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Antimony-induced cardiomyopathy in guinea-pig and protection by L-carnitine

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1 Antimony (Sb) is the mainstay for the treatment of Leishmaniasis. It has serious, often lethal, cardiovascular side effects. The objective of this study was to examine the effects of Sb treatment upon the electrocardiogram (ECG), myocyte contractility (assessed by monitoring sarcomere length during field stimulation), whole-cell action potential (AP) and calcium current (I_{Ca}) of the guinea-pig and to evaluate L-carnitine as a cardioprotective agent.

2 Guinea-pigs received daily injections of either saline, Sb(V), Sb(III), L-carnitine or L-carnitine with Sb(III). Eight lead ECGs were recorded under halothane anaesthesia every 4 days. At the end of each treatment regime, animals were killed and ventricular myocytes were enzymatically isolated.

3 Treatment with Sb(V) for 26 days prolonged the QT interval of the ECG. Treatment with Sb(III) was lethal within 2 days for $\sim$50% of the animals. The survivors showed ECG alterations similar to those described in man: T wave flattening and/or inversion, depression of the ST segment, and elongation of RR and QT intervals. Their ventricular myocytes showed impaired contraction responses to changes in stimulus frequency, elongated AP and reduced I_{Ca}.

4 Combined treatment with L-carnitine and Sb(III) delayed mortality. Prior treatment with L-carnitine followed by combined treatment with L-carnitine and Sb(III) reduced mortality to $<$10% over 12 days and these animals showed normal ECG. Their myocytes showed normal contractility and AP.

5 It is concluded that L-carnitine has a preventive cardioprotective role against antimony-induced cardiomyopathy. The mechanism of action of L-carnitine may be to counter oxidative stress caused by Sb(III).

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Keywords: Sb(III); electrocardiogram (ECG); isolated ventricular myocytes; sarcomere length; contraction; cardioprotection

Abbreviations: AP, action potential; I_{Ca}, L-type calcium current; SL, sarcomere length

Introduction

Leishmaniasis is a parasitic disease, which afflicts 12 million people worldwide. Symptomatic visceral (kala-azar) and cutaneous and muco-cutaneous leishmaniasis cause 2 million new cases each year and is often fatal if not treated. However, no vaccine is yet available (Handman, 2001; UNDP-World Bank-WHO Special Programme for Research and Training in Tropical Diseases) and the mainstay of treatment of leishmaniasis remains the pentavalent antimony compound (Sundar, 2001; Davis *et al*., 2004) Sb(V), despite the development of increasing resistance. The current view is that Sb(V) is a prodrug that generates the more effective trivalent Sb(III) (Roberts *et al*., 1995; Sereno *et al*., 1998; Shaked-Mishan *et al*., 2001) whose leishmanicide activity involves interactions with thiol groups (Sun *et al*., 2000; Frezard *et al*., 2001) in mammals and in parasite using low molecular thiols and/or antimony reductase (Ferreira *et al*., 2003; Yan *et al*., 2003; Zhou *et al*., 2004).

Sb(III) was originally the primary agent for the treatment of leishmaniasis and schistosomiasis. However, Lu & Liu (1963) estimated that 70–90% of deaths resulting from antimony therapy were due to cardiac intoxication and therefore treatment switched to Sb(V) to reduce the cardiovascular risk. Sb(V) is better tolerated but has been repeatedly reported to have threatening effects in human close to those described for Sb(III): hypotension, modifications of the electrocardiogram (ECG) including T wave flattening and/or inversion and prolonged corrected QT interval eventually leading to ST segment changes and P, R and T wave amplitude reductions, torsade de pointes and sudden death (Chulay *et al*., 1985; Hepburn *et al*., 1994; Ortega-Canicer *et al*., 1997; Takur *et al*., 1998; Ribeiro *et al*., 1999). These effects are related to the dose and duration of the treatment. It would thus be desirable to get some insight in these threatening effects of Sb since leishmania resistance to antimony could be overcome by increasing doses of Sb (Sereno *et al*., 1998).

There has been little analysis of the effects of antimony in experimental models. Bradley & Frederick (1941) showed that all forms of Sb evoked degeneration of the rat myocardium, heart failure and death. In the dog, Cotton & Logan (1966) reported that chronic (3–5 daily) injections of Sb(III) evoked a

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tenting of the T wave, a depression of the ST segment, hypotension and sudden death but no effect on contraction in contrast with the depression of cardiac contractility observed by Bromberger-Barnea & Stephens (1965) in the same species and the reported T wave flattening in man. Depressed contractility was also inferred from calcium transient measurements in cultured rat myocytes (Toraason *et al*., 1997; Wey *et al*., 1997). The cardiac effects of Sb(III) could be related to an oxidative stress producing lipid peroxidation, glutathione (GSH) decrease, inhibition of glutathione peroxidase, significant alterations in cellular thiol homeostasis and lactate deshydrogenase (LDH) release (Tirmenstein *et al*., 1995; 1997). On the contrary, Snawder *et al*. (1999) showed increased GSH levels as well as a decrease in cytochrome P450 and an induction of heat–shock stress proteins (HSP70 and HSP25/27) upon exposure to nonlethal doses of Sb(III).

Reports on the human ECG give a coherent descriptive picture of the chronic effects of antimony *in vivo*, whereas little is known in experimental models. The objective of this study was thus to examine the effects of chronic injections of antimony upon the *in vivo* ECG of the guinea-pig and to assess cellular effects of antimony *ex vivo* as revealed by contraction and electrical activity on isolated cardiomyocytes from these animals. The effect of L-carnitine, a cofactor for beta-oxidation of long chain fatty acids, which has been successfully used in many cardiomyopathies including those involving oxidative stress (Strauss *et al*., 1998; Lango *et al*., 2001; Pauly & Pepine, 2003), was also tested with the aim of preventing antimony-induced cardiomyopathy.

Methods

Animals and experimental protocols

Studies were performed on male Dunkin–Hartley guinea-pigs 150–200 g (St Antoine, Pleudaniel, France) housed in the University standard animal care facilities (agreement: B37-261-4). They were fed on standard diet *ad libitum* and observed three times a day. The experimental procedures were performed in accordance with European guidelines (Authorizations: 7741 (COM), 37–045 (FG), 7320 (JML)) and with the Regional Centre-Limousin ethical committee for animal experimentation. Guinea-pigs were randomly assigned to experimental groups.

In the first series of experiments, the test group was injected (i.m.) with 16 mg kg^{-1} Sb(V) (meglumine antimonyl, Glucantime®; sligthly less than the 20 mg kg^{-1} used in man) daily for 26 days while the control group was injected with saline (9‰ NaCl).

In the second series of experiments, animals were injected (i.m.) daily with either saline (9‰ NaCl), 180 mg kg^{-1} L-carnitine and/or 10 mg kg^{-1} Sb(III) (potassium antimonyl tartrate). These doses were based upon values given by Strauss *et al*. (1998) and Cotton & Logan (1966). In a first test group, Sb(III) was injected for 8 days. In a second test group, L-carnitine was injected for 12 days. In a third test group, L-carnitine was injected together with Sb(III) for 8 days. In the last test group, L-carnitine was injected for 4 days prior to combined injections of Sb(III) plus L-carnitine for either 6 or 12 days. At the end of each treatment regime, surviving animals were killed and myocytes were isolated for either mechanical or electrical recording experiments.

