

Antimony-Based Antileishmanial Compounds Prolong the Cardiac Action Potential by an Increase in Cardiac Calcium Currents^[S]

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ABSTRACT

Antimonial agents are a mainstay for the treatment of leishmaniasis, a group of protozoal diseases that includes visceral leishmaniasis, or Kala Azar. Chemotherapy with trivalent potassium antimony tartrate (PAT) and, more importantly, pentavalent antimony-carbohydrate complexes, such as sodium stibogluconate (SSG), has been reported to prolong the QT interval and produce life-threatening arrhythmias. PAT is chemically related to As₂O₃, which alters cardiac excitability by inhibition of human *ether a-go-go* related gene (hERG) trafficking and an increase of cardiac calcium currents. In this study, we report that PAT does not block hERG currents on short-term exposure but reduces current density on long-term exposure (IC₅₀, 11.8 μM) and inhibits hERG maturation on Western blots (IC₅₀, 62 μM). Therapeutic concentrations of 0.3 μM PAT increase cardiac

calcium currents from -4.8 ± 0.7 to -7.3 ± 0.5 pA/pF at 10 mV. In marked contrast, pentavalent SSG, the drug of choice for the treatment of leishmaniasis, did not affect hERG/I_{Kr} or any other cardiac potassium current at therapeutic concentrations. However, both cardiac sodium and calcium currents were significantly increased on long-term exposure to 30 μM SSG in isolated guinea pig ventricular myocytes. We propose that the increase in calcium currents from -3.2 ± 0.3 to -5.1 ± 0.3 pA/pF at 10 mV prolongs APD₉₀ from 464 ± 35 to 892 ± 64 ms. Our data suggest that conversion of Sb(V) into active Sb(III) in patients produces a common mode of action for antimonial drugs, which define a novel compound class that increases cardiac risk not by a reduction of hERG/I_{Kr} currents but—for the first time—by an increase in cardiac calcium currents.

Leishmaniasis represents a complex of parasitic diseases caused by 20 different protozoa in the genus *Leishmania* that are pathogenic for humans and transmitted by an insect vector, the phlebotomine sandfly. It is thought that approximately 12 million people are currently infected worldwide, according to the World Health Organization, mostly in tropical and subtropical regions of the Americas, Africa, and India. However, with increasing travel to and from endemic regions, more and more patients with leishmaniasis are seen by physicians in Western countries (Herwaldt, 1999; Murray et al., 2000; Guerin et al., 2002).

Leishmaniasis presents in three major forms with a wide

range of cutaneous, mucocutaneous, and visceral symptoms. Visceral leishmaniasis or Kala Azar is the most severe form of the disease, with a mortality rate of nearly 100% if not treated properly (Herwaldt, 1999). Treatment still relies largely on pentavalent antimony compounds such as meglumine antimonate (Glucantime; Sanofi-Aventis, Bridgewater, NJ) and sodium stibogluconate (Pentostam; GlaxoSmithKline, Uxbridge, Middlesex, UK) (Herwaldt, 1999; Guerin et al., 2002). Although pentavalent antimony compounds have proven to be very effective, drug use is often limited in patients because of toxic side effects, such as nausea, abdominal pain, chemical pancreatitis, renal toxicity, and electrocardiographic abnormalities, which are especially worrisome (Guerin et al., 2002). The cardiotoxicity of pentavalent antimony compounds, which may include inversion of the ST-segment on the electrocardiogram, QTc prolongation, torsade de pointes arrhythmias, and sudden cardiac arrest (Chulay et al., 1985; Ortega-Carnicer et al., 1997; Thakur, 1998; Berhe et al., 2001; Cesur et al., 2002), severely limits pro-

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ABBREVIATIONS: PAT, potassium antimony tartrate; ECG, electrocardiogram; HEK, human embryonic kidney; hERG, human *ether a-go-go* related gene; HA, hemagglutinin; SSG/Sb(V), sodium stibogluconate; PAT/Sb(III), trivalent potassium antimony tartrate; NAC, *N*-acetyl-L-cysteine; E4031, 1-[2-(6-methyl-2-pyridyl)ethyl]-4-(4-methylsulfonyl aminobenzoyl) piperidine; I-V, current-voltage; APD, action potential duration; ER, endoplasmic reticulum; ROS, radical oxygen species.

longed treatment courses in patients, particularly when high concentrations are indicated to combat and overcome resistance.

Although antimonial compounds have been used in the treatment of leishmaniasis for at least 100 years, their precise mechanism of action remains unknown. Goodwin and Page (1943) were the first to propose that pentavalent antimony Sb(V) might act as a pro-drug that has to be converted into active, trivalent Sb(III) which is thought to interfere with thiol-dependent redox systems of *Leishmania* parasites (Wyllie et al., 2004). In fact, a trivalent Sb(III) compound, tartar emetic [or potassium antimony (III) tartrate (PAT)], was one of the first chemotherapeutic agents used in the treatment of leishmaniasis but ultimately had to be abandoned because of its high toxicity, with changes on the electrocardiogram (ECG) observed in nearly 100% of patients. In comparison, pentavalent Sb(V) is generally much better tolerated, and ECG abnormalities develop in only approximately 10% of patients, usually late during the course of treatment (Chulay et al., 1985; Sundar et al., 1998).

In the present study, we examined the cellular mechanisms by which antimonial compounds induce ECG changes. Based on chemical similarities, we hypothesized that trivalent potassium antimony (III) tartrate/PAT might prolong cardiac repolarization by mechanisms similar to those more recently described for trivalent antineoplastic arsenic trioxide (As_2O_3), which produced inhibition of hERG/ I_{Kr} channel trafficking together with an increase of cardiac calcium currents (Ficker et al., 2004). In a second step we extend our studies to the closely related, clinically important pentavalent antimony compound sodium stibogluconate SSG/Sb(V). We found that both antimonial compounds—regardless of their different oxidation states—increase cardiac calcium currents at therapeutic concentrations, whereas three major cardiac potassium currents (I_{Kr} , I_{Ks} , and I_{K1}) are not affected. This is the first example of drug-induced LQTS in which an increase in depolarizing cardiac calcium currents represents the main mechanism of cardiotoxic drug action leading to QT prolongation and torsade de points tachycardia in patients.

Materials and Methods

Western Blot Analysis. Stably transfected HEK/hERG cells and antibodies used in the present study have been described previously (Ficker et al., 2003, 2004). In brief, HEK/hERG cells were solubilized for 1 h at 4°C in a lysis buffer containing 1% Triton X-100 and protease inhibitors (Complete; Roche Biochemicals, Indianapolis, IN). Protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Proteins were separated on SDS polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and developed using anti-hERG antibody (HERG 519) and ECL Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK). For quantitative analysis, chemiluminescence signals were captured directly on a Storm PhosphorImager (GE Healthcare) (Ficker et al., 2003).

Chemiluminescence Detection of Cell Surface hERG Protein. A hemagglutinin (HA) tag was inserted into the extracellular loop of hERG between transmembrane domains S1 and S2 (Ficker et al., 2003, 2004). Stably transfected HEK/hERGWT HA_{ex} cells were plated at 40,000 cells/well in a 96-well plate. After overnight incubation with test compounds, cells were fixed, blocked, and incubated for 1 h with rat anti-HA antibody (Roche). After washing, horseradish peroxidase-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA) and the double-stranded

DNA stain SYBR Green (Invitrogen, Carlsbad, CA) were added for 1 h (Ficker et al., 2004). SYBR Green fluorescence was measured to determine cell numbers. Chemiluminescent signals were developed using SuperSignal (Pierce) and captured in a luminometer. Luminescence signals were corrected for cell loss as measured by SYBR Green fluorescence with the data presented as normalized surface expression relative to control.

