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A novel steroid-like compound F90927 exerting positive-inotropic effects in cardiac muscle

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1 Here we report a novel steroid-like compound F90363, exhibiting positive inotropy *in vivo* and *in vitro* in various cardiac muscle preparations.

2 F90363 is a racemic mixture composed of the stereoisomers (−)-F90926 and (+)-F90927. Only F90927 exerted positive inotropy, while F90926 induced a weak negative inotropy, but only at concentrations 10^3 times higher than F90927 and most likely resulting from an unspecific interaction.

3 The rapid time course of the action of F90927 suggested a direct interaction with a cellular target rather than a genomic alteration. We could identify the L-type Ca^{2+} current $I_{Ca(L)}$ as a main target of F90927, while excluding other components of cardiac Ca^{2+} signalling as potential contributors. In addition, several other signaling pathways known to lead to positive inotropy (e.g. α- and β-adrenergic stimulation, cAMP pathways) could be excluded as targets of F90927.

4 However, vessel contraction and stiffening of the cardiac muscle at high doses ($>30\,\mu$M, 0.36 mg kg^{-1}, respectively) prevent the use of F90927 as a candidate for drug development. Since the compound may still find valuable applications in research, the aim of the present study was to identify the cellular target and the mechanism of inotropy of F90927.
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Abbreviations: CVR, coronary vascular resistance; DAP, diastolic arterial pressure; LVDP, left ventricular diastolic pressure; LVP, left ventricular pressure; MAP, mean arterial pressure; NCX, Na^+–Ca^{2+} exchanger; RyR2, ryanodine receptor type 2; SAP, systolic arterial pressure; SERCA, sarcoendoplasmic Ca^{2+} ATPase; SR, sarcoplasmic reticulum

Introduction

Congestive heart failure is one of the most frequent and potentially fatal heart diseases. The pathophysiology of heart failure is multifaceted, but in some cases there is an initial weakening of the cardiac function, which has been suggested to be due to a disruption of the excitation–contraction (EC) coupling on the cellular level, leading to a decreased EC-coupling gain (Gomez *et al*., 2001). It is well established that cardiomyocytes undergo extensive cellular remodelling during early phases of cardiac dysfunction, which can be interpreted as a cellular adaptation to compensate for the loss of cardiac function (Ito *et al*., 2000; Sjaastad *et al*., 2003). Possible strategies to overcome this deficiency are to increase Ca^{2+} cycling during each action potential (AP), by stimulation of the L-type Ca^{2+}current (increased Ca^{2+} influx), by increasing the release of Ca^{2+} from the sarcoplasmic reticulum (SR) *via* the Ca^{2+} release channels (ryanodine receptor type 2 (RyR2)) or by increasing the Ca^{2+} sensitivity of myofilaments. Additionally, there is a Ca^{2+} pathway modulated by the intracellular Na^+ concentration *via* the Na^+–Ca^{2+} exchanger (NCX), which can both import and remove Ca^{2+} (Weisser-Thomas *et al*., 2003). All strategies increasing SR Ca^{2+} content are expected to lead to an increase in EC-coupling gain, which would ideally compensate for the deficient cardiac function. As a complication, compounds elevating SR Ca^{2+} load may exhibit an inherent arrhythmogenicity. Unfortunately, until today only few substances, such as cardiac glycosides, are in clinical use to increase cardiac muscle force, and it would be desirable to develop substances acting similarly but without serious side effects.

It is also well established that hormones and hormone-like substances can influence cardiac function (Bers, 2002). Examples are oestrogen, which was found to be an inhibitor of the L-type Ca^{2+} current ($I_{Ca(L)}$) (Meyer *et al*., 1998), or heterosides, like ouabain, which are blockers of the Na^+, K^+-ATPase (Schatzmann, 1965; Schoner, 2002). Yet other steroids, such as veratridine, have been found to act as Na^+-channel openers (Sutro, 1986).

Here we report the discovery and initial characterization of a novel, steroid-like compound exerting positive inotropic effects in cardiac muscle by unknown mechanisms. The goal of the present study was to examine the positive inotropy *in vivo* as well as *in vitro* and to identify the underlying mechanism.

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Methods

Animals

Male New-Zealand White rabbits (for *in vivo* measurements; 2.2–2.7 kg, Elevage des Dombes, Romans, Chatillon sur Chalaronne, France), male Sprague–Dawley rats (for isolated atria and isolated rings from the aorta and saphenous vein; 400–450 g, OFA, Iffa-Credo, France), female guinea-pigs (for Langendorff measurements; 500–600 g, SPF, Hartley, Charles River, France) and male guinea-pigs (for intracellular Ca^{2+} measurements; 450–600 g, Schneider, Bern, Switzerland) were housed at $20\pm3^{\circ}C$ with $55\pm10\%$ humidity, 12 h light/dark cycle, and had free access to species-specific food and tap water. All experiments were carried out according to the guidelines of the Swiss Animal Protection Law or French Law and with the permission of The State Veterinary Office, Bern, Switzerland or in agreement with the local French Ethical Committee guidelines.

Cardiac function measurements in vivo *(ECG, various blood pressures)*

Left ventricular pressure (LVP) was measured from male New-Zealand White rabbits using established protocols (Verscheure *et al*., 1995). In brief, animals were anaesthetized by i.v. injection of sodium pentobarbital (25 mg kg^{-1}). A polyethylene fluid-filled catheter was introduced into the left ventricle *via* the carotid artery to obtain LVP and its first derivative, or into the left femoral artery for blood pressure measurements (systolic and diastolic arterial pressure (SAP and DAP)). The mean arterial pressure (MAP) was calculated as MAP = DAP + (SAP−DAP)/3. A four-limb electrocardiogram (ECG) was recorded in lead II for determination of heart rate (R–R interval) (Verscheure *et al*., 1995). Arrhythmic events were defined as stated with the Lambeth convention (Walker *et al*., 1988).

Measurements of resting membrane potential in guinea-pig papillary muscle

Hearts were rapidly excised from euthanized animals and immersed in an oxygenated modified Krebs solution of the following composition (in mM): 113.1 NaCl, 4.6 KCl, 1.2 $MgCl_2$, 2.45 $CaCl_2$, 3.5 NaH_2PO_4, 21.9 $NaHCO_3$, 5 D(+)- glucose (pH 7.4). Papillary muscles <1 mm in diameter were dissected from the right ventricle of the hearts. The preparations were transferred to a tissue bath maintained at $36\pm0.5^{\circ}C$ and superfused at a rate of 4–6 ml min^{-1} with a modified Krebs solution continuously gassed with 95% O_2 and 5% CO_2.

The papillary muscles were electrically stimulated (Model 6BP, FHC, Pulsar, Brunswick, ME, U.S.A.) with rectangular pulses of 1 ms duration and 1.5 times the threshold voltage through a bipolar Ag electrode. The preparations were allowed to equilibrate for at least 1 h at a stimulation rate of 1 Hz. Membrane resting potential was recorded from cells on the surface by conventional glass microelectrodes (5–20 MΩ) filled with 3 M KCl and coupled to a high-input impedance preamplifier (VF 102 Biologic, Echirolles, France). Membrane potential was simultaneously digitized (20 kHz) and analysed (Venturis FP 466, Digital, Maynor, MA, U.S.A.) using interactive computer software.

After a 1 h equilibration period, control values of resting membrane potential were recorded. Each concentration of every drug tested was applied for 20 min (Le Grand *et al*., 1995a).

