Modulation of atrial fibrillation
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Neuropeptide substance-P causes action potential prolongation and resting membrane depolarization in rabbit atrial myocytes

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Abstract

Background: The cardiac nervous system plays an important role in the genesis of atrial fibrillation (AF). Apart from acetylcholine and norepinephrine, various neuropeptides are released in atrial myocardium of which the direct effects on atrial electrophysiology are largely unknown. We therefore investigated the effects of various neuropeptides including substance-P (SP) on atrial electrophysiology.

Methods and Results: Enzymatically isolated left atrial rabbit myocytes were superfused with SP, neuropeptide-Y, somatostatin-14 or vasoactive intestinal peptide, and studied with patch-clamp and calcium-fluorescence methodologies. With exception of SP, the neurotransmitters did not directly affect atrial action potential (AP) characteristics (at 1 µM). SP reduced both resting membrane potential (RMP) and action potential amplitude, and increased action potential duration at 90% of repolarization (APD$_{90}$) by 40%. The effects on APD$_{90}$ were dose-dependent and occurred at concentrations from 10 nM, but did not increase vulnerability to triggered activity. Voltage-clamp analysis revealed that SP significantly diminished the L-type Ca$^{2+}$ current (I$_{Ca,L}$), the inward rectifier K$^+$ current (I$_{K1}$) and a steady-state outward current. The transient K$^+$ outward current, the Ca$^{2+}$-activated Cl$^-$ current and the Na$^+$-Ca$^{2+}$ exchanger current were unaffected, as was the intracellular Ca$^{2+}$ handling. The reduction in RMP and increase in APD$_{90}$ are due to the decrease in I$_{K1}$ and steady-state outward current, respectively. The latter is probably carried by background-like K$^+$ channels.

Conclusion: SP directly causes a substantial action potential prolongation due to inhibition of a background K$^+$ current. Since a lengthening of atrial repolarization is potentially anti-arrhythmic, we speculate that SP can prevent and/or terminate AF.
**Introduction**

The intrinsic cardiac nervous system is located in the fat pads of the heart, especially on the atria, and contains highly complex networks of ganglia, also known as ganglionic plexus. These ganglia may contain up to 200 neurons, which in addition to the well-known neurotransmitters acetylcholine and norepinephrine, also release a wide range of neuropeptides in the atria of the human heart.

The cardiac autonomic nervous system has been implicated in the genesis of atrial fibrillation (AF). Indeed, electrical stimulation of the ganglionic plexus in the fatty pads causes a slowing of heart rate and provokes AF. Ablation of ganglionic plexus is used to treat patients with lone AF.

Although the effects of noradrenalin (NA) and acetylcholine (ACh) on atrial cellular electrophysiology have been investigated, little is known of the electrophysiological effects of various other neuropeptides/neurotransmitters on atrial electrophysiology. We therefore examined the electrophysiological response of single atrial myocytes to several neuropeptides, particularly substance-P (SP). Substance-P, the first mammalian tachykinin that was identified, has been associated with cardiovascular regulation. The cardiac (physiological) response to SP has been mainly characterized by a decrease in heart rate and ventricular contractility, through stimulation of cholinergic neurons. SP has also been implicated in various cardiovascular diseases such as ischemia, heart failure and atrial fibrillation. For example, degeneration of SP immuno-reactive nerves has been reported in a dog model of atrial fibrillation. In addition, the occurrence of post-operative atrial fibrillation was associated with a decrease in SP serum levels in patients that had undergone coronary artery bypass surgery.

Thus, we determined the electrophysiological effects of SP, neuropeptide-Y (NPY), somatostatin-14 (SOM-14), and vasoactive intestinal peptide (VIP) on isolated rabbit atrial myocytes. We established that SP significantly modulates atrial action potential characteristics through alterations in several types of membrane currents, while NPY, SOM-14 and VIP did not elicit a response (supplement figure and table).

**Methods**

The study conformed to the ‘Guide for the Care and Use of Laboratory Animals’
published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local ethical committee.

**Single cell preparation**

Male New Zealand White specified pathogen free rabbits (2.5-3.5 kg) were anaesthetized with a combination of 20 mg xylazine and 100 mg ketamine (intramuscularly) and heparinized with a bolus of 1000 IU heparin (intravenously). Subsequently, the animals were killed by 200 mg pentobarbital intravenously, after which the heart was quickly excised and mounted on a Langendorff perfusion apparatus. Single atrial cells were isolated from the left atrium by enzymatic dissociation using the protocol that was described previously (for the isolation of ventricular myocytes)\(^1\), with a modification consisting of complementing the enzymatic solution with 6.6 µg/mL protease.