Cellular Electrophysiology. HEK/hERG WT cells were recorded using patch pipettes filled with 100 mM K-aspartate, 20 mM KCl, 2 mM MgCl_2 , 1 mM CaCl_2 , 10 mM EGTA, and 10 mM HEPES, pH 7.2. The extracellular solution was 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 10 mM HEPES, and 10 mM glucose, pH 7.4. To study long-term drug effects on hERG currents, drug was added for 16 to 20 h (overnight) before recording. Sodium stibogluconate [SSG/Sb(V) (Pentostam)], was a gift from GlaxoSmithKline; PAT/Sb(III) was from Sigma (St. Louis, MO). Stock solutions for both compounds were prepared in water. I_{Kr} , I_{Ks} , and I_{K1} currents and action potentials were recorded in isolated ventricular guinea pig myocytes cultured overnight in M199 medium using the following intracellular solution: 119 mM potassium gluconate, 15 mM KCl, 3.75 mM MgCl_2 , 5 mM EGTA, 5 mM HEPES, 4 mM K-ATP, 14 mM phosphocreatine, 0.3 mM Tris-GTP, and 50 U/ml creatine phosphokinase, pH 7.2. The extracellular solution was 132 mM NaCl, 4 mM KCl, 1.2 mM MgCl_2 , 1.8 mM CaCl_2 , and 5 mM HEPES, pH 7.4. L-type Ca^{2+} currents were blocked with 1 μM nisoldipine. The specific hERG/ I_{Kr} blocker E4031 was used to isolate I_{Kr} currents. Cardiac sodium currents were recorded using the following extracellular solution: 40 mM NaCl, 55 mM *N*-methyl-D-glucuronate, 20 mM CsCl, 5.4 mM KCl, 2 mM MgCl_2 , 0.02 mM CaCl_2 , 30 mM tetraethylammonium chloride, 5 mM 4-aminopyridine, and 10 mM HEPES, pH 7.4. The intracellular solution was 120 mM CsCl, 2 mM MgCl_2 , 1 mM CaCl_2 , 11 mM EGTA, 10 mM HEPES, 10 mM glucose, and 1 mM Mg-ATP, pH 7.2. Cardiac calcium currents were recorded using the following extracellular solution: 137 mM NaCl, 5.4 mM CsCl, 1.8 mM MgCl_2 , 1.8 mM CaCl_2 , 10 mM glucose, and 10 mM HEPES, pH 7.4. The intracellular solution was 130 mM Cs MeSO₄, 20 mM tetraethylammonium chloride, 1 mM MgCl_2 , 10 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, 14 mM Tris-phosphocreatine, 0.3 mM Tris-GTP, and 50 U/ml creatine phosphokinase, pH 7.2. To analyze current densities, membrane capacitance was measured using the analog compensation circuit of an Axon 200B patch clamp amplifier (Molecular Devices, Sunnyvale, CA). Pclamp software (Molecular Devices) was used for generation of voltage-clamp protocols and data acquisition. All current recordings were performed at room temperature (20–22°C).

Data Analysis. Data are expressed as mean \pm S.E.M. of *n* experiments or cells studied. Differences between two means were usually tested using a two-tailed Student's *t* test. When indicated, Dunnett's test was used to determine whether multiple treatment groups were significantly different from control. *P* values <0.05 were considered statistically significant. Concentration-response relationships were fit to Hill equations of the following form: $I_{\text{drug}}/I_{\text{control}} = 1/(1 + [D/IC_{50}]^{n_H})$, where *I* indicates current or image densities, *D* is the drug concentration, *n_H* is the Hill coefficient, and *IC*₅₀ is the drug concentration necessary for half-maximal inhibition.

Results

Trivalent Antimony Inhibits Maturation of the Cardiac Potassium Channel hERG. Because both antimony and arsenic are metalloids belonging to group V of the periodic table, they share many chemical properties and are thought to interfere with biological processes in a similar manner. To explore whether trivalent potassium antimony tartrate (PAT/Sb(III)) directly blocks hERG currents or instead reduces hERG processing as has been described for trivalent arsenic trioxide [$\text{As}_2\text{O}_3/\text{As(III)}$; Ficker et al., 2004],

we applied 30 μM PAT/Sb(III) rapidly with the extracellular bath solution and found that hERG tail current amplitudes were not affected (Fig. 1, A and B). Next, we exposed stably transfected HEK/hERG cells overnight to increasing concentrations of PAT/Sb(III) to test for processing-mediated changes in hERG current amplitudes. Similar to experiments with As_2O_3 , current amplitudes were reduced in a concentration-dependent manner with an IC_{50} of 11.8 μM (Fig. 1, C and D). Changes in current amplitudes developed slowly and were accompanied by small changes in deactivation kinetics. After a 3-h exposure to 30 μM PAT/Sb(III), we measured hERG tail current densities of 26.5 ± 4.6 pA/pF ($n = 6$) in treated cells and of 26.4 ± 5.3 pA/pF ($n = 8$) in control cells. At -50 mV, current deactivation was fit best by two exponential time constants that were significantly accelerated from 287 ± 37 and 1851 ± 150 ms under control conditions to 227 ± 35 and 1403 ± 103 ms after 3-h exposure to PAT/Sb(III) (Student's t test, $p < 0.05$, $n = 5-7$). Overnight exposure to PAT/Sb(III) produced similar changes in deactivation kinetics together with a prominent decrease in current amplitudes (Fig. 1, C and D). In these experiments hERG tail currents were significantly reduced from 56.2 ± 7.1 pA/pF measured under control conditions ($n = 9$) to 14.2 ± 2.5 pA/pF after overnight exposure to 30 μM PAT/Sb(III) (Dunnett's test, $p < 0.05$, $n = 8$, Fig. 1E). Because many of the biological effects of trivalent antimony compounds are thought to be mediated via an increase in ROS production,

we tested whether the effects of PAT/Sb(III) on hERG currents could be reversed by a ROS scavenger, the antioxidant *N*-acetyl-L-cysteine (NAC). In experiments with both 30 μM PAT/Sb(III) and 5 mM NAC in the incubation medium, hERG tail current amplitudes were 38.1 ± 6.4 pA/pF and not significantly different from current amplitudes measured under control conditions (Dunnett's, $n = 6$; Fig. 1E). To exclude the possibility that nonspecific effects of NAC may underlie the observed reversal of antimony mediated effects, we incubated HEK/hERG cells with 5 mM NAC in the absence of PAT/Sb(III) and found that hERG tail current amplitudes were not affected. We measured current densities of 38.0 ± 6.5 pA/pF under control conditions and of 40.3 ± 5.5 pA/pF in HEK/hERG cells incubated overnight with 5 mM NAC ($n = 15-16$).