In vivo *ECG recording*

ECGs were recorded under halothane anaesthesia induced with 3% halothane. The animal was then placed in a supine position in a Faraday cage with a mask to stabilise the anaesthesia (halothane 2.5%). Electrodes of thin microneedles were implanted subcutaneously in the forearms and the left leg. Two other thin subcutaneous needles were implanted close to the sternum and on the left of the chest. These needles were connected *via* coaxial cables to the input of two differential Isodam amplifiers (WPI. Inc.). The signal was amplified and filtered (bandwidth: 1 Hz–10 kHz) and continuously monitored on a Gould oscilloscope (20 MHz; type 1421) and on a Dash IV (Astro-Med) chart recorder that also allowed for analog–digital conversion (sampling frequency: 5 kHz). The six classical leads (D1, D2, D3, aVR, aVF, aVL) were recorded together with the precordial leads V1 and V6. The amplitude and duration of ECG waves were manually measured from digitalised recordings with Origin 6.0 software (Microcal Inc.). QT interval values were corrected for the heart rate to give QTc with Bazett ($QTcb = QT/(RR)^{1/2}$) and Fridericia formulae ($QTcf = QT/(RR)^{1/3}$) in which RR is taken in seconds to take into account inaccuracy at high and low heart rates (Molnar *et al*., 1996). The ECG was collected before the first injection (saline or drug) and every 4 days after the beginning of treatment.

Drugs and reagents

L-carnitine and protease XIV were purchased from Sigma (Saint Quentin Fallavier, France). Potassium antimonyl tartrate and Collagenase B were, respectively, purchased from Aldrich Chemical Co. (Saint Quentin Fallavier, France) and Boehringer-Roche (Meylan, France). Halothane (Belamont, Neuilly-sur-Seine, France) and glucantime® (Rhone Poulenc-Rorer, France) were obtained from the regional hospital. For *in vivo* injections, potassium antimonyl tartrate and L-carnitine were dissolved in sodium chloride solution (9‰).

Tyrode solution had the following composition (in mM): NaCl, 140; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1; glucose, 11; NaH_2PO_4, 0.33; HEPES, 10. pH was ajusted to 7.3 with NaOH.

Myocyte isolation

At the end of each treatment regime, guinea-pigs were killed by cervical dislocation. Ventricular myocytes from the left ventricle were enzymatically isolated as previously described (Le Guennec *et al*., 1993). Briefly, the heart was perfused retrogradely for 5 min at 37°C with Tyrode solution gassed with 100% O_2. Perfusion was switched to a 0-Ca^{2+} solution and followed by a solution containing enzymes (protease 12 mg ml^{-1} plus collagenase 0.66 mg ml^{-1}). Enzymes were washed out and Ca^{2+} concentration of the perfusate was progressively increased to 1.8 mM. The minced ventricle was then gently agitated to separate cells. Percentage of rod-shaped cell after isolation, determined by counting the ventricular cells on a Mallassez chamber, usually represented 75–80% of total cell count in control or saline-injected guinea-pigs.

Sarcomere length measurements

Dissociated cells were placed in an orientable perfusion chamber on the stage of an inverted microscope (Nikon, Diaphot 300). Cells were illuminated with white light. Transmitted light was directed to two CCD (charges coupled device) cameras. The circular perfusion chamber had a central point symmetry, which allowed one to bring the cell longitudinal axis into alignment with that of the CCD cameras. Cells were continuously perfused by gravity with Tyrode solution equilibrated with air at 25°C. Cells were field stimulated with 5 ms duration constant current pulses (25% above threshold) delivered from two platinum wires at a reference frequency of 0.5 Hz. The frequency could be changed at will to obtain sarcomere shortening–frequency relationships.

Contraction was monitored by following the sarcomere length (SL) as described by Gannier *et al*. (1993). Briefly, the periodicity of the cell striation pattern (A and I bands) was analysed with a Fast Fourier Transform (FFT) of a video image of the cell during the course of the experiment. A longitudinal fraction of the cell image was recorded with a commercial CCD camera (Micam VS 500, Digital Vision Technology), digitised at 50 Hz (temporal resolution 20 ms), then transiently stored on a computer (Pentium 75 or 486 DX2 66) and used to calculate the spectrum corresponding to the distribution of the SLs. The spectrum fundamental, continuously detected by a software routine, enabled on-line visualization of SL during cell contraction. To improve time resolution to 2 ms for fast events in some experiments, SL was also calculated by an FFT of a video image of the cell recorded at 500 Hz with a nonstandard 218×145 pixel array two stage CCD camera (Gannier *et al*., 1998) set in parallel with the first camera. Cells were selected for experiments on the basis of a diastolic SL of between 1.84 and 2.0 μm during stimulation at 0.5 Hz and clearcut spectrum. Off-line measurement of SL, contraction and relaxation rates and contraction duration were performed with a computer program written to accommodate Origin 6.0 software.

Electrophysiological recordings

Dissociated cells were placed on the stage of an inverted microscope (Olympus IX70, Tokyo Japan) and continuously superfused by gravity with Tyrode at room temperature (22–25°C). Patch pipettes (1–2.5 MΩ) were pulled from thick wall borosilicate glass capillaries (Clark Electromedical Instruments, Reading, U.K.) with a Narishige PB7 puller (Tokyo, Japan). The composition of the pipette solution was (in mM): KCl, 140; NaCl, 5; ATP-Mg, 5; HEPES, 10; pH 7.25 (adjusted with KOH). An Axopatch 200A amplifier (Axon Instruments Inc., Union City, CA, U.S.A.), connected to a Pentium 75 computer equipped with pClamp 6.0.4. software (Axon Instruments), through a Digidata 1200A interface (Axon Instruments), was used to record voltage and calcium current (I_{Ca}). Action potentials (APs) and I_{Ca} were acquired with Clampex at 10 and 6.7 kHz, respectively, and analysed with Clampfit 6.0.5.4. Cells were stimulated at 0.2 Hz by applying a 1 ms suprathreshold current (1–2 nA) to record AP and at 0.1 Hz for I_{Ca}. To record I_{Ca} in normal Tyrode solution, a 100 ms prepulse applied from −80 to −40 mV aimed to inactivate the sodium current was followed by 150 ms depolarising pulses to voltages −40 to +100 mV (10 mV steps) during which I_{Ca} was measured as the net peak inward or outward current. For this study, small cells were chosen to improve control of the voltage clamp. AP plateau amplitude was measured 200 ms after the AP upstroke, and duration at 100% repolarisation. Owing to the relative instability of AP duration in isolated cells, AP parameters in each cell was taken as the mean values of 15 consecutive APs.