Small aliquots of single cell suspension were introduced into a recording chamber on the stage of an inverted microscope. Cells were allowed to adhere for 5 minutes after which superfusion was started. Single quiescent rod-shaped myocytes with clear cross-striations and smooth surfaces were selected for measurements.

**Cellular electrophysiology**

*Data acquisition and analysis*

APs and membrane currents were recorded at 36.5 °C with the amphotericin-B perforated or ruptured patch-clamp technique, using an Axopatch 200B Clamp amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA). Voltage control, data acquisition, and analysis were performed using custom-made software.

Series resistance was compensated for by 70%-80% and potentials were corrected for liquid junction potential.\(^1\) Signals were low-pass filtered (cut-off frequency: 5 kHz) and digitized at 40 kHz. Cell membrane capacitance \((C_m)\) was estimated by dividing the decay time constant of the capacitive transient in response to 5 mV hyperpolarizing voltage clamp steps from a holding potential of –40 mV, by the series resistance.

*Current-clamp experiments*

APs were measured with the amphotericin-B-perforated patch clamp technique
using normal Tyrode’s solution containing (in mM): NaCl 140, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 1.0, glucose 5.5, HEPES 5.0, pH 7.4 (NaOH). The patch-pipettes (borosilicate glass; 1-3 MΩ) were filled with a ‘standard’ solution containing (in mM): K-gluconate 110, KCl 30, NaCl 5, MgCl$_2$ 1, amphotericin-B 0.22, HEPES 10, pH 7.3 (KOH).

APs were elicited at 1 to 4 Hz by 2-ms (1.5× diastolic stimulation threshold) current pulses applied through the patch pipette. The following action potential characteristics were determined: resting membrane potential (RMP), maximal upstroke velocity ($V_{\text{max}}$), AP amplitude (APA), and AP duration (APD) at 20%, 50% and 90% repolarization (APD$_{20\%}$, APD$_{50\%}$ and APD$_{90\%}$, respectively). Values obtained from 10 consecutive APs were averaged.

Atrial myocytes were allowed to equilibrate for a 5-minute period of continuous stimulation (1 Hz) after which the wash-in of the following neurotransmitters and neuropeptides: 1 µM NA, 1 µM ACh, 1 µM Som-14, 1 µM VIP, 1 µM NPY, and 1 µM SP was started. Effects were assessed after equilibration, but not earlier than 3 minutes after application. In dose-response experiments, SP (1 nM – 10 µM) was applied in a cumulative sequence with 5 minutes intervals between increments in concentration. In addition, to assess the arrhythmogenic (triggered activity) effects of SP, atrial myocytes were rapidly paced at a rate of 5-6 Hz by a train of 20 consecutive stimuli, followed by a pause of 10 s. The occurrence of spontaneous depolarizations during the pause was counted in 4 consecutive tracings and averaged.

Voltage-clamp experiments
Steady state currents, L-type Ca$^{2+}$ current ($I_{\text{Ca,L}}$), transient outward K$^+$ current ($I_{\text{to}}$), Ca$^{2+}$-activated Cl$^-$ current ($I_{\text{Cl(Ca)}}$) and Na$^+$-Ca$^{2+}$ exchange current ($I_{\text{NCX}}$) were measured with solutions specified below and using voltage-clamp protocols depicted in the corresponding figures.