Pentavalent Sodium Stibogluconate Does Not Affect hERG Maturation. Because better tolerated pentavalent antimony compounds such as SSG have supplanted the more toxic trivalent PAT/Sb(III), which is tolerated in patients only up to a serum concentration of approximately 1 μM (Schulert et al., 1966; Doenges, 1968), we sought to determine whether our findings with PAT/Sb(III) could be extended to SSG/Sb(V). In cultures of *Leishmania donovani* parasites in human macrophages, 10 to 15 $\mu\text{g/ml}$ SSG/Sb(V) was able to eliminate 80 to 90% of the parasite load. These concentrations are thought to be less than or equal to peak serum concentrations obtained in humans during therapy

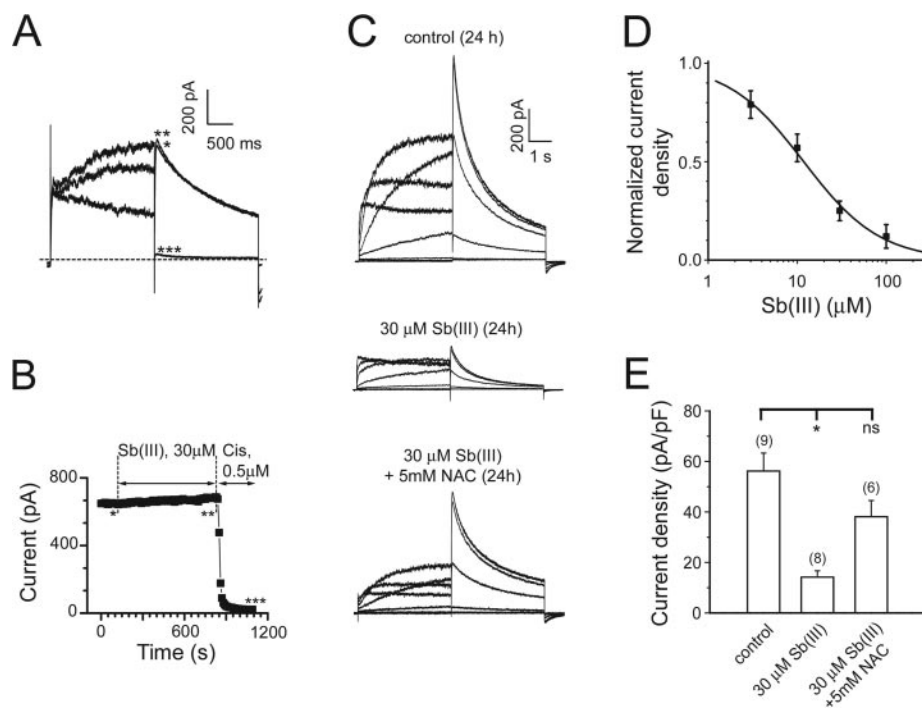


Fig. 1. Prolonged exposure to trivalent potassium antimony tartrate PAT/Sb(III) reduces hERG currents stably expressed in HEK293 cells. A, hERG tail currents recorded under control conditions (*) or in the presence of extracellularly applied 30 μM PAT/Sb(III) (**) or 0.5 μM cisapride (***). Currents were elicited from a holding potential of -80 mV with depolarizing voltage pulses to $+20$ mV. Tail currents were recorded on return to -50 mV. Note that during the depolarizing test pulse hERG currents show small run-up during application of PAT/Sb(III). B, hERG tail currents are not affected by short-term extracellular application of 30 μM PAT/Sb(III) but are reduced by the hERG blocker cisapride. C, ensemble of hERG currents recorded after overnight culture (24 h) under control conditions or after overnight exposure (24 h) to either 30 μM Sb(III) or a combination of 30 μM Sb(III) + 5 mM NAC (5 mM) using depolarizing voltage steps from -60 to $+60$ mV (20 mV increments) from a holding potential of -80 mV. Tail currents were recorded on return to -50 mV. D, concentration-dependent reduction of hERG tail current density by overnight exposure to PAT/Sb(III). The IC_{50} , determined using a Hill equation, is 11.8 μM ($n = 6-9$). E, quantification of maximal tail current densities under control conditions, in cells exposed overnight to PAT/Sb(III) or PAT/Sb(III)/NAC. PAT/Sb(III) significantly reduced hERG tail current densities (Dunnett's test, $*p < 0.05$), whereas tail current densities recorded in PAT/Sb(III)/NAC treated cells were not significantly (ns) different from control (Dunnett's test, ns at 0.05 level). Cell numbers analyzed given in parenthesis.

and correspond to approximately 12 to 16 μM SSG/Sb(V) (Berman and Wyler, 1980; Berman et al., 1982; Jaser et al., 1995).

To test for direct block, we applied 100 μM SSG/Sb(V) with the extracellular perfusate in voltage-clamp experiments and found that hERG tail currents elicited on return to -40 mV were not affected (Fig. 2, A and B). Next, we tested for SSG/Sb(V)-induced trafficking block by exposing stably transfected HEK/hERG cells to 30 μM SSG/Sb(V) overnight. We used a concentration somewhat higher than peak serum concentrations because we limited long-term exposure times to 24 h, whereas adverse cardiac events were usually observed late in treatment regimens, after several days of drug exposure. Although 30 μM PAT/Sb(III) induced prominent changes in hERG trafficking, hERG currents were not affected by long-term exposure to 30 μM SSG/Sb(V). Tail current densities averaged 39.9 ± 7.8 pA/pF in control cells and 42.5 ± 5.4 pA/pF in SSG-treated cells ($n = 6-7$, Fig. 2, C and D).

To confirm the differences in hERG processing observed on exposure to comparable concentrations of PAT/Sb(III) and

SSG/Sb(V), we performed Western blot experiments (Fig. 3, A and B). hERG channels are synthesized as an immature ER-resident protein of approximately 135 kDa that matures to a fully-glycosylated protein of approximately 155 kDa that is transported to the cell surface (Zhou et al., 1998). Incubation with compounds that produce processing or trafficking defects in the ER should be reflected in a decrease of mature, fully-glycosylated hERG protein. In line with our electrophysiological experiments, we found that overnight exposure to PAT/Sb(III) decreased the amount of fully glycosylated mature hERG with an IC_{50} of 62.3 μM (Fig. 3C, $n = 3-4$). However, the level of mature, fully-glycosylated hERG protein was not altered by SSG up to 300 μM in accordance with electrophysiological observations. At higher SSG/Sb(V) concentrations, moderate hERG trafficking defects were detected. We extrapolated an IC_{50} value of approximately 10 to 11 mM based upon measurements with concentrations up to 3 mM SSG/Sb(V) (Fig. 4C). The disparate results obtained with PAT/Sb(III) and SSG/Sb(V) were further confirmed using a chemiluminescence assay that monitors the expression of cell surface protein directly. In these experiments, HEK cells stably expressing hERG tagged with an extracellular hemagglutinin (HA) epitope (Ficker et al., 2003) were treated overnight with either PAT/Sb(III) or SSG/Sb(V). Although overnight treatment with PAT/Sb(III) reduced hERG surface