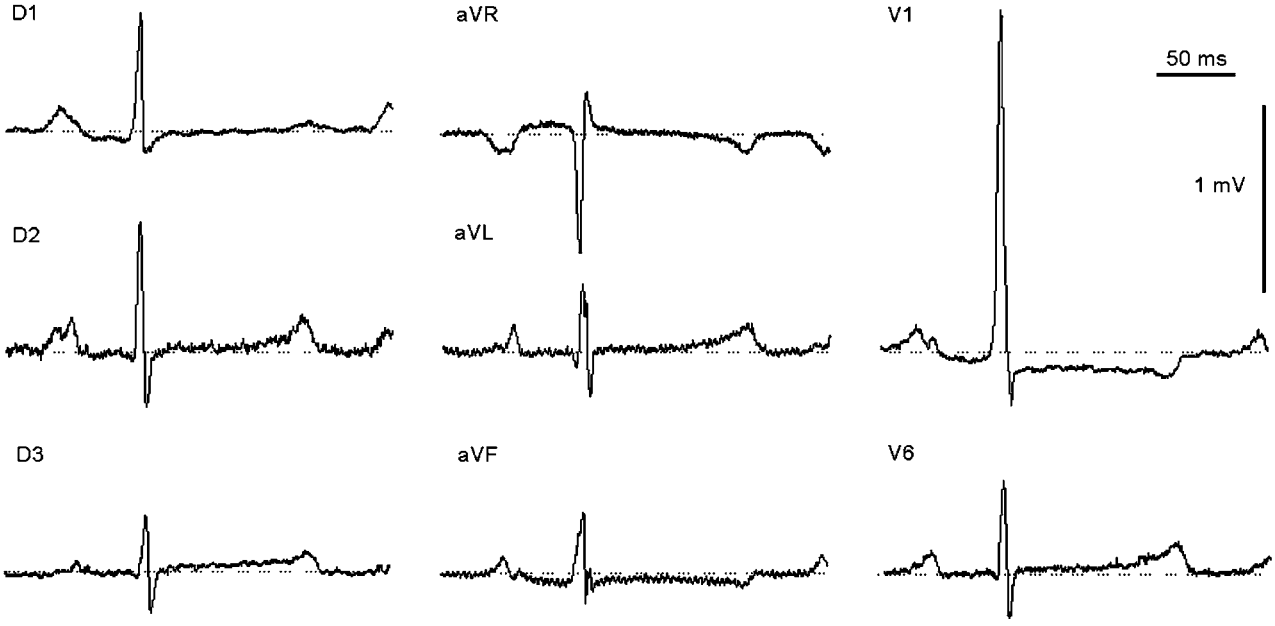

Figure 1 Typical eight lead electrocardiogram from a guinea-pig. The three classical frontal leads D1, D2 and D3 were recorded simultaneously. The three augmented leads aVR, aVL and aVF were recorded consecutively. The two precordial leads V1 and V6 were recorded simultaneously. Time and voltage scale apply to all traces. Dotted lines represent 0 mV.

Table 1 Mean values of amplitudes and waves (intervals) durations of guinea-pig ECG

Lead	*Amplitudes* (mV)							
	P	*Q*	*R*	*S*	*QRS*	*ST*	*T*	*Jpoint*
D2	0.146±0.010	−0.073±0.016	0.779±0.074	−0.419±0.055	1.198±0.107	0.027±0.006	0.122±0.014	−0.008±0.008
D1			0.564±0.071	−0.114±0.019	0.677±0.081			
V1			1.167±0.111	−0.345±0.089	1.512±0.153	−0.108±0.017		
V6			0.495±0.038	−0.426±0.085	0.921±0.115	0.055±0.014		

Intervals (ms)							
RR	*PQ*	*Pd*	*QRS*	*JT+*	*QT*	*QTcb*	*QTcf*
229.1±4.6	59.7±1.5	25.3±1.1	22.6±0.7	102.2±2.3	139.5±2.4	291.6±3.4	227.9±2.9

Data represent mean values±s.e. of the ECG recorded in 50 guinea-pigs.
QTcb: Bazett correction for QT.
QTcf: Fridericia correction for QT.
QRS: measured from beginning of Q to J point.
Pd: duration of the P waves.

Table 2 Effects of injections of antimony and carnitine on the survival of guinea-pigs

	Treatment					
	Saline	*Sb(III)*	*L-carnitine*	*L-carnitine + Sb(III)*		
				a	*b*	*c*
N_s	12	27	10	12	21	9
N_e	12	13	10	5	19	8
% survival	100	48	100	42	90	89

N represents the number of animals at the beginning ($_s$) and at the end ($_e$) of the treatment period.
a. L-carnitine and Sb(III) for 8 days.
b. 4 days of L-carnitine alone followed by 6 days of L-carnitine and Sb(III).
c. 4 days of L-carnitine alone followed by 12 days of L-carnitine and Sb(III).

Statistics

All results are expressed as means±s.e. Results were analysed with paired Student's *t*-test and the Student–Newman–Keuls for multiple comparisons following *F*-score on analysis of variance. Sb-induced mortality was analysed with Fischer's exact test. Fit to gaussian distribution was tested with the Anderson–Darling test of normality. $P<0.05$ was considered to reflect a statistical difference.

Results

Effects of saline and Sb(V) on the ECG

The normal eight leads ECG of guinea-pigs is illustrated in Figure 1 and quantitative values are reported in Table 1. The general aspect is close to that of the human ECG if the higher heart rate (265±5 beats min^{-1}, $n=50$) is taken into account. However, noteworthy is the very large R wave in V1 in guinea-pig as opposed to adult man. RS is often symmetrical in D2 and S is often larger than R in D3. In most cases, the ST segment is upsloping but it is generally flat and depressed in the V1 and aVF leads. Most measurements were performed on the D2 lead in which T wave was negative in only two out of 50 cases. Owing to the upsloping of the ST segment, ST interval was taken from the J point (where the QRS complex joins the ST segment (Hurst, 1998)) to the maximum of the T wave (T^+) and ST amplitude was taken at the midpoint between J point and T^+. QT was measured from the beginning of the Q wave to the end of T wave (Tend).

In saline-injected guinea pigs, ECG recorded every 4 days for up to 26 days showed no significant changes with time indicating that repeated anaesthesia had no effect ($n=9$).

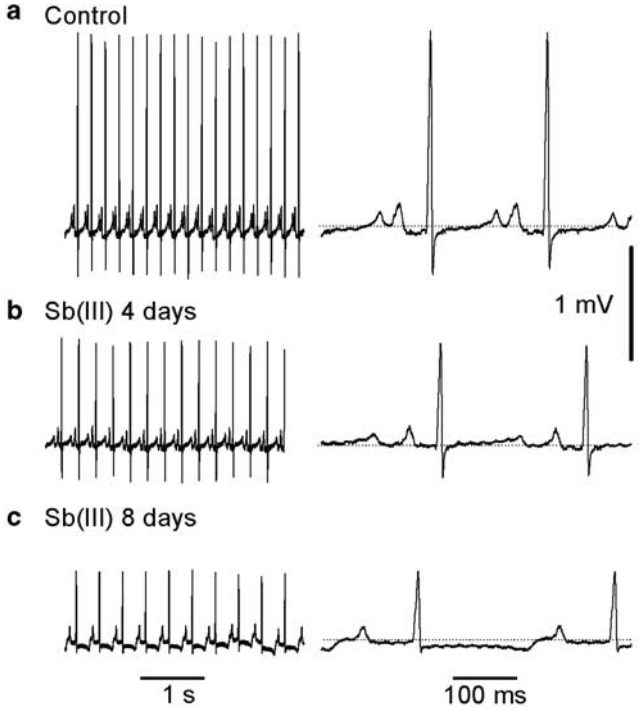

Figure 2 Effects of Sb(III) on the guinea-pig ECG. ECGs were recorded from one animal before (a) and 4 days (b) and 8 days (c) following the onset of daily injections of Sb(III). All traces represent the D2 lead of the ECG, details of which are shown upon an expanded time scale to the right. Time scales apply to traces in the respective columns. The vertical scale applies to all traces. The dotted lines indicate 0 mV.

