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The new compound, LASSBio 294, increases the contractility of intact and saponin-skinned cardiac muscle from Wistar rats

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1 A new compound designated as LASSBio 294 (L-294), 3,4-methylenedioxybenzoyl-2-thienylhydrazone, was synthesized as an alternative therapeutic for cardiac dysfunction.

2 L-294 increased in a dose-dependent manner the spontaneous contractions of isolated hearts from Wistar rats with maximal effect ($128.0 \pm 0.7\%$ of control) observed at 25 μM.

3 The positive inotropic effect of L-294 was also observed in electrically stimulated cardiac tissues from Wistar rats. The maximal increment of twitches, at 200 μM, was $163.1 \pm 18.4\%$ for atrial, $153.5 \pm 28.5\%$ for papillary and $201.5 \pm 18.5\%$ for ventricular muscles.

4 In saponin skinned ventricular cells: (a) L-294 present in the period of sarcoplasmic reticulum (SR) loading with Ca^{2+} shifted the dose and caffeine-induced contracture curve; (b) L-294 (100 μM) increased 40% the Ca^{2+} uptake into SR; (c) L-294 did not significantly alter the sensitivity of contractile proteins to Ca^{2+} in SR-disrupted skinned ventricular cells.

5 Retrograde perfusion of the isolated heart from Wistar rats with L-294 (100 μM) did not cause any significant change in rhythm, heart rate (control, 220 ± 14.7 b.p.m.; 246 ± 24.6 b.p.m. for L-294), PR interval (control, 66.0 ± 2.4 ms; 64.0 ± 2.3 ms for L-294) or QRS duration (control, 28.8 ± 3.4 ms; 32.0 ± 2.0 ms for L-294).

6 These results suggest a novel mechanism for a positive cardioinotropic effect through an interaction with the Ca^{2+} uptake/release process of the SR. The effect of L-294 could be explained by a pronounced increased accumulation of Ca^{2+} into the SR.

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Abbreviations: CHF, congestive heart failure; L-294, LASSBio 294; SR, sarcoplasmic reticulum

Introduction

Positive inotropic agents are used in the treatment of the clinical syndrome of congestive heart failure. Potential cellular mechanisms for the increased cardiac performance include increased intracellular Ca^{2+} or increased sensitivity of contractile proteins to Ca^{2+}. Both factors interfere with the regulation of cardiovascular homeostasis and can induce toxic effects. It is essential to identify more effective and non-toxic pharmacotherapeutic agents that could improve cardiac contractility through an increase in intracellular free calcium concentration. Recently, new generations of bioactive compounds, N-heteroaryl-hydrazone and acyl-(*N*-heteroaryl)-hydrazone have been synthesized (structure 1, Figure 1) in which some of them contained a 2-furyl ring at the hydrazone terminal portion (structure 2, Figure 1) (Ribeiro *et al*., 1998; Barja-Fidalgo *et al*., 1999; Fraga *et al*., 2000; Figueiredo *et al*., 2000). These last derivatives have been shown to produce an anti-platelet activity related to an alteration in the intracellular Ca^{2+} mobilization (Todeschini *et al*., 1998). Further structural modifications in these groups generated the acyl-(3,4-metiledioxy-phenyl)-arylhydrazone class (structure 1, A = 3,4-methylenedioxy-phenyl, Figure 1), resulted from the replacement of the heterocycle ring from the acyl moiety to 3,4-metilenedioxy-phenyl ring originated from the safrole (4-allyl-1,2-methyldioxybenzene, structure 3, Figure 1), an abundant natural product obtained from *Ocotea pretiosa*. A novel chemical compound, 3,4-methylenedioxybenzoyl-2-thienyl hydrazone, is an isoster of 2-furyl compound (structure 2, Figure 1), which contains in its structure a 2-thienyl ring instead of an oxygenated ring (Lima *et al*., 2000). This compound designated as LASSBio 294 (L-294) is evaluated in the present study on: (i) the isometric tension in rat ventricular, papillary and atrial cardiac muscles; (ii) the electrocardiogram (EKG) and spontaneous contractions of isolated rat hearts; (iii) the calcium release and uptake by sarcoplasmic reticulum (SR) in chemically skinned ventricular muscle; (iv) the calcium sensitivity of the contractile proteins in cardiac skinned fibres. One possible mechanism underlying the cardiotonic effect of L-294 is related to the increase of intracellular Ca^{2+} concentration in myocardium by enhanced Ca^{2+} accumulation into the SR.

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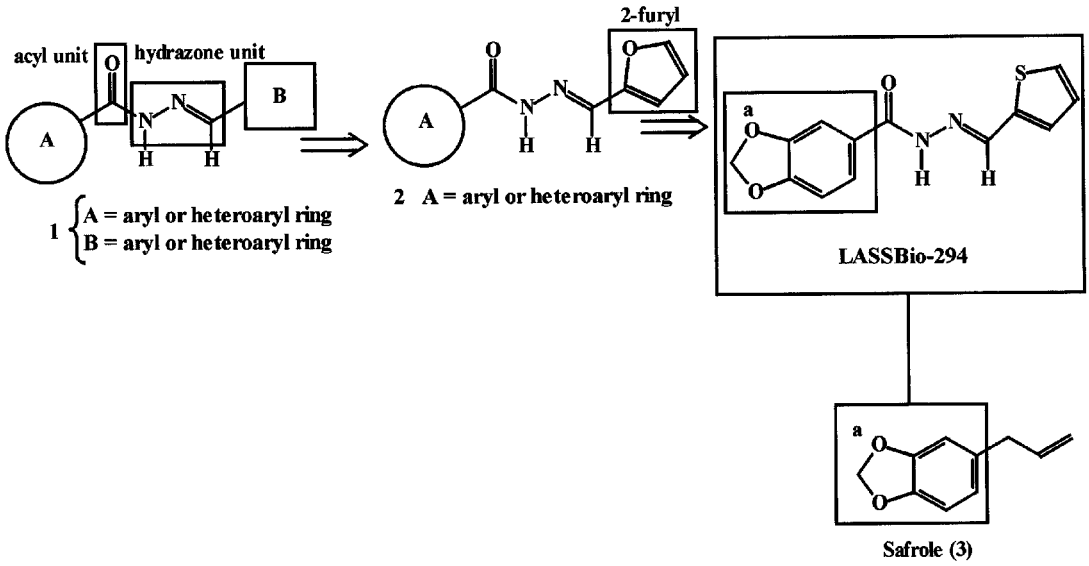


Figure 1 Retrosynthetic analysis for preparation of LASSBio-294 (L-294) from safrole.