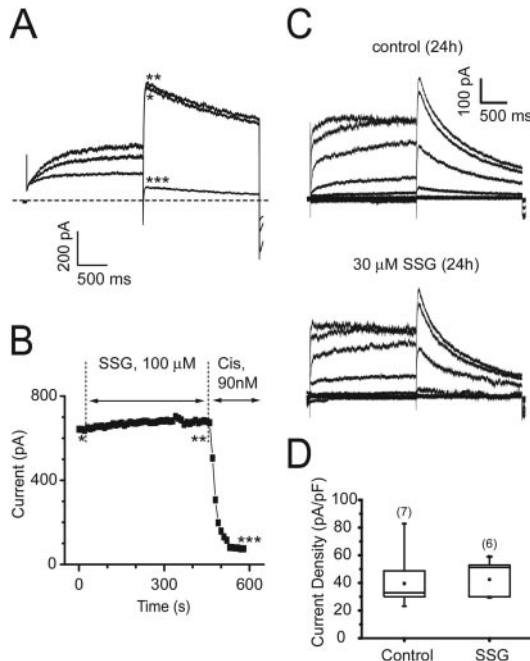


Fig. 2. Prolonged exposure to pentavalent sodium stibogluconate SSG/Sb(V) has no effect on hERG currents stably expressed in HEK293 cells. A, hERG tail currents recorded under control conditions (*), in the presence of 100 μM SSG/Sb(V) (**) and of 90 nM cisapride, which blocks approximately 90% of control currents (***). Currents were elicited from a holding potential (h.p.) of -80 mV with depolarizing voltage pulses to $+20$ mV. Tail currents were recorded on return to -40 mV. Note that during the depolarizing test pulse, hERG currents show small run-up with application of SSG/Sb(V). B, hERG tail currents are not affected by short-term extracellular application of 100 μM SSG but are reduced by cisapride. C, ensemble of hERG currents elicited by depolarizing voltage commands from -60 to $+60$ mV in increments of 20 mV from a holding potential of -80 mV under control conditions, and after overnight exposure (24 h) to 30 μM SSG/Sb(V). Tail currents were recorded and quantified on return to -50 mV. D, maximal hERG tail current densities measured after overnight culture under control conditions and in the presence of 30 μM SSG. Data are represented as statistical box charts with asterisks representing outliers, whiskers determining the 5th and 95th percentile, boxes determining the 25th and 75th percentile. Means are represented by squares. Current density was not significantly different in control and SSG-treated cells (Student's t test, ns at 0.05 level). Cell numbers analyzed given in parenthesis.

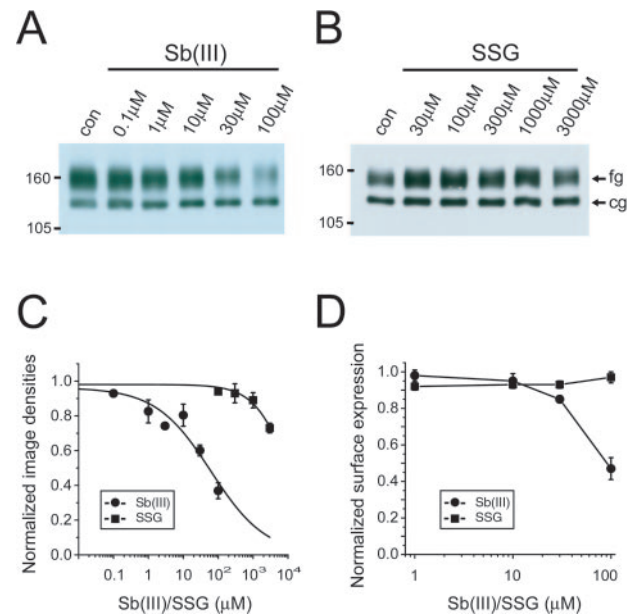


Fig. 3. Effects of PAT/Sb(III) and SSG/Sb(V) on surface expression of hERG. A, Western blot analysis showing effects of overnight exposure to increasing concentrations of PAT/Sb(III) on hERG WT stably expressed in HEK293 cells. B, Western blots of hERG after overnight exposure to increasing concentrations of SSG/Sb(V). Arrows: fg indicates the fully glycosylated mature 155-kDa form of hERG; cg indicates the core-glycosylated ER-resident 135-kDa form of hERG. C, concentration-dependent reduction of fully glycosylated mature 155-kDa form of hERG (fg) on Western blots after overnight exposure to either PAT/Sb(III) or SSG/Sb(V). PAT/Sb(III) reduces mature form of hERG in a dose-dependent manner. IC_{50} is 62 μM ($n = 3-4$). SSG causes a small reduction in fully-glycosylated mature hERG protein (fg) at unphysiologically high concentrations. Image densities on Western blots were analyzed directly on a Storm PhosphorImager. D, overnight exposure to PAT/Sb(III) reduces surface expression levels of externally HA-tagged hERG protein in a concentration-dependent manner as determined by chemiluminescence measurements ($n = 3$). Surface expression of hERG is not altered by SSG/Sb(V) at concentrations up to 100 μM .

expression in a concentration-dependent manner, SSG/Sb(V) was without effect at concentrations up to 100 μ M (Fig. 3D).

Thus, SSG/Sb(V) represents a rare example of a therapeutic compound that has been associated with drug-induced long QT syndrome but does not affect hERG currents. To gain further insight in SSG/Sb(V) mediated cardiac toxicity, we analyzed the effects of SSG/Sb(V) on cardiac action potentials to identify alternative ion channel targets in cardiomyocytes that may be directly linked to the reported prolongation of the QT interval during therapy with pentavalent antimony compounds.

Long-Term Exposure to Both PAT/Sb(III) and SSG/Sb(V) Prolongs the Cardiac Action Potential. In a first series of experiments, we studied the short-term effects of extracellularly applied SSG/Sb(V) on action potentials evoked at a stimulation frequency of 0.1 Hz in freshly isolated guinea pig ventricular myocytes and compared those results with those of PAT/Sb(III) to address the possibility that a major membrane current other than hERG may be directly blocked by antimonial compounds. We found that action potential duration APD_{90} was not affected by rapid application of extracellular PAT/Sb(III) (Fig. 4A). APD_{90} was 486 ± 129 ms under control conditions and 492 ± 133 ms after 5-min superfusion with 30 μ M PAT/Sb(III) ($n = 3$). Likewise, 100 μ M SSG was without effect. APD_{90} was 428 ± 77 ms in control cells and 429 ± 75 ms after perfusion with SSG/Sb(V) ($n = 4$, Fig. 4B). To address the possibility that complex interactions of PAT/Sb(III) and/or SSG/Sb(V) with multiple cardiac membrane currents may obscure short-term block of individual current components in action potential recordings, we applied both 30 μ M PAT/Sb(III) and 100 μ M SSG/Sb(V) rapidly with the extracellular perfusate to freshly

isolated ventricular myocytes and determined short-term block of three major cardiac current components: I_{K_r} , I_{K_s} , and I_{Ca} . We found that none of these currents was blocked in the short term by either PAT/Sb(III) or SSG/Sb(V) (see Supplementary Table 1).

Next, we examined changes in cardiac excitability after prolonged drug exposure. In these experiments, isolated ventricular myocytes were cultured overnight in the presence of either 30 μ M PAT/Sb(III) or 30 μ M SSG/Sb(V) (Fig. 5). Action potential duration APD_{90} was significantly prolonged from 464 ± 35 ms ($n = 7$) measured in myocytes cultured under control conditions to 748 ± 30 ms after overnight exposure to PAT/Sb(III) ($n = 6$). Thirty micromolar SSG/Sb(V) prolonged APD_{90} to 655 ± 35 ms ($n = 4$), providing evidence that SSG increases cardiac excitability as predicted from clinical observations. Because our experiments in heterologous expression systems have identified hERG/ I_{K_r} as a possible target

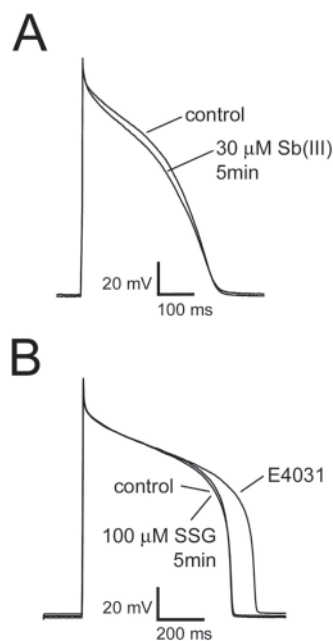


Fig. 4. Short-term exposure to neither PAT/Sb(III) nor SSG/Sb(V) prolongs the cardiac action potential. A, current clamp recordings of cardiac action potentials (AP) elicited in a freshly isolated ventricular myocyte and 5 min after start of extracellular perfusion with 30 μ M PAT/Sb(III). Membrane potential (m.p.) was -70 mV. B, APs elicited in a freshly isolated ventricular myocyte and 5 min after start of extracellular perfusion with 100 μ M SSG/Sb(V). E4031 (5 μ M) was used to block native I_{K_r} /hERG currents and prolong the cardiac AP. m.p. was -70 mV. Stimulation frequency was 0.1 Hz.

