

**SIGMA RECEPTOR ACTIVATION BLOCKS POTASSIUM CHANNELS AND DEPRESSES
NEUROEXCITABILITY IN RAT INTRACARDIAC NEURONS***

by

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Abbreviations: σ receptor: Sigma receptor; Kv: voltage-activated K⁺ channels; K_(DR): delayed outwardly-rectifying K⁺ channels; K_(Ca): Ca²⁺-sensitive K⁺ channel; BK: large conductance K_(Ca) channels; SK: small conductance K_(Ca) channels; IK: intermediate conductance K_(Ca) channels I_M: M-current; BK_i: fast inactivating BK current; BK_s: non-inactivating BK currents; AHP: afterhyperpolarization; TTX: tetrodotoxin; GDP- β -S: guanosine 5'-O-(2-thiodiphosphate) trilithium salt; DTG: 1,3-Di-O-tolyguanidin; TEA: tetraethylammonium chloride.

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ABSTRACT

Sigma (σ) receptors have been implicated in the regulation of the cardiovascular system and σ -1 receptor transcripts have been found in parasympathetic intracardiac neurons. However, the cellular function of σ -1 receptors in these cells remains to be determined. Effects of σ receptor activation on voltage-activated K^+ channels and action potential firing were studied in isolated intracardiac neurons using whole-cell patch-clamp recording techniques. Activation of σ receptors reversibly blocked delayed outwardly-rectifying potassium channels, large conductance Ca^{2+} -sensitive K^+ channels, and the M-current with maximal inhibition >80%. The inhibition of K^+ channels by σ ligands was dose-dependent, and the rank order potency of (+)-pentazocine > ibogaine > DTG suggests that the effect is mediated by σ -1 receptor activation. Preincubation of neurons with the irreversible σ receptor antagonist, metaphit, blocked DTG-mediated inhibition of K^+ channels, confirming that the effect is mediated by σ receptor activation. While bath application of σ ligands depolarized intracardiac neurons, the number of action potentials fired by the cells in response to depolarizing current pulses was decreased in the presence of these drugs. Neither dialysis of the neurons nor application of intracellular GDP- β -S inhibited the effect of σ receptors on K^+ channels, which suggests that the signal transduction pathway does not involve a diffusible cytosolic second messenger or a G-protein. Taken together, these data suggest that σ -1 receptors are directly coupled to K^+ channels in intracardiac neurons. Furthermore, activation of σ -1 receptors depresses the excitability of intracardiac neurons, and is thus likely to block parasympathetic input to the heart.

INTRODUCTION

Sigma receptors are non-opioid, non-phencyclidine receptors that are distributed throughout the central and peripheral nervous system. These receptors have high affinity for numerous drugs in clinical use including psychotherapeutic agents such as antipsychotics (Su, 1982) and selective serotonin reuptake inhibitors (Narita et al., 1996). At least two subtypes of σ receptors have been classified based on the findings from biochemical and radioligand binding experiments, and have been designated as σ -1 and σ -2 (Hellewell and Bowen, 1990). However, to date, only the σ -1 receptor has been cloned (Hanner et al., 1996). These receptors have been implicated in physiological processes such as learning and memory and in pathophysiological disorders such as schizophrenia and depression (Snyder and Largent, 1989). More recently, σ receptors have been linked to the development of drug dependence (Ujike et al., 1996).

Sigma receptors also appear to play a role in the regulation of the cardiovascular system, as suggested by pharmacological studies using σ receptor ligands. For example, it has been shown that the σ receptor ligand, DuP 754, raises the threshold of ventricular fibrillation in rats (Lishmanov et al., 1999). Conversely, the σ receptor agonists, (+) SKF-10,047, 1,3-Di-O-tolyguanidin (DTG), and (+)-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine, have been shown to produce tachycardia by activating central and peripheral σ receptors (Wu and Martin, 1989; Lishmanov et al., 2000). Some of the effects of σ receptor ligands on the cardiovascular system are likely due to activation of σ receptors on cardiac muscle. Sigma receptors have been detected in cardiac myocytes from neonatal and adult rats, and stimulation of these receptors was shown to elicit direct inotropic and chronotropic effects on cardiac muscle (Ela et al., 1994; Novakova et al., 1995). However, σ receptors have also been found in autonomic neurons that regulate the cardiovascular system (Zhang and Cuevas, 2002), and modulation of the electrical

activity of these neurons by endogenous or exogenous σ ligands is likely to have profound effects on cardiovascular function.

Sigma receptor ligands, including haloperidol, (+) pentazocine, DTG and ibogaine, were previously shown to inhibit high voltage-activated calcium channels in intrinsic cardiac neurons (Zhang and Cuevas, 2002). The pharmacological profile of the sigma receptor mediating these effects was consistent with a σ -2 receptor. While σ -1 receptor transcripts were detected in intracardiac neurons, no cellular effects have been attributed to this receptor. One possible role for σ -1 receptors in these cells is the modulation of voltage-activated K^+ channels. Recent experiments have shown that currents mediated by Kv1.4 and Kv1.5 are depressed by the σ ligand (+)-SKF 10,047 in oocytes injected with Kv subunits and the σ -1 receptor, but not in oocytes injected with the Kv subunits alone (Aydar et al., 2002). Furthermore, σ -1 receptors were shown to alter the kinetic properties of Kv1.4 channels in the absence of ligand, and Kv1.4 and Kv1.5 coimmunoprecipitated with the σ -1 receptor (Aydar et al., 2002), which led those investigators to propose that the σ -1 receptor functions as a ligand-regulated auxiliary K^+ channel subunit. The inhibition of delayed outwardly rectifying K^+ channels, such as Kv1.5, by verapamil has been shown to depress action potential firing in intrinsic cardiac neurons, and this phenomenon has been implicated in the tachycardia associated with this type IV antiarrhythmic drug (Hogg et al., 1999). However, it remains to be determined if σ -1 receptors couple to voltage-activated K^+ channels in intracardiac neurons, and if σ receptor activation can alter the active membrane properties of these cells. By regulating the function of K^+ channels in parasympathetic intracardiac neurons, σ -1 receptors, and therefore drugs that act on these receptors, may have significant influence on the heart.

Experiments were undertaken to determine the effects of σ receptors on currents mediated by voltage-activated K^+ channels (I_K) and the active membrane properties of intracardiac neurons from neonatal rats. Sigma receptors were shown to decrease the peak

amplitude of currents mediated by delayed outwardly-rectifying K^+ channels ($I_{K(DR)}$), large conductance Ca^{2+} -sensitive K^+ channels (I_{BK}), and the M-current (I_M). The rank-order potency of K^+ channel inhibition by different sigma ligands suggest that the effect is mediated by σ -1 receptors. Neither cell dialysis nor intracellular application of GDP- β -S blocked the effects of σ -1 receptors on I_K , consistent with a direct interaction between σ -1 receptors and the K^+ channels. Activation of σ -1 receptors also decreased action potential firing and changed the action potential configuration in intracardiac neurons. Therefore, σ -1 receptor activation depresses the excitability of intracardiac neurons and is likely to attenuate parasympathetic input to the heart.

METHODS

Preparation

The isolation and culture of neurons from neonatal rat intracardiac ganglia has been described previously (Cuevas and Adams, 1994). Briefly, neonatal rats (2-7 day old) were euthanized by decapitation and the hearts excised and placed in a physiological saline solution containing (mM): 140 NaCl, 3 KCl, 2.5 CaCl₂, 0.6 MgCl₂, 7.7 glucose and 10 histidine; pH to 7.2 with NaOH. Atria were removed and incubated for 1 h at 37 °C in physiological saline solution containing 1 mg/ml collagenase (Type 2, Worthington). Following enzymatic treatment, clusters of ganglia were dissected from the epicardial ganglion plexus and dispersed by trituration in a high glucose culture medium (DMEM), 10% fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The dissociated neurons were then plated onto glass cover slips coated with poly-L-lysine, and incubated at 37 °C under a 95% air-5% CO₂ atmosphere for 48-72 hours.

Electrical recordings

Electrophysiological recording methods used were similar to those previously described (Cuevas et al., 1997). Active membrane properties and voltage-activated K⁺ channel currents in intracardiac neurons were studied under current-clamp and voltage-clamp mode, respectively, using the whole cell patch-clamp technique. Electrical access was achieved through the use of the amphotericin B perforated-patch method to preserve the intracellular integrity and prevent the loss of cytoplasmic components and subsequent alteration of the functional responses of these neurons (Cuevas and Adams, 1994). For perforated-patch experiments, a stock solution of amphotericin B (60mg/ml) in dimethyl sulfoxide (DMSO) was prepared and diluted in pipette solution immediately prior to use to yield a final concentration of 198 µg/ml amphotericin B in

0.33% DMSO. Final patch pipette resistance was 1.0-1.3 M Ω to permit maximal electrical access under the present recording configuration.

Action potentials were elicited by depolarizing current pulses (+100 pA) in absence and presence of sigma ligands. Currents through depolarization-activated K⁺ channels were elicited by step depolarizations from -90 mV to more positive potentials (-50 to +70 mV). For experiments studying the effects of σ receptor activation on I_M, cells were held at -30 mV to activate the channel and repolarized to -60 mV to deactivate the channels. Membrane voltages and currents were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA), filtered at 5 kHz (-3 dB; 4-pole Bessel filter), and digitized at 20 kHz (Digidata 1200 B).

Data analysis

Analyses of these data were conducted using the SigmaPlot 2000 program (SPSS Science, Chicago, IL). Data points represent means \pm SEM. Statistical difference was determined using paired *t*-test for within-group experiments, and unpaired *t*-test for between groups experiments, and was considered significant if $p < 0.05$.

Solutions and reagents

The bath solution for action potential experiments was a physiological saline solution (PSS) containing (in mM) 140 NaCl, 1.2 MgCl₂, 3 KCl, 2.5 CaCl₂, 7.7 glucose and 10 HEPES, pH to 7.2 with NaOH. Potassium channel currents were isolated by adding tetrodotoxin (TTX, 400 nM) and cadmium chloride (CdCl₂, 100 μ M) to the PSS to inhibit voltage-activated Na⁺ channel and Ca²⁺ channel currents, respectively. Pipette solution used for perforated-patch experiments contained (in mM): 75 K₂SO₄, 55 KCl, 5 MgSO₄, and 10 HEPES (adjust to pH 7.2 with N-methyl-d-glucamine). For studies using conventional (dialyzing) whole cell recording configuration, the

pipette solution contained (in mM) 140 KCl, 2 MgCl₂, 2 ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 Mg₂ATP, 0.1 GTP lithium salt (GTP), and 10 HEPES-KOH, pH to 7.2. In some experiments GTP was replaced with 100 μM guanosine 5'-O-(2-thiodiphosphate) trilithium salt (GDP-β-S) to inhibit G protein activation. All chemical reagents used were of analytical grade. Haloperidol; ibogaine hydrochloride; (+)-pentazocine; 1,3-Di-O-tolylguanidine (DTG); 2*S*-(2*a*,6*a*,11*R*^{*}]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride ((+)-SKF 10,047); tetrodotoxin (TTX); tetraethylammonium chloride (TEA) and linopirdine were purchased from Sigma Chemical (St. Louis, MO).