Recording of spontaneous Ca^{2+} oscillations in isolated rat myocytes

Single cardiomyocytes were isolated from rat hearts by enzymatic digestion techniques using a Ca^{2+}-free perfusion solution containing (in mM): 117 NaCl, 5.7 KCl, 4.4 $NaHCO_3$, 1.5 KH_2PO_4, 1.7 $MgCl_2$, 21 HEPES, 11.7 glucose and 20 taurine, pH 7.2 (NaOH) (Mitra & Morad, 1985). For cytosolic Ca^{2+} ($[Ca^{2+}]_i$) measurements, cells were kept in a solution containing (in mM): 140 NaCl, 0.5 $MgCl_2$, 5 KCl, 5.5 glucose, 5 HEPES and 1.8 $CaCl_2$, pH 7.4 (NaOH). Quiescent, rod-shaped, Ca^{2+}-tolerant myocytes were loaded with fluo-3 in the presence of fluo-3 AM (5 μM, 20 min). Fluo-3 fluorescence was excited by a xenon lamp at 460–490 nm and emission was detected at 520 nm with a photomultiplier tube. Cells were field stimulated at 1 Hz and oscillations were identified by spontaneous elevations of $[Ca^{2+}]_i$ in between field stimulations.

Cardiac function measurements on Langendorff mounted hearts

Cardiac function measurements using guinea-pig hearts were performed as described previously (Le Grand *et al*., 1995b). In brief, hearts excised from guinea-pigs were placed in cold (4°C) modified Krebs medium containing (in mM): 124.6 NaCl, 4 KCl, 1.1 $MgSO_4$, 0.3 NaH_2PO_4, 1.8 $CaCl_2$, 24.9 $NaHCO_3$, and 11.1 D(+)-glucose, pH 7.4, continuously gassed with 95% O_2 + 5% CO_2 and mounted on a Langendorff system. A latex balloon was inserted through the left atrium and mitral valve into the left ventricle. Iso-volumetric systolic and diastolic LVP, left ventricular developed pressure (LVDP = systolic-diastolic pressure), left ventricular end diastolic pressure, heart rate (HR), positive $dP\,dt^{-1}_{max}$, negative $dP\,dt^{-1}_{max}$ and coronary flow (CF) were measured at $37\pm1^{\circ}C$ with a constant perfusion pressure of 80 cm H_2O.

Contractile force measurements in isolated atria

Force measurements of rat atria were performed using established protocols (Letienne *et al*., 2001). In brief, left atria were isolated from male Wistar rats and mounted vertically, while avoiding tissue stretch, in an organ bath filled with Krebs solution containing (in mM): 119 NaCl, 5.6 KCl, 1.17 $MgSO_4$, 2.1 $CaCl_2$, 1.0 NaH_2PO_4, 25.0 $NaHCO_3$, 10.0 glucose, pH 7.4, gassed with a mixture of 95% O_2 + 5% CO_2 and maintained at 34°C. The atria were stimulated electrically with 4 Hz (impulse duration 1 ms, two-fold threshold current) *via* two electrodes (Campden, Stimulator 915, Phymep, Paris, France). After 30 min of equilibration, a single concentration of the tested drug was injected into the organ bath, and the maximal increase of isometric contractions was measured.

Measurements of vessel contraction (thoracic aorta and saphenous vein)

Measurements of vessel contraction were performed as described previously (Valentin *et al*., 1996). Briefly, thoracic aortae were isolated from male Sprague–Dawley rats and saphenous veins from New-Zealand white rabbits. Vessels were placed in Krebs solution containing (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, D(+)-glucose 10 (pH: 7.4), gassed with 95% O_2 + 5% CO_2 and cut in segments (rings) of 3–5 mm in length. Each ring was then suspended between two stainless steel wire hooks and mounted in an organ bath with constant flow of bath solution kept at 37°C. The tissue was maintained under 2 g basal tone and allowed to stabilize for at least 40 min before experimentation.

Single-cell patch clamp in whole-cell configuration and confocal Ca^{2+} imaging

Cardiac myocytes were isolated from male adult guinea-pig hearts using established protocols (Keller *et al*., 2004). Rapid changes of the extracellular solution containing (in mM): 140 NaCl, 5 KCl, 10 glucose, 1 $CaCl_2$, 1 CsCl, 0.5 $BaCl_2$, 10 HEPES, pH 7.4 (NaOH) were carried out with a gravity-driven superfusion system ($t_{1/2} < 500$ ms). All experiments were performed at room temperature (20–22°C). Currents were recorded in the whole-cell configuration of the patch-clamp technique using an Axopatch-200B voltage-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.) driven by a data acquisition board and commanded by software developed under LabView (both from National Instruments, Austin, TX, U.S.A.). Filamented borosilicate glass capillaries (G150F-3, Warner Instruments Inc., Hamden, CT, U.S.A.) were used to pull micropipettes on a horizontal puller (DMZ, Zeitz Instrumente, Augsburg, Germany). The final tip diameter was 1–2 μm and resistance was 1–2.5 MΩ with the following pipette solution (in mM): 120 CsAsp, 20 HEPES, 20 TEA-Cl, 5 K_2-ATP, 2 GSH, 0.5 $CaCl_2$, 0.05 fluo-3 $5K^+$, 2 DM-nitrophen-$4Na^+$, pH 7.2 (CsOH). Recorded membrane potentials were not corrected for the junctional potential calculated to be ≈12 mV for this pipette solution. Current measurements were digitized with 5–10 kHz. For the measurements of L-type Ca^{2+} current ($I_{Ca(L)}$), Na^+ current ($I_{Na(V)}$) was inactivated by a pre-pulse from −80 to −50 mV. Data were analysed using Igor Pro software (WaveMetrics, Lake Oswego, OR, U.S.A.). Peak current amplitudes were determined after baseline correction from the mean of a 0.5-ms interval around the most negative data point. Confocal Ca^{2+} imaging was performed as described previously (Niggli & Lederer, 1993; Keller *et al*., 2004).

Chemicals

Stock solutions of (−)-F90926, (+)-F90927 and F90363 were made in dimethyl sulphoxide (DMSO) and used within 2 days. Final solutions never contained more than 0.1% DMSO.

Statistical analysis

Data are presented as means ± s.e.m. Inter-group comparisons were analysed by one-way analysis of variance (ANOVA) followed by the Dunnett *post hoc* test. $P \leqslant 0.05$ was considered significant.

