exponential ($n = 5$). Recovery was complete in about 1 s. CON, control; CRN, canrenone.

papillary muscles of humans (Mugge *et al.*, 1984), guinea pigs (Mugge *et al.*, 1984) and rats (Cargnelli *et al.*, 2001). This effect was also seen with other spironolactone metabolites, such as sodium canrenoate (Coraboeuf and Deroubaix, 1974) and potassium canrenoate (Baskin *et al.*, 1973; Vassallo *et al.*, 1998). The acute negative inotropic effect of canrenone represents a non-genomic effect of this drug on cardiac contractility. Moreover, another study showed that spironolactone produces a negative effect on the isolated working heart of the rat (Moreau *et al.*, 1996).

We also studied the acute action of canrenone on cardiac lusitropism. We observed that only the highest concentration of canrenone reduced –dT/dt in papillary muscles preparations whereas the RT_{50} remained unchanged in all concentrations studied. In addition, we have not observed any change on $[Ca^{2+}]_i$ transient kinetics induced by canrenone at lower concentrations. The effect observed on –dT/dt with the highest tested canrenone concentration might still be due to a direct effect of canrenone on relaxation. Alternatively, it is possible that the effect is secondary to reduced after load, that is, it may be due to the reduction of DT produced by the canrenone-treated muscle (Konishi *et al.*, 1992).

During the cardiac action potential, Ca^{2+} enters the cardiomyocyte mainly through L-type calcium channels triggering the release of additional calcium by the activation of ryanodine-sensitive calcium channels of the SR, the so-called Ca^{2+}-induced Ca^{2+} release (Fabiato, 1983). This process results in a rapid increase of $[Ca^{2+}]_i$, which activates myofilament shortening and cellular contraction. A reduced amplitude of the Ca^{2+} transient may result from lower amplitude of $I_{Ca\text{-}L}$, decreased $[Ca^{2+}]_{SR}$ or both. Our experiments showed that canrenone exerts an acute inhibition of the $I_{Ca\text{-}L}$, causing a reduced Ca^{2+} influx. We consider that this reduced Ca^{2+} influx is the primary cause for the reduced $[Ca^{2+}]_{SR}$ and diastolic $[Ca^{2+}]_i$ at steady state, which we found after exposure of cardiomyocytes to canrenone. As a result, the amplitude of the $[Ca^{2+}]_i$ transient was reduced in the presence of canrenone and this was responsible for the depressed contractility induced by canrenone both in the cardiomyocyte and in the isolated papillary muscle preparation. Our results at the cellular level corroborated the previously described negative inotropic action of canrenone at the muscle level.

Alternatively, the effects of canrenone on diastolic Ca^{2+} concentration could be attributed to changes in rapid and slow Ca^{2+} transport systems. However, we did not find evidence of increased Ca^{2+} transport by SR Ca^{2+} ATPase or NCX, as canrenone did not change the time course of $[Ca^{2+}]_i$ decline during twitches and during transients evoked by caffeine in the presence of extracellular Na^+ respectively. Additionally, we did not observe any visible effect of canrenone on the decay of the $[Ca^{2+}]_I$ transients induced by caffeine in $0Na^+$-$0Ca^{2+}$ Tyrode solution, which rules out major effects of canrenone on the slow Ca^{2+} transport systems, such as mitochondrial Ca^{2+} uniporter and sarcolemmal Ca^{2+} ATPase (Bassani *et al.*, 1992; 1994). Thus, our present data do not identify important effects of canrenone on the mechanisms that promote cytosolic Ca^{2+} removal, particularly the rapid transporters, namely SR Ca^{2+} ATPase and NCX (Bassani *et al.*, 1992; 1994; Bassani and Bassani, 2002).

The positive inotropic effect of canrenone at low concentrations observed by Vassallo *et al.* (1998) was previously reported by Coraboeuf and Deroubaix (1974). These authors suggested that this positive inotropic effect was a consequence of the increased coronary flow induced by sodium canrenoate. They concluded that the negative inotropic effect of sodium canrenoate at low concentrations is overtaken by

the increase in the ventricular pressure induced by the increased coronary flow. The increase in the coronary flow is a consequence of the vascular dilatation induced by canrenone (Sorrentino *et al.*, 2000) through L-type calcium channel blockade in smooth muscle cell (Dacquet *et al.*, 1987).