RESULTS

Sigma receptors activation inhibit delayed outward rectifying K⁺ currents

Mammalian intracardiac neurons exhibit currents mediated by delayed outwardly rectifying K⁺ channels (Xi-Moy and Dun, 1995; Hogg et al., 1999) and express various subunits that contribute to these channels, including KCNA5 and KCNA6 (Cuevas, unpublished observation). Given that σ -1 receptors have been shown to depress heterologously expressed KCNA5 channels (Aydar et al., 2002), and that inhibition of these channels may have significant effects on the function of intracardiac neurons, it seemed prudent to test the effects of σ receptor activation on currents mediated by voltage-activated K⁺ channels in these cells. Figure 1A shows a family of depolarization-activated K⁺ currents recorded from a single intracardiac neuron in the absence and presence of DTG (100 μ M). Bath application of 100 μ M DTG depressed peak voltage-activated K⁺ current amplitude at potentials positive to +20 mV. The voltage-dependence and kinetics of the currents observed are consistent with the delayed outwardly rectifying K⁺ current ($I_{K(DR)}$) previously reported in intracardiac neurons (Xi-Moy and Dun, 1995). Figure 1B shows a plot of the mean peak $I_{K(DR)}$ amplitude in the absence and presence of DTG as a function of voltage. The DTG-induced block of $I_{K(DR)}$ was voltage-dependent, with the current being blocked by $26.5 \pm 6.2\%$ at +70 mV, but only by $1.5 \pm 0.1\%$ at +10 mV ($n = 7$). This difference was statistically significant ($P < 0.01$).

Concentration-dependent inhibition of $I_{K(DR)}$ by sigma ligands

Further pharmacological experiments were undertaken to confirm that σ receptors inhibit $I_{K(DR)}$ in intracardiac neurons and to identify the σ receptor subtype mediating the observed effects. Figure 2A shows representative currents recorded from three intracardiac neurons in the absence (Control) and presence of (+) pentazocine, ibogaine, and DTG, respectively, at the

indicated concentrations. All of the σ ligands tested depressed peak $I_{K(DR)}$ in a concentration-dependent manner. A plot of the mean peak $I_{K(DR)}$ as a function of drug concentration for several σ ligands is shown in Figure 2B. Fits of the data using the Hill equation gave half-maximal inhibitory concentration (IC_{50}) values for haloperidol, (+)-pentazocine, ibogaine, (+) SKF10,047 and DTG of 9.7 ± 0.5 , 76.4 ± 7.7 μ M, 218.1 ± 7.0 μ M, 295.4 ± 45.4 μ M and 341.3 ± 26.0 μ M, respectively, and Hill coefficients of ~ 0.9 for all drugs. Maximum inhibition of $I_{K(DR)}$ by sigma ligands was over 80%. The IC_{50} values and rank-order potency, in particular the observation that (+) pentazocine is significantly more potent than ibogaine ($p < 0.001$) suggests that the effect on $I_{K(DR)}$ is mediated by activation of σ -1 receptors.

The sigma receptor antagonist, metaphit, depresses the effect of DTG on K^+ channels

To confirm the fact that the effect of sigma ligands on $I_{K(DR)}$ was mediated by activation of σ receptors and not the result of direct channel block, experiments were done using the irreversible σ receptor antagonist, metaphit. Metaphit rapidly and specifically acetylates σ receptors, and inhibits ligand binding to the receptor (Bluth et al., 1989). In intracardiac neurons, metaphit has been shown to block σ -2 receptor mediated attenuation of voltage-gated Ca^{2+} channel currents (Zhang & Cuevas, 2002). Isolated intracardiac neurons were preincubated in 50 μ M metaphit (in PSS) for 10 min at room temperature. Following wash out of drug, $I_{K(DR)}$ was activated by depolarizing cells to +50 mV from a holding potential of -90mV in the absence and presence of 100 μ M DTG. Figure 3A shows representative currents recorded from two different intracardiac neurons without (upper) or with (lower) metaphit preincubation. Upon application of DTG, $I_{K(DR)}$ peak amplitude was depressed under both conditions, but in cells preincubated in metaphit the response to DTG was obtunded. Figure 3B shows a bar graph of degree of block of $I_{K(DR)}$ by 100 μ M DTG in control neurons (DTG; n=4) or neurons preincubated in metaphit (Metaphit + DTG; n=5). DTG decreased mean $I_{K(DR)}$ by $31 \pm 3\%$ in

control cells, whereas in cells exposed to metaphit the decrease was $18 \pm 3\%$. The difference in DTG attenuation of $I_{K(DR)}$ for the two conditions was statistically significant ($p < 0.05$). Several other σ receptor antagonists including SM-21, BD-1047 and BD-1067 were tested to determine if these agents could block the effects of DTG on $I_{K(DR)}$. However, all of these drugs themselves depressed peak amplitude of $I_{K(DR)}$, suggesting that they are either acting as partial agonists of the σ -1 receptors, as reported previously for BD-1047 (Zambon et al., 1997), or having direct effects on the K^+ channels.

Effect of intracellular dialysis with GTP and GDP- β -S on sigma receptor inhibition of $I_{K(DR)}$

Controversy exists in the literature as to whether σ receptors couple to G proteins and if these second messengers are involved in the regulation of ion channels by σ receptors. To determine if σ receptor-mediated inhibition of K^+ channels in intrinsic cardiac neurons is dependent on G protein activation, neurons were dialyzed with pipette solution containing either GTP (100 μ M) or the G protein inhibitor, GDP- β -S (100 μ M). Cell dialysis with 100 μ M GDP- β -S has previously been shown to successfully block G protein mediated signal transduction in intrinsic cardiac neurons (Cuevas and Adams, 1994, 1997; Zhang and Cuevas, 2002). Figure 4A shows representative currents in response to step depolarizations from -90 to +50 mV recorded from two neurons dialyzed with either GTP (top traces) or GDP- β -S (bottom traces) in the absence and presence of 100 μ M DTG. The inhibition of $I_{K(DR)}$ evoked by this concentration of DTG under dialyzing conditions, with GTP in the pipette, was similar to that observed when the intracellular milieu was preserved (~30%). The DTG induced attenuation of $I_{K(DR)}$ was present when the cells were dialyzed with GDP- β -S (Fig 4A, bottom terraces). A summary of the peak $I_{K(DR)}$ amplitudes elicited upon depolarization to +50 mV under the different experimental conditions and normalized to their respective control values is presented in Figure 4B. The difference observed between the two groups was not statistical significant. The I-V

relationships for 6 neurons dialyzed with either GTP or GDP- β -S are shown in Figure 4C and Figure 4D, respectively. While dialyzing cells with GDP- β -S decreased the peak $I_{K(DR)}$ amplitude, as compared with the cells dialyzed with GTP, DTG attenuated peak amplitudes under both conditions to a similar extent. These data suggest that neither cell dialysis nor inhibition of G-protein activation blocks σ -1 receptor-mediated inhibition of $I_{K(DR)}$. Thus, neither a diffusible cytosolic second messenger nor a G protein couples σ -1 receptors to $I_{K(DR)}$.

Effects of sigma receptor ligands on action potential firing

Studies have shown that inhibition of $I_{K(DR)}$ by verapamil in intracardiac neurons can depress action potential firing (Hogg et al., 1999). Experiments were thus conducted to determine if σ receptor modulation $I_{K(DR)}$ has a similar effect on neuroexcitability in these cells. The effects of σ ligands on the active membrane properties of isolated intracardiac neurons were studied using the amphotericin B perforated-patch method under current clamp mode. Figure 5A shows a family of action potentials elicited from a single intracardiac neuron in response to depolarizing current pulses (100 pA; 300 ms) in the absence (control) and presence of 100 μ M DTG (DTG), and following washout of drug (wash). DTG depolarized the neuron and decreased the number of action potentials evoked by the current injection in a rapidly reversible manner. In similar experiments, 100 μ M DTG depolarized neurons from a control value of -50.8 ± 2.1 mV to -49.2 ± 2.4 mV ($n=9$). DTG also decreased the number of action potentials evoked by depolarizing membrane pulses by 85% (Fig 5B). Both of these changes were statistically significant ($p<0.01$ and $p<0.001$, respectively). DTG also altered the action potential configuration by decreasing both the action potential overshoot and afterhyperpolarization, and by slowing both the rate of depolarization and rate of repolarization (inset of figure 5A and table1). The effects of DTG were mimicked by haloperidol (10 μ M), ibogaine (200 μ M), and (+) pentazocine (50 μ M), which depressed action potential firing by 65%, 85% and 65%,

respectively and changed the configurations of the waveform in a reversible manner. The effects of the σ ligands on the action potential firing and configuration are summarized in Table 1.

Role of M-currents in the effects of sigma receptor activation.

One possible mechanism by which σ -1 receptor activation depolarizes intracardiac neurons is via a block of the M-current. Previous studies have shown that inhibition of I_M by muscarinic receptor activation depolarizes intracardiac neurons (Cuevas et al., 1997). The M-channels mediating I_M are non-inactivating and close slowly in response to membrane repolarization from depolarizing holding potentials. The closing of these channels in intracardiac neurons upon repolarization results in a characteristic inward current relaxation (Cuevas et al., 1997). Figure 6A shows whole-cell currents recorded from a single cell in response to repolarizing steps to -60 mV from a holding potential of -30 mV in the absence (Control) and presence of 1 mM DTG. The inward relaxation observed under control conditions is abolished by application of DTG. Similarly, linopirdine (20 μ M), a specific blocker of I_M (Wallace et al., 2002), eliminates the inward relaxation (Figure 6B). Figures 6C and 6D show bar graphs of mean peak I_M recorded under control conditions (absence of drug) and when either DTG (n = 4) or linopirdine (n = 6) were added, respectively. I_M was completely blocked by application of either drug, and this effect was reversible after wash out in both cases (data not shown).

Effects of sigma receptor activation on BK currents

In central and peripheral neurons, action potential afterhyperpolarizations (AHP) are mediated by the opening of Ca^{2+} -activated K^+ channels ($I_{K(Ca)}$). Thus far, three types of Ca^{2+} -activated K^+ channels have been identified on the basis of their pharmacology and single

channel conductances. These channels have been named according to their unitary conductance: large (big) conductance $K_{(Ca)}$ channels (BK), small conductance $K_{(Ca)}$ channels (SK) and intermediate conductance (IK). While both BK and SK channels have been found in mammalian intracardiac neurons, neonatal rat intracardiac neurons only express BK channels (Franciolini et al., 2001; Jelson et al., 2003). The fact that σ receptor activation decreases the AHP suggests that σ receptors may modulate these channels. TEA, at micromolar concentrations (200-500 μ M), has been shown to preferentially block BK channels in intracardiac neurons and to block the AHP more effectively than either charybdotoxin or iberiotoxin (Franciolini et al., 2001). Thus, 500 μ M TEA was used to distinguish between outward K^+ currents mediated by BK and other K^+ channel types (i.e. $I_{K(DR)}$, I_M). Outward currents were evoked by stepping to +50 mV from a -90 mV holding potential in the absence and presence of 500 μ M TEA. The net TEA-sensitive current, and thus the current mediated by BK channels, was determined by subtracting the current remaining after TEA application from that observed under control conditions (absence of TEA). Two distinct TEA-sensitive currents, which differed in their time-dependent inactivation kinetics, were seen in our experiments. These currents were expressed in different populations of neurons, with the fast inactivating current (BK_i) being expressed in 6 of 10 cells and the slowly-inactivating or noninactivating currents (BK_s) being expressed in the remaining 4 cells. Currents representative of each BK channel subtype are shown in Figures 7A and 7B. Both the rapidly decaying and the slowly decaying TEA-sensitive currents were blocked by 1 mM DTG. Figure 7C and 7D show bar graphs of the mean peak and sustained current amplitude obtained from the BK_i ($n = 6$) and BK_s ($n = 4$) species, respectively. Both phases of the current were reduced in the two current types when 1 mM of DTG was applied.