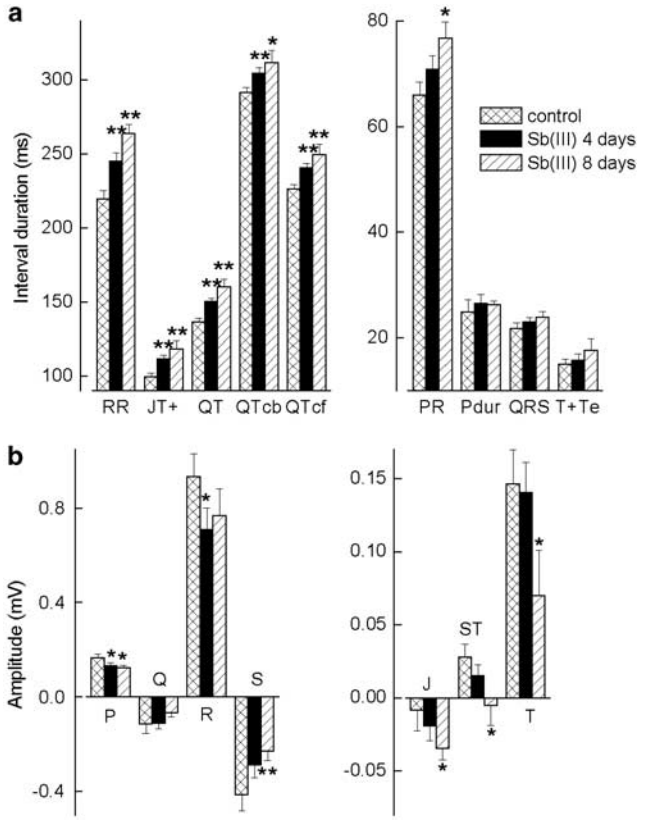

Figure 3 Effects of Sb(III) on wave and interval durations (a) and wave amplitudes (b) of the guinea-pig ECG. Measurements were performed before and following 4 days and 8 days of Sb(III) injections. All measurements were performed on the D2 lead. (a) RR, PQ, QRS and QT are the classical intervals, QRS being measured as a QJ interval. Classical QT, measured from the beginning of Q to the end of T, is presented with its corrected values using either Bazett formula (QTcb) or Fridericia formula (QTcf). Pdur: duration of the P waves. JT+: interval from the J point to the maximum of the T wave. T+Te: interval from the maximum to the end of the T wave. (b) In addition to the classical waves (P, Q, R, S and T), J point and ST elevation are also shown. Columns and bars represent mean ± s.e. ($n = 13$). Significance according to paired Student's *t*-test between control and Sb(III) treatments are indicated as * ($P < 0.05$) or ** ($P < 0.001$).

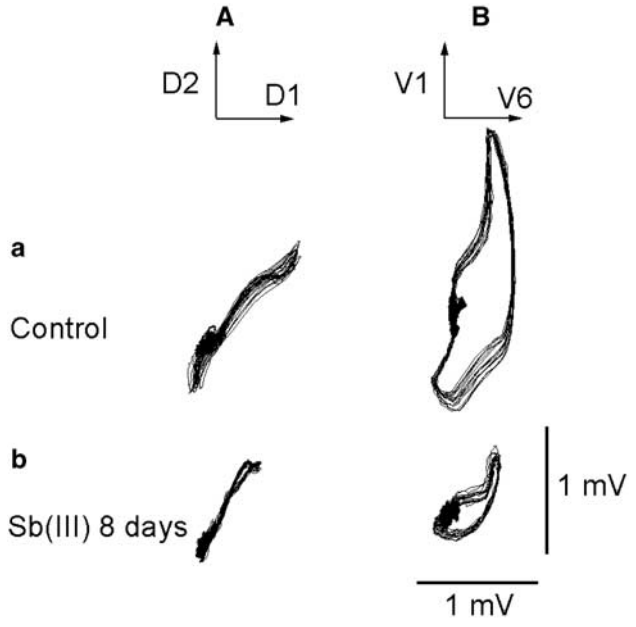

Figure 4 Cartesian representation of the guinea-pig ECG and its modification by Sb(III). Coupled derivations are in (A), D2 *versus* D1 and in (B), V1 *versus* V6. Data were recorded from the same animal before (a) and following 8 days (b) treatment with Sb(III). Each trace contains about 25 cycles (loops) corresponding to 30 s acquisition.

When guinea-pigs were injected with Sb(V) during 26 days ($n = 10$), no mortality was recorded. There were no qualitative changes in their eight leads ECG but slight quantitative changes were noticed in RR interval, T wave amplitude and QT duration. RR interval initially decreased from 236.8 ± 6.1 to 224.8 ± 3.8 ms ($P = 0.009$) at 12 days and lengthened to 244.1 ± 8.0 ms by the 26th day. The T wave amplitude initially increased from 0.083 ± 0.29 to 0.106 ± 0.09 mV at day 8 and then flattened to 0.068 ± 0.024 mV at day 26, but these effects were not significant. In contrast, QT significantly increased from 128.5 ± 3.8 to 144.2 ± 6.4 ms ($P = 0.04$). Both corrected QT values, QTcb and QTcf, significantly increased from 264 ± 5 ms and 207 ± 5 ms to 292 ± 11 ms ($P = 0.03$) and to 231 ± 9 ms ($P = 0.04$), respectively.

Treatment with Sb(III)

Sb(III) had very significant effects on guinea-pig viability ($P = 0.0013$) since 14 of 27 animals injected with Sb(III) died within 2 days (Table 2). No obvious symptoms or behaviour changes were noticed prior to mortality. The remaining 13 animals lived apparently normally though towards the end of the treatment, two of them showed weakness and had diarrhoea. When they were killed for cell isolation, the hearts appeared normal but the cell yield was much reduced to less than 40% of the standard.