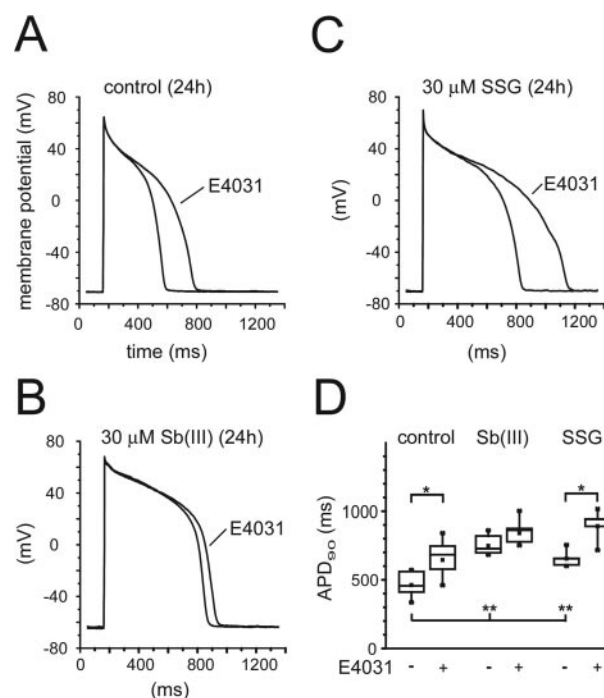


Fig. 5. Long-term exposure to both PAT/Sb(III) and SSG/Sb(V) prolongs the cardiac action potential in isolated guinea pig ventricular myocytes. A, current clamp recordings of action potentials (AP) elicited in an isolated ventricular myocyte cultured overnight (24 h) under control conditions and after extracellular perfusion of 5 μ M E4031. Membrane potential (m.p.) was -70 mV. B, cardiac APs elicited in the absence and presence of 5 μ M E4031 in a ventricular myocyte cultured overnight (24 h) in medium containing 30 μ M PAT/Sb(III) (m.p. -65 mV). C, cardiac APs elicited in the absence and presence of 5 μ M E4031 in a ventricular myocyte cultured overnight in medium containing 30 μ M SSG/Sb(V) (m.p. -70 mV). Action potentials shown in A–C were elicited at a stimulation rate of 0.1 Hz. D, quantitative analysis of action potential duration APD_{90} under control conditions and after exposure to either 30 μ M PAT/Sb(III) or 30 μ M SSG/Sb(V) ($n = 4$ –7). E4031 (5 μ M) was used to determine prolongation of cardiac action potentials on blockade of native I_{K_r} /hERG currents. APD_{90} was significantly prolonged in myocytes cultured overnight in the presence of either 30 μ M Sb(III) or 30 μ M SSG (Dunnett's test, $** p < 0.05$). Block of hERG/ I_{K_r} currents by E4031 prolonged the cardiac action potentials significantly in myocytes cultured under control conditions and in the presence of 30 μ M SSG (Student's t test, $* p < 0.05$) but not on long-term exposure to 30 μ M PAT/Sb(III). Data are represented as statistical box charts with asterisks representing outliers, whiskers determining the 5th and 95th percentile, and boxes determining the 25th and 75th percentile. Means are represented by squares.

for PAT/Sb(III) but not SSG/Sb(V), we used the specific blocker E4031 to evaluate the contribution of hERG/I_{Kr} currents to cardiac repolarization after long-term drug exposure. Ventricular myocytes cultured under control conditions express robust hERG/I_{Kr} currents (Ficker et al., 2003). Consequently, 5 μ M E4031 prolonged APD₉₀ from 464 ± 34 to 645 ± 50 ms ($n = 7$; Fig. 5A). In myocytes cultured in the presence of 30 μ M PAT/Sb(III), APD₉₀ was not significantly prolonged by E4031 (PAT/Sb(III): 748 ± 30 ms; PAT/Sb(III) + E4031: 841 ± 38 ms, $n = 6$, Fig. 5B) commensurate with a PAT/Sb(III)-induced reduction of hERG/I_{Kr} currents. In contrast, action potentials remained exquisitely sensitive to E4031 in SSG/Sb(V)-treated myocytes (Fig. 5C). Five micromolar E4031 prolonged APD₉₀ significantly from 655 ± 35 to 892 ± 64 ms ($n = 4$) underscoring the idea that hERG/I_{Kr} currents are not modified by long-term exposure to physiologically relevant concentrations of SSG/Sb(V).

Effects of PAT/Sb(III) and SSG/Sb(V) on Cardiac Membrane Currents. Our analysis of cardiac action potentials elicited in the absence and presence of the specific blocker E4031 indicated that hERG/I_{Kr} currents may be reduced in cardiomyocytes on long-term exposure to PAT/Sb(III). To test directly for changes of hERG/I_{Kr}, we performed voltage clamp experiments in isolated guinea pig ventricular myocytes. hERG/I_{Kr} currents were activated with depolarizing test pulses to +60 mV and isolated as E4031-sensitive tail currents on return to -40 mV. On long-term exposure to 30 μ M PAT/Sb(III), I_{Kr} current density was dramatically reduced from 0.36 ± 0.05 pA/pF to 0.04 ± 0.02 pA/pF ($n = 3$). Because PAT/Sb(III) is toxic and not well tolerated by myocytes, we controlled for specificity by analyzing the cardiac inward rectifier current I_{K1}. We found that I_{K1} currents were not affected by long-term exposure to PAT/Sb(III). At -100 mV, we measured I_{K1} current densities of -13.3 ± 1.6 pA/pF in myocytes cultured under control conditions and -12.3 ± 1.2 pA/pF on overnight exposure to 30 μ M PAT/Sb(III) ($n = 6-7$).

Given the chemical similarities between trivalent antimony and arsenic, which increases cardiac calcium currents on long-term exposure, we also examined the effects of PAT/Sb(III) on calcium currents. On overnight exposure, calcium current density more than doubled at 10 mV from -3.2 ± 0.3 pA/pF ($n = 6$) in control myocytes to -7.8 ± 1.4 pA/pF ($n = 16$) in myocytes cultured in the presence of 30 μ M PAT/Sb(III) (Fig. 6). The increase in amplitudes was accompanied by small gating changes affecting current activation as well as inactivation and could be prevented by inclusion of 5 mM NAC in PAT/Sb(III) containing incubation medium. Overnight incubation with 5 mM NAC alone did not alter calcium current amplitudes (Supplementary Fig. S1). In myocytes exposed to PAT/Sb(III)/NAC, current density was -3.8 ± 0.3 pA/pF, on average, and was not significantly different from current densities recorded under control conditions (Dunnett's, $n = 4$, Fig. 6D). To study the time-dependent development of PAT/Sb(III) effects more closely, we exposed ventricular myocytes for 3 h to 30 μ M PAT/Sb(III) and compared cardiac calcium current densities to time-matched controls. In these experiments, we found a small increase in peak calcium current densities from -4.6 ± 0.2 to -5.6 ± 0.6 pA/pF when measured at +20 mV ($n = 11$, Fig. 6E) which points toward a fast post-translational modification such as

phosphorylation of the calcium channel complex by PAT/Sb(III).