DISCUSSION

The results presented here show that in neonatal rat intracardiac neurons activation of σ -1 receptors reversibly inhibited $I_{K(DR)}$, $I_{K(Ca)}$ and I_M through a pathway that involved neither a diffusible cytosolic second messenger nor a G-protein. Furthermore, activation of σ receptors resulted in the depolarization of the neurons and depression of neuroexcitability.

Studies in our laboratory have demonstrated that σ -1 receptors are expressed in parasympathetic intracardiac neurons, but the cellular function of these receptors remained unknown. Whereas σ -2 receptors in these cells couple to Ca^{2+} channels, σ -1 receptors modulate the function of various K^+ channels in these neurons. While the modulation of Ca^{2+} channels in intracardiac neurons by neurotransmitters is well documented, less is known about the regulation of K^+ channels in these cells (see Adams and Cuevas, 2004). Activation of M_1 muscarinic receptors in neonatal rat intracardiac neurons has been shown to block I_M in these cells (Cuevas et al., 1997), and activation of a PGE_2 receptor inhibits a small-conductance $I_{K(Ca)}$ in adult guinea pig intracardiac neurons (Jelson et al., 2003). Sigma-1 receptor activation, however, inhibits multiple K^+ channels subtypes in intracardiac neurons. The modulation of K^+ channels by σ receptors has been reported in mouse sympathetic neurons, frog melanotrophs, and mouse neurohypophysial nerve terminals (Kennedy and Henderson, 1990; Soriani et al., 1998; Soriani et al., 1999a&b; Wilke et al., 1999). The K^+ channel types that have been shown to be modulated by σ receptor activation include A-Type K^+ channels (I_A), $I_{K(Ca)}$, $I_{K(DR)}$, and I_M , and multiple K^+ channels types are frequently affected in individual cells (Soriani et al., 1999a, Wilke et al., 1999a, Soriani et al., 1999b). All of those K^+ channel subtypes, except A-Type K^+ channels which are not expressed in intracardiac neurons, were shown to be affected by σ receptor activation in our study.

While σ receptors have been shown to block K^+ channels in native cells, the σ receptor subtype mediating these effects has not been definitively identified. The rank order potency for

I_K inhibition by σ ligands reported here, haloperidol > (+)-pentazocine > ibogaine > DTG, suggests that the effect is mediated by σ -1 receptor activation. In contrast, the rank order potency for σ ligand inhibition of Ca^{2+} channels in intracardiac neurons is: haloperidol > ibogaine > (+)-pentazocine > DTG, which is consistent with a σ -2 receptor mediated effect. The IC_{50} values for the various σ ligands tested here are in agreement with those reported in the literature for modulations of voltage-gated K^+ channels. For example, in frog pituitary melanotrophs, (+)-pentazocine inhibited delayed outwardly rectifying K^+ channels with an IC_{50} of 37 μM , compared to the IC_{50} of 42 μM reported here. Similarly, 100 μM (+)-SKF10047 blocked approximately 50% of the voltage-activated K^+ currents recorded in neurohypophysial nerve terminals (Wilke et al., 1999a). Thus, σ -1 receptors are likely to be responsible for inhibition of K^+ channels in intracardiac neurons and in other native cells studied. The conclusion that σ receptors mediate the inhibition of I_K by σ ligands in intracardiac neurons is strengthened by the observation that metaphit, an irreversible, antagonist of σ receptors, blocks the effects of DTG on I_K in these cells.

Considerable controversy exists as to the mechanisms by which σ receptors modulate K^+ channels. In frog pituitary melanotrophs, cell dialysis with GTP- β -S and preincubation in cholera toxin were shown to inhibit σ receptor effects on I_A and $I_{K(DR)}$ (Soriani et al., 1999 a&b), suggesting that σ receptors couple to the K^+ channels via a G protein. G proteins have also been implicated in both σ receptor-mediated activation of phospholipase C and regulation of Ca^{2+} release from intracellular stores (Morin-Surun et al., 1999; Hayashi et al., 2000). However, neither cell dialysis nor application of intracellular GTP- β -S blocked the inhibition of I_K by σ -1 receptors in intracardiac neurons. Thus, σ receptors couple to K^+ channels in these cells via a membrane-delimited signal transduction cascade that does not involve a G protein. Our

observation is in agreement with the direct protein-protein interaction between σ -1 receptors and K^+ channels shown by Jackson and colleagues (Lupardus et al., 2000; Aydar et al., 2002).

The net effect of σ receptor modulation of ion channels in intracardiac neurons at rest is a depolarization of the membrane potential. We have previously shown that the I_M contributes to the resting membrane potential of intracardiac neurons and that inhibition of this channel depolarizes the cells (Cuevas et al., 1997). Given that application of DTG blocks I_M , σ receptor modulation of these channels likely accounts for the depolarizations reported here. Further support for this conclusion comes from the fact that neither inhibition $I_{K(DR)}$ nor $I_{K(Ca)}$ has been linked to changes in the resting membrane potential of these neurons (Hogg et al., 1999; Xu and Adams, 1992; Jelson et al., 2003). Inhibition of voltage gated Ca^{2+} channels by σ -2 receptor activation is also unlikely to account for this depolarization since removal of extracellular Ca^{2+} does not alter the resting membrane potential of these cells (DeHaven and Cuevas, 2004).

The block of I_K by σ receptors in intracardiac neuron is associated with decreased action potential firing, whereas previous studies have shown that σ receptor block of I_K enhances neuroexcitability (Soriani et al., 1998). Our laboratory has shown that inhibition of I_M is associated with increased excitability of intracardiac neurons (Cuevas et al., 1997), and thus σ receptor modulation of non- I_M K^+ channels must be responsible for the decreased excitability observed. The depressed neuroexcitability reported here likely results from modulation of $I_{K(DR)}$ by σ receptors. Verapamil has been shown to convert tonic and adapting intracardiac neurons into phasic neurons via a direct block of $I_{K(DR)}$ (Hogg et al., 1999). However, high concentrations of verapamil failed to abolish action potential firing in intracardiac neurons, whereas high concentrations of σ ligands completely blocked the genesis of action potentials in these cells. The concentrations of (+) pentazocine (50 μ M) and ibogaine (200 μ M) required to appreciably decrease action potential firing and alter the action potential configuration in our study suggest

that the effects of σ ligands on the active membrane properties of these cells are primarily mediated by σ -1 receptors. However, inhibition of voltage-activated K^+ channels alone cannot explain the complete block of action potential firing at high concentrations of σ ligands, and thus σ receptors are likely to affect other channel types that regulate action potential firing (e.g. voltage-gated sodium channels).

The inhibition of $I_{K(Ca)}$ by σ receptors is also likely to contribute to changes in the active membrane properties of intracardiac neurons. Two types of σ receptor-regulated BK currents were found in rat intracardiac neurons, one which exhibited rapid time-dependent inactivation, BK_i , and a second which showed little or no inactivation, BK_s . The presence of distinct subpopulations of BK channels with dissimilar inactivation kinetics has been reported in rat adrenal chromaffin cells and mouse neocortical pyramidal neurons and (Solaro et al., 1995; Sun et al., 2003). The inhibition of BK currents by σ receptors would account, at least in part, for the increase in action potential duration and decrease in AHP reported here, since inhibition of BK by 200 μ M TEA produces similar effects on the action potentials of these cells (Franciolini et al., 2001). While inhibition of voltage-gated Ca^{2+} channels may contribute to the decrease in $I_{K(Ca)}$ amplitude observed in our studies, it is unlikely to exclusively account for the block of BK channels observed here. Evidence for this conclusion comes from the fact that concentrations of Cd^{2+} (100 μ M) that inhibit all Ca^{2+} channels in these neurons (Cuevas and Adams, 1997) fail to eliminate the AHP in these cells (Franciolini et al., 2001). Furthermore, in our study, 100 μ M Cd^{2+} blocked < 5% of I_K , whereas 500 μ M TEA or Ca^{2+} -free extracellular solution blocked ~25% of I_K (data not shown). Taken together, these data suggest that σ -1 receptors are likely having a direct effect on BK channels in intracardiac neurons. However, σ receptor modulation of other mechanisms of Ca^{2+} entry or homeostasis must also be examined to confirm a direct effect on $I_{K(Ca)}$.

There has been considerable speculation about the role of σ receptors in the cardiovascular system. While the endogenous ligand responsible for activating σ receptors under physiological and pathophysiological conditions remains to be determined, there is data suggesting that some putative σ receptor ligands may affect the heart and coronary vasculature. For example, pregnenolone, a putative σ ligand (Maurice et al., 2001), increases heart rate and cardiac output in anesthetized dogs (Hogskilde et al., 1991). Such effects may in part be due to activation of σ receptors on intracardiac neurons. Similarly, various drugs that act on σ receptors, such as haloperidol, have significant effects on the heart (Monassier and Bousquet, 2002). Our observations raise the possibility that the cardiovascular effects of these compounds may be mediated by their modulation of the electrical activity of intrinsic cardiac neurons. In conclusion, stimulation of σ -1 receptors inhibits multiple voltage-gated K^+ channel subtypes and depresses excitability in intracardiac neurons. Thus, activation of σ -1 receptors in these cells is likely to attenuate parasympathetic input to the heart and, consequently, affect cardiovascular function.

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REFERENCES

Adams DJ and Cuevas J (2004) Electrophysiological properties of cardiac neurons-the foundation of neurocardiology, In *Basic and Clinical Neurocardiology* (Armour JA and Ardell JL eds) pp 1-60, Oxford University Press.

Aydar E, Palmer CP, Klyachko VA and Jackson MB (2002) The sigma receptor as a ligand-regulated auxiliary potassium channel subunit. *Neuron*. 34(3):399-410.

Bluth LS, Rice KC, Jacobson AE and Bowen WD (1989) Acylation of sigma receptors by Metaphit, an isothiocyanate derivative of phencyclidine. *Eur J Pharmacol* 161: 273-277.

Cuevas J and Adams DJ (1994) Local anaesthetic blockade of neuronal nicotinic ACh receptor-channels in rat parasympathetic ganglion cells. *Br J Pharmacol*. 111: 663-672.

Cuevas J and Adams DJ (1997) M4 muscarinic receptor activation modulates calcium channel currents in rat intracardiac neurons. *J Neurophysiol* 78:1903-1912.

Cuevas J, Harper AA, Trequattrini C, and Adams DJ (1997) Passive and active membrane properties of isolated rat intracardiac neurons: regulation by H- and M-currents. *J. Neurophysiol*. 78: 1890-1902.

Dehaven WI and Cuevas J (2004) VPAC receptor modulation of neuroexcitability in intracardiac neurons: dependence on intracellular calcium mobilization and synergistic enhancement by PAC₁ receptor activation. *J. Biol. Chem*. 279: 40609-40621

Ela C, Barg J, Vogel Z, Hasin Y, Eilam Y (1994) Sigma receptor ligands modulate contractility, Ca⁺⁺ influx and beating rate in cultured cardiac myocytes. *J Pharmacol Exp Ther.* 269(3):1300-1309.

Franciolini F, Hogg R, Catacuzzeno L, Petris A, Trequatrini C and Adams DJ (2001) Large-conductance calcium-activated potassium channels in neonatal rat intracardiac ganglion neurons. *Pflugers Arch.* 441(5):629-638.

Hanner M, Moebius FF, Flandorfer A, Knaus HG, Striessnig J, Kempner E and Glossmann H (1996) Purification, molecular cloning, and expression of the mammalian sigma1-binding site. *Proc. Natl. Acad. Sci. U.S.A* 93: 8072-8077.