Sb(III) induced noticeable changes in the ECG wave amplitude and duration of treated animals as shown in Figures 2 and 3. Most evident were a highly significant bradycardia and prolongation of the QT interval. The latter observation also holds for the corrected values of the QT (QTcb and QTcf) ($P < 0.01$; Figure 3a). QT changes were largely accounted for by JT+ increase ($P < 0.01$), while the T+T end duration was not modified. There was a small increase in PQ interval by the 8th day ($P < 0.05$) but neither the P wave nor the QRS durations were changed. Sb(III) decreased the amplitudes of all waves (Figures 2 and 3b), the effect being most obvious for the S and T waves. T wave flattening and widening by the 4th day is illustrated in Figure 2. By the 8th day, the ST segment was depressed and downsloping and the T wave became negative. Such negative T waves were recorded in 6 out of the 13 guinea-pigs that survived Sb(III) treatment but was never observed before Sb(III) injection ($n = 27$). Negative ST segment was also recorded 10 times during Sb(III) treatment but only once before its onset. They were even more frequent from the 4th day in lead D1. The decrease in ECG wave amplitude is not limited to the D2 lead. In D1, decreases in QRS from 0.97 ± 0.07 to 0.78 ± 0.07 mV at 4 days and to 0.76 ± 0.07 mV at 8 days were observed ($P < 0.01$). Indeed, cartesian representations of D2 *versus* D1 and V1 *versus* V6 loop representations of 10–20

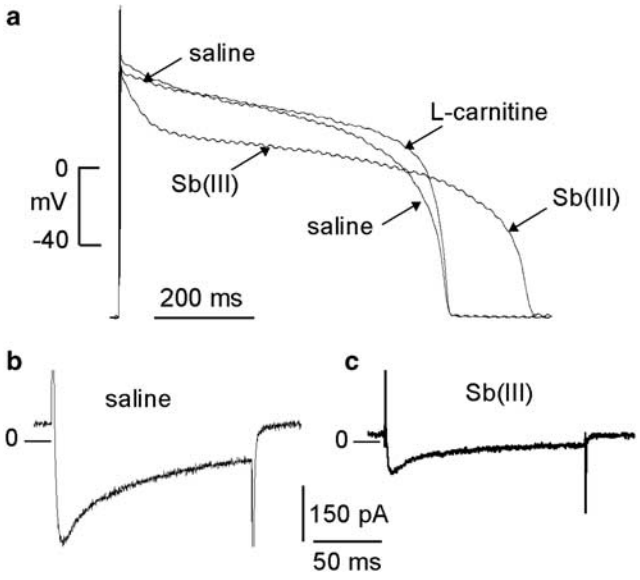

Figure 5 AP and calcium currents recorded from enzymatically dissociated ventricular myocytes. (a) Superimposed typical AP recorded in myocytes from three different guinea-pigs treated with either saline, L-carnitine or Sb(III) for 8 days. (b, c) Typical calcium currents recorded in normal Tyrode solution, in myocytes from the same guinea-pigs as in (a), with a voltage step to 0 mV following a 100 ms prepulse from −80 to −40 mV: saline (b) or Sb(III) (c)-treated guinea-pigs. The cell's capacitances were 37 and 35 pF for the saline and Sb(III) myocytes, respectively. Time and amplitude scales apply to both (b, c) panels. The zero current level is indicated by the horizontal line near each current trace. The stimulation frequency was 0.2 Hz in (a), and 0.1 Hz in (b, c).

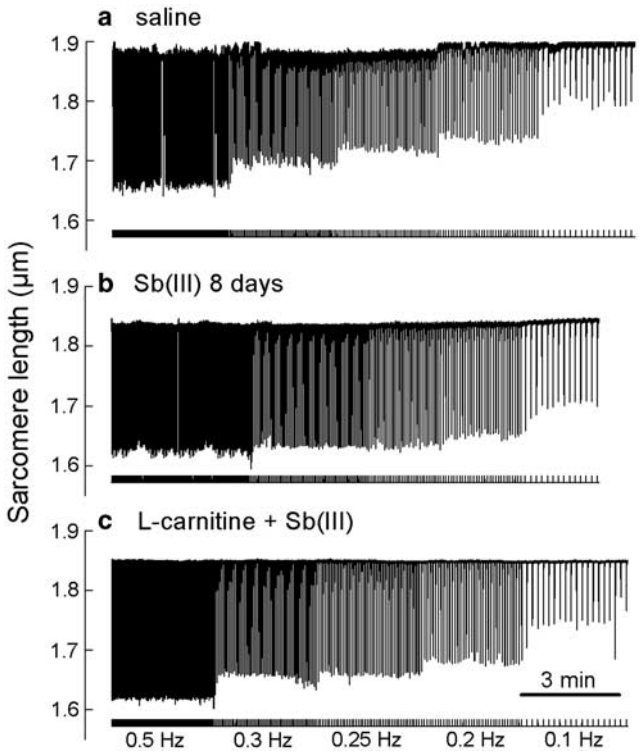

Figure 6 Effect of Sb(III) upon stimulus frequency-dependent changes of SL in isolated ventricular myocytes of guinea-pig. SL was measured by an on-line video detection method (see Methods) where downward deflection of the trace represents sarcomere shortening upon field stimulation which is schematically indicated below each recording. (a) A myocyte obtained from a control animal injected daily with saline. (b) A myocyte obtained from an animal injected daily with Sb(III) for 8 days. (c) A myocyte obtained from an animal injected with L-carnitine alone for 4 days followed by injection with L-carnitine and Sb(III) for 12 days. The time scale in (c) is applicable to all three traces. In each cell, stimulus frequency was progressively reduced from 0.5 Hz to 0.3 Hz, 0.25 Hz, 0.2 Hz and 0.1 Hz as indicated below the recordings.

ECGs show that Sb(III) decreases QRS amplitude in all derivations (Figure 4).

All *ex vivo* measurements were performed upon dissociated myocytes from the heart of guinea-pigs whose ECG had been previously recorded. Myocyte electrical activity was recorded in cells isolated from four guinea-pigs in each group (saline, Sb(III) and L-carnitine-injected animals). Figure 5a illustrates APs recorded from myocytes obtained from saline and Sb(III)-treated animals after 8 days. Whereas saline-injected animal ventricular myocytes showed normal APs with an elevated plateau ($+36.2\pm2.9$ mV, $n=6$), myocytes from Sb(III)-injected guinea-pigs had APs with a depressed plateau ($+9.5\pm1.7$ mV, $n=6$, $P<0.001$) and a prolonged duration of 832 ± 12 ms as compared to 549 ± 26 ms in the saline group ($P<0.001$). It should be mentioned that the resting potential was not changed by Sb(III) (saline: -77.4 ± 0.5 mV; Sb(III): -77.5 ± 0.4 mV; $P=0.849$). Moreover in Sb(III)-treated guinea-pigs, the maximum peak inward I_{Ca} recorded at 0 mV was consistently depressed (-2.8 ± 0.2 pA pF^{-1}, $n=6$) as compared to saline-treated guinea-pigs (-7.3 ± 0.3 pA pF^{-1}, $n=5$; $P<0.001$; Figures 5b and c) with no significant changes in the voltage-dependence of the current–voltage relationships nor in the apparent reversal potential of the calcium current (not shown).