Results

Chemistry of F90363, (−)-F90926 and (+)-F90927

The structure of F90363 contains four stereogenic centres. (−)-F90926 and (+)-F90927 are pure enantiomers, the relative configuration of which is shown in Figure 1c. F90363 was prepared by total synthesis and differs from all natural steroids

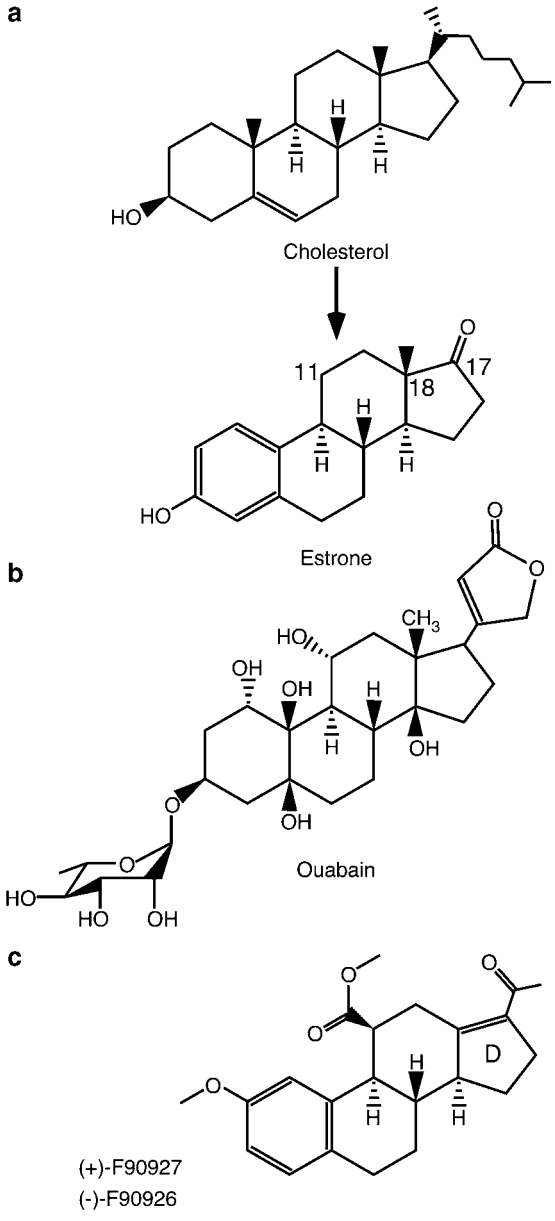

Figure 1 Structure of (−)-F90926 and (+)-F90927. (a) Structural similarities among two known hormones, with a 4 C-ring (3 × C_6, 1 × C_5) basic structure. (b) Structure of ouabain. (c) Structure of (+)-F90927 and (−)-F90926. Being that only the absolute configuration is known, it is just possible to state that (+)-F90927 is the active and (−)-F90926 the inactive enantiomer.

and sterols by the absence of the β-methyl group at C-18. Its closest natural relative would be estrone (Figure 1a) although further structural divergences do exist (i.e. hydroxymethyl function in position 2; a β-methoxycarbonyl group at C-11; a β-methyl ketone at C-17). The rationale for testing the impact of F90363 and related molecules in the cardiovascular domain stems from the presence of an activated double bond in the vicinity of the D ring, which is a hallmark of cardiac glycosides (e.g. ouabain, Figure 1b). Both F90926 and F90927 exhibited no affinity for steroid hormone receptors ($IC_{50} > 10\,\mu$mol, data not shown).

Positive inotropic effects of F90363

The first series of experiments with the racemic mixture F90363 were performed in order to confirm the positive inotropy and to determine the potency of the compound. A marked positive inotropy of F90363 administered intravenously was observed in an anaesthetized rabbit (Figure 2). Doses up to $0.04\,mg\,kg^{-1}$ F90363 only moderately affected the blood pressure, but $0.16\,mg\,kg^{-1}$ and higher lead to a clear increase in both SAP and LVP. Interestingly, with $0.63\,mg\,kg^{-1}$ F90363, and even more prominent with $2.5\,mg\,kg^{-1}$, the LVP did not return to the baseline during diastole, suggesting incomplete relaxation and an increase in resting tension of the cardiac muscle. Furthermore, at $2.5\,mg\,kg^{-1}$ F90363 the LVP exhibited a pronounced variability arising from arrhythmic contractions (also visible in the SAP recording, but less pronounced).

For this reason F90363 was compared with the cardiac steroid ouabain, which also has positive inotropic and arrhythmogenic actions, mediated by inhibition of the Na^+,K^+-ATPase (Schatzmann, 1965; Schoner, 2002). Figure 3 shows examples of simultaneous recordings of the electrocardiogram (ECG) and LVP from a rabbit. Similar to $0.04\,mg\,kg^{-1}$ ouabain, $0.63\,mg\,kg^{-1}$ F90363 also increased systolic LVP (SLVP) (Figure 3B, inset b). Increasing the doses

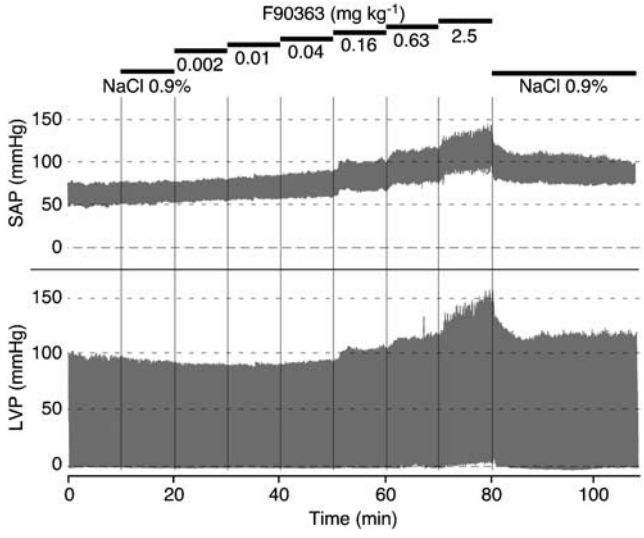

Figure 2 *In vivo* measurements of SAP and LVP recorded from an anaesthetized rabbit during i.v. application of increasing doses of F90363 for 10 min each. Depending on the dose, both parameters increased reversibly, suggesting positive inotropy.

of each substance four-fold completely changed the picture (Figure 3A and B, inset c): While $0.16\,mg\,kg^{-1}$ ouabain was fatal for the animals, $2.5\,mg\,kg^{-1}$ F90363 caused serious arrhythmias without being fatal (Figure 3C, inset d). Analysis of the pooled results of $n = 4$–6 animals (Figure 3C) showed a similar dose–response curve for F90363 and ouabain on SLVP, although F90363 doses were required to be 10 times higher than with ouabain to obtain a corresponding increase in SLVP (Figure 3C, inset a). In contrast, low doses of F90363 and ouabain both elevated MAP to a similar extent (Figure 3C, inset b), despite the more pronounced elevation of SLVP induced by ouabain. Figure 3C, inset c offers an explanation for this observation: Application of ouabain resulted in the appearance of arrhythmias (observed in the ECG). Furthermore, F90363 did not induce a depolarization of the resting membrane potential in guinea-pig papillary muscle (control: -90.9 ± 0.5 mV, 10 μM F90363: -89.2 ± 2 mV; $n = 6$; $P = 0.18$), as could be observed when inhibiting Na^+,K^+-ATPase (e.g. by digoxin: control: -91.4 ± 0.6 mV; 10 μM digoxin: -66.4 ± 5.3 mV; $n = 6$; $P = 0.0065$). Taken together, the different pharmacological profiles for cardiac steroids and F90363 suggested a target other than the Na^+,K^+-ATPase for the new compound, despite their structural similarities.

It is well established that the cardiac function can be modulated in various ways by α- and β-adrenergic signaling pathways (Barki-Harrington *et al*., 2004), notably that stimulation of either pathway can have a positive inotropic effect. Therefore, F90363 was tested in combination with either an α-adrenergic receptor antagonist (prazosine, 1 μM) or a β-adrenergic receptor antagonist (propranolol, 1 μM) (Figure 4a). Figure 4a shows that none of the two receptor antagonists significantly inhibited the positive inotropy of F90363 on the tension of isolated atria ($n = 8$).