Hayashi T, Maurice T and Su TP (2000) Ca(2+) signaling via sigma(1)-receptors: novel regulatory mechanism affecting intracellular Ca(2+) concentration. *J Pharmacol Exp Ther* 293(3):788-798.

Hellewell SB and Bowen WD (1990) A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. *Brain Res* 527(2):244-253.

Hogg RC, Trequatrini C, Catacuzzeno L, Petris A, Franciolini F and Adams DJ (1999) Mechanisms of verapamil inhibition of action potential firing in rat intracardiac ganglion neurons. *J Pharmacol Exp Ther* 289(3):1502-1508.

Hogskilde S, Wagner J, Strom J, Sjontoft E, Olesen HP and Bredgaard SM (1991)

Cardiovascular effects of pregnenolone emulsion: an experimental study in artificially ventilated dogs. *Acta Anaesthesiol Scand* 35(7):669-675.

Jelson GS, DeMasi GM, Sager KL and Hardwick JC (2003) Modulation of guinea pig intrinsic cardiac neurons by prostaglandins. *Am J Physiol Regul Integr Comp Physiol*. 285(3):R682-689.

Kennedy C and Henderson G. (1990). Inhibition of potassium currents by the sigma receptor ligand (+)-3-(3-hydroxyphenyl)-N-(1-propyl)piperidine in sympathetic neurons of the mouse isolated hypogastric ganglion. *Neuroscience*. 35(3):725-733

Lishmanov YB, Maslov LN, Naryzhnaya NV and Tam SW (1999) Ligands for opioid and sigma-receptors improve cardiac electrical stability in rat models of post-infarction cardiosclerosis and stress. *Life Sciences* 65(1):PL13-PL17.

Lishmanov YB, Maslov LN, Krylatov AV, Ugdyzhekova DS, Gilligan P and Tam SW (2000) The anti-arrhythmia and proarrhythmia properties of sigma-receptor ligands. *Eksp Klin Farmakol* 63: 39-43.

Lupardus PJ, Wilke RA, Aydar E, Palmer CP, Chen Y, Ruoho AE and Jackson MB (2000) Membrane-delimited coupling between sigma receptors and K⁺ channels in rat neurohypophysial terminals requires neither G-protein nor ATP. *J Physiol*. 526(3):527-539.

Maurice T, Urani A, Phan VL and Romieu P (2001) The interaction between neuroactive steroids and the sigma1 receptor function: behavioral consequences and therapeutic opportunities. *Brain Res Rev* 37(1-3):116-132

Monassier L and Bousquet P (2002) Sigma receptors: from discovery to highlights of their implications in the cardiovascular system. *Fundam Clin Pharmacol.* 16(1):1-8

Morin-Surun MP, Collin T, Denavit-Saubié M, Baulieu EE and Monnet FP (1999) Intracellular σ_1 receptor modulates phospholipase C and protein kinase C activities in the brainstem. *Proc Nat Acad Sci* 14: 8196-8199.

Narita N, Hashimoto K and Tomitaka S (1996) Minabe Y. Interactions of selective serotonin reuptake inhibitors with subtypes of sigma receptors in rat brain. *Eur J Pharmacol* 307(1):117-119.

Novakova M, Ela C, Barg J, Vogel Z, Hasin Y and Eilam Y (1995) Inotropic action of sigma receptor ligands in isolated cardiac myocytes from adult rats. *Eur J Pharmacol* 286: 19-30.

Solaro CR, Prakriya M, Ding JP and Lingle CJ (1995) Inactivating and noninactivating $\text{Ca}(2+)$ - and voltage-dependent K^+ current in rat adrenal chromaffin cells. *J Neurosci.* 15(9):6110-23.

Soriani O, Foll FL, Roman F, Monnet FP, Vaudry H and Cazin L (1999a) A-Current down-modulated by sigma receptor in frog pituitary melanotrope cells through a G protein-dependent pathway. *J Pharmacol Exp Ther.* 289(1):321-328

Soriani O, Le Foll F, Galas L, Roman F, Vaudry H and Cazin L (1999b) The sigma-ligand (+)-pentazocine depresses M current and enhances calcium conductances in frog melanotrophs. *Am J Physiol.* 277(1):E73-E80.

Soriani O, Vaudry H, Mei YA, Roman F and Cazin L (1998) Sigma ligands stimulate the electrical activity of frog pituitary melanotrope cells through a G-protein-dependent inhibition of potassium conductances. *J Pharmacol Exp Ther* 286: 163-171.

Snyder SH and Largent BL (1989) Receptor mechanisms in antipsychotic drug action: focus on sigma receptors. *J Neuropsychiatry Clin Neurosci* 1(1):7-15.

Su T P (1982) Evidence for sigma opioid receptor: binding of [3H]SKF-10047 to etorphine-inaccessible sites in guinea-pig brain. *J Pharmacol Exp Ther* 223: 284-90.

Ujike H, Kuroda S and Otsuki S (1996) sigma Receptor antagonists block the development of sensitization to cocaine. *Eur J Pharmacol.* 296(2):123-128.

Wallace DJ, Chen C and Marley PD (2002) Histamine promotes excitability in bovine adrenal chromaffin cells by inhibiting an M-current. *J Physiol.* 540(3):921-939.

Wilke RA, Lupardus PJ, Grandy DK, Rubinstein M, Low MJ and Jackson MB (1999) K⁺ channel modulation in rodent neurohypophysial nerve terminals by sigma receptors and not by dopamine receptors. *J Physiol.* 517(2):391-406

Wu KM and Martin W R (1989) Cardiovascular and respiratory effects of an opioid kappa agonist ethylketazocine and sigma agonist N-allylnormetazocine in acutely decerebrated dogs. *Pharmacol.Biochem.Behav* 34: 405-411.

Xi-Moy SX and Dun NJ (1995) Potassium currents in adult rat intracardiac neurones. *J Physiol.* 486:15-31.

Xu ZJ and Adams DJ (1992) Voltage-dependent sodium and calcium currents in cultured parasympathetic neurones from rat intracardiac ganglia. *J Physiol* 456:425-441.

Zambon AC, De Costa BR, Kanthasamy AG, Nguyen BQ and Matsumoto RR. (1997). Subchronic administration of N-[2-(3,4-dichlorophenyl) ethyl]-N-methyl-2-(dimethylamino) ethylamine (BD1047) alters sigma 1 receptor binding. *Eur J Pharmacol.* 324:39-47.

Zhang H and Cuevas J (2002) Sigma receptors inhibit high-voltage-activated calcium channels in rat sympathetic and parasympathetic neurons. *J Neurophysiol* 87(6):2867-2879.

Zhang H and Cuevas J (2003) Sigma receptors activation depresses delayed outwardly rectifying K⁺ channels in rat intracardiac neurons. Abstracts, Society for Neuroscience 32nd Annual Meeting, 923.17, New Orleans, LA.

FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Sigma receptor ligands inhibit $I_{K(DR)}$ in rat intracardiac neurons. A: Depolarization-activated (-50 to +70 mV; 300 ms) K^+ currents recorded from a single neuron held at -90 mV in the absence (Control) and presence of 100 μ M DTG (DTG) or after washout of drug (Wash). B: Peak $I_{K(DR)}$ current density as a function of voltage recorded in a similar manner from 7 neurons in the absence (o) and presence (●) of 100 μ M DTG.

Figure 2. Dose-dependent inhibition of $I_{K(DR)}$ by sigma receptor ligands in rat intracardiac neurons. A: Whole-cell K^+ currents evoked from 3 intracardiac neuron by step depolarizations to +50 mV from a holding potential of -90 mV in the absence (Control) and presence of (+) pentazocine, ibogaine, and DTG at the indicated concentrations. B: Peak whole-cell $I_{K(DR)}$ current amplitude, evoked by depolarizing to +50 mV from -90 mV, normalized to control and plotted as a function of sigma ligands concentration. Data points represent mean \pm SEM for ≥ 4 observations. The curves represent best fit to the data using the Hill equation. Half-maximal inhibition was $9.7 \pm 0.5 \mu$ M for haloperidol, $76.4 \pm 7.7 \mu$ M for (+)-pentazocine, $218.1 \pm 7.0 \mu$ M for ibogaine, $295.4 \pm 45.4 \mu$ M for SKF10,047 and $341.3 \pm 26.0 \mu$ M for DTG. The corresponding Hill coefficients were 1.01 ± 0.05 , 0.69 ± 0.05 , 1.14 ± 0.04 , 0.84 ± 0.11 , 0.97 ± 0.07 , respectively.

Figure 3. Attenuation of DTG-mediated inhibition of $I_{K(DR)}$ by the sigma receptor antagonist, metaphit. A: depolarization-activated (-90 to 50mV) $I_{K(DR)}$ recorded from 2 intracardiac neurons in the absence (Control) and presence of 100 μ M DTG (DTG). Bottom traces are from a neuron preincubated in metaphit (50 μ M in PSS, 10 min). B: bar graph of the percent inhibition of mean peak $I_{K(DR)}$ (\pm SE) produced by 100 μ M DTG in control cells that were not exposed to metaphit

(DTG) or cells preincubated in metaphit (50 μ M, 10 min; Metaphit + DTG). $I_{K(DR)}$ was evoked by step depolarizations (-90 to +50 mV). Data were collected from 7 neurons for each condition, and asterisk denotes significant difference between the groups ($p < 0.05$)

Figure 4. Sigma receptor inhibition of $I_{K(DR)}$ is not blocked by intracellular GDP- β -S. A: Depolarization activate (-90 to +50 mV) K^+ currents recorded from neurons dialyzed with pipette solutions containing either 100 μ M GTP or GDP- β -S in the absence (Control) or presence of 100 μ M of DTG (DTG). B: Mean (\pm SEM) peak $I_{K(DR)}$ recorded from neurons dialyzed with either GTP (n=6) or GDP- β -S (n=6) in the presence of 100 μ M DTG. Currents are normalized to their respective controls (absence of drug). Mean (\pm SE) peak $I_{K(DR)}$ current density as a function of depolarization voltage (-90 mV, holding potential) recorded from neurons dialyzed with pipette solution containing either 100 μ M GTP (C, n = 6) or 100 μ M GDP- β -S (D, n = 6) in the absence (o) and presence (•) of 100 μ M DTG.

Figure 5. Inhibition of action potential firing by sigma receptor ligand, DTG, in rat intracardiac neurons. A: Action potentials fired from an isolated intracardiac neuron in response to 100 pA depolarizing current pulses in the absence (Control) and presence of 50 μ M DTG (DTG), and following washout of drug (Wash). Inset is superimposition of first action potential from Control and DTG traces. B: Bar graph of the number of action potentials fired in the absence (Control) or presence of 100 μ M DTG (DTG) and following washout of the drug (Wash). Asterisk denotes significant difference from Control and DTG ($p < 0.01$) (n=7).

Figure 6. The sigma receptor ligand, DTG, inhibits the M-current in rat intracardiac neurons. A: Slow inward current relaxation (M-current) induced by repolarizing step (-30 mV to -60 mV, 250 ms) recorded from a single intracardiac neuron before (Control) and after application of 1 mM of DTG (DTG). B: M-current recorded in the absence (Control) and in the presence of 20

μM linopirdine (Linopirdine). Bar graph of mean current amplitudes in the absence (Control) or presence of 1mM DTG (C, $n = 4$) and 20 μM linopirdine (D, $n= 6$). The amplitude of the M-current was defined as the difference between the peak current observed at the start of the relaxation (panel A, left trace, black arrow) and the holding current at the end of the 250 ms repolarizing step (panel A, left trace, gray arrow). Asterisks denote significant difference from Control ($p<0.001$).