Methods

EKG and isometric tension recordings in isolated rat heart

The Animal Care and Use Committee at the Universidade Federal do Rio de Janeiro approved the protocol described as follows. Male Wistar rats (250–350 g) were killed by cervical dislocation under ether anaesthesia. The heart was rapidly removed, mounted onto a Langendorff system through the aorta and submersed into a chamber filled with Tyrode solution (see Solutions) at 32.0 ± 1.0°C. EKG recordings were obtained through three glass pipette electrodes, filled with 1 M KCl solution, positioned close to the heart. The heart was perfused with Tyrode solution (8 ml min^{-1}) in the absence (control) or presence of L-294 (5–100 μM). EKG was recorded on a polygraph (Gould model 2400) before (control) and 10 min after addition of each concentration of L-294 and also 30 min after washout. In some experiments in which the isometric tension was recorded, the isolated heart, mounted in the Langendorff system, was pinned at the base of the left ventricle and attached to a force transducer (Grass, model FT-03). Spontaneous contractions were recorded continuously on a chart recorder (Gould model 2400) and amplitudes were measured under a pre-load of 1 g before and after addition of L-294 (5–100 μM).

Recording of isometric tension in papillary muscle and fascicles of atrial and ventricular muscles

Left papillary muscles (2–3 mm-diameter and 6–7 mm-length), and fascicles of atrial (2-mm-diameter and 5 mm-length) and left ventricular (4 mm-diameter and 8 mm-length) muscles were dissected for isometric tension recording. One end of each muscle was attached to a force transducer (Grass, model FT-03) and the other end to a hook fixed at the bottom of the experimental chamber. The chamber was filled with Tyrode solution and oxygenated with carbogen gas (95% $O_2$2 plus 5% $CO_2$2) at 37.5°C. Muscles were field stimulated at a rate of 1 Hz, 3 ms duration and the twitches were recorded on a polygraph (Grass model 7). The muscle length and the intensity of stimulation current were increased to optimize the amplitude of muscle twitches. L-294 was added into the Tyrode solution from a stock solution of 50 mM dissolved in DMSO to obtain final concentration of 10–500 μM. Twelve experiments for each type of muscle were performed for each L-294 concentration.

Skinned muscle fibre preparation

Male Wistar rats (200–250 g) were sacrificed under ether anaesthesia and their hearts rapidly removed. Small fascicles, approximately 250–350 μm diameter and 10 mm length were dissected from the sub-endocardium of the left ventricular wall at room temperature in an oxygenated, nominally Ca^{2+}-free Tyrode solution. The ends of the fascicles were attached to two hooks, one connected to a micromanipulator and the other to a force transducer (Grass, model FT-03). After mounting in a horizontal chamber (internal volume of 1 ml), the fascicles were stretched to 120% of original resting position using a graded binocular stereo microscope. To destroy the sarcolemma and obtain skinned myocardial fibres, the fascicles were immersed for 5 min in 0.5% v v^{-1} saponin (Merck Chemical Co.) (Bangham & Horne, 1962; Miller & Smith, 1985) diluted in relaxing solution (solution R, see Solutions) containing 5 mM $K_2$2-ethylene glycol-bis (β-amino-ethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA). The isometric tension was recorded in a Grass polygraph (model 7400). In the experiments in which we investigated the direct effect of L-294 on the Ca^{2+} sensitivity of contractile system, the SR membranes were also disrupted by a further 60 min exposure to solution R containing the non-ionic detergent octyl phenoxy polyethoxyethanol (Triton X-100, 1% v v^{-1}, Sigma Chemical Co.) (Best, 1983; Miller *et al.*, 1985). This treatment did not interfere with the maximal activated tension (Po) of the fibre (Miller & Smith, 1985). Throughout all experiments, the temperature was maintained at 22 ± 0.5°C.

Experimental protocols Maximal response of the skinned cardiac cells (Po) was determined by exposure to 0.5 mM $CaCl_2$ added to a washout solution (solution W, see Solutions). At the plateau of the Ca^{2+}-induced contracture, the fibres were relaxed by replacing the solution in the chamber with solution R. Po was measured in the beginning and at the end of experiments. If the initial Po decreased >20% with time, these preparations were discarded. After the initial Po measurement, the SR was depleted of Ca^{2+} by solution R containing 20 mM caffeine. The presence of 5 mM EGTA in solution R prevents Ca^{2+} re-uptake into the SR. Ca^{2+} depletion of SR induced by caffeine preceded each step of the SR Ca^{2+} loading procedure with pCa 6.6/2.57×10^{-7} M Ca^{2+}). Ca^{2+} loading (uptake) into SR was evaluated by the contracture induced by exposure of the fibres to solution W containing 20 mM caffeine (Su & Hasselbach, 1984).

Two different protocols were designed to investigate the effect of L-294 on Ca^{2+}-accumulation into SR. First, we compared the caffeine-induced contractures at different periods of SR loading time from 15 s to 8 min in the absence or presence of L-294. Second, the dose-response curves for caffeine induced Ca^{2+} release were obtained after loading the SR for 3 min with pCa 6.6 (2.57 ± 10^{-7} M Ca^{2+}) in the absence or presence of L-294 (100 μM).

Effect of L-294 on Ca-sensitization of the contractile proteins was studied in SR-disrupted preparations. The efficiency of treatment with Triton X-100 was confirmed for each experiment by the inhibition of more than 90% of caffeine-induced tension (20 mM) after SR loading with pCa 6.6 (2.57×10^{-7} M Ca^{2+}). A pCa *versus* tension curve was performed in the absence and in the presence of 100 μM L-294.

Solutions and drugs

For experiments performed in isolated hearts, the Tyrode solution had the following composition (in mM): NaCl, 130; $CaCl_2$, 1.25; KCl, 5; $MgCl_2$, 1; NaH_2PO_4, 0.5; $NaHCO_3$, 24; Glucose, 5.6 equilibrated with carbogen mixture (95% O_2/5% CO_2, pH 7.4 at room temperature). The same composition was used for atrial, papillary and ventricular experiments, except, for $CaCl_2$ concentration which was increased to 2.5 mM. During dissection of small fascicles for skinned fibre experiments, no $CaCl_2$ was added into the Tyrode solution. The composition of washing solution (W) was, in (mM): potassium propionate, 185; magnesium acetate, 2.5; imidazole propionate, 10 and K_2Na_2ATP, 5 (pH 7.0). The skinning and relaxing (R) solutions had the same composition of solution W except that each contained 5 mM K_2-ethylene glycol-bis (β-amino-ethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA). Solutions of different pCa values were prepared by replacing K_2EGTA with CaK_2EGTA keeping the total EGTA concentration constant at 5 mM. Association constants used to obtain the different ratios of K_2EGTA/CaK_2EGTA were those reported by Orentlicher *et al.* (1974) and for others ligands by Fabiato & Fabiato (1979). Caffeine was purchased from Sigma (St. Louis, MO, and U.S.A.) and added to the working solutions immediately before use. L-294 (Figure 1) was synthesized by LASSBio laboratory at the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. Due to low solubility in water, L-294 was dissolved in dimethyl sulphoxide (DMSO) in a 50 mM stock solution and aliquots were added to the working solutions.