Steady state currents, $I_{\text{to}}$ and $I_{\text{Cl(Ca)}}$ were measured using the amphotericin-B perforated patch clamp technique with standard pipette solution (see above) and normal Tyrode’s solution as external solution. For accurate $I_{\text{to}}$ measurements, 30 µM tetrodotoxin (TTX), 0.25 mM CdCl$_2$, 5 µM E-4031, and 100 µM chromanol 293B was added to the normal Tyrode’s solution. For $I_{\text{Cl(Ca)}}$ measurements, we added 10 µM TTX and 2 mM 4-aminopyridine (4-AP) to the normal Tyrode’s solution.
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\( I_{Ca,L} \) was measured using the ruptured patch clamp technique with pipette solution containing (in mM): CsCl 145, K\(_2\)-ATP 5, EGTA 10, HEPES 10, pH 7.2 (NMDG-OH). The extracellular solution contained (mM): TEA-Cl 145, CsCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 1.0, glucose 5.5, HEPES 5.0; pH 7.4 (NMDG-OH). \( I_{Ca,L} \) was measured in the presence of 0.25 mM 4,4’diisothiocyanatostilbene-2,2’-disulfonic acid (DIDS; Sigma-Aldrich, MO, USA) to block \( I_{Cl(Ca)} \). \( I_{NCX} \) was measured using the ruptured patch clamp technique with pipette solution containing (in mM): CsCl 145, NaCl 5, Mg-ATP 10, TEA-Cl 10, EGTA 20, CaCl\(_2\) 10, HEPES 10, pH 7.2 (NMDG-OH). The bath solution consisted of a K\(^+\)-free Tyrode’s solution to which 1 mM BaCl\(_2\), 2 mM CsCl\(_2\), 5 µM nifedipine, 100 µM ouabain, and 200 µM DIDS was added to suppress membrane currents other than \( I_{NCX} \). \( I_{NCX} \) was measured as 10 mM Ni\(^{2+}\)-sensitive current during a descending voltage ramp protocol. Since the effects of Ni\(^{2+}\) on \( I_{NCX} \) are reversible, \( I_{NCX} \) measurements in the absence and presence of SP were carried out in the same cell.

Voltage-dependencies of (in)activation were determined by fitting a Boltzmann function \( y=A/[1+\exp((V-V_{1/2})/k)] \) to the individual curves, yielding a half-maximal voltage \( V_{1/2} \) (mV) and a slope factor \( k \) (mV). Time constants of inactivation were obtained by fitting current decay with a bi-exponential function \( y=y_0+A_f \exp(-t/\tau_f)+A_s \exp(-t/\tau_s) \), where \( A_f \) and \( A_s \) are the amplitudes of the fast and slow inactivating components, and \( \tau_f \) and \( \tau_s \) their respective inactivation time constants.

Currents densities were calculated by dividing the current amplitude by \( C_m \).

In voltage clamp experiments the effect of SP on the various membrane currents was assessed at a concentration of 10 µM.

Cytosolic Ca\(^{2+}\) transients

Intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) was measured in indo-1 loaded myocytes as described previously. Dual wavelength emission of indo-1 was recorded ((405-440)/(505-540) nm, excitation at 340 nm) and free Ca\(^{2+}\) was calculated. Ca\(^{2+}\) transients were elicited at 5 Hz using field stimulation. For determination of diastolic and systolic Ca\(^{2+}\) concentrations, and Ca\(^{2+}\) transient amplitudes were, data from 10 consecutive Ca\(^{2+}\) transients were averaged.

The effect of SP on Ca\(^{2+}\) was assessed at a concentration of 10 µM 3 minutes after application.
Neuropeptide substance-P

Drugs
All drugs used for cellular measurements were obtained from Sigma-Aldrich (MO, USA), except for E-4031 (Tocris, MN, USA), SP (Enzo Life Sciences, NY, USA), TTX (Abcam Biomedicals, Cambridge, UK) and NA (Centrafarm, Etten-Leur, The Netherlands).

DIDS was freshly prepared as a 0.5 M and chromanol 293B as a 0.1 M stock solution in DMSO. Nifedipine was prepared as a 5 mM stock solution in ethanol. E-4031 and TTX were prepared as a 5 and 30 mM stock solution in distilled water. All stock solutions were diluted appropriately before use. SP was freshly diluted at its final concentration. DIDS and nifedipine were stored in the dark.

Statistics
Data are presented as mean±SEM. A student t-test or Two-Way Repeated measures ANOVA followed by pairwise comparison using the Student-Newman-Keuls method was used where appropriate. $P < 0.05$ was defined as statistical significant.

Results
Effect of noradrenalin and acetylcholine on action potential characteristics
Single atrial myocytes demonstrated an intact signal transduction system as evidenced by their characteristic response to these classical neurotransmitters as shown in supplement figure and table. Application of NA slightly hyperpolarized the resting membrane, and increased $V_{\text{max}}$, APA and APD. ACh also hyperpolarized the resting membrane and increased $V_{\text{max}}$ and APA, but decreased APD.