Figure 7. Sigma receptor activation blocks large conductance Ca^{2+} -activated K^+ channel (BK) in rat intracardiac neurons. Traces of BK mediated currents recorded from two neurons, expressing fast inactivating (A) and slowly-inactivating BK channels (B), in the absence (Control) and presence of 1 mM DTG (DTG). BK mediated currents were evoked by step depolarizations to +50 mV from a holding potential of -90 mV, and defined as the net TEA-sensitive current. The net TEA-sensitive current was determined by subtracting the outward current recorded in the presence of 500 μM TEA (with or without DTG) from the outward current obtained in the absence of TEA (with or without DTG). Bar graph of the peak and sustained BK current amplitudes recorded in the absence (Control) and presence of DTG (DTG, 1mM) in cells expressing fast-inactivating (C, $n = 6$) or slowly-inactivating (D, $n = 4$) BK channels. Asterisks denote significant difference from control ($p<0.05$).

Table 1: Effects of sigma ligands on the resting membrane potential and action potential waveform parameters. Action potentials were evoked by 100 pA current injections. All drugs were bath applied and the data are shown as: mean \pm SE for the number of cells (n) shown in the table. Asterisks denote significant difference. (*: $p < 0.05$ and **: $p < 0.01$).

	n	RMP (mV)	Peak Amplitude (mV)	Rising Slope (mV/sec)	Decay Slope (mV/sec)	AHP (mV)
Control	6	-50.8 \pm 2.1	34.4 \pm 5.7	55.9 \pm 19.9	-30.4 \pm 5.9	-21.7 \pm 1.3
DTG (100 μ M)	6	-49.2 \pm 2.4*	4.6 \pm 5.6**	11.8 \pm 6.3**	-8.6 \pm 3.6**	-12.3 \pm 2.1**
Control	6	-51.0 \pm 2.1	25.1 \pm 5.2	36.4 \pm 8.1	-20.3 \pm 2.9	-22.2 \pm 2.2
PTZ (50 μ M)	6	-48.9 \pm 2.3*	11.7 \pm 4.9**	12.7 \pm 4.0*	-7.6 \pm 1.7**	-16.6 \pm 1.6**
Control	5	-54.5 \pm 1.3	35.9 \pm 3.5	59.4 \pm 6.0	-26.7 \pm 2.7	-22.8 \pm 1.1
IBO(200 μ M)	5	-51.0 \pm 2.2*	-3.9 \pm 6.3**	4.7 \pm 1.7**	-4.7 \pm 1.6**	-6.3 \pm 2.7**

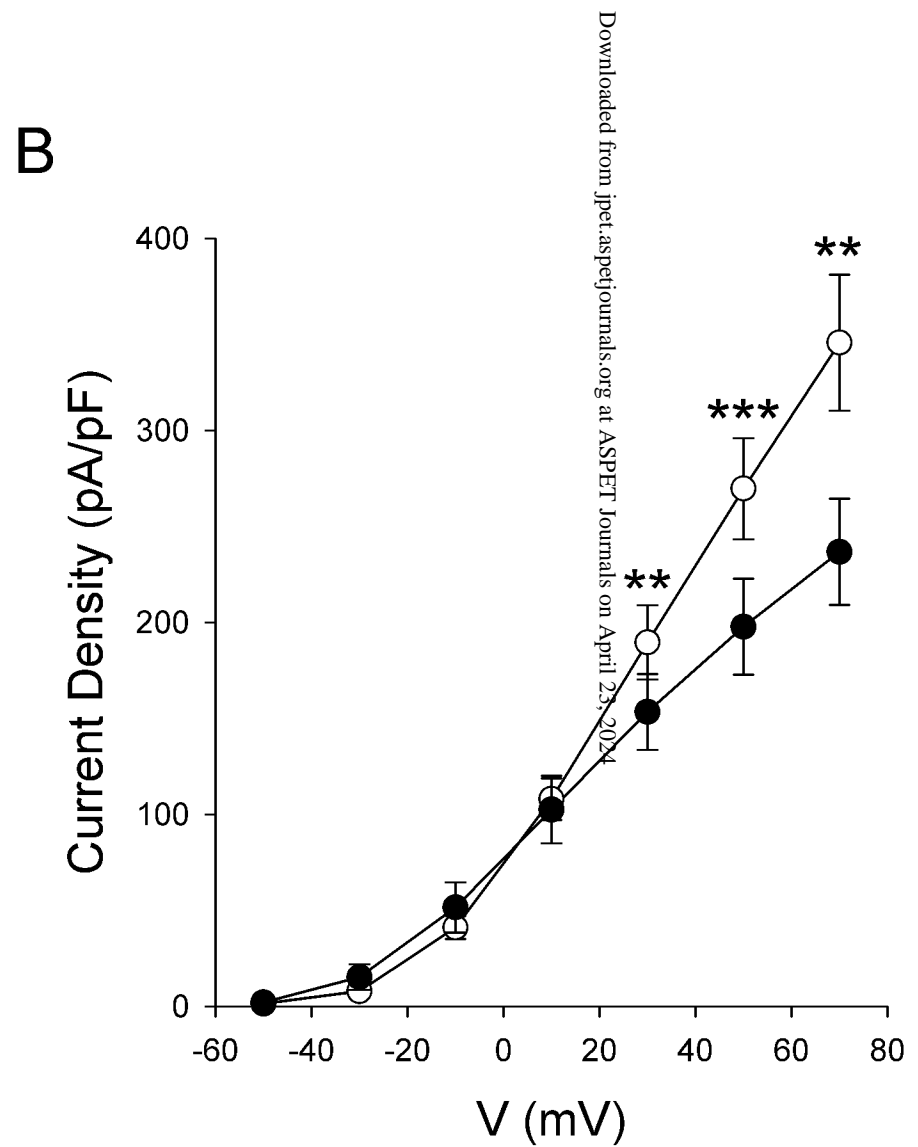
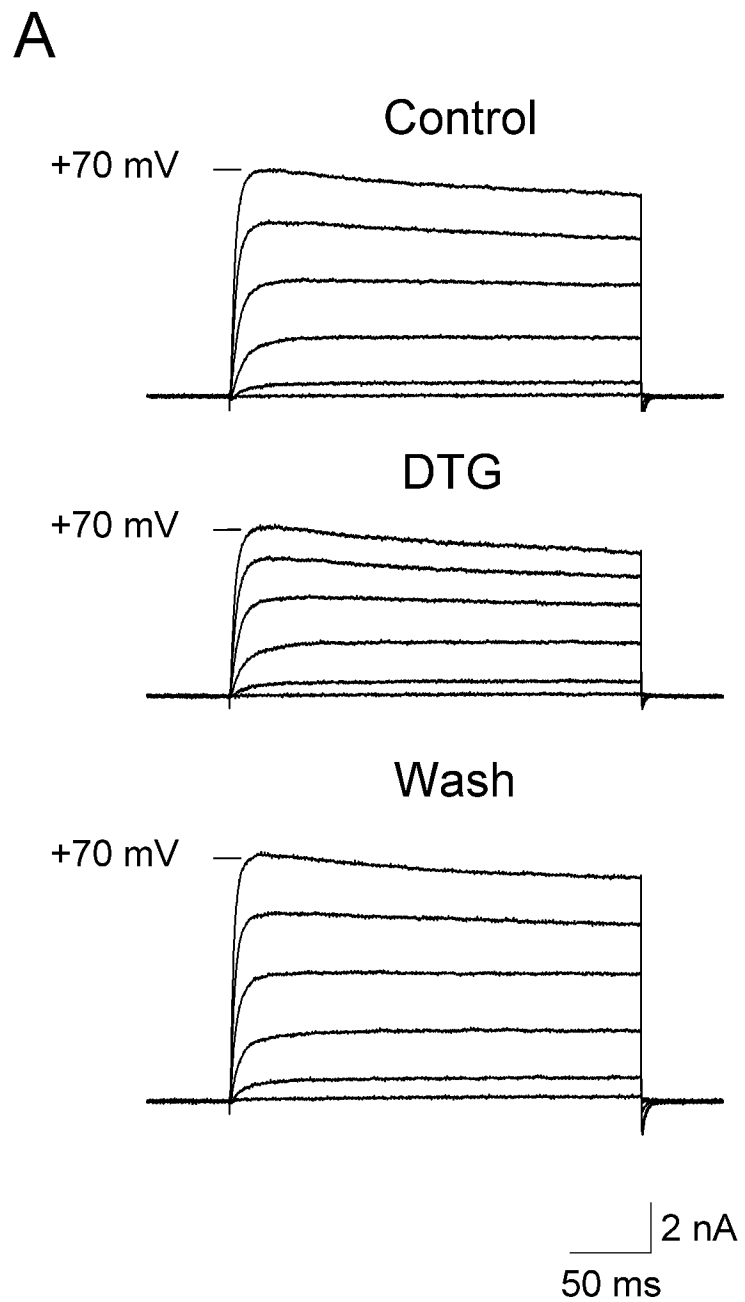


