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

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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/I₉, 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/I₉, 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 (Glucantine; Sanofi-Aventis, Bridgewater, NJ) and sodium stibogluconate (Pentostam; GlaxoSmith-Kline, Uxbridge, Middlesex, UK) (Herwaldt, 1999; Guerin et al., 2002). Although pentavalent antimonial 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...
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 antipyrine arsenic trioxide (As₂O₃), which produced inhibition of hERG/IKr 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 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_{K_r}, I_{K_s}, and I_{K_1}) are not affected. This is the first example of drug-induced LQTSs 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<sub>α</sub> 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 MgCl₂, 1 mM CaCl₂, 10 mM EGTA, and 10 mM HEPES, pH 7.2. The extracellular solution was 140 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES, and 10 mM glucose, pH 7.4. To study the duration of drug effects on hERG currents, drug was added for 16 to 20 h (overnight) before recording. Sodium atibigluconate [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_{K_r}, I_{K_s}, and I_{K_1} 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 MgCl₂, 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 MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.4. L-type Ca<sup>2+</sup> currents were blocked with 1 μM nisoldipine. The specific hERG/IKr blocker E4031 was used to isolate IK<sub>r</sub> currents. Cardiac sodium currents were recorded using the following extracellular solution: 40 mM NaCl, 55 mM N-methyl-D-glucamine, 20 mM CaCl₂, 5.4 mM KCl, 2 mM MgCl₂, 0.02 mM CaCl₂, 30 mM tetraethylammonium chloride, 5 mM 4-aminopyridine, and 10 mM HEPES, pH 7.4. The intracellular solution was 120 mM CaCl₂, 2 mM MgCl₂, 11 mM CaCl₂, 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 CaCl₂, 1.6 mM MgCl₂, 1.8 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4. The intracellular solution was 130 mM CsMeSO₄, 20 mM tetraethylammonium chloride, 1 mM MgCl₂, 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 ± 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/I<sub>max</sub> = [1/(1 + IC₅₀<sup>1/n</sup>)], where I indicates current or image densities, D is the drug concentration, n<sub>H</sub> is the Hill coefficient, and IC₅₀ 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 [As₂O₃/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 As2O3, current amplitudes were reduced in a concentration-dependent manner with an IC50 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 μg/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

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**Fig. 1.** Prolonged exposure to trivalent potassium antimony tartrate PAT/Sb(III) reduces hERG currents stably expressed in HEK293 cells. A, hERG tail currents elicited 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 under control conditions (±) or in the presence of extracellularly applied 30 μM PAT/Sb(III) (Student’s t test, p < 0.05, n = 5–7). 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).

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<tr>
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<th>Concentration (μM)</th>
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<td>30</td>
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**Notes:**
- Student’s t test, p < 0.05.
- ns at 0.05 level.
- Cell numbers analyzed given in parenthesis.
and correspond to approximately 12 to 16 \mu M SSG/Sb(V) (Berman and Wyler, 1980; Berman et al., 1982; Jaser et al., 1995).

To test for direct block, we applied 100 \mu M 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 \mu M 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 \mu M PAT/Sb(III) induced prominent changes in hERG trafficking, hERG currents were not affected by long-term exposure to 30 \mu M 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 IC50 of 62.3 \mu M (Fig. 3C, n = 3–4). However, the level of mature, fully-glycosylated hERG protein was not altered by SSG up to 300 \mu M in accordance with electrophysiological observations. At higher SSG/Sb(V) concentrations, moderate hERG trafficking defects were detected. We extrapolated an IC50 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 expression, 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 \mu M 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 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 \mu M 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 \mu M 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. 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 \mu M 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 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 \mu M 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 \mu M 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 155-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). Although overnight treatment with PAT/Sb(III) reduced hERG surface expression, SSG/Sb(V) has no effect on hERG currents stably expressed in HEK293 cells.

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expression in a concentration-dependent manner, SSG/Sb(V) was without effect at concentrations up to 100 μM (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 ADP₉₀ was not affected by rapid application of extracellular PAT/Sb(III) (Fig. 4A). ADP₉₀ was 486 ± 129 ms under control conditions and 492 ± 133 ms after 5-min superfusion with 30 μM PAT/Sb(III) (n = 3). Likewise, 100 μM SSG was without effect. ADP₉₀ 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 μM PAT/Sb(III) and 100 μM SSG/Sb(V) rapidly with the extracellular perfusate to freshly isolated ventricular myocytes and determined short-term block of three major cardiac current components: Iₖᵣ, Iₖₛ, and Iₖᵣ. 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 μM PAT/Sb(III) or 30 μM SSG/Sb(V) (Fig. 5). Action potential duration ADP₉₀ 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 ADP₉₀ 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/Iₖᵣ as a possible target for SSG/Sb(V), we further investigated the long-term effects of SSG/Sb(V) on cardiac action potentials in myocytes cultured overnight in the presence of SSG/Sb(V) and compared those results with those of PAT/Sb(III) to establish the extent to which the long QT syndrome may be directly linked to the reported prolongation of the QT interval during therapy with SSG/Sb(V).