Effect of neuropeptides on action potential characteristics; SP causes membrane depolarization and APD prolongation.
Atrial myocytes did not respond to the presence of the neuropeptides Som-14, VIP and NPY at a concentration of 1 µM (supplemt figure and table). Application of SP on the other hand, resulted in substantial alterations in AP morphology in comparison to control conditions (CTRL), particularly a depolarization of the resting membrane and an increase in APD (Figure 1A). Figure 1B summarizes the effects of SP on AP parameters. At 1 µM SP, the initial fast repolarization (APD$_{20}$) was not
significantly affected, but APD$_{50}$ (CTRL: 21.2±7.5 ms vs. SP: 43.4±14.6 ms, n=6) and APD$_{90}$ (CTRL: 89.0±2.0 ms vs. SP: 125.2±2.5 ms, n=6) were significantly increased (Figure 1B). The RMP (CTRL: -68.0±1.5 ms vs. SP: -62.0±2.1 ms, n=6) and the APA (CTRL: 108.6±3.4 ms vs. SP: 97.0±6.1 ms, n=6) were both significantly decreased (Figure 1B$_{II}$). The slight reduction in $V_{\text{max}}$ was not statistically significant (P=0.3, Figure 1B$_{III}$). Furthermore, AP prolongation by SP was present at all stimulation frequencies (1 to 4 Hz) (Figure 1C), and occurred in a dose-dependent fashion from a concentration of 10 nM (Figure 1D).

Figure 1. Effect of substance-P on action potential (AP) characteristics of rabbit atrial myocytes

(A) Atrial action potentials elicited at 1 Hz under control conditions and in the presence of 1 µM substance-P (SP). (B) Bar histograms showing average values for AP duration at 20, 50, and 90% of repolarization (APD$_{20}$, APD$_{50}$, and APD$_{90}$) (B$_1$), resting membrane potential (RMP) and action potential amplitude (APA) (B$_{II}$), and maximal upstroke velocity ($V_{\text{max}}$) (B$_{III}$) before (control) and after application of 1 µM SP. (C) Plot showing the frequency-dependence of APD$_{90}$ before (control) and after application of 1 µM SP. (D) Plot showing concentration-dependence of relative APD$_{90}$ before (control) and after application of increasing concentrations of SP. * P < 0.05

Effects of substance-P on membrane currents

The depolarization of the resting membrane and prolongation of the AP can result from a decrease in outward current, an increase in inward current, or both. To establish the mechanism(s) underlying the effect of SP on the AP characteristics, we
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investigated the major membrane currents underlying rabbit atrial APs.

**Steady-state current.** Steady-state currents were measured in the perforated-patch configuration at the end of hyper- and depolarization steps of 500 ms duration (holding potential -60 mV) in the absence of blockers (Figure 2A). Representative examples of current tracings recorded at -120 and +40 mV show a moderate decrease in steady-state in- and outward current in the presence of 10 µM SP (Figure 2B), which was confirmed by the average current-voltage (I-V) relationships in Figure 2C. The steady-state inward current at -120 mV, considered to be carried by $I_{K1}$ channels, was significantly decreased by SP (CTRL: -6.8±1.1 pA/pF vs SP: -6.0±1.3 pA/pF, n=6). Besides, a significant decrease in steady-state outward current at all voltages positive to -40 mV was seen. At +50 mV the outward current decreased by 20% from 6.9±0.5 pA/pF (CTRL) to 5.5±0.6 pA/pF (SP). At voltages more positive than -40 mV the outward current is considered to be composed of different types of $K^+$ currents, including the sustained component of the transient outward current ($I_{to}$), and of the slow, rapid and ultra-rapid components of the delayed rectifier $K^+$ current ($I_{Kr}$, $I_{Kur}$ and $I_{Kur}$, respectively).

Figure 2. Effect of substance-P on steady state currents. 
(A) Voltage protocol. (B) Current tracings recorded at -120 and +40 mV before (left panel) and after application of 10 µM substance-P (SP, right panel). (C) Average current-voltage relationships of the steady-state current before (control) and after application of 10 µM SP. * $P < 0.05$
Transient outward $K^+$ current ($I_{to}$). Figure 3B shows representative $I_{to}$ peak currents upon depolarization to 50 mV (protocol as in Figure 3A). On average, $I_{to}$ peak densities (Figure 3C) were not altered by SP, nor was the time course of current decay, as illustrated in Figure 3D. Figures 3E-G summarize the voltage-dependency of $I_{to}$ activation and inactivation. SP caused a small, but significant shift in $V_{1/2}$ of inactivation by -3.5 mV (Figure 3F, n=5, P=0.02) as well as a steepening of the slope of the voltage dependency of activation (CTRL: $k=14.2\pm0.9$ vs. SP: $k=9.8\pm0.1$ mV, Figure 3G, n=5, P<0.01).