Coraboeuf and Deroubaix (1974) also reported a dose-dependent increase in amplitude and duration of the plateau phase of the action potential induced by sodium canrenoate. The authors considered that these effects could be caused by either an increase in depolarizing currents (sodium and calcium) or a reduction in repolarizing currents (chloride and potassium). The blockade of the $I_{Ca\text{-}L}$ by canrenone reported by us excludes this current as a possible contributor to the effect of canrenone on the action potential. Coraboeuf and Deroubaix (1974) also provided evidence against a role for sodium and chloride currents in the action potential changes induced by canrenone. They concluded that the increase in amplitude and duration of the action potential plateau induced by sodium canrenoate is due to potassium current blockade. Gomez *et al.* (2005) demonstrated that spironolactone and canrenoic acid block IK_s, IK_{ur} and I_{to} currents from cultured cells expressing hERG, $K_v1.5$ and $K_v4.3$. In our study, calcium current was recorded under conditions that suppress repolarizing currents (4-AP in the extracellular solution and K^+ replaced by Cs^+ in the intracellular solution). Therefore, our data do not exclude a possible effect of canrenone on potassium currents (I_{to1}, IK_{slow} and IK_{ur}) from rat ventricular cardiomyocytes.

The previously described aldosterone action on $I_{Ca\text{-}L}$ occurs only after 6 h of aldosterone exposure (Benitah and Vassort, 1999). Therefore, we considered that the acute effect of canrenone on $I_{Ca\text{-}L}$ may be independent of its effect on aldosterone receptors. However, the precise pathways linking canrenone with the inhibition of the $I_{Ca\text{-}L}$ are currently unknown and warrant further investigation.

Our results on $I_{Ca\text{-}L}$ kinetics indicate that canrenone action on $I_{Ca\text{-}L}$ may be indirect, rather than direct on the channel as canrenone did not alter any of the kinetic parameters of $I_{Ca\text{-}L}$ studied by us. The decrease in $I_{Ca\text{-}L}$ amplitude induced by canrenone reported by us was in agreement with results from a previous study (Dacquet *et al.*, 1987) that described $I_{Ca\text{-}L}$ inhibition by spironolactone in smooth muscle cells from rat portal vein. Moreover, the percentage of reduction in the $I_{Ca\text{-}L}$ caused by spironolactone – 42% (Dacquet *et al.*, 1987) – was similar to the one found by us with canrenone – 40%. Dacquet *et al.* (1987) also demonstrated that spironolactone action on $I_{Ca\text{-}L}$ was reversible. We observed no significant recovery of $I_{Ca\text{-}L}$ amplitude after washout, but we cannot conclude that this action of canrenone on $I_{Ca\text{-}L}$ was irreversible, as our period of washout was not as long as that used by Dacquet *et al.* (1987).

Our finding that canrenone reduces the $[Ca^{2+}]_{SR}$ induced by caffeine in a $0Na^+$-$0Ca^{2+}$ solution is in accordance with those from a previous study (Dacquet *et al.*, 1987), showing that spironolactone reduced the transient contractions induced by noradrenaline and acetylcholine in rat portal vein strips in a Ca^{2+}-free solution.

We are aware that our results obtained under acute administration of canrenone to normal animals should not be extrapolated to disease models and may not reflect canrenone's effect after its chronic administration. Another limitation of the present work was that canrenone was studied at the cellular level at a single concentration. However, we and others (Cargnelli *et al.*, 2001) have shown that increasing canrenone concentration in multicellular preparations produced greater negative inotropic effect.

In summary, we have demonstrated for the first time that canrenone reduces the amplitude of cardiac $I_{Ca\text{-}L}$ and intracellular Ca^{2+} stores in cardiomyocytes. These effects reduce the amplitude of intracellular Ca^{2+} transients which, in turn, contributes to the negative inotropic action of canrenone on the heart.

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Conflict of interest

None.

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