Figure 1

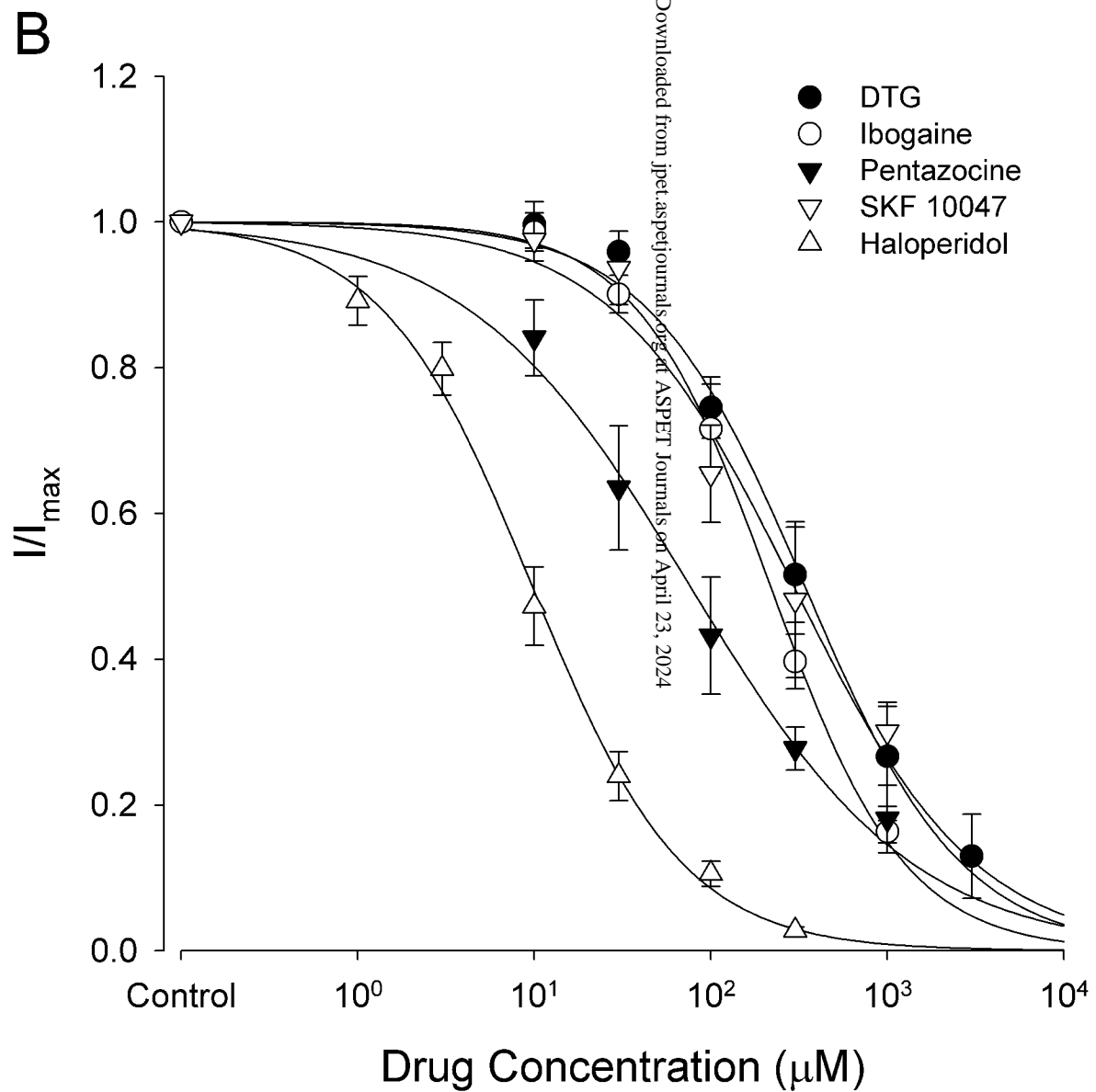
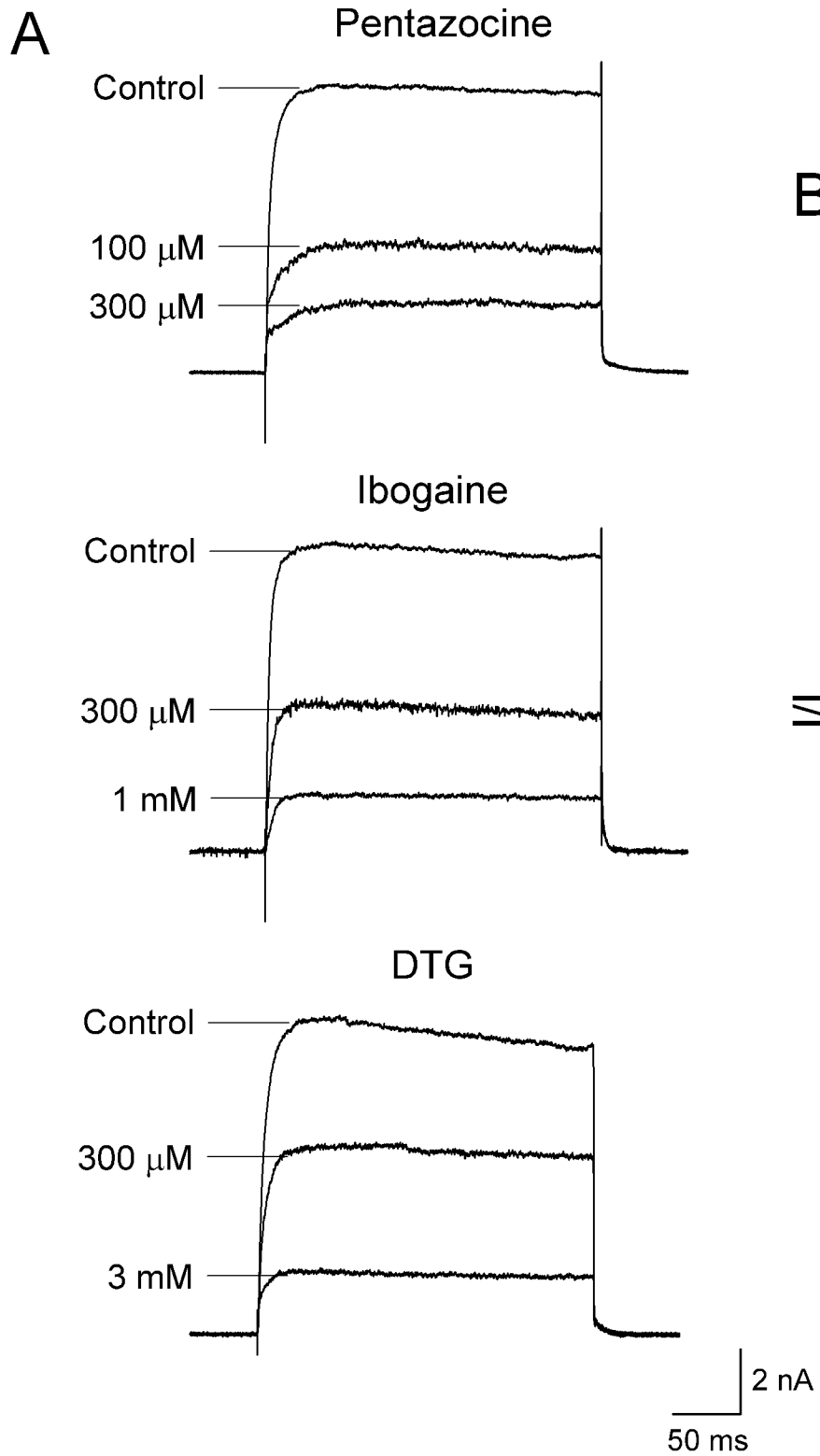


Figure 2

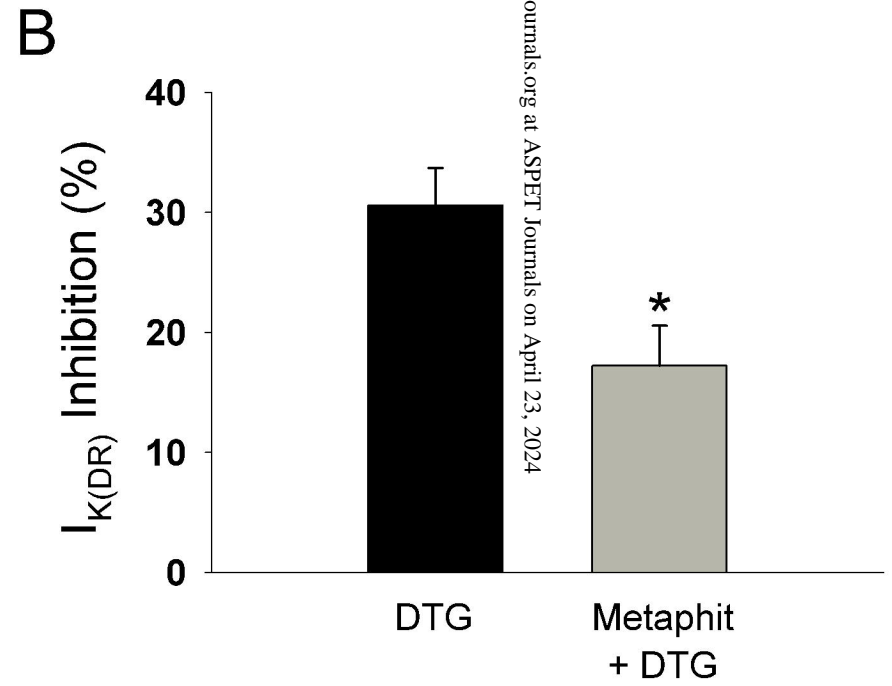
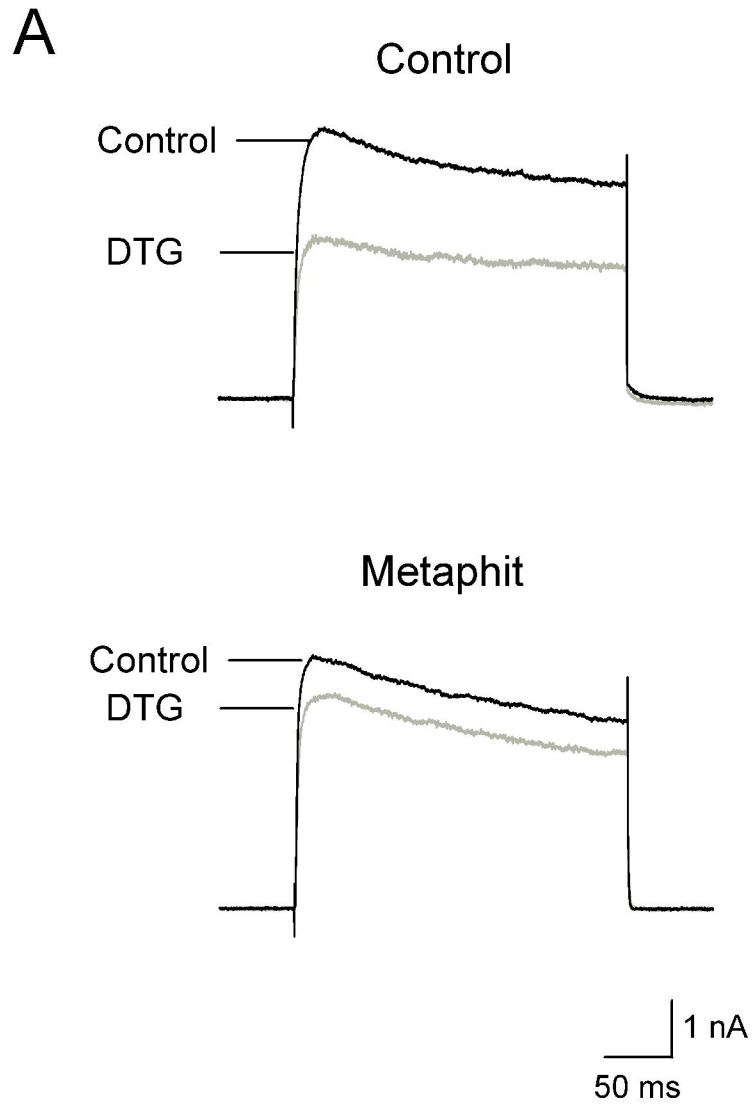


