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Original Article

Gold nanoparticle-spermidine complex blocks the inward rectifier potassium channel

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Abstract: A previous study showed that negatively charged gold nanoparticles block ion pores by binding to the sulfur group of the cysteine loop of the ion channel when small molecules like amine lead the nanoparticles inside the ion pore. Cells were voltage clamped at -100 mV. Subsequently a bath application of 30 μM Ach produced a current followed by the extracellular application of 100 mM spermidine and 50 nM of nanoparticle complex. Peak amplitude was then recorded. The addition of Ach (30 uM) reversed the effect, and we recorded inhibition of the peak amplitude. We also recorded electrocardiogram (EKG) and the atria effective refractory period (AERP) after treatment with the complex in the atrium of a rabbit heart in a Langendorff apparatus. Upon external application of the complex, the Ach-activated current was blocked by 48.8% ± 3.1% with 82.7% ± 3.1% reversal. In recording the EKG and the AERP after the addition of the complex including 30 mM spermidine with 50 nM nanoparticles, the complete resolution of atrial fibrillation at 50 s and the elongation of AERP from 46 to 52 was observed, which unveils a new class 3 anti arrythmic agent using gold nanoparticles with spermidine. Negatively charged gold nanoparticles (0.8 nm) block ion pores after penetrating the cell membrane with spermidine, thus entering the cells with a polyamine transporter and acting at the intracellular face of the channel via binding to the sulfur group of the human inward rectifying potassium channel- I(KAch).

Keywords: Negatively charged gold nanoparticle, spermidine, inward rectifier potassium channel, cell penetration, atrial fibrillation

Introduction

Spermidine is an organic polycation that is protonated at physiologic pH and can potentially interact with 3 negatively charged nanoparticles [1]. Spermidine is non-toxic to mammalian cells [2]. Intracellular spermidine is responsible for the intrinsic gating and rectification of strong inward- rectifier K channels through direct plugging of the ion channel pore [3]. These K channels control the resting membrane potential and the excitability threshold [4]. Gold nanoparticles (0.8 nm) spontaneously adhere to the lipid bilayer surface and penetrate the lipid bilayer interior by passive diffusion [5, 6]. Spermidine enhances and facilitates the cellular uptake of ultrasmall 0.8 nm nanostructures [7].

Stimulation of the vagus nerve decreases heart rate by raising potassium permeability, while the activated K current-I(KAch)- is carried by the G-protein-activated inward- rectifier potassium channel (Kir) [8, 9]. Acetylcholine (Ach) binds to muscarinic receptors, thus triggering a heterotrimeric G-protein cascade that results in activation of the muscarinic activated potassium channel- IKACh [10, 11]. Atrial fibrillation (AF) is related to increasing Kir channel activity with shortened atrial action potential [12].

The Ach-activated potassium current-I(KAch) [13] flows through the Kir channel [14]. Its activation causes some degree of hyperpolarization according to the existing vagal tone. Drugs that selectively block the I(KAch) current are used to treat AF. We suggest the use of a selective I(KAch) blocker that blocks the ion pore directly, an action that can be reversed by Ach. This complete I(KAch) blockade decreases the resting membrane potential without having a marked effect on repolarization. Selective blo-