Sarcomere shortening during stimulated contraction of isolated ventricular myocytes is illustrated in Figure 6. In myocytes obtained from saline-injected animals, a decrease in stimulation frequency led to a decrease in sarcomere shortening, that is, in contraction amplitude (Figure 6a). To take into account its variability from cell to cell, contraction amplitude was normalised to that obtained at a stimulation frequency of 1 Hz (Figure 7), which represented a sarcomere shortening of 0.219 ± 0.007 μm ($n=73$) from a resting length of 1.852 ± 0.003 μm. In myocytes obtained from saline-treated animals, the normalized frequency histogram of the ratio of the contraction amplitudes (0.5 Hz/1 Hz) showed a gaussian distribution with a mean value of ~70% (69.3 ± 1.3%, $n=73$; Figure 7a). Indeed, this mean value of ~70% decreased when test frequency is lowest (e.g., 30% at 0.1 Hz). The kinetics of contraction (shortening and lengthening) at two stimulation frequencies of 0.3 and 1 Hz are illustrated in Figure 8a. Upon increasing frequency, a classical increase in sarcomere shortening and relengthening rates occurred while the time to peak and total duration of the contraction decreased.

In myocytes obtained from Sb(III)-treated animals, resting SL (1.867 ± 0.006 μm, $n=65$) was not different from that of saline-injected guinea-pigs (1.852 ± 0.003 μm, $n=73$). Although the sarcomere shortening at 1 Hz was similar (0.215 ± 0.009 μm, $n=65$) to that of saline-injected guinea-pigs, only a few cells, however, showed the normal relationship between sarcomere shortening and frequency of stimulation.

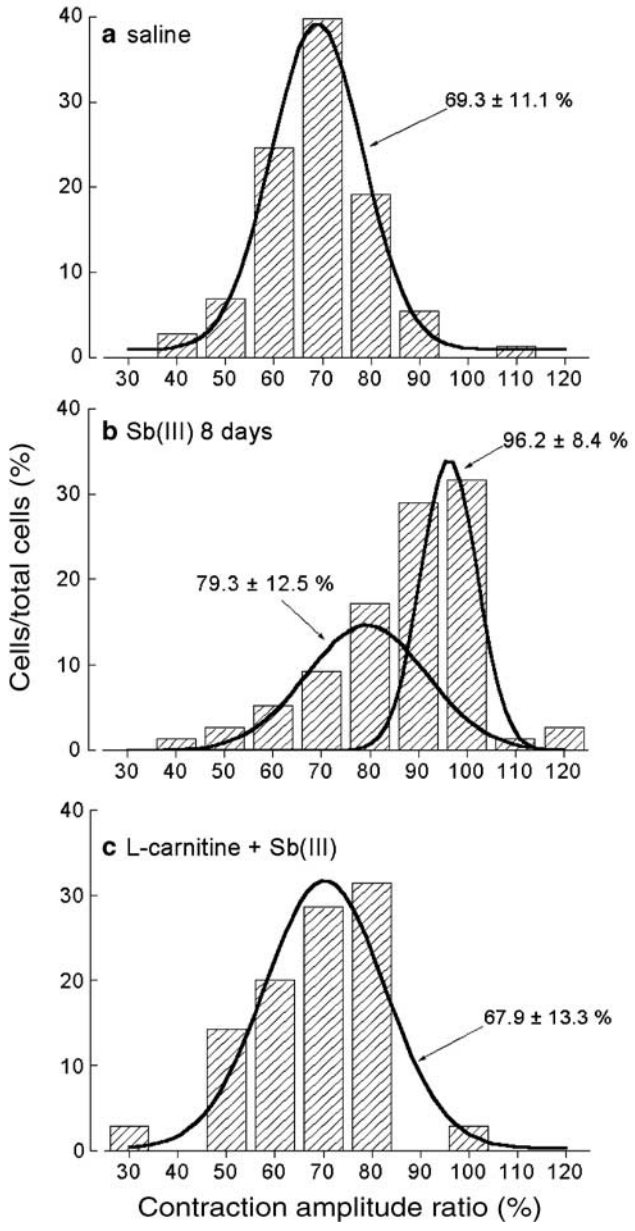

Figure 7 The effect of Sb(III) upon the normalised contraction–amplitude ratio of isolated venticular myocytes from guinea-pig. Contraction–amplitude ratio represents sarcomere shortening recorded at 0.5 Hz over sarcomere shortening recorded at 1 Hz. Results are shown for myocytes from control animals injected with saline (a; $N=9$ guinea-pigs; $n=75$ cells), myocytes from guinea-pigs injected with Sb(III) for 8 days (b; $N=8$; $n=76$) and myocytes from guinea-pigs treated for 4 days with L-carnitine and then for 12 days with L-carnitine plus Sb(III) (c; $N=7$; $n=70$). The lines represent gaussian functions fit to the data with their associated mean and s.d. values. Distributions were tested for normality with the Anderson–Darling normality test. In myocytes from Sb(III)-injected animals, the distribution needs two gaussians. A contraction–amplitude ratio of 100% indicates no change in amplitude with frequency at 0.5 and 1 Hz.

In most cells, it was virtually constant whatever the frequency of stimulation larger than 0.2 Hz (Figure 6b). Contraction amplitude decreased only at 0.1 Hz. The distribution of cell's relative contraction amplitude with frequency was no longer fitted with a single gaussian (Figure 7b). The mode of the distribution was 100% which indicates no change of amplitude with changing frequency from 0.5 to 1 Hz. The overall mean was $86.9\pm1.7\%$ ($n=76$) and could be distributed into a low-peak gaussian ($79.3\pm12.5\%$) and a high-peak gaussian ($96.2\pm8.3\%$), the latter showing a virtually null inotropic effect of frequency. Superimposition of contractions at 0.3 and 1 Hz showed that sarcomere shortening and relengthening rates are virtually independent of frequency (Figure 8b). These rates decreased only at very low frequencies (0.1 Hz; not shown). However, contraction duration was still reduced as in saline-injected animals when frequency increased, as did the AP duration (not shown). In addition, 10% of the cells from Sb(III)-treated animals showed oscillations of sarcomere shortening in response to a single stimulus. These occurred as either early or delayed after-contractions (Figure 8d–f). This was never observed in cells from saline-treated animals. It should be noted that the whole spectrum of events, after-contractions, the flat sarcomere shortening–frequency relationship and a normal increase in sarcomere shortening with frequency, was recorded in different myocytes in each heart from Sb(III)-treated animals.

Effects of L-carnitine

The injection of L-carnitine alone for 12 days had little effect upon the ECG except for a significant increase of the Q wave amplitude in D2 (-0.042 ± 0.012 to -0.086 ± 0.014 mV: $P=0.04$) and R wave amplitude in D1 (0.513 ± 0.103 to 0.778 ± 0.113 mV: $P=0.01$; $n=10$). There was a slight but not significant decrease of PQ interval. Electrical parameters of L-carnitine-treated guinea-pigs were not statistically different from those of saline-injected ones. Resting potential, plateau amplitude, AP duration and I_{Ca} values were -77.7 ± 0.9 mV, $+32.3\pm1.6$ mV, 520 ± 36 ms and -6.8 ± 0.4 pA pF^{-1} ($n=6$), respectively. There was no significant change in either the distribution or the mean of sarcomere shortening (not shown) as compared to saline injected guinea-pigs.

The protective effect of L-carnitine

The simultaneous onset of treatment with L-carnitine and Sb(III) did not change the overall mortality (Table 2) but delayed the fatal issue: guinea-pigs died between the fourth and the seventh day of treatment without any sign of illness. In the five guinea-pigs that survived, none of the duration and amplitude parameters of the ECG were significantly changed but for the QT that was significantly increased after 4 days (139 ± 3 to 148 ± 4 ms: $P=0.05$). However, the change in QTc was no longer significant whatever the correction used.