Since F90363 is known to be a racemic mixture, the purified stereoisomers named (+)-F90927 and (−)-F90926 (see also Figure 1c) were separately tested on isolated rat atria (Figure 4b). As it turned out, F90927 is the active enantiomer responsible for the gain in function described above, whereas F90926 barely changed the developed tension, except a slight decrease at very high concentrations above 10 μM. This observation was also paralleled by a corresponding change of the EC_{50} (1.33 ± 0.1 μM for F90363 *versus* 0.76 ± 0.1 μM for F90927). Therefore, F90926 does not interfere antagonistically with F90927 and does not exhibit negative inotropy. Taken together, the purified enantiomer F90927 was identified as the active compound.

Characterization of the stereoisomer F90927

In order to determine the mechanism behind the F90927 inotropy, a series of experiments were performed on guinea-pig isolated hearts, mounted on a Langendorff apparatus. While perfusing the hearts with different concentrations of F90927 (10 nM–1 μM), the contraction velocity (expressed as $dP\,dt^{-1}_{max}$), the LVDP, the heart rate and the coronary flow were recorded (Figure 5). As expected, the contraction velocity increased in parallel with the concentration of F90927, but only up to 300 nM. Above this concentration the contraction velocity surprisingly started to decline (Figure 5a). In addition, the results shown in Figure 5a may suggest a biphasic effect, since there was an initial increase in $dP\,dt^{-1}_{max}$ from 10–30 nM, followed by a less prominent acceleration when increasing the

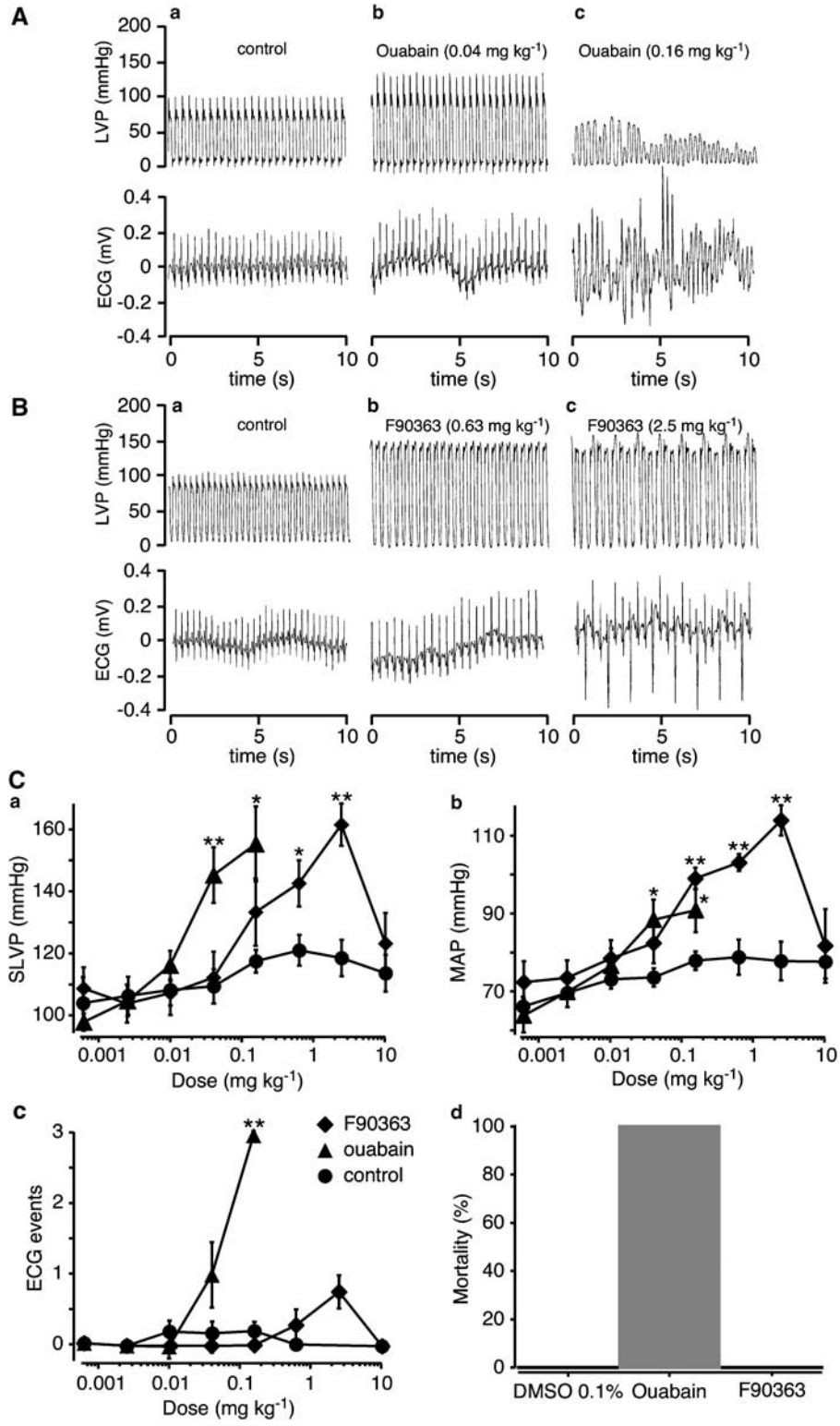

Figure 3 Simultaneous recordings of the ECG and LVP from anaesthetized rabbits. (A) Effect of ouabain (i.v. application), (a) control, (b) 0.04 mg kg^{-1} ouabain, (c) 0.16 mg kg^{-1} ouabain. While 0.04 mg kg^{-1} ouabain showed a positive inotropic effect, 0.16 mg kg^{-1} ouabain induced ventricular fibrillations. (B) Effect of F90363 (i.v. application), (a) control, (b) 0.63 mg kg^{-1} F90363, (c) 2.5 mg kg^{-1} F90363. F90363 exhibited positive inotropy; however, at higher concentrations (nonfatal) arrhythmias occurred, limiting the positive inotropy. (C) Changes of systolic LVP in the presence of F90363 (SLVP, a), MAP (b), ECG events (c) and mortality (d). Dose–response curves of F90363 ($n=4$), ouabain ($n=5$); DMSO 0.1% was used as control ($n=6$). (a) SLVP dose–response relationship, (b) MAP dose–response relationship, (c) Number of ECG events (i.e. arrhythmias) observed while applying the indicated concentrations, (d) mortality of F90363 (2.5 mg kg^{-1}, $n=4$), vehicle (DMSO 0.1%, $n=6$) and ouabain (0.16 mg kg^{-1}, $n=5$) in rabbits. Mean ± s.e.m., $*P<0.05$, $**P<0.01$; both *versus* vehicle.