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 μM 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 μM SSG/Sb(V). E4031 (5 μM) was used to block native Iₖᵣ/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 μM E4031. Membrane potential (m.p.) was −70 mV. B, cardiac APs elicited in the absence and presence of 5 μM E4031 in a ventricular myocyte cultured overnight (24 h) in medium containing 30 μM PAT/Sb(III) (m.p. −65 mV). C, cardiac APs elicited in the absence and presence of 5 μM E4031 in a ventricular myocyte cultured overnight in medium containing 30 μM 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 ADP₉₀ under control conditions and after exposure to either 30 μM PAT/Sb(III) or 30 μM SSG/Sb(V) (n = 4–7). E4031 (5 μM) was used to determine prolongation of cardiac action potentials on blockade of native Iₖᵣ/hERG currents. ADP₉₀ was significantly prolonged in myocytes cultured overnight in the presence of either 30 μM Sb(III) or 30 μM SSG (Student’s t-test, *p < 0.05). Block of hERG/Iₖᵣ currents by E4031 prolonged the cardiac action potentials significantly in myocytes cultured under control conditions and in the presence of 30 μM SSG (Student’s t test, **p < 0.05). 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 expressed robust hERG/I_{Kr} currents (Ficker et al., 2003). Consequently, 5 μM E4031 prolonged APD_{90} from 464 ± 34 to 645 ± 50 ms (n = 7; Fig. 5A). In myocytes cultured in the presence of 30 μM PAT/Sb(III), APD_{90} 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 APD_{90} 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 μM 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_{Kr}. We found that I_{Kr} currents were not affected by long-term exposure to PAT/Sb(III). At −100 mV, we measured I_{Kr} 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 μM 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 cardiac 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 μM 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 cardiac 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 μM 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 μM 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 As_{2}O_{3} 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 μM 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...
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 μM 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 $I_{Kr}$, were not affected by long-term exposure to SSG/Sb(V). For $I_{Kr}$, 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 μM SSG/Sb(V) (n = 4–5, Fig. 7, C and D). For $I_{Ks}$, 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 potential targets. Sodium currents were elicited using short depolarizing test pulses that followed on a conditioning prepulse to −140 mV. I-V relationships showed increases in current amplitudes on exposure to 30 μM 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 μM 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 IC50 values measured between SSG/Sb(V) and PAT/Sb(III) (10–11 mM and 62.6 μM, 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 μM PAT/Sb(III) calculated according to a conversion rate of 1% with the original dose of 30 μM SSG/Sb(V) as a reference. We found that 0.3 μM PAT/Sb(III) was sufficient to increase cardiac calcium currents on long-term exposure (Fig. 9A).

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 μM 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 μM 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 μM 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 μM E4031 in guinea pig ventricular myocytes. Myocytes were cultured overnight (24 h) either under control conditions or in the presence of 30 μM SSG/Sb(V). $I_{Kr}$ current amplitudes were measured in the presence of 30 μM SSG/Sb(V). $I_{Kr}$ current densities were measured at +60 mV measured in myocytes cultured overnight under control conditions and in the presence of 30 μM SSG/Sb(V) (n = 4–6). $I_{Kr}$ 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.

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 μM 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 $-4.8 \pm 0.7$ pA/pF ($n = 7$) in control myocytes and increased by approximately 52% to $-7.3 \pm 0.5$ pA/pF ($n = 10$) in the presence of 0.3 $\mu$M PAT/Sb(III) (Fig. 9B), which is similar to an increase of 56% measured in myocytes cultured overnight in the presence of 30 $\mu$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 Cav1.2 (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 $\mu$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 cardiac 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; Freizard et al., 2001; Miekeley et al., 2002). Consequently, during therapy regimens with daily applications of up to 16 $\mu$M SSG/Sb(V), Sb(III) should accumulate at rates high enough to produce pro-arrhythmic 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 Sb(V) or As$_2$O$_3$/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 pro-arrhythmic cardiac events because it was not accompanied by any obvious change in channel gating that is known to prolong cardiac repolarization (Balser, 2001).