In contrast to calcium currents, changes in cardiac sodium currents were negligible. When measured at -20 mV, which corresponds to the peak of the I-V relationship, current density was -136 ± 15 pA/pF in control myocytes ($n = 9$) and -166 ± 23 pA/pF on exposure to 30 μ M PAT/Sb(III) ($n = 6$) attesting to the specificity of the observed effects. Taken together, long-term exposure to PAT/Sb(III) increases cardiac excitability in vitro in a manner similar to As₂O₃ albeit at much higher concentrations.

Pentavalent antimony differs substantially from this pattern, because in current clamp experiments with E4031, hERG/I_{Kr} currents seemed to be preserved in myocytes treated overnight with 30 μ M SSG/Sb(V), although action potentials were prolonged. To confirm and expand on this observation we recorded hERG/I_{Kr} currents as E4031-sensitive tail current components and found that current density was not different between control myocytes and myocytes

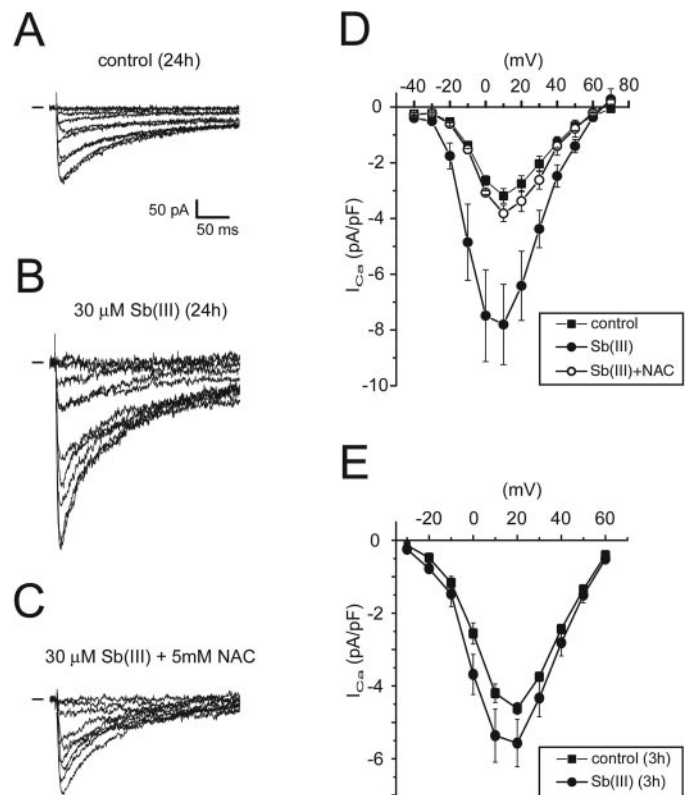


Fig. 6. Long-term exposure to PAT/Sb(III) increases cardiac calcium currents. A, series of cardiac calcium currents recorded in a guinea pig ventricular myocyte after overnight culture under control conditions. B, cardiac calcium currents measured in a ventricular myocyte after overnight exposure to 30 μ M PAT/Sb(III). C, the antioxidant *N*-acetyl-L-cysteine (NAC) attenuates increases in calcium currents on long-term exposure to PAT/Sb(III). Current traces were elicited using 300-ms step depolarizations (-30 to +70 mV) in increments of 10 mV from a holding potential of -40 mV. D, averaged I-V relationships ($n = 4-6$) of calcium currents recorded in ventricular myocytes cultured overnight under control conditions or in the presence of either 30 μ M PAT/Sb(III) or 30 μ M PAT/Sb(III)/5 mM NAC. Note that overnight incubation with 5 mM NAC alone does not affect cardiac calcium currents. E, increase in cardiac calcium currents induced by 3-h exposure to 30 μ M PAT/Sb(III). Averaged I-V relationships were determined from cardiac calcium currents elicited with 300-ms depolarizations (-30 to +60 mV) in increments of 10 mV from a holding potential of -40 mV in myocytes cultured for 3 h in the absence and presence of 30 μ M PAT/Sb(III) ($n = 11$). Zero current lines indicated by dash to the left of current traces shown in A to C.

cultured overnight in the presence of SSG/Sb(V). We measured 0.36 ± 0.05 pA/pF under control conditions and 0.43 ± 0.08 pA/pF on long-term exposure to $30 \mu\text{M}$ SSG/Sb(V) ($n = 3-5$, Fig. 7, A and B). Likewise, two additional cardiac potassium currents, the slow delayed rectifier I_{Ks} and the inward rectifier I_{K1} , were not affected by long-term exposure to SSG/Sb(V). For I_{Ks} , we measured at the end of depolarizations to $+60$ mV current densities of 9.1 ± 1.2 pA/pF under control conditions and 7.8 ± 0.4 pA/pF on exposure to $30 \mu\text{M}$ SSG/Sb(V) ($n = 4-5$, Fig. 7, C and D). For I_{K1} , we measured -12.4 ± 1.1 pA/pF in SSG/Sb(V)-treated myocytes at -100 mV, which was not different from -13.3 ± 1.6 pA/pF measured in controls ($n = 6-9$).

Because three major cardiac potassium currents were not affected, the prolongation of action potential duration by long-term exposure to SSG/Sb(V) exposure prompted us to study depolarizing cardiac sodium and calcium currents as

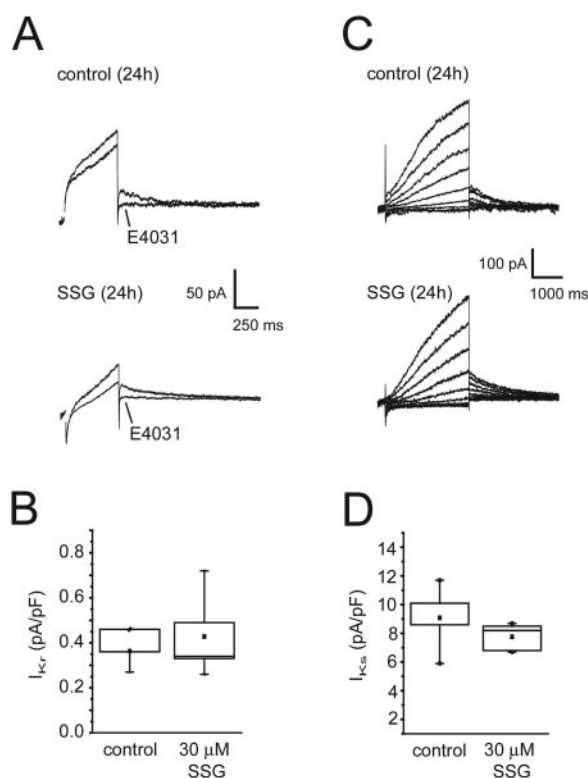


Fig. 7. Long-term exposure to SSG/Sb(V) does not affect amplitude of native hERG/ I_{Kr} or I_{Ks} currents in cultured guinea pig ventricular myocytes. **A**, isolation of E4031-sensitive I_{Kr} current in ventricular myocytes cultured overnight under control conditions or in the presence of $30 \mu\text{M}$ SSG/Sb(V). Current traces were elicited with voltage steps to $+60$ mV from a holding potential (h.p.) of -40 mV in the absence and presence of $5 \mu\text{M}$ E4031. **B**, quantification of I_{Kr} current densities measured as E4031 sensitive tail currents on return to -40 mV in ventricular myocytes cultured overnight under control conditions or in the presence of $30 \mu\text{M}$ SSG/Sb(V) ($n = 3-6$). I_{Kr} current density was not significantly different in control and SSG treated myocytes at 0.05 level (Student's t test). **C**, outward currents elicited with depolarizing step pulses (-30 to $+60$ mV, 10 -mV increments, h.p. -40 mV) after extracellular application of $5 \mu\text{M}$ E4031 in guinea pig ventricular myocytes. Myocytes were cultured overnight (24 h) either under control conditions or in the presence of $30 \mu\text{M}$ SSG/Sb(V). I_{Ks} current amplitudes were measured in the presence of E4031 at the end of depolarizing test pulses to $+60$ mV. **D**, quantification of I_{Ks} current densities at $+60$ mV measured in myocytes cultured overnight under control conditions and in the presence of $30 \mu\text{M}$ SSG/Sb(V) ($n = 4-6$). I_{Ks} current density was not significantly different in control and SSG treated myocytes at 0.05 level (Student's t test). Data in **B** and **D** are represented as statistical box charts.