Figure 3H depicts the average steady state I-V relationships, obtained by current amplitude measurement at the end of the 500 ms voltage steps, in accordance with Figure 2C, but now in the presence of blockers for Na$^+$ current ($I_{Na}$), $I_{Ca,L}$, $I_{Kr}$, and $I_{Ks}$. At -120 mV the steady-state inward current decreased from -6.9±1.6 pA/pF (CTRL) to -5.75±1.5 pA/pF (SP, n=5, P<0.01), while at +50 mV a 25% reduction in steady-state outward current was observed from 6.1±0.85 pA/pF (CTRL) to 4.6±0.46 pA/pF (SP, n=5, P=0.02).

$L$-type Ca$^{2+}$ current ($I_{Ca,L}$). $I_{Ca,L}$ was measured using a 2 step protocol (Figure 4A). During the first depolarizing pulses (P1) $I_{Ca,L}$ activates, while the second pulse (P2) is used for measurement of voltage dependency of inactivation. The representative $I_{Ca,L}$ recordings upon a depolarizing pulse to 0 mV (Figure 4B), and the average I-V relationships of $I_{Ca,L}$ in Figure 4C, show a decrease in peak $I_{Ca,L}$ in the presence of SP. At 0 mV, the reduction in peak $I_{Ca,L}$ amounted to ~20%, from -21±3.3 pA/pF in control conditions to -16.6±2.3 pA/pF in the presence of SP (n=6, P<0.05). Neither the voltage dependencies of (in)activation (Figure 4D), nor the time course of current decay (Figure 4B, inset) were affected by SP.

Ca$^{2+}$-activated Cl$^-$ current ($I_{Cl(Ca)}$). $I_{Cl(Ca)}$ was elicited by depolarizing steps to +30, +40 and +50 mV from a holding potential of -60 mV (Figure 5A), and defined as the early transient peak (Figure 5B). SP did not affect $I_{Cl(Ca)}$ as peak amplitudes were similar under control conditions and in the presence of SP (Figure 5C). Comparable to the data shown in Figure 2C and 3H, SP reduced steady-state outward currents at the end of the 250 ms depolarizing steps by ~25% (Figure 5D, at +50 mV: CTRL 7.2±0.8 pA/pF vs. SP 5.7±0.5, n=6, P=0.01) in the presence of 2 mM 4-AP. This
rules out the possibility that the SP effect is mediated by a reduction in sustained component of $I_{to}$ or $I_{Kur}$.

**Figure 3. Effect of 10 µM substance-P (SP) on transient outward $K^+$ current ($I_{to}$).**

(A) Voltage protocol. (B) Current tracings recorded at -120 and +50 mV before (left panel) and after application of 10 µM substance-P (SP, right panel). (C) Average current-voltage (I-V) relationships of $I_{to}$ peak currents before (control) and after application of 10 µM SP. (D) Bar histogram showing average values for time constants of fast and slow inactivation before (control) and after application of SP. (E) Plot showing average voltage-dependencies of activation and steady-state inactivation before (control) and after application of 10 µM SP. Solid lines indicate the Boltzmann fits of the average data. (F,G) Bar histograms showing average values for half-maximal voltage ($V_{1/2}$) and slope factor ($K$) of voltage-dependence of inactivation and activation resp. before (control) and after application of 10 µM SP. (H) Average I-V relationships of steady-state current before (control) and after application of 10 µM SP. Blockers for Na$^+$-, Ca$^{2+}$-, rapid and slow delayed rectifier $K^+$ currents were continuously present. * $P < 0.05$
**Figure 4. Effect of 10 µM substance-P on L-type Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)).**

(A) Voltage protocol. (B) Current tracings recorded under control conditions and in the presence of 10 µM substance-P (SP). Insets: voltage protocol (left) and bar histogram showing the average time constants of fast and slow inactivation before (control) and after application of SP (right). (C) Average current-voltage relationships of I\(_{\text{Ca,L}}\) before (control) and after application of 10 µM SP. (D) Average voltage-dependencies of activation and steady-state inactivation before (control) and after application of SP. Solid lines indicate the Boltzmann fits of the average data. * indicates \(P < 0.05\)