When injections of L-carnitine were initiated 4 days before the onset of simultaneous treatment with Sb(III), mortality was significantly reduced to 10% ($P=0.002$) at 6 days. Combined L-carnitine–Sb(III) treatment was thus prolonged for up to 12–15 days (Table 2). Mortality was still significantly reduced to 10% ($P=0.035$) and differences with controls as observed with Sb(III) alone were no longer significant ($P=0.428$). The eight derivations ECG did not show the changes that had been observed with 8 days of Sb(III) alone (not shown; $n=10$). After 12 days of combined L-carnitine-Sb(III) treatment, and thus 16 days since the onset of injections of L-carnitine, bradycardia was insignificant (1%) and there were no significant prolongation of either QT (4%), QTc (3%)

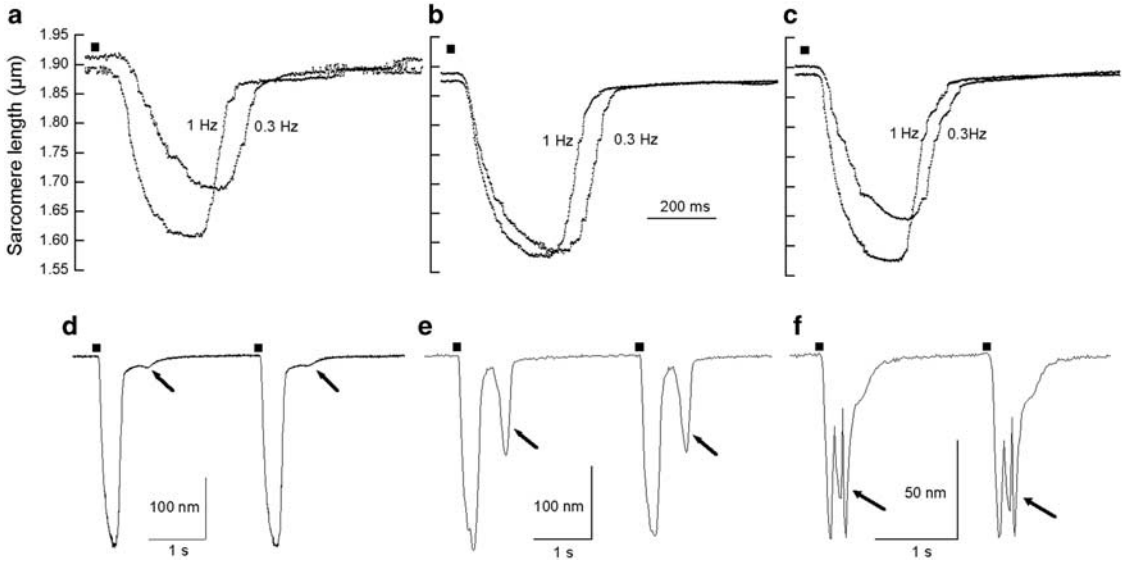

Figure 8 Contractions of isolated ventricular myocytes of guinea-pig. Typical contractions elicited by field stimulation (squares) from myocytes isolated from a guinea-pig injected with (a) saline, (b) Sb(III) for 8 days and (c) 4 days with L-carnitine and then for 12 days with L-carnitine plus Sb(III). Each set in (a, b and c) represents superimposed contractions obtained at stimulation frequencies of 0.3 and 1 Hz. Horizontal bar (200 ms) and SL scale apply to all three sets. (d–f) Multiphasic contractions obtained under similar conditions from myocytes isolated from 8 days Sb(III)-treated guinea-pigs. Contractions induced by field stimulation (squares) are followed by after-contractions (arrows). Contractions illustrated in (b and d) are from the same guinea-pig.

or JT+ (1%). The only significant change in the ECG was the PQ interval which increased from 56.2 ± 3.0 to 68.9 ± 6.5 ms ($P = 0.04$) as for 8 day Sb(III) treatment (Figure 2c). Changes in P and T waves amplitudes were not significant but there was still a significant reduction in QRS and S wave amplitudes from 1.19 ± 0.07 to 0.89 ± 0.16 mV ($P = 0.04$) and from -0.60 ± 0.08 to -0.40 ± 0.11 mV ($P = 0.02$), respectively, although these were less than had been seen after 8 days of Sb(III) alone.

The yield of cell isolation could not be distinguished from that of saline-injected animals. APs were of normal duration with an elevated plateau (Figure 5a). Resting SL was $1.867 \pm 0.006\,\mu$m ($n = 70$) and sarcomere shortening at 1 Hz was $0.210 \pm 0.010\,\mu$m: these values are not different from those of saline-treated animals. When frequency of stimulation was changed, contraction amplitude decreased or increased as in saline-injected guinea-pigs. The positive sarcomere shortening–frequency relationship was seen in all cells (Figure 6c). The distribution of cell's relative contraction amplitude with frequency was gaussian ($67.9 \pm 2.2\%$, $N = 7$; $n = 70$; Figure 7c) with a mean value that was not different from the saline-injected group (Figure 7a). Both sarcomere shortening and relengthening rates increased with increasing frequency and time to peak and contraction duration decreased as in saline-injected conditions (Figure 8c). None of the cells showed multiple oscillations of contraction upon stimulation as observed with Sb(III) alone.

Discussion

The main points arising from the results presented are as follows. Treatment of guinea-pigs with antimony induces mortality and/or ECG changes that could be prevented by prior treatment with L-carnitine. Ventricular myocytes isolated from Sb(III)-treated animals showed impaired responses to changes in stimulus frequency, elongated AP with depressed plateaux and reduced I_{Ca}. These phenomena were not seen in myocytes isolated from animals treated also with L-carnitine. It is concluded that L-carnitine can protect the heart from the deleterious effects of the antileishmaniasis agent Sb(III).

The results obtained from eight leads recording of the guinea-pig ECG were in general agreement with the more commonly used D1 D2 derivations and show a large similitude with the human ECG but for the large R wave in V1. The negative ST segment in the V1 lead was unlikely to result from anaesthesia since all other leads showed normal ST segment. Moreover, telemetry recordings along the anteroposterior leads exhibited similar ST depression (Matz *et al*., 1998). The absence of any significant effect of injections of saline or L-carnitine showed the stability of the measured parameters of the guinea-pig ECG despite repetitive anaesthesia.

In the present study, we used both Sb(III) and Sb(V) to assess the effects of antimony on the ECG. Treatment with Sb(V) induced QT prolongation that has also been described in man (Chulay *et al*., 1985; Hepburn *et al*., 1994; Takur *et al*., 1998). The most threatening effects of antimony observed in man (ECG changes and high mortality) with Sb(V) (Chulay *et al*., 1985; Ortega-Canicer *et al*., 1997; Ribeiro *et al*., 1999) and Sb(III) were routinely obtained in guinea-pig with Sb(III), an outcome that could be expected from the observed conversion of Sb(V) to Sb(III) (Roberts *et al*., 1995; Frezard *et al*., 2001; Ferreira *et al*., 2003).