potential targets. Sodium currents were elicited using short depolarizing test pulses that followed on a conditioning pre-pulse to -140 mV. I-V relationships showed increases in current amplitudes on exposure to $30 \mu\text{M}$ SSG/Sb(V) without major changes in current gating or kinetics (Supplementary Fig. S2). At -20 mV, current density increased from -136 ± 15 pA/pF in control myocytes to -213 ± 22 pA/pF measured in SSG/Sb(V)-treated cells ($n = 9$). In addition, we found a marked increase in cardiac calcium currents that developed slowly overnight (Fig. 8A). At $+10$ mV, calcium current density increased from -3.2 ± 0.26 to -5.0 ± 0.31 pA/pF on long-term exposure to $30 \mu\text{M}$ SSG/Sb(V) ($n = 6$). It is noteworthy that SSG/Sb(V)-induced increases in calcium currents could be blunted by inclusion of 5 mM NAC in SSG/Sb(V) containing incubation media. In experiments with NAC, current density was -2.54 ± 0.46 pA/pF and not significantly different from control values ($n = 3$, Fig. 8B).

The observation that SSG/Sb(V) effects were reduced by the antioxidant NAC in a manner similar to trivalent PAT/Sb(III) was unexpected, because pentavalent SSG/Sb(V) represents the highest oxidation state of antimony and should not readily partake in NAC sensitive redox reactions. However, it has been suggested that pentavalent antimony has to be reduced into the more toxic trivalent Sb(III) to gain therapeutic activity (Goodwin and Page, 1943). We considered this possibility and estimated a putative conversion rate using our Western blot analysis of hERG trafficking. We assumed that the approximately 100-fold difference in IC_{50} values measured between SSG/Sb(V) and PAT/Sb(III) ($10-11$ mM and $62.6 \mu\text{M}$, respectively; see Fig. 3C) on Western blots might be caused by small amounts of inactive Sb(V) reduced in vitro into active Sb(III). This assumption led us to expose isolated guinea pig ventricular myocytes to $0.3 \mu\text{M}$ PAT/Sb(III) calculated according to a conversion rate of 1% with the original dose of $30 \mu\text{M}$ SSG/Sb(V) as a reference. We found that $0.3 \mu\text{M}$ PAT/Sb(III) was sufficient to increase cardiac calcium currents on long-term exposure (Fig. 9A).

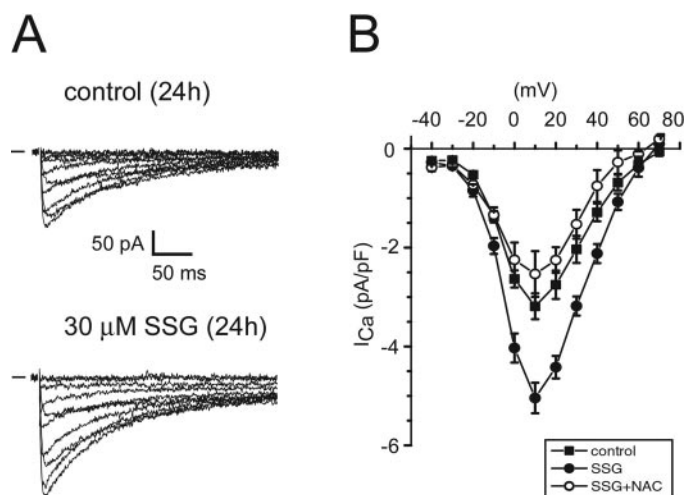


Fig. 8. Long-term exposure to SSG/Sb(V) increases cardiac calcium currents. **A**, series of cardiac calcium currents recorded in guinea pig ventricular myocytes after overnight culture under control conditions or on long-term exposure to $30 \mu\text{M}$ SSG/Sb(V). Current traces were elicited using 300 -ms step depolarizations (-30 to $+70$ mV) in increments of 10 mV (h.p. -40 mV). **B**, averaged I-V relationships ($n = 4-6$). Note that the antioxidant NAC (5 mM) attenuates the increase in calcium currents observed on long-term exposure to SSG/Sb(V). Zero current lines indicated by dash to the left of current traces shown in **A**.

When measured at +10 mV, calcium current density was -4.8 ± 0.7 pA/pF ($n = 7$) in control myocytes and increased by approximately 52% to -7.3 ± 0.5 pA/pF ($n = 10$) in the presence of $0.3 \mu\text{M}$ PAT/Sb(III) (Fig. 9B), which is similar to an increase of 56% measured in myocytes cultured overnight in the presence of $30 \mu\text{M}$ SSG/Sb(V) (see Fig. 8). Thus, the possibility exists that SSG/Sb(V) indeed acts as a prodrug for Sb(III) in our experiments.

Discussion

In the present study, we analyzed the effects of two antimony-based antileishmanial compounds, trivalent PAT/Sb(III) and pentavalent SSG/Sb(V), on cardiac membrane currents. We show that both compounds prolong the action potential of guinea pig ventricular myocytes at therapeutically relevant concentrations for the treatment of leishmaniasis via an increase in cardiac calcium currents. The proarrhythmic role played by an increase in cardiac calcium currents has been recognized and described in detail more recently in Timothy syndrome, a novel arrhythmia disorder associated with gain-of-function mutations in the cardiac L-type calcium channel gene $\text{Ca}_v1.2$ (Splawski et al., 2004, 2005). In the heart, calcium currents regulate the plateau phase of the cardiac action potential and increased amplitudes produce a delay in cardiac repolarization, which may explain the propensity of patients treated with antimonial compounds to develop QT prolongation and life-threatening arrhythmias. In our experiments, cardiac calcium currents proved to be especially sensitive to the trivalent antimony compound PAT. In fact, $0.3 \mu\text{M}$ PAT/Sb(III) was sufficient to increase cardiac calcium currents by approximately 50%. This provides the rationale for proarrhythmic events reported in the past with therapeutic concentrations of PAT/Sb(III) during therapy of schistosomiasis, another tropical parasitic infection (Schulert et al., 1966). PAT/Sb(III) induced increases in calcium currents could be blunted by the

antioxidant NAC consistent with the well known property of trivalent antimony to produce oxidative stress via an increase in ROS production (Mueller et al., 1998). Although further studies will be necessary to elucidate the precise molecular mechanism(s) underlying the increase in calcium currents observed with trivalent antimony as well as trivalent arsenic compounds (Ficker et al., 2004), it seems that fast post-translational modifications of the channel protein complex are involved as judged from the increase in calcium currents observed on short-term exposure (3 h) to PAT/Sb(III). Both compound classes possess high affinity for sulfhydryl groups and may affect calcium channels by oxidizing cysteine residues located either directly on the channel protein or on a closely associated protein (Miller et al., 2002).