**Na\(^+\)-Ca\(^{2+}\) exchange current (I\(_{\text{NCX}}\)).** I\(_{\text{NCX}}\) was measured in Ca\(^{2+}\)-buffered conditions as the Ni\(^{2+}\)-sensitive current during a descending voltage ramp protocol (Figure 6A, inset). Figure 6B shows a representative example of I\(_{\text{NCX}}\) in control conditions and in the presence of SP. On average, SP had no effect on the reverse (outward) mode, nor on the forward (inward) mode of I\(_{\text{NCX}}\) (Figure 6B).
Figure 5. Effect of 10 μM substance-P on Ca\(^{2+}\) activated Cl\(^{-}\) current (I\(_{\text{CaCl}}\)).

(A) Voltage protocol. (B) Current tracings recorded during a depolarizing step to +30mV under control conditions and in the presence of 10 μM substance-P (SP). (C,D) Average current-voltage relationships of peak I\(_{\text{CaCl}}\) (C) and steady-state current at t=300 ms (D) before (control) and after application of 10 μM SP. Blockers for Na\(^{+}\)-, transient outward and ultra-rapid delayed rectifier K\(^{+}\) currents were continuously present. * indicates P < 0.05.

Figure 6. Effect of 10 μM substance-P on the Na\(^{+}\)-Ca\(^{2+}\) exchange current (I\(_{\text{NCX}}\)).

(A) Current tracings recorded under control conditions and in the presence of 10 μM substance-P (SP). Insert: Voltage protocol. (B) Plot showing the average current-voltage relationships of I\(_{\text{NCX}}\) before (control) and after application of 10 μM SP. * P < 0.05
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**SP sensitive steady-state outward current.** To obtain more insight in the nature of the steady-state outward current inhibited by SP, we deduced the SP sensitive current by subtracting the current amplitude in the presence of SP from the current amplitude measured under control conditions, using the data shown in Figure 2, 3 and 5. Figure 7 shows that the resultant SP sensitive I-V curves are largely similar, despite the presence of the various ion channel blockers. They are virtually linear and reverse sign at ~ -75 mV, close to the calculated Nernst potential for K⁺ ions (-85mV), indicating that an outward background (or leakage) K⁺ current is involved. The additional observation that the SP sensitive current in the voltage-range where I_{Ca,L} is active, is less pronounced in the presence of a Ca²⁺ channel blocker (open circles vs. black-filled circles, Figure 7), may point also to a contribution of a Ca²⁺-regulated K⁺ current.

**Figure 7. Effects of various membrane current blockers on the substance-P sensitive steady-state current.** Substance-P (SP) sensitive currents were extracted from Figure 2 (black-filled circles), Figure 3 (open circles) and Figure 5 (grey filled circles), by subtraction of the current in the presence of SP from the current measured under control conditions.

**Effects of SP on spontaneous action potentials and calcium homeostasis**

To investigate the effects of SP on the incidence of spontaneous APs on the one hand, and modifications in Ca²⁺ homeostasis on the other hand, atrial myocytes were rapidly paced at a rate of 5 Hz by a train of 20 consecutive stimuli, followed by a pause of 10 s. Figure 8 shows representative examples of the effects of SP on transmembrane potentials (Figure 8A) and Ca²⁺ concentration (Figure 8B,C) measured in separate atrial myocytes during and after rapid pacing. The last 2 stimulated APs (Figure 8A) and Ca²⁺ transients (Figure 8B) of the rapid pacing protocol are shown. Under control conditions (Figure 8A, black line), burst pacing
resulted in a transient depolarization directly after termination of stimulation and incidentally induced a spontaneous AP. In the presence of 10 µM SP (Figure 8A, grey line), there was an increase in the amplitude of the transient depolarization following the last stimulated AP and a depolarization of the resting membrane. Rapid pacing did not result in Ca\textsuperscript{2+} aftertransients (Figure 8B). Figure 8C,D shows that the diastolic, systolic and peak Ca\textsuperscript{2+} were not different under control conditions and in presence of SP.

These findings indicate that 1) the SP induced membrane depolarization does not result in an increased incidence of spontaneous AP’s and 2) the reduction I\textsubscript{Ca,L} does not affect Ca\textsuperscript{2+} handling.