Statistical analysis

All data were presented as mean ± s.e.mean Student's *t*-test was applied for comparison of two individual averaged groups. Analysis of Variance (ANOVA) was used for comparison of more than two groups of study. Difference between groups were considered significant when $P<0.05$. The dose-response curve for caffeine was fitted to the contractile response data using the equation: $y = y_{max} \cdot [caffeine]^n/([caffeine]^n + k_{0.5})$, where y was the percentage of isometric tension, n was the Hill coefficient, and $k_{0.5}$ was the caffeine concentration causing a 50% of maximal tension.

Results

Effects of L-294 on spontaneously beating isolated heart

A reversible increase in the isometric tension of spontaneous beating hearts under pre-load condition (1 g) was observed when they were treated with L-294. As shown in Figure 2, perfusion of the heart with L-294 (50 μM) caused an immediate increase in contractility, reaching a steady state within 2 min. L-294 did not change either the baseline tension nor the heart rate. Five minutes after L-294 washout, the contractility recovered to control value. The maximal effect was reached at a concentration of 25 μM in which L-294 increased the contractility by 28.0 ± 0.7% (from 3.71 ± 0.76 to 4.75 ± 0.90 g, $n=7$) (Figure 2b).

Effects of L-294 on tension in papillary, atrial and ventricular muscles

L-294 produced a marked positive inotropic effect when applied to isolated papillary muscle or to atrial and ventricular fascicles. Figure 3a illustrates the twitch tension of electrically stimulated cardiac muscles before and during exposure to L-294 (10 and 50 μM). As seen in the isolated heart, the effect of L-294 on isolated tissues was completely reversed after washout (Figure 3a). Pooled data, shows significant increases in twitch tensions at concentrations as low as 10 μM (Figure 3b). At 200 μM, twitches from ventricular, atrial and papillary muscles were increased to 201.5 ± 18.5% ($n=12$), 163.1 ± 18.4% ($n=12$) and 153.5 ± 28.5% ($n=12$) of control, respectively. To investigate if the effect of L-294 on cardiac contractility was related to activation of beta-adrenergic activity, papillary muscle was pre-treated with propranolol (10 μM) and then, L-294 was added in incremental concentrations of 25, 50 and 100 μM. As shown in the Figure 4, propranolol did not interfere to the inotropic effect of L-294.

Effect of L-294 on EKG in isolated whole rat hearts

Electrical activity of spontaneously beating isolated hearts in a Langendorff system was observed before and after treatment with L-294. As shown in the tracing of Figure 5, no significant changes of EKG pattern was observed in the range of concentrations that caused a significant increase in

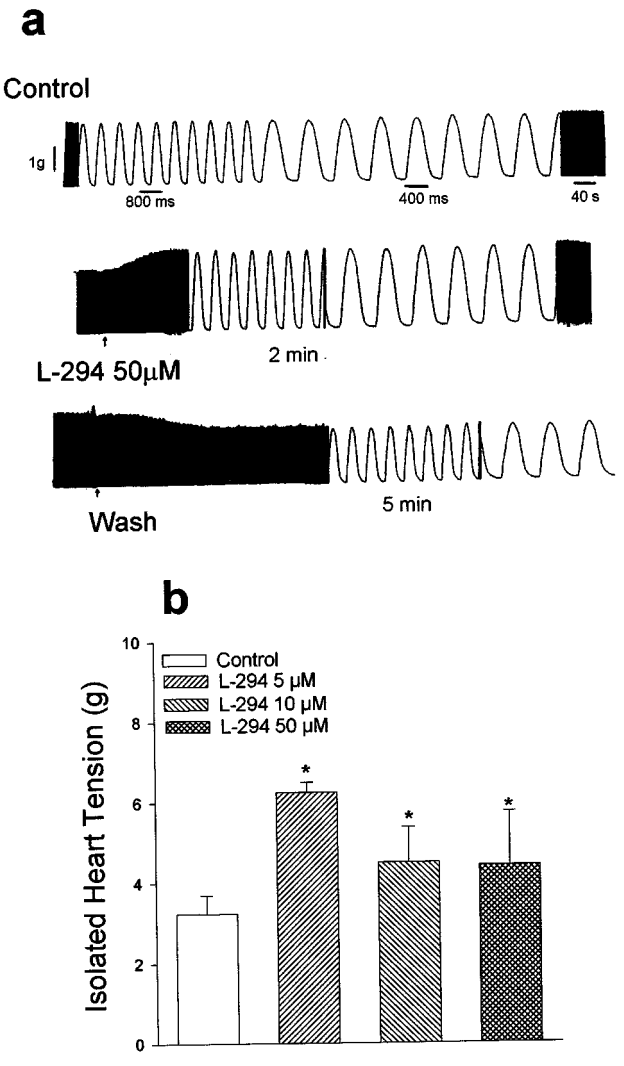


Figure 2 Effect of L-294 on spontaneous contractions in the rat isolated heart. (a) Representative continuous recordings of isolated heart contractions before and during addition of 50 μM L-294. The arrows indicate the beginning of drug perfusion and washout. Second trace shows gradual increase of the amplitude of contractions by L-294 and the maximal effect after 2 min of perfusion. The last trace shows the contraction-relaxation cycles 5 min after washout of L-294. (b) Data represent maximal contraction observed before and after treatment with incremental concentrations of L-294 ($n=7$). *Significantly different compared with control value ($P<0.05$).

cardiac contractility. Analysis of EKG from six experiments, revealed that heart rate (HR) (control: 220 ± 14.7 b.p.m.; L-294: 246 ± 24.6 b.p.m.), PR interval (control: 66.0 ± 2.4 ms; L-294: 64.0 ± 2.3 ms) and QRS duration (control: 28.8 ± 3.4 ms; L-294: 32.0 ± 2.0 ms) were not significantly altered by perfusion with 100 μM L-294. Cardiac arrhythmia was not observed in any experiment during perfusion with L-294.

Effect of L-294 on Ca^{2+} sensitivity of the contractile proteins in saponin-skinned rat cardiac fibres

Saponin-skinned ventricular fibres were treated with Triton X-100 for 60 min to destroy the SR membranes. In this preparation, the effect of L-294 was observed on the contractures induced by Ca^{2+} directly activating the myofibrils. The exposure of cardiac fibres to this detergent disrupted the SR membrane but did not affect the ability to contract when exposed to Ca^{2+} (Orentlicher *et al.*, 1974). The damage to the SR membranes was confirmed by the inhibition of caffeine-induced contracture, indicating that the only Ca^{2+} source to activate the contractile proteins was derived from the solution added to the chamber. The Ca^{2+}-induced tensions were not altered by L-294 (Figure 6a). The presence of 100 μM L-294 in the pCa solutions neither changed the threshold for Ca^{2+} activation of cross-bridge formation nor shifted the pCa-tension curve (Figure 6b). These results demonstrated that the inotropic effect of L-294 was not correlated to an increase in the Ca^{2+} sensitivity of the contractile proteins.