Figure 3

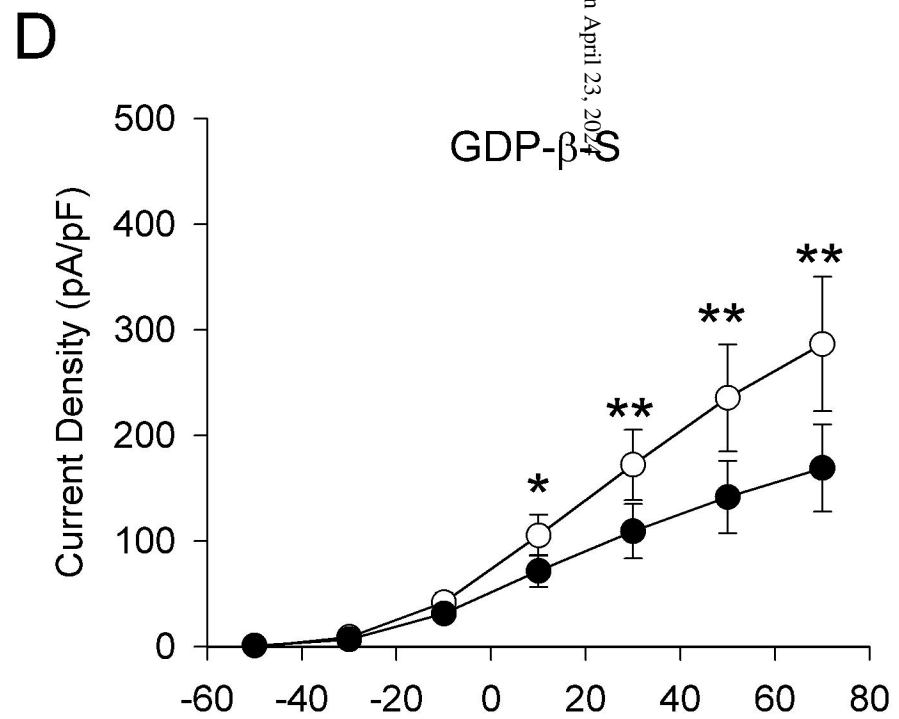
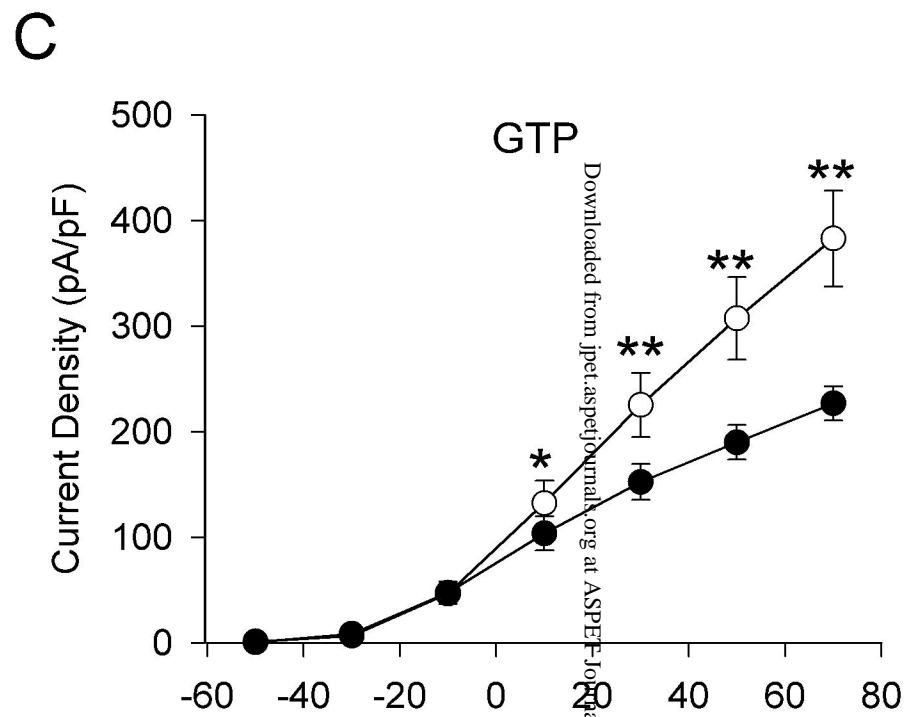
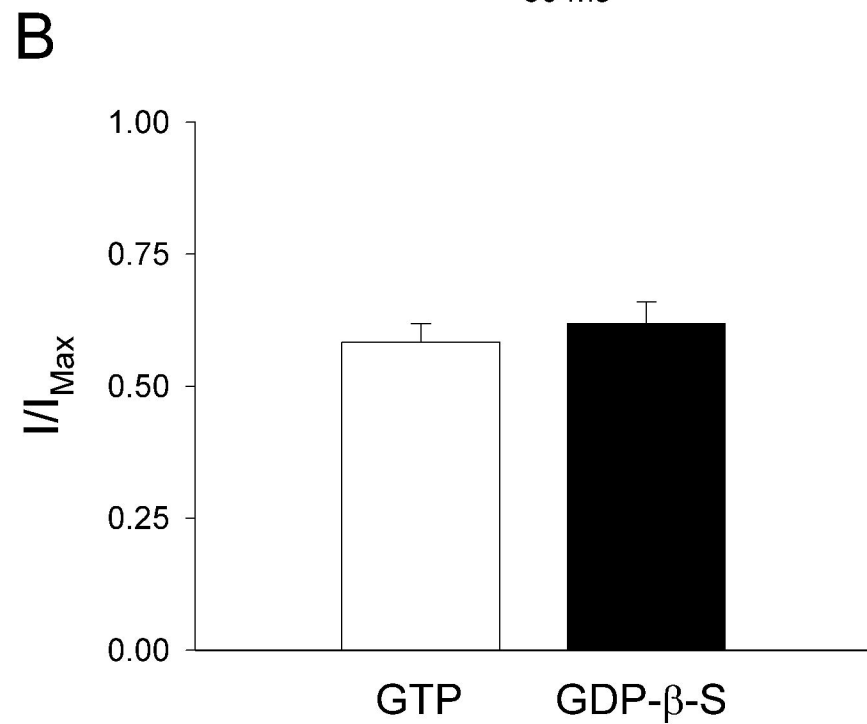
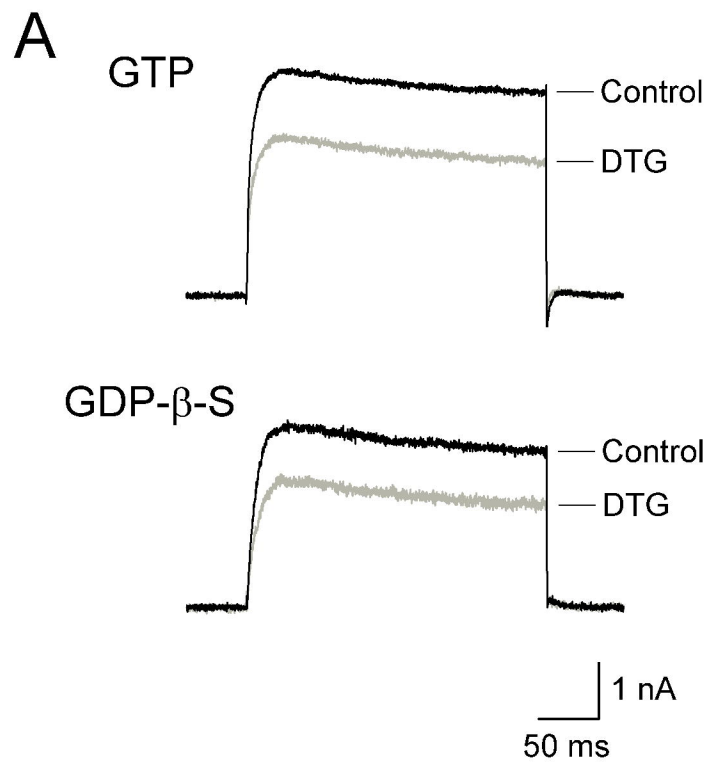