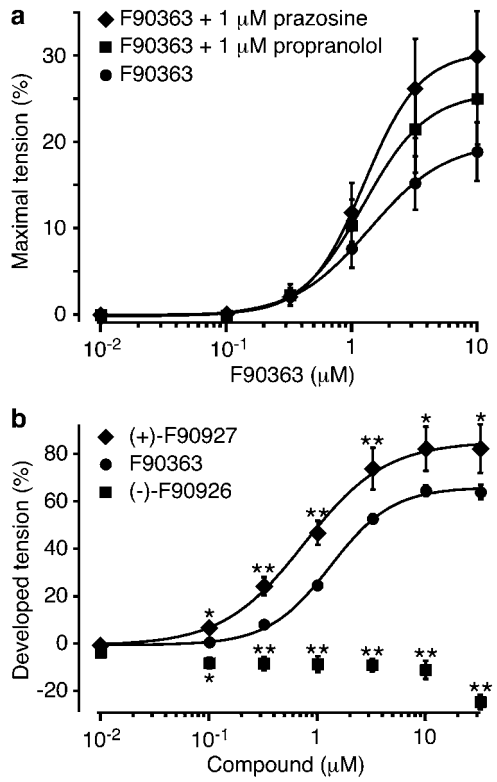

Figure 4 (a) Influence of α- and β-adrenergic antagonists on the positive inotropy of F90363 in paced, isolated rat atria. F90363 ($EC_{50}=1.39\pm8.9\,\mu$M, $n=8$), F90363 + 1 μM propranolol ($EC_{50}=1.25\pm1.1\,\mu$M, $n=8$) and F90363 + 1 μM prazosine ($EC_{50}=1.25\pm3.6\,\mu$M, $n=8$). Concomitant application of α- and β-adrenergic antagonists together with F90363 did not decrease the dose–response relationship of F90363. (b) Discrimination between the two enantiomers of F90363 ((−)-F90926 and (+)-F90927) in paced, isolated rat atria. F90363 ($EC_{50}=1.33\pm0.09\,\mu$M, $n=1.56$, $n=8$), F90927 ($EC_{50}=0.76\pm0.1\,\mu$M, $n=1.13$, $n=4$) and F90926 ($n=4$). F90927 is the enantiomer exerting the positive inotropy, whereas high concentrations of F90926 showed a slight reduction of force. Mean ± s.e.m., $^*P<0.05$, $^{**}P<0.01$; both *versus* F90363.

concentration of F90927 to 100 nM, and again a steeper increase when 300 nM F90927 was applied. At 1 μM F90927, where a cut-off in the increase of contraction velocity occurred, a stiffening of the cardiac muscle could be observed, which would counteract any further positive inotropy. This can also explain a similar cut-off in the elevation of LVDP at the same concentration (Figure 5b). Simultaneous measurements of the heart rate (Figure 5c) revealed no significant changes between 10 and 300 nM; only at the concentration of 1 μM an acceleration could be observed. The coronary flow also remained constant in the range from 10 to 300 nM (Figure 5d), but at 1 μM F90927 it was significantly reduced. Since the perfusion of the heart was carried out at a constant pressure, a reduced coronary flow indicates an elevated coronary vascular resistance (CVR), resulting from a reduced diameter of the coronary vessels. Thus, the reduction in coronary flow and the increase in heart rate could explain the bell-shaped concentration–response curve of F90927 for $dP\,dt^{-1}_{max}$ and LVDP.

Since secondary changes resulting from a stimulation of vessel contraction by F90927 could underlie the reduction of coronary flow, this possibility was examined by carrying out measurements of tension changes induced by F90927 in isolated rings of saphenous veins from rabbits and thoracic aorta from rats (Figure 6). Both vessel types contracted during the application of F90927, but a significant tension development was only noticed at concentrations above 3 μM. In saphenous veins tension did not decline at high concentrations of F90927, unlike the tension in the thoracic aorta and the LVDP in isolated guinea-pig hearts. Yet, vessels of different size and/or originating from different tissues might have different pharmacological sensitivities; thus, a contribution to the decrease of coronary flow by constriction of the resistance vessels at lower concentrations of F90927 cannot be completely excluded. Additionally, stiffening of the cardiac muscle can contribute to the compression of the resistance vessels and both mechanisms can thus account for the decrease in coronary flow (Figure 5d).

Potential mechanism of action

Further experiments were carried out in order to evaluate different mechanisms known to exhibit positive inotropy, such as PKA-mediated phosphorylation of a multitude of Ca^{2+} signalling proteins (Antos *et al*., 2001) or enhanced Ca^{2+} release from the SR (Suarez *et al*., 2004) and their role in the mechanism of action of F90927 (Figure 7). These experiments were carried out with electrically paced rat atria. To examine whether PKA-mediated phosphorylation was involved, atria were pre-incubated with 100 μM of the membrane permeant db-cAMP in order to fully activate PKA. As a control, 0.1 μM isoprenaline was applied to further activate PKA *via* β-adrenergic stimulation. When compared to untreated controls, atria pre-incubated with db-cAMP showed a reduced tension increment as a reaction to 0.1 μM isoprenaline, as expected (Figure 7a). However, when 10 μM F90927 was applied, the tension increment was not significantly different between control and db-cAMP pretreated atria, suggesting a different pathway for F90927. It is important to note, that the PKA activation by db-cAMP was apparently not complete, allowing for some residual β-adrenergic stimulation. Since the atria were not perfused, it is likely that not all cells in this thick preparation were exposed to the full concentration of 100 μM db-cAMP. Therefore, a small residual stimulation of the twitch force by isoprenaline was still visible despite the db-cAMP treatment. The same difficulty has to be expected for the experiments with thapsigargin pretreatment (see below).

Dependence of the positive inotropy on Ca^{2+} release from the SR was examined by pretreating the atria with 10 μM thapsigargin to block the Ca^{2+} uptake into the SR *via* the SR Ca^{2+} ATPase (SERCA). Subsequent application of 1 mM caffeine (Blayney *et al*., 1978) elicited a smaller increase in developed tension in pretreated atria compared to untreated controls (Figure 7b). However, when the atria of both groups were exposed to F90927, the observed increase in developed tension was still comparable, suggesting that a modulation of Ca^{2+} release from the SR is not needed for the action of F90927 (Blatter *et al*., 2003). Taken together, PKA-mediated phosphorylation as well as modulation of SR Ca^{2+} release could be excluded as mechanisms underlying the inotropy mediated by F90927.