Effect of L-294 on Ca^{2+} loading and release from SR in skinned fibres

Maximal response (Po) of cardiac skinned fibres was determined by exposure to $CaCl_2$ (0.5 mM). Afterwards, the fibre was exposed to a solution containing Ca^{2+} 2.57×10^{-7} M (pCa 6.6) during 3 min. The SR Ca^{2+} loading was evaluated by the contracture induced by 20 mM caffeine which completely releases Ca^{2+} from SR. The same procedure for SR Ca^{2+} loading was repeated in the presence of L-294 (1–200 μM). The presence of 1 μM L-294 during loading time did not cause any change in the caffeine-induced contracture. However, 5 μM L-294 increased caffeine response by $15.6\pm2.5\%$ ($P<0.05$, $n=6$) and the maximal effect was $44.4\pm8.6\%$ of control. The EC_{50} for this effect was 5.4 μM of L-294 (Figure 7). The amount of Ca^{2+} accumulated in SR increases with time of exposure to Ca^{2+} loading solution and L-294 accelerates this process (Figure 8a). Caffeine response relative to Po as a function of SR loading time is shown in Figure 8b. The continuous line was derived from a nonlinear regression of the means to the equation: $E=E_o\ (1-e^{-kt})$, where the k was 0.74 ± 0.18 and 2.21 ± 0.29 min^{-1} for the curves in the absence and presence of L-294, respectively. The k values were obtained for each experiment and averaged. The time to reach 50% of maximal effect, $t_{1/2}$, was reduced from 0.94 to 0.31 min by L-294 (100 μM). Caffeine induced tension in a dose-dependent manner (0.05–10.0 mM) after 3 min of SR loading with solution of pCa 6.6, Ca^{2+} 2.57×10^{-7} M (Figure 9a). L-294 (100 μM) added in the loading solution decreased the threshold of caffeine-induced contracture (Figure 9a, b). The lowest caffeine concentration tested (0.05 mM) did not cause any tension in the control fibres, however, the contracture in presence of L-294 was about 30% of Po. The same amplitude of tension was observed with approximately 1 mM caffeine in non-treated fibres (Figure 9b). At low concentrations of caffeine, oscillations were observed when L-294 was present during SR loading process (Figure 9a). We have observed this response to caffeine in several others experiments using skinned fibres preparation of cardiac tissues. No oscillation was observed in the absence of L-294 during the SR loading, however, in the presence of the drug it occurred in 60% of the experiments. The dose-response relationship for the caffeine effect after previous treatment with L-294 is summarized in Figure 9b. Caffeine concentrations to produce 50% of maximal tension were 0.78 ± 0.11 and 0.34 ± 0.07 in

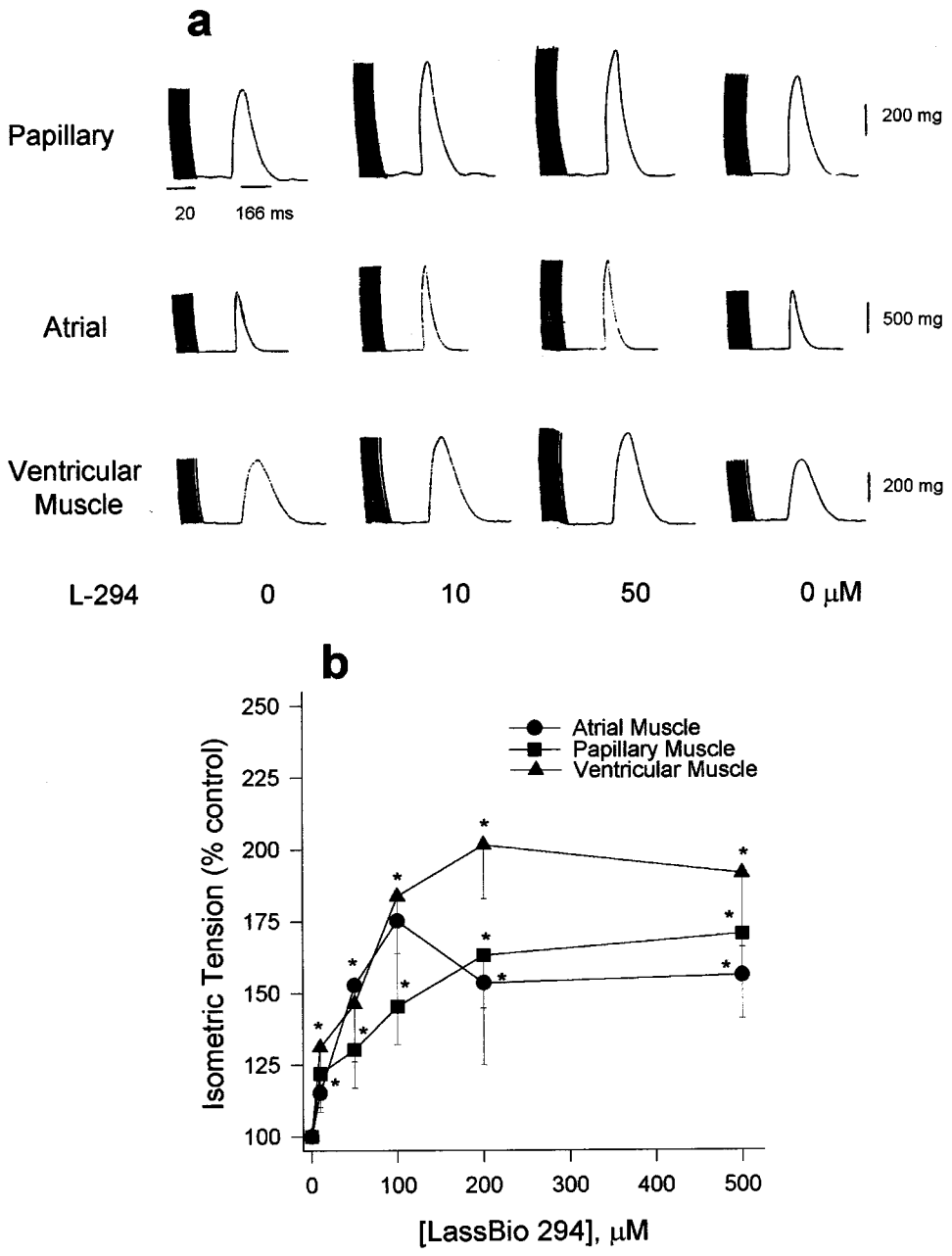


Figure 3 (a) Typical tracings of isometric tension in papillary, atrial and ventricular muscles before and after exposure to L-294 (10 and 50 μM) and 5 min after washout. (b) Tension as percentage of control (before addition of L-294) *versus* L-294 concentration. Data are mean$\pm$s.e.mean. *$P<0.05$, significant difference from the value before L-294 application.