In those animals that survived the initial period of high mortality, treatment with Sb(III) affected T wave and QT and ST segments, even after correction for changes in RR interval. QT prolongation and T wave flattening are accounted for by JT+ segment prolongation, whereas QRS duration was not changed. These results suggest that there was no significant effect of Sb on either conduction or depolarisation while the

drug markedly affected the repolarisation process. Thus, Sb(III) treatment should prolong AP but the extent of this prolongation may not be the same in different cell types since T+ and Tend are meant to sign the repolarisation of epicardial and M cells, respectively (Yan & Antzelevitch, 1998). Indeed, cells isolated from Sb(III)-treated animals showed prolonged AP with very low plateaux, which resulted from a reduced I_{Ca}, likely leading to a decrease in delayed potassium current activation. Whether this K^+ current is itself depressed or not in Sb(III)-treated animals will need further investigations. Shimizu & Antzelevitch (1999) have shown that low external calcium and ryanodine could suppress negative T wave and alternans, and give rise to torsade de pointes. Reduced entry of calcium could therefore account for torsade de pointes that have been described during antileishmanian treatment with Sb in man (Ortega-Canicer *et al*., 1997; Takur *et al*., 1998) and explain death of guinea-pigs in the first days of Sb treatment. Although no histological studies have been performed on hearts from Sb(III)-treated guinea-pigs (which appeared normal upon excision), cells death and/or increased fibrosis might account for the reduced yield of cells upon isolation and might contribute to the reduced amplitude of the ECG waves that was noticed on all the leads that were used in this study.

Wey *et al*. (1997) reported an increased diastolic Ca^{2+} with complete loss of Ca^{2+} transient and excitability in rat cultured cardiac myocytes exposed to 200 μM Sb(III). In Sb(III)-treated guinea-pigs, resting calcium is unlikely to have much increased since the resting SL were not different from those of saline-injected ones ($1.867 \pm 0.006\,\mu$m ($n = 65$) and $1.852 \pm 0.003\,\mu$m ($n = 73$), respectively) and excitability was perfectly maintained as was the resting potential. Moreover, the apparent reversal potential of the Ca^{2+} current was similar in myocytes from control and Sb(III)-treated guinea-pigs (not shown). Toraason *et al*. (1997) reported a reduced Ca^{2+} transient in rat cultured cardiac myocytes exposed to 6 μM Sb(III) and multiphasic Ca^{2+} transients at 8 μM. They assumed a reduced Ca^{2+} availability and a decreased influx of Ca^{2+} across the sarcolemma. In agreement with part of their hypothesis, we showed here that I_{Ca} and the plateau of the AP were actually depressed by Sb(III) treatment of guinea-pig *in vivo*.

Changes in contractility with frequency that have been described by Koch-Weser & Blinks (1963) in terms of negative (NIEA) and positive (PIEA) inotropic effect of activation, likely to represent ryanodine restitution time on one hand and calcium influx, sarcoplasmic reticulum (SR) Ca load and fractional SR Ca release on the other hand (Maier *et al*., 2000; Bers, 2001), were blunted with Sb(III) treatment of guinea-pigs. A blunted response occurred in human failing heart (Pieske *et al*., 1999) that otherwise showed, as in rabbit and guinea-pig, a positive relationship because PIEA more than overcome refractoriness of the SR Ca release process (Pieske *et al*., 1999; Maier *et al*., 2000). However, the blunted response in myocytes from Sb(III)-treated guinea-pigs is closer to that described by Sipido *et al*. (2000) for hypertrophied canine ventricular myocytes, since in this model it results from an increased contractility at low frequency due to an increased SR Ca load.

The Sb(III) reduced I_{Ca} should decrease the Ca^{2+} release from the SR. On the other hand, Sb(III) treatment also promoted after-contractions and multiphasic contractions that are known to occur with calcium overload of the SR (Bers, 2001). This suggests that the Ca load of the SR might be higher in Sb(III)-treated guinea-pigs than in control ones (likely because of an increased calcium influx *via* reverse mode Na/Ca exchange during the prolonged AP duration, although this remained to be determined). Thus, a higher Ca load of the SR associated with the decreased I_{Ca} could maintain contractility close to normal and might account for the blunted changes in contractility with frequency (Figures 6 and 7). Further investigations are required to explain these observations leading to a maximal twitch contraction rate at low frequency and to determine more precisely which aspects of the excitation–contraction coupling pathway are influenced by Sb(III).

The effective antileishmania agent is Sb(III) that has strong affinity for thiol groups (Sun *et al*., 2000; Frezard *et al*., 2001; Ferreira *et al*., 2003). In rat myocytes, this led to a decreased GSH content, inhibition of GSH peroxidase and induction of an oxidative stress (Tirmenstein *et al*., 1995; 1997) and pointed to a free radical-mediated alteration of Ca^{2+} mobilisation. Indeed, oxidant stress has been shown to induce mechanical oscillations and after-contractions, to prolong the cardiac AP and to reduce I_{Ca} (Beresewicz & Horackova, 1991; Cerbai *et al*., 1991; Josephson *et al*., 1991; Kourie, 1998) as observed in Sb(III)-treated guinea-pigs in the present study. It also delayed the onset of potassium current, increased Ca^{2+} flux *via* Na/Ca exchange in guinea-pig myocytes (Beresewicz & Horackova, 1991; Tokube *et al*., 1996) and increased the cardiac SR Ca release channel open probability (Boraso & Williams, 1994; Balshaw *et al*., 2001). All these reported effects of an oxidant stress might occur in isolated myocytes from the Sb(III)-treated guinea-pigs and explain our results, although additional experiments are needed to assess intracellular Ca^{2+} handling under these conditions.

Beneficial effects of L-carnitine reported here would also be in agreement with the involvement of free radicals. Cardioprotection by L-carnitine is well documented. Although its mechanism of action is not fully understood, it was shown to protect the perfused myocardium against oxidative stress and to be a free radical scavenger (Lango *et al*., 2001; Pauly & Pepine, 2003). We found in our study that L-carnitine also reduced Sb(III) associated mortality from $\sim$50% to less than $\sim$10% and corrected most ECG and contractility changes. Such a protective effect of L-carnitine was also obtained with the anticancer drug adriamycin (Strauss *et al*., 1998; Zeidan *et al*., 2002). Singal *et al*. (1988) and De Beer *et al*. (2001) suggested free radical generation as possible mechanism of action of adriamycin-induced cardiomyopathy. L-carnitine prevented adriamycin from increasing diastolic calcium (Mijares & Lopez, 2001) and promoted HSP25 synthesis (Strauss *et al*., 1998). Both HSP synthesis and the free radical scavenging properties of L-carnitine point to oxidative stress as a possible initial step of Sb(III)-induced cardiomyopathy. This hypothesis is strengthened by the reported Sb(III)-induced lipid peroxidation (Toraason *et al*., 1997) as well as induction of HSP upon exposure to nonlethal doses of Sb(III) (Snawder *et al*., 1999). It remains to be seen whether L-carnitine also protects the Leishmania parasite from Sb(III).

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