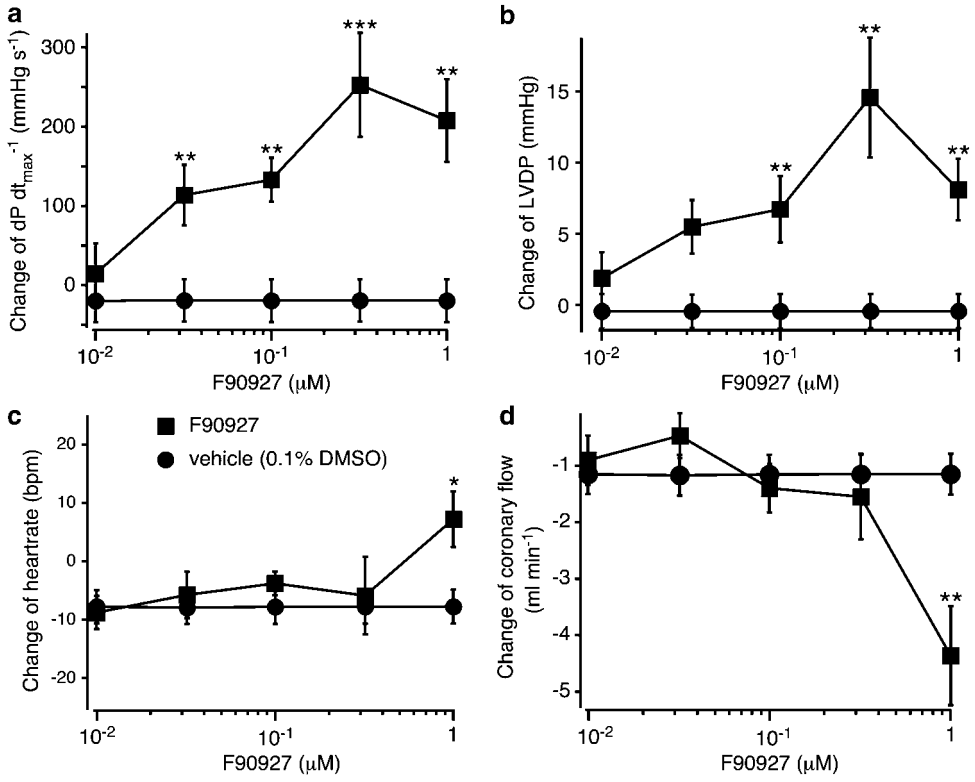

Figure 5 Application of F90927 on spontaneously beating, isolated guinea-pig hearts. F90927 ($n=5$–7) as well as the vehicle (0.1% DMSO, $n=7$) were added for at least 15 min. (a) Changes in contraction velocity, shown as $dP\,dt_{max}^{-1}$. (b) Changes of LVDP. (c) Changes in heart rate. (d) Changes in coronary flow. Mean±s.e.m., $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; all *versus* vehicle.

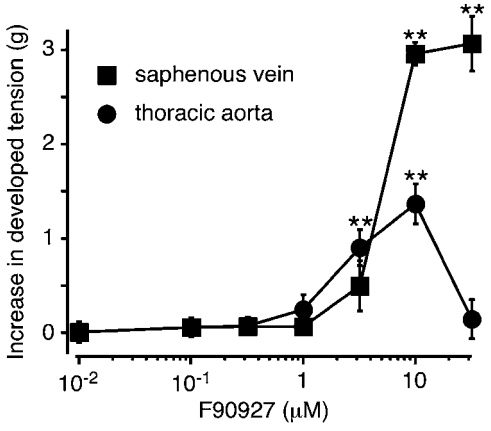

Figure 6 Vessel contraction in the presence of F90927 in the saphenous vein (endothelium-denuded; $n=8$) and the thoracic aorta ($n=4$) of male rats. The constant tension of 2 g on the vessel rings and the resting tension of the vessels were subtracted, revealing the increase in developed tension induced by F90927. Mean±s.e.m., $^{**}P<0.01$, both *versus* 0.

F90927 and intracellular Ca^{2+}

To confirm that the positive inotropy was caused by alterations in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) cycling, isolated rat ventricular myocytes were field stimulated (1 Hz) and $[Ca^{2+}]_i$ was recorded with the fluorescent Ca^{2+} indicator fluo-3 (Figure 8). An elevation of fluorescence and therefore also of the resting $[Ca^{2+}]_i$ became apparent upon application of F90927, preceding the occurrence of the spontaneous Ca^{2+} oscillations (Figure 8a). Furthermore, a correlation between increasing concentrations of F90927 and the number of cells with spontaneous Ca^{2+} oscillations (independent of electrical stimulation) was found (Figure 8b). These spontaneous oscillations may underlie the variability in systolic pressure at high concentrations of the compound.

Among a variety of possible pathways that could underlie the positive inotropy of F90927 on the cellular level, potential alterations of the cardiac sodium current ($I_{Na(V)}$) and the L-type Ca^{2+} current ($I_{Ca(L)}$) were studied with the patch-clamp technique in the whole-cell configuration. A protocol delivering a depolarizing voltage ramp was applied to initially activate $I_{Na(V)}$, followed by $I_{Ca(L)}$ (Figure 9a). The most prominent change in current amplitude coincided with $I_{Ca(L)}$. The current peak was larger and was shifted towards more negative potentials. Peak $I_{Na(V)}$ decreased only slightly in the presence of 10 μM F90927. The current recorded in the presence of Cd^{2+} was used to further discriminate between $I_{Na(V)}$ and $I_{Ca(L)}$, because $I_{Ca(L)}$ is much more sensitive towards Cd^{2+} than $I_{Na(V)}$ (DelPrincipe *et al*., 2000). Overall we measured an increase in peak $I_{Ca(L)}$ in the presence of 10 nM F90927 by 75.8±15.9% ($n=11$) and with 10 μM F90927 by 190.4±15.6% ($n=14$; both mean±s.e.m., $P<0.05$).

To confirm the stimulation of $I_{Ca(L)}$, and to assess the resulting consequences on Ca^{2+} transients, both signals were recorded simultaneously on a laser-scanning confocal microscope (Figure 9b). Both, 1 and 10 μM F90927 increased $I_{Ca(L)}$ and the Ca^{2+} transient (130±2 and 160±6%, respectively, $n=4$) in a concentration-dependent manner.

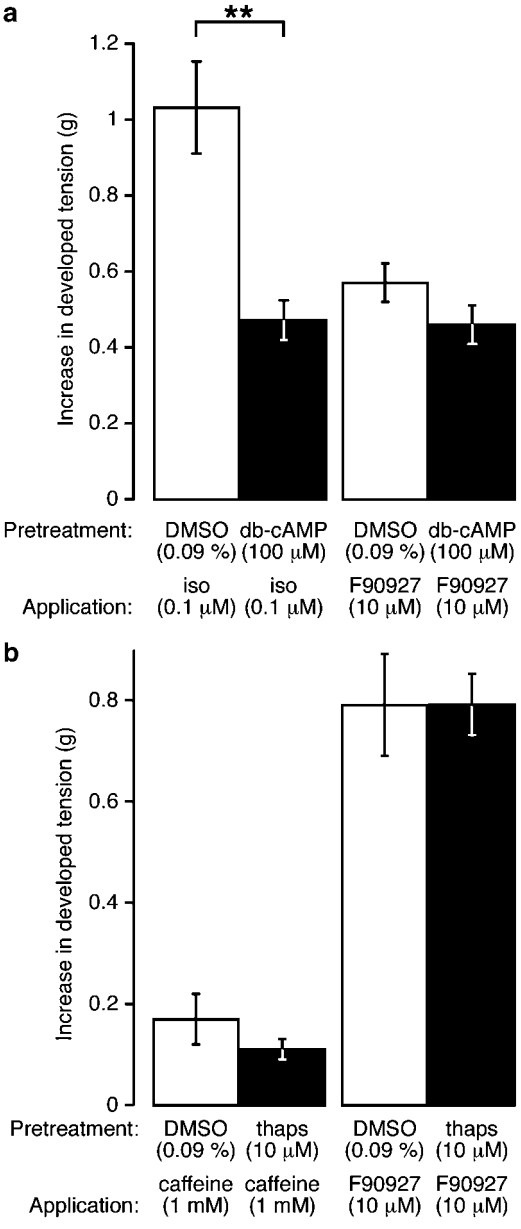

Figure 7 Twitch (4 Hz) tension recordings in isolated rat atria. (a) Atria were pretreated for 15 min with either 0.09% DMSO (empty bars) or 100 μM db-cAMP (filled bars) to activate PKA. The increase in developed tension after application of either 0.1 μM isoprenaline (empty bar: 0.09% DMSO, 1.03 ± 0.12 g, $n=6$; filled bar: 100 μM db-cAMP, 0.48 ± 0.05 g, $n=6$) or 10 μM F90927 (empty bar: 0.09% DMSO, 0.57 ± 0.05 g, $n=6$; filled bar: 100 μM db-cAMP, 0.46 ± 0.05 g, $n=6$) was measured. (b) Atria pretreated for 15 min with either 0.09% DMSO (empty bars) or 10 μM thapsigargin (thaps, filled bars) to block the SERCA. The increase in developed tension induced by either 1 mM caffeine (empty bar: 0.09% DMSO, 0.17 ± 0.05 g, $n=6$; filled bar: 10 μM thapsigargin, 0.11 ± 0.02 g, $n=6$) or 10 μM F90927 (empty bar: 0.09% DMSO, 0.79 ± 0.1 g, $n=6$; filled bar: 10 μM thapsigargin, 0.79 ± 0.06 g, $n=6$) was measured. Mean $\pm$ s.e.m., $^{**}P<0.01$ *versus* DMSO.