the absence and presence of L-294 during SR loading, respectively. Caffeine dose-response curve for fibres previously treated with L-294 during SR loading was markedly shifted to the left. The response of the fibres to 20 mM caffeine was also increased by L-294 (control: 72.34$\pm$2.74%; L-294: 102.17$\pm$4.30% Po). L-294 significantly altered the Hill coefficient ($P<0.01$), where the calculated n values were 1.34$\pm$0.10 and 0.62$\pm$0.09 for drug-free and L-294-treated fibres, respectively. In Figure 10, after SR-loading with 2.57×10^{-7} M Ca^{2+} (pCa 6.6) during 3 min, the fibres were washed twice with solution W and then, treated with L-294 (100 μM). L-294 did not induce contracture in skinned cardiac fibres indicating that this compound did not release Ca^{2+} from SR. In another experimental protocol, the fibres were exposed to caffeine (2 mM) in the presence of L-294 (100 μM) after SR pre-load with Ca^{2+}. As shown in Figure 11, L-294 did not increase the caffeine-induced contracture. These results suggest that L-294 has no effect on the Ca^{2+} release from SR through the Ca^{2+} release channels.

Discussion

The important finding of this study is that the new compound, L-294, is an effective positive inotropic agent and is potentially interesting in the treatment of certain heart-

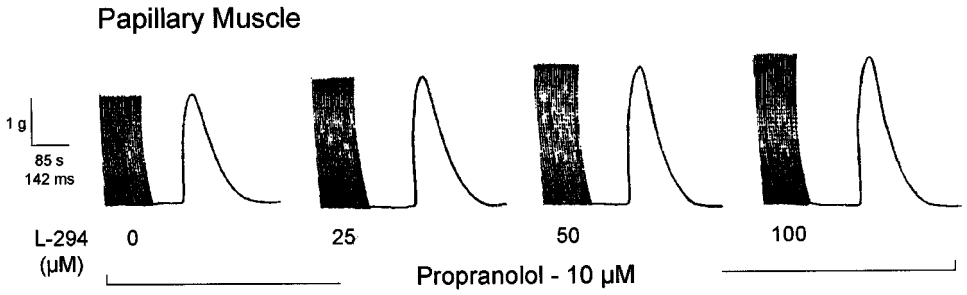


Figure 4 Positive inotropic effect of L-294 on papillary muscle from rat was not altered by pre-treatment with beta-adrenergic antagonist agent. Muscle twitches elicited by electric field stimulation at 1.0 Hz were recorded in the presence of propranolol (10 μM). L-294 was added at concentrations as indicated. The traces were recorded 5 min after exposure to L-294. Same results were observed in six experiments.

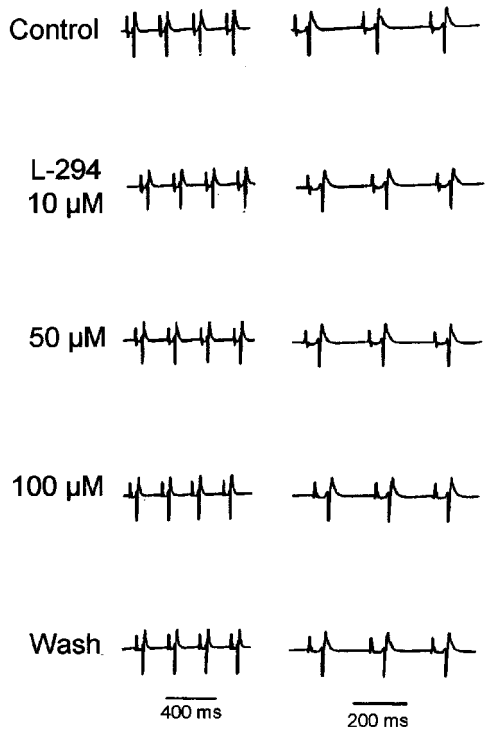


Figure 5 Electrocardiograph tracings of isolated hearts before and after retrograde perfusion with Tyrode solution containing 10, 50 and 100 μM L-294.

failure syndromes. To demonstrate the effect of L-294 on the contractility of cardiac muscle, we analysed isometric tension of isolated whole hearts in a modified Langendorff preparation and contractility of isolated cardiac tissues (papillary muscle and fascicles of atrial and ventricular muscles). In spontaneously beating hearts, after 2 min of perfusion, L-294 caused sustained increase in heart contractility with no change in the resting tension. This action was immediately reversed after washout suggesting a rapid diffusion and on/off binding of L-294. The positive inotropic effect of L-294 was also observed in stimulated atrial, papillary and ventricular muscles. The greater effect of L-294 on isolated tissues compared to the intact heart could be explained by the establishment of optimal length-tension only in the tissues. The greater efficacy of L-294 in ventricular than atrial muscle can be related to a specific intracellular action of L-294, in which the source of Ca^{2+} for contraction is less dependent on Ca^{2+} released from SR, in this tissue (Mill *et al*., 1992). The relative selective effect on the ventricular muscle suggests that the mechanism of L-294 is related to an effect on intracellular Ca^{2+} handling. This greater inotropic effect of L-294 on the left ventricle can be hemodynamically important due to the relevant function of that muscle in generating systolic blood flow. There are two major groups of inotropic drugs: (1) Those that increase the intracellular Ca^{2+} concentration (Fleischer & Inui, 1989): digitalis-like compounds (Kelly & Smith, 1996), β-adrenergic agonists (Weber *et al*., 1982; Roubin *et al*., 1984) and type III phosphodiesterase inhibitors (Weishaar *et al*., 1985); (2) Those that increase the sensitivity of contractile proteins to Ca^{2+} (Scholz, 1984); carnosine (Zaloga *et al*., 1997) and levosimendan, presently in clinical phase testing (Toivonen *et al*., 2000). Our results show that within the range of concentrations used in this study the cardiac effect of L-294 is not mediated *via* mechanisms similar to the inotropic agents described above. Lack of changes in the EKG parameters of isolated hearts such as rhythm, heart rate, PR interval and QRS duration suggests that L-294 does not alter the gating of ionic channels present in the sarcolemma. The contribution of β receptor activation to the efficacy of L-294 was eliminated because the mean increase in the peak isometric tension of isolated cardiac tissue previously treated with 10 μM propranolol was not significantly different from that observed in non-treated muscles. A Ca^{2+}-sensitizing effect of L-294 on contractile proteins was excluded by the pCa-tension data from Triton X-100 treated skinned ventricular fibres. The inotropic effect of L-294 may be related to an increase of intracellular Ca^{2+} mobilization as a consequence of increased Ca^{2+} uptake into SR and/or release of Ca^{2+} from SR through the ryanodine receptor calcium release channels (RyR2).