**Figure 8. Effect of substance-P on triggered activity and intracellular calcium concentration.** (A) Action potential (AP) recordings after burst pacing under control conditions and in the presence of 10 µM substance-P (SP). Insets: (left) Stimulation protocol (a train of 20 consecutive stimuli at 5 or 6 Hz was followed by a pause of 10 s, (right) Bar histogram showing the total number of triggered APs in 4 consecutive tracings before (control) and after application of 10 µM SP. (B) Intracellular calcium (Ca\textsuperscript{2+}) transients measured after burst pacing before (control) and after application of 10 µM SP. (C) Ca\textsuperscript{2+} transients under control conditions and in the presence of 10 µM SP. (D) Bar histograms showing average diastolic, systolic and peak Ca\textsuperscript{2+} before (control) and after application of 10 µM SP.* P < 0.05

**Discussion**

The cardiac autonomic nervous system plays a significant role in the induction of atrial fibrillation. Particularly, alterations in the sympatho-vagal balance and excessive intrinsic cardiac nerve activity have been implicated to trigger atrial arrhythmias.\textsuperscript{19-21} In addition to the classical neurotransmitters, the wide range of neuropeptides Harbour by the atrial intracardiac ganglia and nerve fibers
potentially modulate atrial electrophysiology.\textsuperscript{3,12,22,23} To date however, the efferent actions on the atrial myocyte directly mediated by the various neuropeptides have scarcely been defined.

In this study we therefore explored the effects of several neuropeptides, most importantly SP, on the electrical properties of single rabbit atrial myocytes. Our study shows that SP exerts a significant effect on atrial action potential morphology, whereas other neuropeptides (i.e. NPY, SOM-14, VIP) do not. Alterations in action potential configuration by SP were characterized by a subtle reduction in resting membrane potential and action potential amplitude, together with a profound increase in action potential duration. The AP prolonging effect was dose-dependent and occurred from a concentration as low as 10 nM, suggesting that the electrophysiological actions of SP on atrial myocytes are receptor-mediated involving the neurokinin cell-surface receptors (NK1-NK3).\textsuperscript{24} Remarkably, the AP prolongation was also present at high pacing rates and was effectuated without an increase in $I_{\text{Ca,L}}$ current and facilitation of spontaneous Ca\textsuperscript{2+} aftertransients. SP therefore may be a powerful anti-fibrillatory agent that is efficacious in the atrium.

**Mechanisms of reduced resting membrane potential and action potential prolongation by substance P**

The effects of SP on membrane currents are diverse and highly tissue-specific. Data on the effect of SP on membrane currents largely stem from studies on central and peripheral neurons, and to a lesser degree from studies on smooth muscle cells originating from the gastro-intestinal tract. To our knowledge no data are available concerning the actions of SP on membrane currents in cardiac myocytes.

We observed a small but significant decrease of steady-state inward current in the negative potential range (considered to be $I_{\text{K1}}$), as well as a ~25% reduction in steady-state outward current in the positive potential range in the presence of SP. A reduction in $I_{\text{K1}}$ is consistent with the observed depolarization of the resting membrane, and to the reduced APA and $V_{\text{max}}$ (although the latter was not statistically significant). Similar to our findings, SP mediated reduction of $I_{\text{K1}}$ has also been reported in central and peripheral neurons from rat and guinea pig.\textsuperscript{25-29} Alternatively, the small decrease in steady-state inward current at negative potentials, could also reflect inhibition of constitutively active (i.e. in the absence
of an agonist) acetylcholine-activated K⁺ current ($I_{K\text{ACh}}$). Indeed, $I_{K\text{ACh}}$ carried by GIRK1/GIRK4 channels expressed in oocytes, is inhibited by SP through a protein kinase C dependent pathway. The reduction in steady-state outward current in the positive potential range complies with the observed AP prolongation in the presence of SP. The ionic mechanism underlying this decrease in steady-state outward current, however, remains to be elucidated. The well-known outward currents that are active in this voltage range in atrial myocytes are $I_{Ks}$, $I_{Kr}$, and $I_{Kur}$.

Yet, our experiments do not indicate a decrease in any of these current types by SP, since the reduction in steady-state outward current persists in the presence of their respective blockers (Figure 7). Changes in outward $I_{Ca(II)}$ and $I_{to}$ do not contribute to the observed action potential prolongation, as peak amplitudes of both current types are unchanged in the presence of SP, despite minor kinetic alterations in the latter.