Figure 4

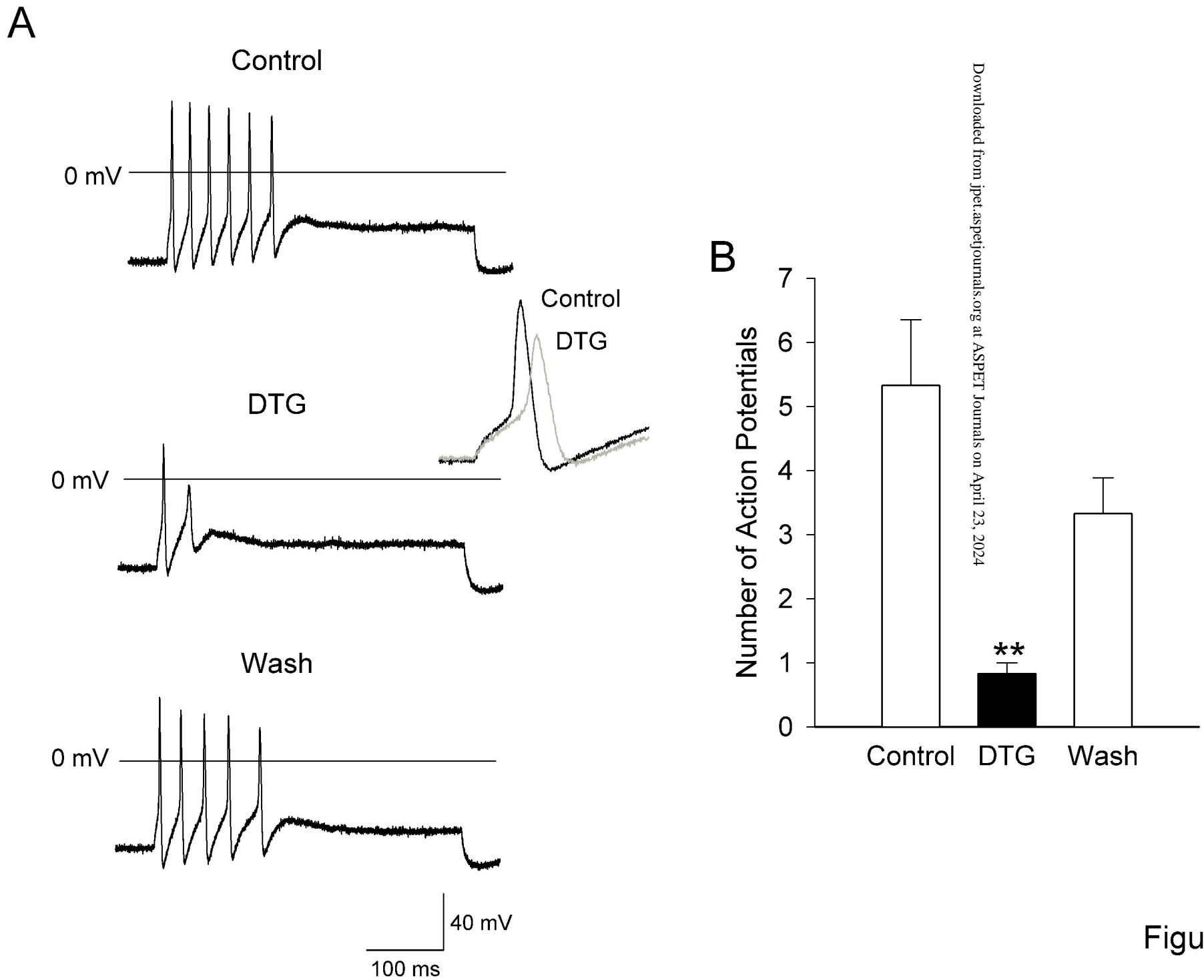


Figure 5

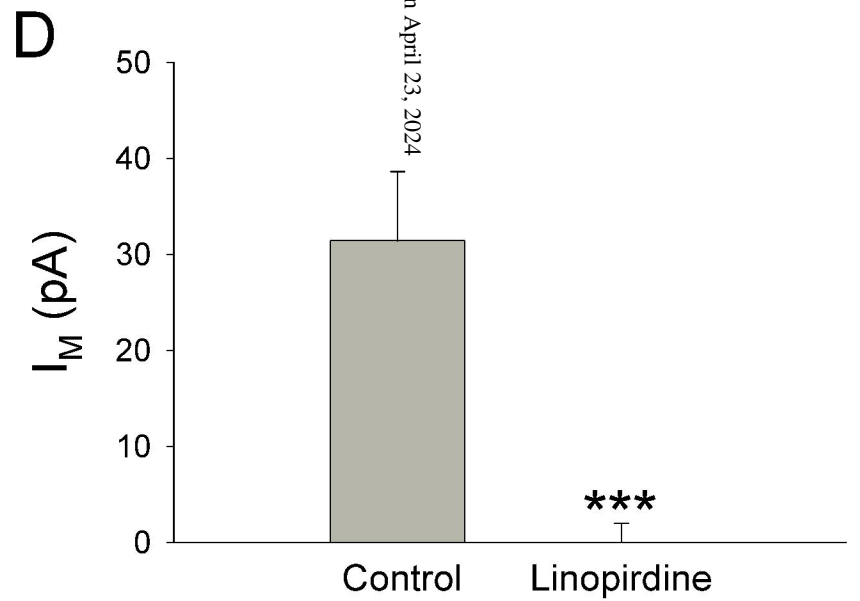
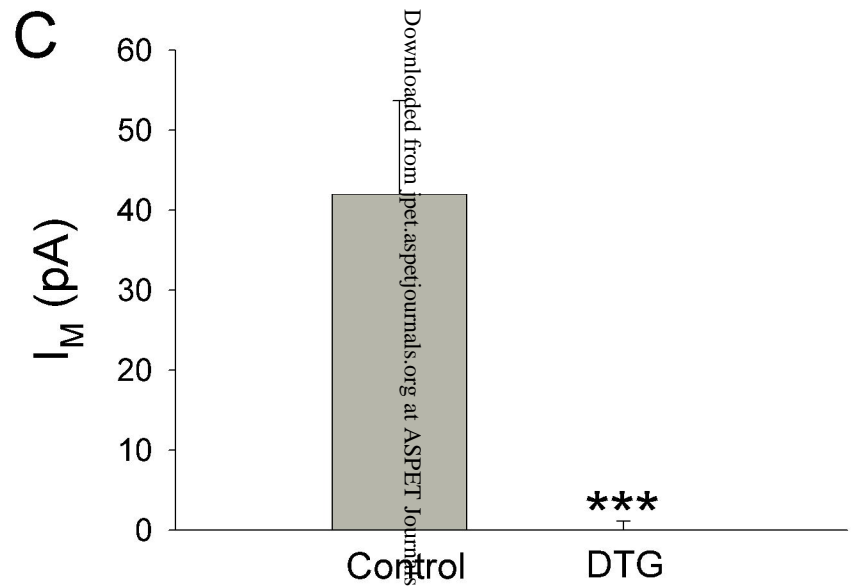
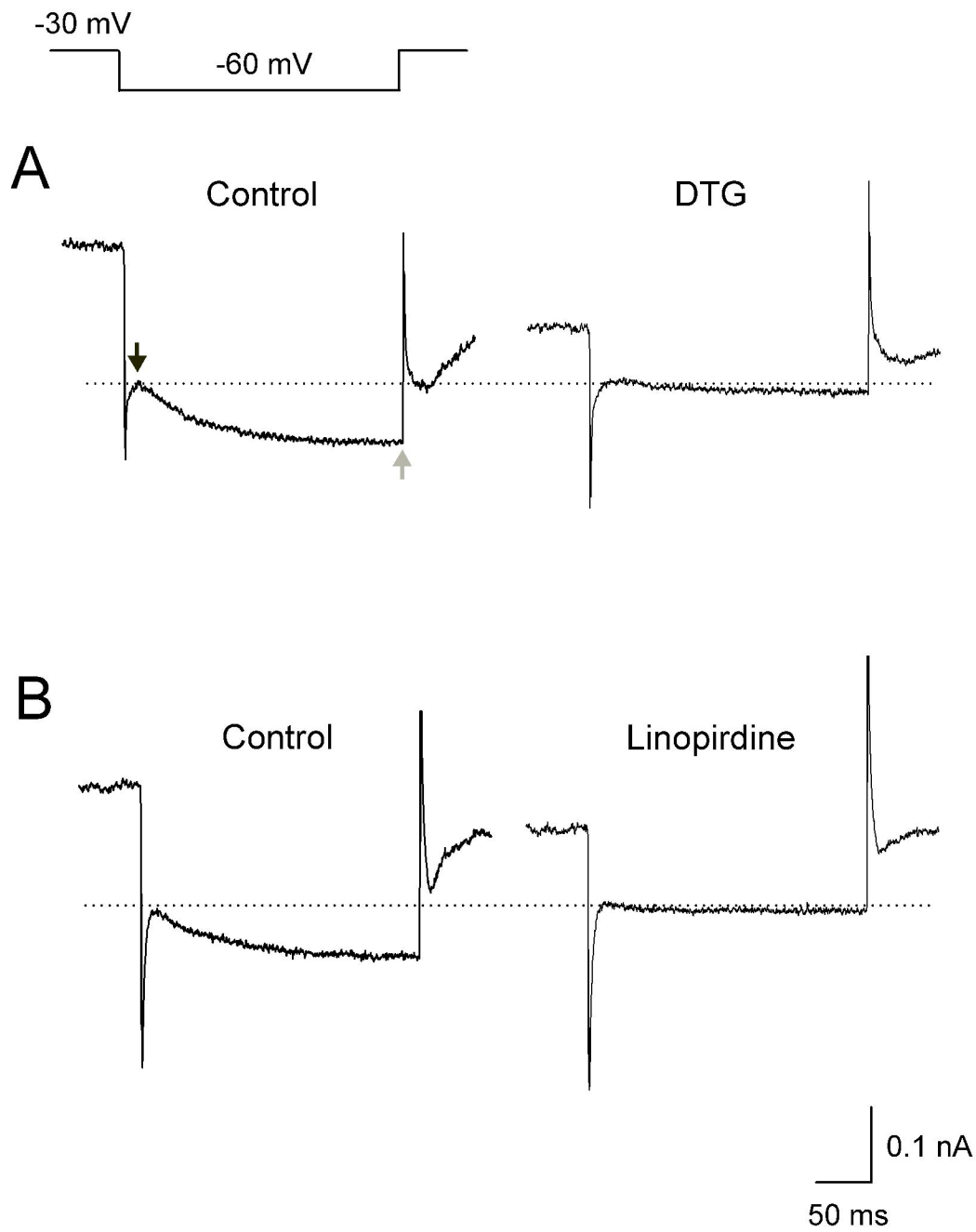


Figure 6

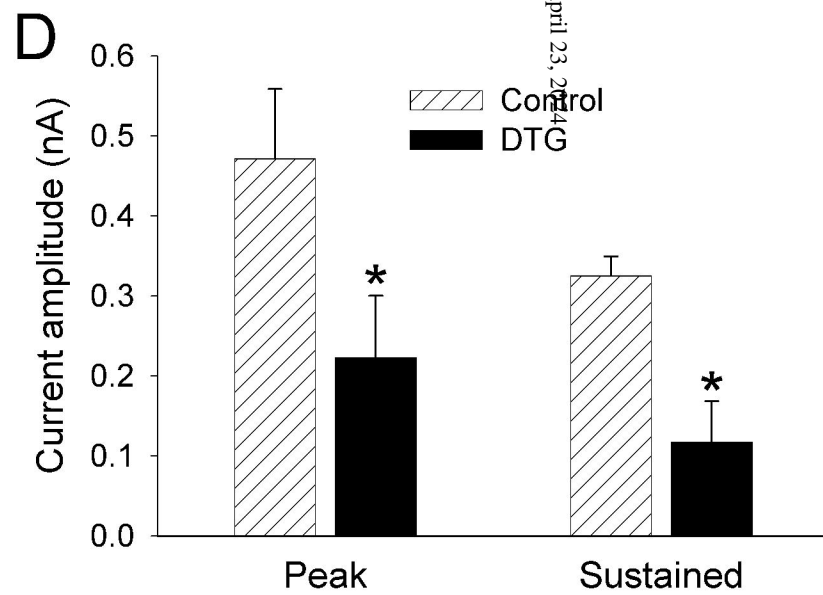
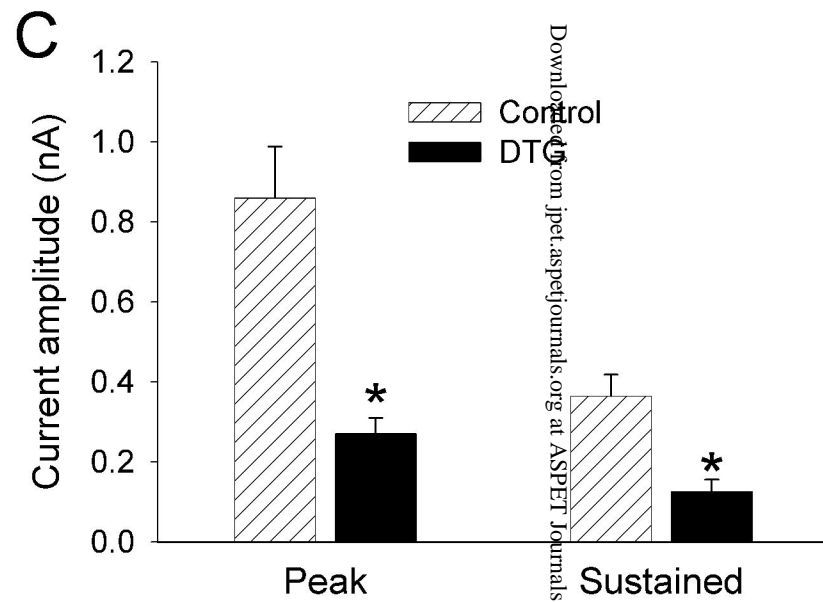
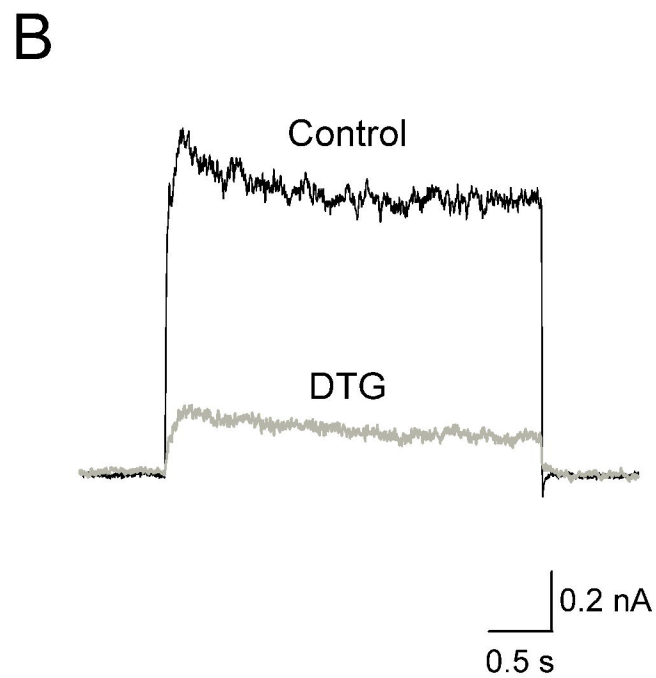
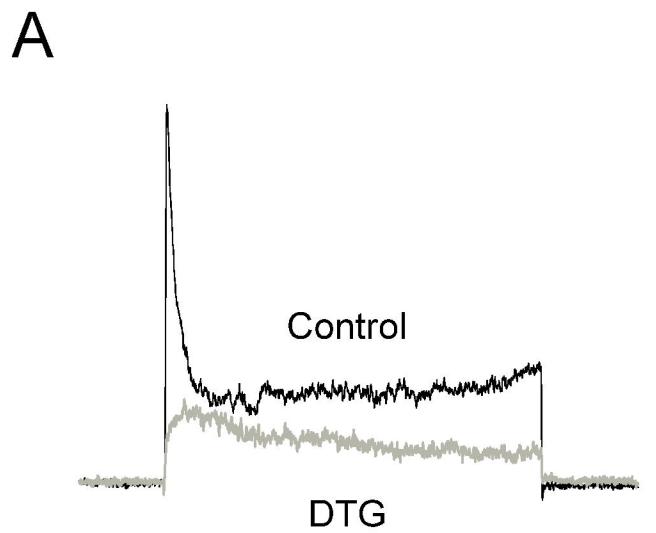


Figure 7