We hypothesize that the mechanism of action of L-294 is due to a change in the Ca-loading process into SR as demonstrated in the skinned fibre preparation. Incremental concentrations of L-294 were added to pCa 6.6 (2.57×10^{-7} M) during the SR loading time (3 min) and the amplitude of tension in response to 20 mM caffeine was evaluated. In the control (absence of L-294), the caffeine-induced tension was about 60% of Po. Therefore, caffeine response increased by 17 and 25% when 10 and 100 μM L-294 was present in the loading solution. In addition to this, a significant increase in the amount of Ca^{2+} available for release was observed when L-294 was present during all

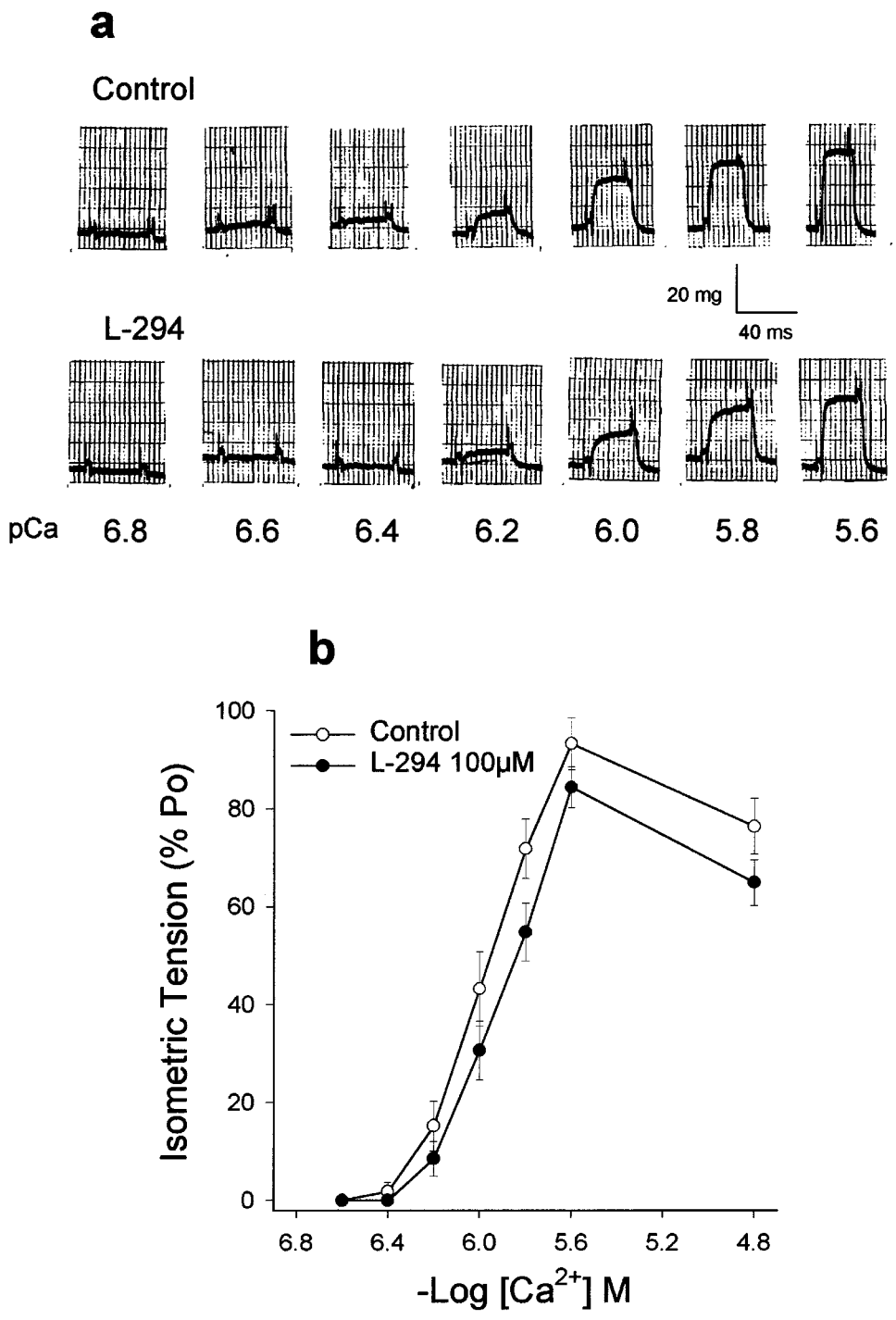


Figure 6 Effect of L-294 on the calcium sensitivity of contractile proteins in chemically skinned rat ventricular myocardium. (a) Isometric force tracings from left ventricular muscle fibres after treatment with Triton X-100 to disrupt the SR membrane function. The skinned fibres were exposed to washing solution containing increasing Ca^{2+} concentrations (pCa 6.8–5.6). (b) Tension-Ca^{2+} concentration relationships were determined in skinned ventricular muscle fibres in the absence or presence of 100 μM L-294. Isometric tensions are relative to maximal response at 0.5 mM $CaCl_2$2 (Po) and are expressed as mean$\pm$s.e.mean of 10 experiments.

tested loading times (15 s to 8 min). The tension developed in fibres exposed to 20 mM caffeine and pre-loaded with or without L-294 (100 μM) for 1 and 8 min was 74.11$\pm$4.12 and 94.71$\pm$2.20% or 48.33$\pm$3.89 and 77.22$\pm$2.78% of Po. Further more, parallel left shift was observed with the dose versus caffeine-induced tension curve when fibres were loaded with Ca^{2+} and L-294. Oscillations induced by low concentrations of caffeine were observed in 60% of our experiments probably as a consequence of an increase of Ca-induced-Ca release process. Trafford *et al*. (2000) described previously that caffeine at concentration $>$0.5 mM increased the frequency and decreased the amplitude of spontaneous release of Ca^{2+} from SR through the ryanodine channels (Ry2). Those authors measured the intracellular Ca^{2+}