In marked contrast to As$_2$O$_3$, which inhibited hERG trafficking within its therapeutic concentration range of 0.1 to 1 $\mu$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 $\mu$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$_2$O$_3$. For example, changes in hERG deactivation were apparent within minutes on exposure to 10 $\mu$M As$_2$O$_3$ but developed slowly over 3 h with 30 $\mu$M PAT/Sb(III). Because changes in hERG gating have been previously linked to ROS production (Tagliatalata et al., 1997; Berube et al., 2001) and because both gating changes and trafficking inhibition were blunted by the ROS...
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 electrophysiological changes has also been reported in a long-term 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 antioxidon 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 antimonal 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 antimonal compounds.

The proarrhythmic action of antimonal 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 presumably shown minimal effects on the paraparasitic 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; Fenerich et al., 2004; Kuryshov 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 myocardocytes and/or carefully performed PTQ studies in whole animals, to produce a fair representation of the actual risk associated with the use of novel therapeutic entities.

References
Mueller S, Miller WH, and Dejean A (1998) Trivalent antimonials induce degradative events in ASPET Journals on June 16, 2017 molpharm.aspetjournals.org Downloaded from


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Fig S1

The figure shows the relationship between membrane potential (mV) and calcium current ($I_{Ca}$) measured in pA/pF. The graph compares control conditions with NAC 5mM treatment.
Figure S1  Chronic exposure to 5mM of the anti-oxidant NAC does not affect cardiac calcium currents. Averaged IV relationships determined from cardiac calcium currents elicited with 300ms depolarizations (-30 to +60mV, 10mV increments) from a holding potential of –40mV in guinea pig ventricular myocytes cultured overnight in the absence and presence of 5mM NAC (n=18).

Figure S2  Chronic exposure to SSG/Sb(V) increases cardiac sodium currents. A, series of cardiac sodium currents elicited in a guinea pig ventricular myocyte after overnight culture under control conditions and in a myocyte exposed overnight to 30µM SSG/Sb(V). Current traces were elicited using step depolarizations (-80 to +20mV) in increments of 10mV following on a pre-pulse to –140mV (h.p. –40mV). B, averaged I-V relationships (n=9). Note that SSG/Sb(V) causes a significant increase in cardiac sodium currents. Zero current lines indicated by dash to the left of current traces shown in A.
**Table 1:**

*Acute block of cardiac membrane currents by SSG/PAT*

<table>
<thead>
<tr>
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<th>Control (pA/pF)</th>
<th>100 µM SSG (pA/pF)</th>
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<tr>
<td>$I_{K_r}$</td>
<td>1.40 ± 0.12</td>
<td>1.34 ± 0.11</td>
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<tr>
<td>$I_{K_s}$</td>
<td>3.28 ± 0.25</td>
<td>3.40 ± 0.20</td>
<td>3</td>
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<tr>
<td>$I_{Ca}$</td>
<td>-6.48 ± 0.55</td>
<td>-6.06 ± 0.43</td>
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<table>
<thead>
<tr>
<th></th>
<th>Control (pA/pF)</th>
<th>30 µM PAT (pA/pF)</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>$I_{K_r}$</td>
<td>1.43 ± 0.26</td>
<td>1.46 ± 0.26</td>
<td>3</td>
</tr>
<tr>
<td>$I_{K_s}$</td>
<td>3.92 ± 0.88</td>
<td>4.00 ± 0.78</td>
<td>3</td>
</tr>
<tr>
<td>$I_{Ca}$</td>
<td>-5.99 ± 0.35</td>
<td>-5.54 ± 0.54</td>
<td>9</td>
</tr>
</tbody>
</table>

$I_{K_r}$ **tail currents** were not affected by acute application (5min) of either 100µM SSG or 30 µM PAT/Sb(III). E4031 sensitive $I_{K_r}$ tail currents were elicited at –40mV following on 650ms, depolarizing test pulses to +60mV in freshly isolated guinea pig ventricular myocytes from a holding potential of −40mV.

$I_{K_s}$ **tail currents** were not affected by acute application (5min) of either 100µM SSG or 30 µM PAT/Sb(III). E4031 resistant tail currents were elicited at –40mV following on 2500ms, depolarizing test pulses to +60mV in freshly isolated guinea pig ventricular myocytes from a holding potential of −40mV.

$I_{Ca}$ **peak inward currents** were not affected by acute application (5min) of either 100µM SSG or 30 µM PAT/Sb(III). Cardiac calcium currents were elicited with 300ms depolarizing test pulses in freshly isolated guinea pig ventricular myocytes from a holding potential of −40mV.

All current amplitudes have been normalized to cell capacitances and are presented as mean ± SEM. Student’s t-test has been used to test for differences in current densities under control condition and in the presence of extracellularly applied SSG or PAT/Sb(III). We did not detect any significant block of cardiac $I_{K_r}$, $I_{K_s}$ or $I_{Ca}$ currents at the 0.05 level on acute perfusion of either 100 µM SSG or 30 µM PAT.