Based on our observation that NAC blunts not only the effects of long-term exposure to Sb(III) but also SSG/Sb(V), we suggest that reductive conversion of Sb(V) into active, cardiotoxic Sb(III) provides the unifying cellular mechanism that links both compounds to produce a common mode of cardiotoxic drug action in patients. In vitro, Sb(III) is present as an impurity in most SSG/Sb(V) preparations (Franco et al., 1995) and/or is generated de novo by reductive conversion of small amounts of Sb(V). Sb(III) contributes prominently, however, to total antimony levels in patients where 10 to 25% of Sb(V) administered during treatment is converted into the active, more toxic Sb(III) (Burguera et al., 1993; Frezard et al., 2001; Miekeley et al., 2002). Consequently, during therapy regimens with daily applications of up to $16 \mu\text{M}$ SSG/Sb(V), Sb(III) should accumulate at rates high enough to produce proarrhythmic increases in cardiac calcium current amplitudes.

Although de novo generated Sb(III) seems to mediate the increase in calcium currents observed with SSG/Sb(V), cardiac sodium currents seem to be modified directly by pentavalent Sb(V) species because sodium currents were not significantly affected by long-term exposure to either trivalent PAT/Sb(III) or $\text{As}_2\text{O}_3/\text{As(III)}$ (Ficker et al., 2004). Pentavalent antimony compounds are known to inhibit cellular phosphatases and further experiments will be required to determine whether this is the mechanism whereby sodium currents are affected (Pathak et al., 2002). It is important to note that the observed increase in sodium current amplitude is not likely to contribute to proarrhythmic cardiac events because it was not accompanied by any obvious change in channel gating that is known to prolong cardiac repolarization (Balsler, 2001).

In marked contrast to As_2O_3 , which inhibited hERG trafficking within its therapeutic concentration range of 0.1 to $1 \mu\text{M}$ (Shen et al., 1997), we were surprised to find that heterologously expressed hERG currents were not affected by long-term exposure to SSG/Sb(V) in concentrations up to $300 \mu\text{M}$. Long-term exposure to PAT/Sb(III) reduced hERG trafficking on Western blots and hERG currents in electrophysiological experiments although with IC_{50} values well beyond free serum concentrations. In general, PAT/Sb(III) seems to have less of an effect on hERG than As_2O_3 . For example, changes in hERG deactivation were apparent within minutes on exposure to $10 \mu\text{M}$ As_2O_3 but developed slowly over 3 h with $30 \mu\text{M}$ PAT/Sb(III). Because changes in hERG gating have been previously linked to ROS production (Taglialatela et al., 1997; Berube et al., 2001) and because both gating changes and trafficking inhibition were blunted by the ROS

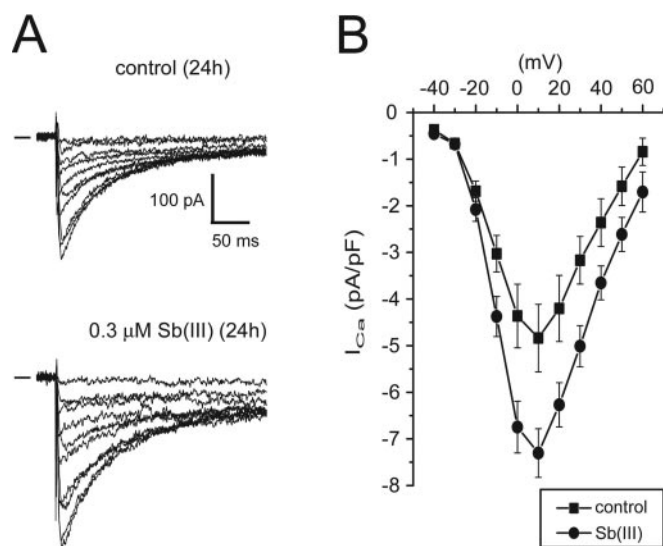


Fig. 9. Cardiac calcium currents are exquisitely sensitive to PAT/Sb(III). A, series of cardiac calcium currents measured in guinea pig ventricular myocytes after overnight culture under control conditions or on long-term exposure to $0.3 \mu\text{M}$ PAT/Sb(III). Current traces were elicited using 300 ms step depolarizations (-30 to $+70$ mV) in increments of 10 mV (h.p. -40 mV). B, averaged I-V relationships ($n = 4-6$). Zero current lines indicated by dash to the left of current traces shown in A.

scavenger NAC, we conclude that PAT/Sb(III) effects on hERG currents are mediated by a modest increase in ROS production (Tirmenstein et al., 1995; Liu et al., 2001; Lecœur et al., 2002). The low sensitivity of hERG to Sb(III) may also explain our failure to detect effects with SSG/Sb(V) because Sb(III) concentrations may simply not accumulate to high enough levels during experiments/therapy with SSG/Sb(V) to affect hERG/ I_{Kr} currents.

Our hypothesis that Sb(III) ions are the main culprit for the cardiotoxic increase in cardiac calcium currents has important consequences for quality assessment and control of therapeutic pentavalent antimony products, because it suggests that variations in toxicity observed between different compound batches/lots may be closely related to residual Sb(III) content and therefore should be monitored carefully (Sundar et al., 1998). Likewise, the slow onset of cardiotoxic events in the clinic days after initiation of therapy with Sb(V) compounds may be related to the predicted slow build-up of more toxic Sb(III) in patients (Rees et al., 1980; Ortega-Carnicer et al., 1997). A slow onset of electrographic changes has also been reported in a long-term exposure guinea pig model (Alvarez et al., 2005). It is noteworthy that in this animal model, the cardiotoxicity of both Sb(III) and Sb(V) could be attenuated by the cardioprotective antioxidant L-carnitine, similar to what we report here for the antioxidant NAC. However, in contrast to our observations, prolongation of action potential duration in guinea pigs was accompanied by a decrease in cardiac calcium currents after an 8-day exposure to Sb(III). This difference may be explained best by the different exposure times used. Moreover, long-term exposure to antimonial compounds was associated with symptoms of cardiomyopathy, including fibrosis, which may cause additional remodeling of cardiac membrane currents (Alvarez et al., 2005). We believe that the increase in calcium currents described in our study reflects the initial change in cardiac excitability on exposure to both trivalent and pentavalent antimony compounds.

The proarrhythmic action of antimonial compounds such as PAT/Sb(III) and SSG/Sb(V) constitutes another paradigm for acquired LQT syndrome. Antimonial compounds represent the first example of a compound class that exclusively targets cardiac calcium currents at therapeutic concentrations. Consequently, prophylactic use of calcium antagonists may be considered beneficial to avert or combat adverse cardiac events precipitated by antimony-based drugs, possibly in combination with cardioprotective antioxidants such as L-carnitine or NAC, which has supposedly shown minimal effects on the antiparasitic action of SSG/Sb(V) (Roberts et al., 1995). Our study also has direct consequences for the preclinical assessment of cardiac risk in the development of novel drug candidates, which is currently focused narrowly on hERG channels as target for either direct block or possibly acquired trafficking defects (European Medicines Agency, 2004; Fenichel et al., 2004; Kuryshev et al., 2005). In contrast, our data favor a careful evaluation of drug-induced cardiac risk that does not rely exclusively on the hERG assay but instead uses more complex systems, such as cardiac myocytes and/or carefully performed QT studies in whole animals, to produce a fair representation of the actual risk associated with the use of novel therapeutic entities.

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