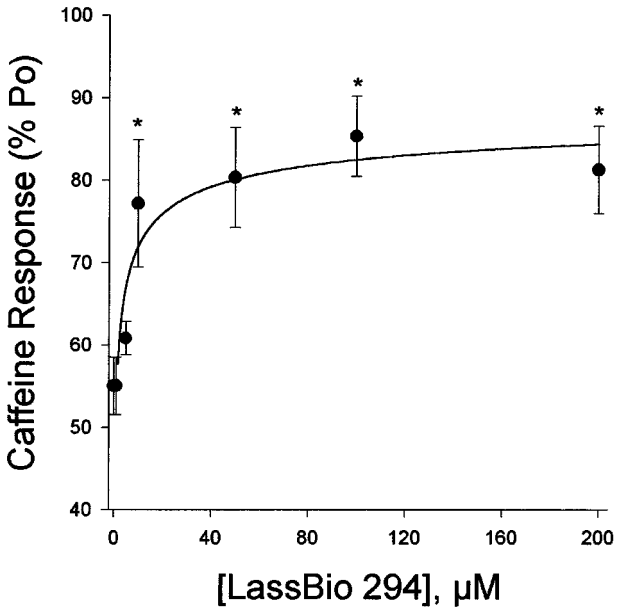
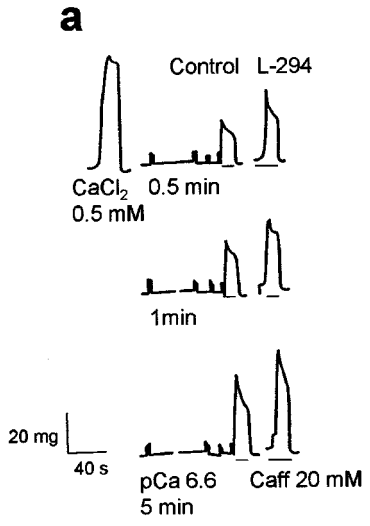


Figure 7 Effect of L-294 on the caffeine-induced contraction in skinned ventricular muscle fibres. The skinned fibres were incubated in a solution of pCa 6.6 for 3 min in the absence or presence of L-294 for Ca^{2+} loading into the SR. Then, the skinned fibres were exposed to 20 mM caffeine to develop tension. Data represent the mean of 10 experiments. *Significantly different compared to control ($P<0.05$).



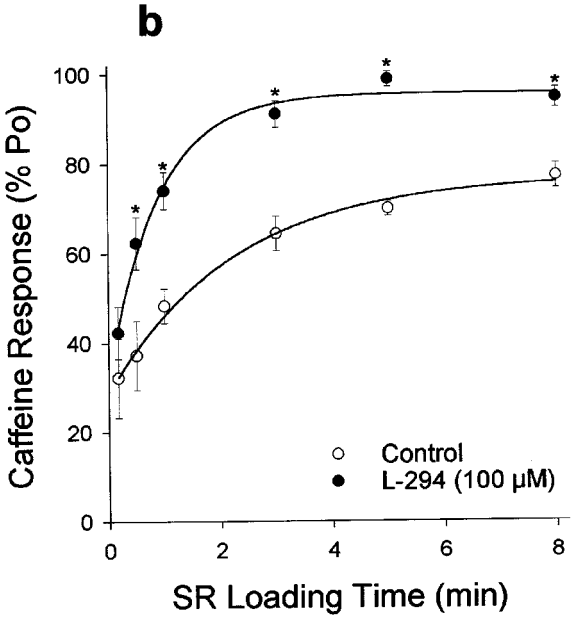


Figure 8 Time dependent Ca^{2+} loading into the SR in the absence or presence of L-294 (100 μM). (a) Representative tracing showing initially the maximal response (Po) of skinned fibres to 0.5 mM $CaCl_2$2 and then, release of stored Ca^{2+} induced by caffeine (20 mM) after SR preload for different periods of time (0.5, 1.0 and 5.0 min) with pCa 6.6 without or with L-294. (b) Amplitudes of caffeine-induced tensions in skinned fibres plotted as function of the loading time in the absence or presence of 100 μM L-294. Contracture amplitudes are expressed as percentage of Po and show mean ± s.e.mean ($n=10$). *Significantly different from control ($P<0.05$).

concentration in isolated rat ventricular myocytes using Fluo-3 or Indo-1 and suggested that caffeine could modulate the Ry2 from ventricular cells. Our results suggest that L-294 induced caffeine to produce oscillations when present during SR loading. We can not describe that L-294 interferes directly with caffeine as L-294 was not present during caffeine contracture (after SR pre-load). Thus, if caffeine-induced oscillations are related to a magnification of Ca-induced-Ca release, L-294 is not directly affecting this process. It is possible that the modification of the Ca^{2+} concentration into the SR can alter the Ca-induced-Ca release process. Further experiments need to be done before we can speculate on these phenomena. All the results suggest an increase in the content of Ca^{2+} accumulated in the SR induced by L-294. As Ca^{2+} accumulation is the net difference between Ca^{2+} uptake into and release from SR, the effect of L-294 could also be explained by an additional action in altering the sensitivity of the SR Ca^{2+} release channel, RyR2, to Ca^{2+} or caffeine. We discarded this hypothesis because L-294 was not able to induce Ca^{2+} release from SR in skinned fibres pre-loaded with Ca^{2+}. Also, it did not change the amplitude of the caffeine-induced tension when added together with caffeine. Although L-294 produced a shift to the left in the caffeine concentration *versus* tension curve, this was probably not due to an effect of L-294 to sensitize RyR2 to caffeine. The reason for this is that, unless L-294 binds tightly to RyR2, if at all, it would not be present when caffeine was added because L-294 was washed out after SR loading in its presence. Consequently, L-294 may be acting on the Ca pump to increase the amount of calcium loaded. There are several interesting implications regarding the new drug, L-294, related to its effect on Ca-uptake from SR. Twitch amplitude developed by cardiac muscle, as occurs with skeletal muscle, depends on a cycling process between Ca^{2+} release and Ca^{2+} uptake from SR. The important difference between both muscles is the time course of this cycling process; being slower in cardiac muscle. In cardiac muscle, due to a limited rate in Ca^{2+} uptake, the twitch amplitude can be decreased by shortening the time between two stimuli or when the muscle is exposed to drugs such as caffeine that cause Ca^{2+} release from SR. L-294 can alter this cycling process by increasing the amount of Ca^{2+} available for contraction and reducing the diastolic Ca^{2+} concentration.