Taken together, our data provide evidence that the positive inotropy caused by F90927 is mainly due to stimulation of the L-type Ca^{2+} current; thus F90927 appears to be a Ca^{2+}-channel agonist with a steroid-like structure.

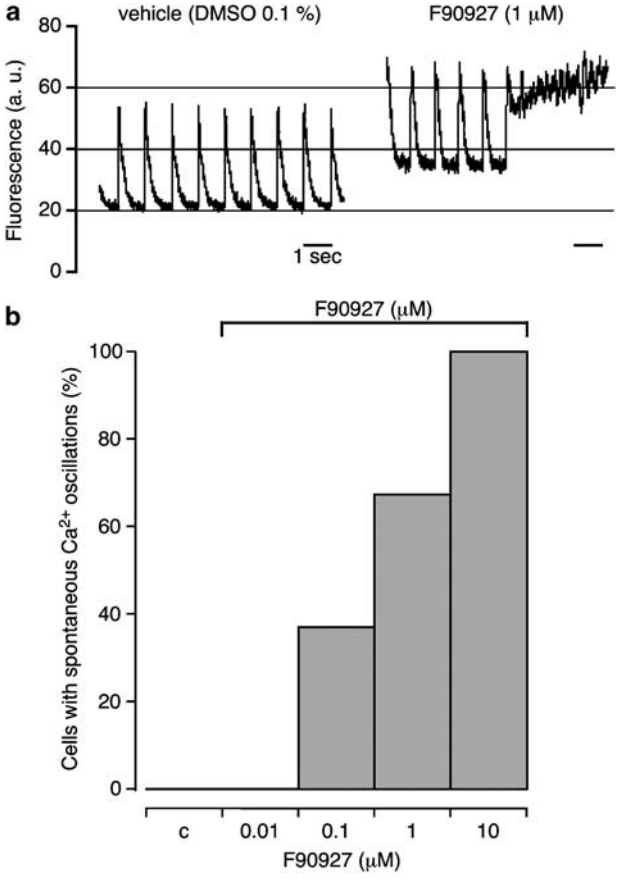

Figure 8 Influence of F90927 on $[Ca^{2+}]_i$. (a) Recordings of $[Ca^{2+}]_i$ transients in electrically stimulated (1 Hz) isolated rat ventricular myocytes, loaded with fluo-3. Left: vehicle (0.1% DMSO); right: 1 μM F90927 (data obtained from the same cell). The right panel shows elevated resting $[Ca^{2+}]_i$ and spontaneous Ca^{2+} oscillations. (b) A correlation was found between the number of cells with spontaneous Ca^{2+} oscillations and increasing concentrations of F90927.

Discussion

Steroid hormones exert multiple biological actions *via* activation of intracellular receptors (belonging to the thyroid hormone receptor super family) and *via* modulation of gene transcription (Truss & Beato, 1993). In addition, steroids are known to exert nongenomic actions. As a general pattern, it appeared that these are prevalent in tissues where the genomic action is less prominent, that is, the cardiovascular and central nervous systems (for a review, see Simoncini & Genazzani, 2003). These actions are also highly specific for a particular chemical structure and any structural modification of a hormone can completely change the picture.

Among several synthesized compounds, F90927 was found to potently affect various parameters of the cardiovascular system. The rapid time course observed in the present study suggested a nongenomic signalling pathway of F90363 and F90927 that could involve L-type Ca^{2+} channels.

Cardiac inotropy

Experiments with F90363 were performed in order to quantify the positive inotropy and to determine the potency of this

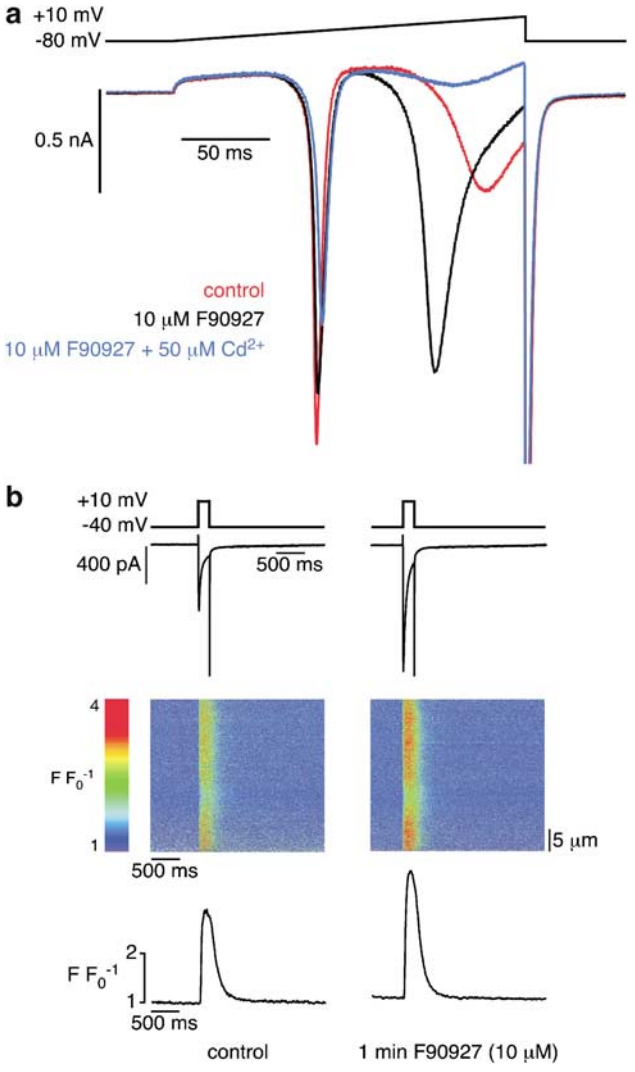

Figure 9 Identification of the ion current stimulated by F90927. (a) Recordings during a ramp protocol in guinea-pig ventricular myocytes voltage clamped in the whole-cell configuration. Ramp: −80–+10 mV in 200 ms. Red trace: control; black trace: after 1 min superfusion with 10 μM F90927; blue trace: after 1 min superfusion with 10 μM F90927 plus 50 μM Cd^{2+}. An increase in peak $I_{Ca(L)}$ of 75.8±15.9% (for 10 nM F90927, $n=11$) and of 190.4±15.6% (10 μM F90927, $n=14$) was observed. (b) Ca^{2+} transients elicited by $I_{Ca(L)}$. Top to bottom: voltage protocol, current recordings, normalized line scan recordings of fluo-3 fluorescence, averaged fluorescence corresponding to the line-scans above (normalized to initial fluorescent value of the control recording; F F_0^{-1} of the right trace is initially 1.1). Overall increases of 130±2% (with 1 mM F90927) and 160±6% (with 10 mM F90927) in the Ca^{2+} transient was observed (both $n=4$). Mean±s.e.m.

compound. Later it was found that (+)-F90927 is the active stereoisomer of the racemic mixture F90363, while the inactive isomer (−)-F90926 exhibited no positive and just a weak negative inotropic effect. In addition, F90926 did not antagonize the positive inotropy of F90927. Intravenous injection of F90363 increased both SAP and LVP in a dose-dependent manner that was readily reversible. The observed effect of F90927 on vessel tension probably contributed to the increase in DAP, since it was more pronounced than the increase in diastolic left-ventricular pressure.