Finally, the reduction of $I_{Ca,L}$ is expected to lead to a shortening of the AP. Therefore, the reduction in steady-state outward current by far outweighs the effect of $I_{Ca,L}$ reduction on AP duration.

The nature of the SP sensitive steady state outward current

Examination of the SP sensitive current (Figure 7) indicates that an outward K⁺ current is involved, since the reversal potential is close to the calculated Nernst potential for K⁺ ions (-85mV). A potential candidate to be at the basis of this K⁺ current is the so-called background or leakage K⁺ current, which was found to be inhibited by SP in neurons. Although it has proven difficult to designate the channel type underlying this background K⁺ current, recent evidence suggests that members of the two-pore-domain K⁺ (K2P) channel family generate such a K⁺ background conductance. At least two members of the K2P family have been identified in cardiac myocytes, TASK-1 and TREK-1, and there is clear evidence that these channels may regulate AP duration. Furthermore, the data from Figure 7 implicate the involvement of another K⁺ current. That is, the SP sensitive current in the voltage-range where $I_{Ca,L}$ is active, is less pronounced in the presence of a Ca²⁺ channel blocker (open circles vs. filled circles), which may point to a contribution of a Ca²⁺-activated K⁺ current ($I_{K,\text{Ca}}$) as well. Small-conductance K⁺,Ca channels (SK1-3) have been demonstrated to underly $I_{K,\text{Ca}}$ in atrial myocytes and shown to have a
significant role in atrial repolarization and fibrillation.\textsuperscript{42,43}

**Conclusion**

The results from this study represent the first evidence that SP can act as a neurotransmitter directly to the atrial myocyte, leading to significant AP prolongation through inhibition of an outward potassium background current. The lengthening of atrial repolarization with consequent increase in the effective refractory period, is considered anti-arrhythmic for re-entrant arrhythmias and may prevent or terminate atrial fibrillation. The AP-prolonging effect of SP was unabated at higher frequencies, contrary to many class III antiarrhythmic drugs known to display reverse rate-dependent effects on cardiac repolarization.\textsuperscript{44}

We speculate that stimulation of SP release may be protective against AF (this study), whereas reduced SP levels may facilitate AF development.\textsuperscript{14,43}
Supplement figure. Effects of various neurotransmitters and neuropeptides on atrial action potential configuration.

(A) Representative examples of APs stimulated at 1Hz under control conditions and in the presence of 1 μM noradrenalin (NA), (B) acetylcholine (ACh), (C) somatostatin-14 (Som-14), (D) neuropeptide-Y (NPY), (E) and vasoactive peptide (VIP).

Supplement table. Effects of NA, ACh, SOM-14, NPY and VIP on action potential characteristics.

<table>
<thead>
<tr>
<th></th>
<th>NA (n=12, 2 Hz)</th>
<th>ACh (n=6, 2 Hz)</th>
<th>SOM-14 (n=3, 1 Hz)</th>
<th>NPY (n=2, 1 Hz)</th>
<th>VIP (n=1, 1 Hz)</th>
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<tbody>
<tr>
<td><strong>RMP</strong> (mV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTRL</td>
<td>-77.8 ± 1.5</td>
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<td>-82.8 ± 1.4</td>
<td>-80.7 ± 1.7</td>
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<td>81.5 ± 1.4</td>
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<td>-79.4 ± 1.6</td>
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<tr>
<td><strong>V_{max}</strong> (V/s)</td>
<td></td>
<td></td>
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<tr>
<td>CTRL</td>
<td>361.5 ± 47.5</td>
<td>268.3 ± 31.2</td>
<td>374.2 ± 30.6</td>
<td>192.8 ± 30.4</td>
<td>542.0 ± 61.6</td>
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<td>NT</td>
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<td><strong>APA</strong> (mV)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>CTRL</td>
<td>113.6 ± 4.7</td>
<td>107.1 ± 8.7</td>
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<td><strong>APD_{90}</strong> (ms)</td>
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<tr>
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<tr>
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<td>109.6 ± 5.9</td>
<td>89.7 ± 8.3</td>
<td>82.1 ± 8.3</td>
</tr>
</tbody>
</table>

RMP, resting membrane potential; V_{max}, maximal upstroke velocity; APA, action potential amplitude; APD_{90}, action potential duration at 90% of repolarization.

* indicates P<0.05
** indicates P<0.01
Chapter 7

References


Neuropeptide substance-P


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