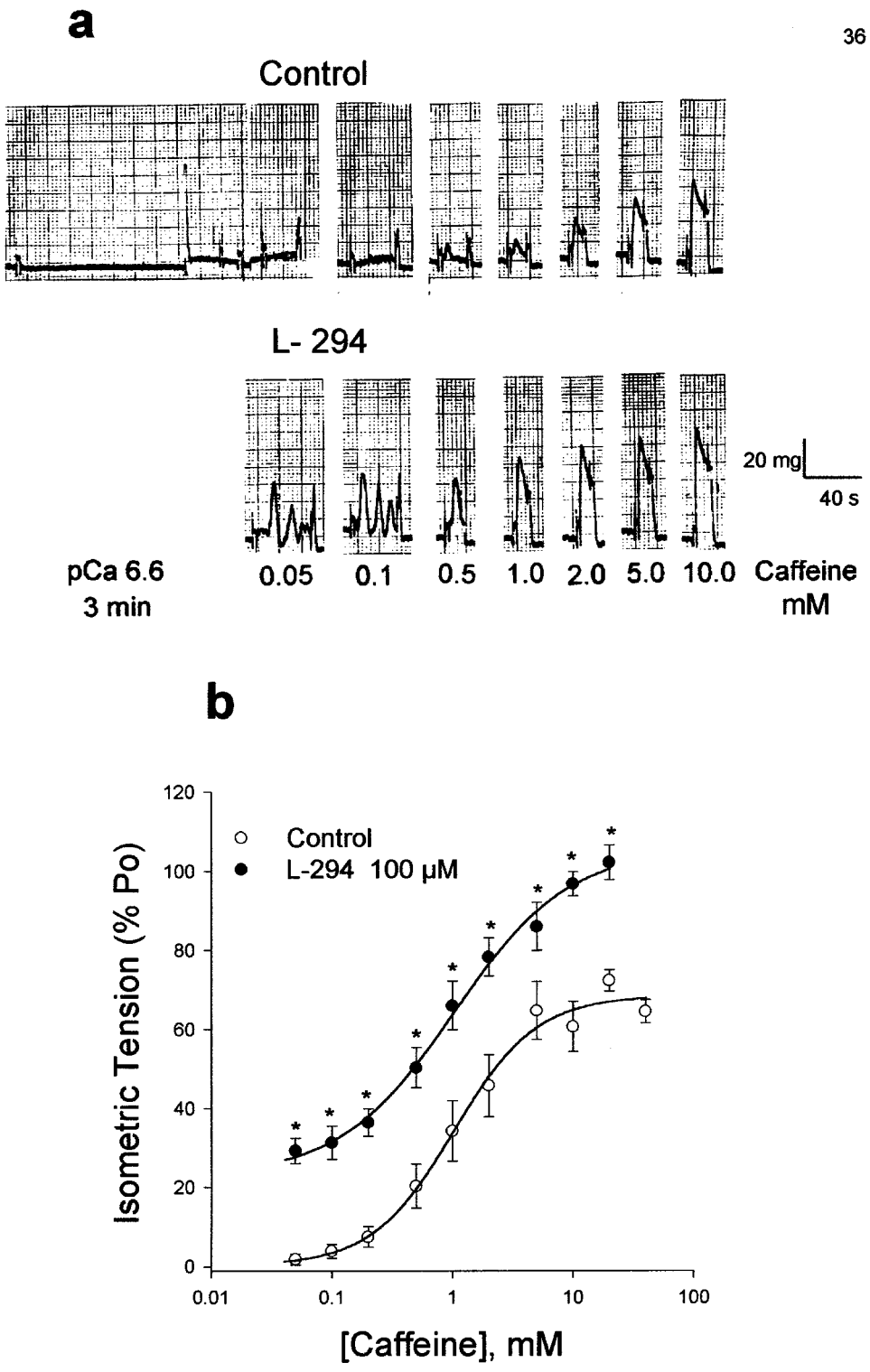


Figure 9 Effect of L-294 on the caffeine dose-response curve in rat cardiac saponin-skinned fibres. (a) Caffeine-induced contractures after SR preload with solution of pCa 6.6 for 3 min in the absence and presence of L-294 (100 μM). (b) Caffeine dose-response curves for fibres in the absence and presence of L-294 as indicated. Isometric tensions are related to the maximal activated response to $CaCl_2$ (0.5 mM - Po). $n=9$ experiments for each concentration except for 5 and 20 mM, $n=18$. *Significantly different from control ($P<0.05$).

In conclusion, new therapeutic strategies are necessary to resolve the mechanical dysfunction associated with reduced cardiac contractile performance. Accordingly, the development of a novel potent cardiotonic agent such as L-294, could have beneficial effect on the left ventricular dysfunction.

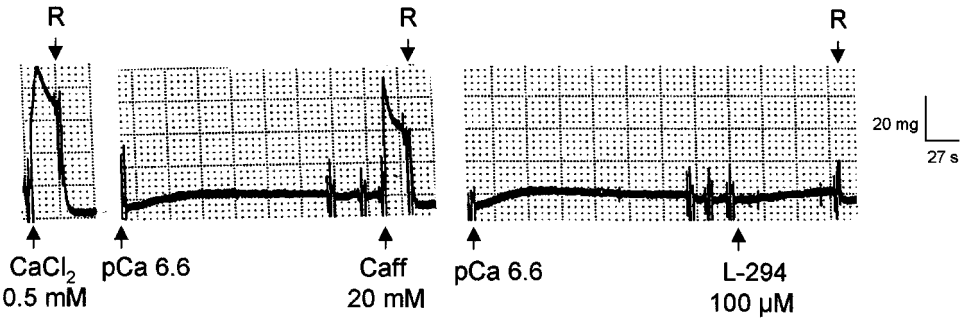


Figure 10 L-294 did not induce tension in ventricular skinned cells from Wistar rats. Maximal tension (Po) was induced by exposing the saponin-treated ventricular cells to $CaCl_2$ (0.5 mM). Solution R containing high concentration of EGTA was used to produce relaxation of the fibres. After SR loading with Ca^{2+} during 3 min, caffeine induced contracture was 85% of Po. Same SR loading procedure was repeated before exposing the fibres to L-294 (100 μM). Lack of contractile response was observed in the presence of L-294. Same result was observed in five experiments.

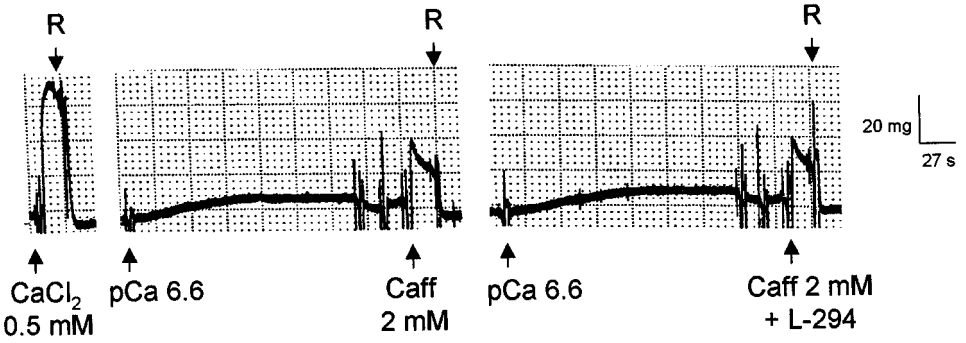


Figure 11 L-294 (100 μM) did not increase the caffeine-induced contracture in ventricular skinned cells. Maximal isometric tension (Po) was induced by exposing the saponin-treated ventricular cells to 0.5 mM $CaCl_2$. Solution R containing high concentration of EGTA was used to produce relaxation of the fibres. The SR of the fibres were loaded with Ca^{2+} by exposure to pCa 6.6 during 3 min. After two wash cycles with solution W, tension was induced by caffeine (2 mM). Note that the presence of L-294 (100 μM) did not alter the amplitude caffeine-induced contracture.

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