Owing to its positive inotropy F90363 was compared to the cardiotonic steroid ouabain, which stimulates cardiac force generation by inhibiting the Na^+,K^+-ATPase (Glitsch, 2001; Schoner, 2002). This comparison revealed that F90363 was much less arrhythmogenic at comparable positive inotropic concentrations. With F90363, only doses above 1 mg kg^{-1} lead to a few extrasystolic ECG events and no lethality was observed, even with very high doses of F90363. In addition, despite the marked positive inotropic effect, no depolarization of the resting membrane potential was noted in guinea-pig papillary muscle, contrary to what has been observed with Na^+,K^+-ATPase inhibitors (Gadsby, 1985; Glitsch, 2001), leading to the conclusion that F90363 does not inhibit the Na^+,K^+-ATPase. Furthermore, the *in vivo* experiments showed a bradycardia when F90363 was applied, which was not observed with the ouabain experiments (visible in the example shown in Figure 3A/B; upon application of ouabain the heart rate remains at 150 b.p.m. while upon application of F90363 the heart rate decreases from 180 b.p.m. in control to less than 150 b.p.m.). In contrast, the Langendorff experiments showed no change in heart rate when applying F90927, except for the highest concentration (see also '*Side effects*'). Since the spontaneously beating heart on the Langedorff apparatus lacks cardiovascular nervous feedback (e.g. baroreceptors and blood pressure regulation), these differing findings could be explained by the absence of blood-pressure regulation in the Langendorff preparation. Based on the pharmacological profile, it seemed reasonable to suggest an alternative mechanism by which F90363 enhances cardiac function.

It is well established that cardiac function and contractile force can be modulated by the α- and β-adrenergic signalling pathways (Barki-Harrington *et al*., 2004). In order to test for this possibility, F90363 was applied in combination with either an α-adrenergic receptor antagonist (prazosine) or a β-adrenergic receptor antagonist (propranolol). Since in the presence of these antagonists no decrease in the dose–response curve was observed, F90363 apparently does not interact with α- and β-adrenergic pathways.

Mechanisms of action

In principle, the regulation of myocardial contractility by cardiotonic agents can be accomplished by three distinct basic mechanisms : (1) a modification of spatio-temporal features of the $[Ca^{2+}]_i$ signal; (2) an enhanced Ca^{2+}-binding affinity of troponin-C and (3) facilitation of the process subsequent to Ca^{2+} binding to troponin-C (for a review, see Endoh, 2002). As examples, cardiac glycosides, sympathomimetics and phosphodiesterase inhibitors increase the $[Ca^{2+}]_i$ signal. Ca^{2+} sensitizer compounds increase myocardial contractility without increasing the energy requirement for Ca^{2+} cycling. Both sympathomimetics and phosphodiesterase inhibitors act *via* increases of intracellular cAMP to activate PKA-dependent phosphorylation of various proteins important in Ca^{2+} signalling.

Even though in our experiments with intact tissue a complete PKA stimulation by db-cAMP was presumably not achieved, a significant blunting of the isoproterenol response was observed after pretreatment with db-cAMP. In contrast, the positive inotropy induced by F90927 was not affected by the db-cAMP pretreatment. Taken together, changes of

PKA-mediated phosphorylation are very unlikely to cause the inotropy of F90927.

Similarly, pretreatment of atria with the SR-Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin partly antagonized the caffeine-induced contractions. However, the increase in developed tension produced by application of F90927 was not modified by thapsigargin pretreatment, suggesting that F90927 pathway does not involve a direct interaction with intracellular Ca^{2+} release from the SR.

Another possible mechanism for the positive inotropy mediated by F90927 would be an alteration of the cellular Ca^{2+} homeostasis. Early evidence for this possibility was found when larger Ca^{2+} transients were observed in electrically paced isolated rat myocytes. Also, the appearance of spontaneous $[Ca^{2+}]_i$ oscillations correlated strongly with increasing concentrations of F90927. Subsequently, several Ca^{2+} signalling pathways that could potentially augment the Ca^{2+} transients by a mechanism independent of SR function were examined. On the level of ionic membrane currents, $I_{Na(V)}$ showed a small reduction, whereas the L-type Ca^{2+} current ($I_{Ca(L)}$) was strongly stimulated. In addition, the voltage dependence of the peak $I_{Ca(L)}$ was shifted towards more negative potentials. Experiments in combination with laser-scanning confocal microscopy of the Ca^{2+} indicator fluo-3 revealed that the larger $I_{Ca(L)}$ was in fact resulting in a more robust Ca^{2+} transient, which would consequently lead to elevated contractility. Taken together, our results strongly suggest that the main target of F90927 is the L-type Ca^{2+} channel.

Arrhythmias: F90927 versus *ouabain*

The apparent discrepancy between the spontaneous Ca^{2+} transients induced by F90927 (Figure 8) which do not induce fatal arrhythmias and the high mortality due to arrhythmias observed with ouabain can be accounted for by the different mechanisms of both drugs. While ouabain inhibits the Na^+,K^+-ATPase and thus changes the $[Na^+]_i$ and $[K^+]_i$ homeostasis, causing a depolarization of the membrane potential (Glitsch, 2001; Schoner, 2002), F90927 mainly increases Ca^{2+} influx by increasing peak $I_{Ca(L)}$ without affecting the membrane potential.

The depolarized membrane potential in the presence of ouabain may make the myocytes more susceptible to delayed afterdepolarizations (DADs) (de Groot *et al*., 2000). Therefore, cells exhibiting spontaneous oscillations may trigger arrhythmias in the whole myocardium, provided the number of synchronously active cells is sufficiently large.

Side effects

In addition to the positive inotropic response, two side effects were noted, both of which would preclude further development of F90927 as a drug candidate for clinical applications. At high concentrations (10 μM) a significant reduction of coronary blood flow occurred, indicating a considerable increase of CVR. This could result either from resistance vessel compression due to incomplete relaxation and stiffening of the cardiac muscle or from resistance vessel contraction. The concentration range where vessel contraction by F90927 was prominent was only slightly different from that which led to reduced coronary flow (1 μM for the decrease in coronary flow *versus* 3 μM for the rise in large vessel tension). Considering that other vessels (e.g. resistance vessels in the heart) might be more sensitive than the vessels investigated in this study, we cannot exclude a contribution of resistance vessel contraction to the increase in CVR. Therefore, both mechanisms will probably contribute to the decrease in coronary flow. Furthermore, the cardiac muscle stiffening itself acts like a basal contraction and during diastole the heart does not relax completely. The incomplete relaxation ultimately leads to reduced ventricular filling and to a decline in the cardiac performance.

Taken together, this study revealed that F90927, a novel synthetic steroid-like compound, is a cardioactive drug exerting positive inotropy. Positive inotropy in the presence of F90927 is mediated by its agonistic effect on the L-type Ca^{2+} current, most likely by a direct pharmacological effect on the Ca^{2+}-channel proteins.

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