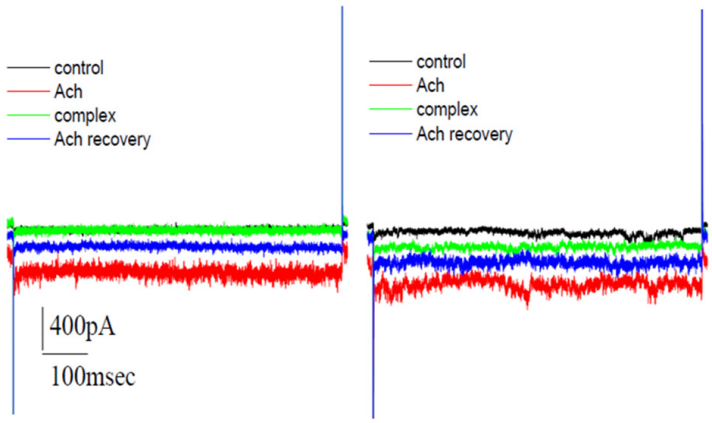


Figure 1. Patch clamp study in human atrial cardiomyocyte: We assessed changes in the Ach activated current upon extracellular and intracellular treatments of the complex containing 100 mM spermidine and 50 nM nanoparticles. Upon internal application of the complex, the peak amplitude was inhibited by 75.4% ± 4.4% (n=5). Upon external application of the complex, the Ach-activated current was blocked by 48.8% ± 3.1% with 82.7% ± 3.1% reversal. Blocking by extracellular treatment was lesser than that by intracellular treatment because of the loss of efficiency incurred when the complex penetrated the lipid bilayer of the cell membrane.

cking of the Ach-activated potassium current-I(KAch) is expressed only in the atrial myocardium and is not present in the ventricles.

The selective I(KAch) blocker, Tertiapin-Q is an effective agent for rhythm control in chronic atrial tachy-pacing-induced AF. However, "Tertiapin-Q" cannot be used in the clinical setting owing to the irreversibility off its binding to the extracellular I(KAch) domain sites [15]. A blockade of the inward- rectifier K(+) channels by antimalarial drugs such as quinidine or chloroquine terminates its reentry in cholinergic AF. Chloroquine interact with the vestibule of the cytoplasmic domain of the inward rectifier potassium channel Kir 2.1 [16]. However, long term use of these drugs generally increases a patient's risk of death and these drugs are therefore not used as first-line therapies because of their extensive side effects including an increase heart rate and pumping efficiency impairments [17].

Materials and methods

Materials

The 0.8 nm negatively charged gold nanoparticles were purchased from Nanoprobes, Inc (New York, NY, USA). The ionic bond was induced by vortexing 2 amples with different concentrations of positively charged spermidine (i.e., 10 mM and 100 nM) and 50 nM of negatively charged gold nanoparticles for approximately 15 min followed by mixing with a pipette for 15 min. The negatively charged COO- particles individually coated the thin film of 3 nanoparticle molecules that were each bound to one NH_2 and 2 NH_1 spermidine molecules. The spermidine hydroxide solution was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Electrophysiology

The right atrial appendages were obtained from patients undergoing cardiac surgery. Sinus rhythm was present in all cases. Atrial specimens were collected and finely minced using irridectomy scissors in Krebs buffer. Enzymatic digestions with collagenase B (1 mg/mL, Roche) were then conducted in Krebs buffer supplemented with 0.5% bovine serum albumin (Invitrogen). Isolated cells were filtered to remove undissociated pieces and gradually resuspended in Dulbecco's modified Eagle's medium (Biowhittaker, Wallkersville, MD, USA) supplemented with 1% antibiotics (100 IU mL^{-1} penicillin-G-Na; 50 IU mL^{-1} streptomycin sulfate), 1 nM insulin and 10% fetal calf serum. This materials were approved by the Institutional Animal Care and Use Committee (IACUC) in Tulane University Campus (LA, USA) and we get informed consent by the Institutional Review Board (IRB) with human studies.

HL-1 cells were cultured as a routine protocol [18]. For automated patch clamp experiments the cells were detached from the coverslips and transferred into extracellular buffer. The cells showed robust inward currents upon application of 100 μM ACh in a 60 mM containing extracellular buffer. The extracellular buffer (EC) was used for cell preparation and cell storage as well as for the dilution of compounds and the patch clamp recordings. The extracellular buffer contained in (mM): 140 NaCl, 2.5 KCl, 2 MgCl , 2 CaCl, 10 HEPES, 10 Glucose, 15 Sucrose. The extracellular buffer was adjusted to pH 7.4 (± pH 0.1) and osmolality 320 (± 5 mOsmol/l), and then stored at 4°C. Before

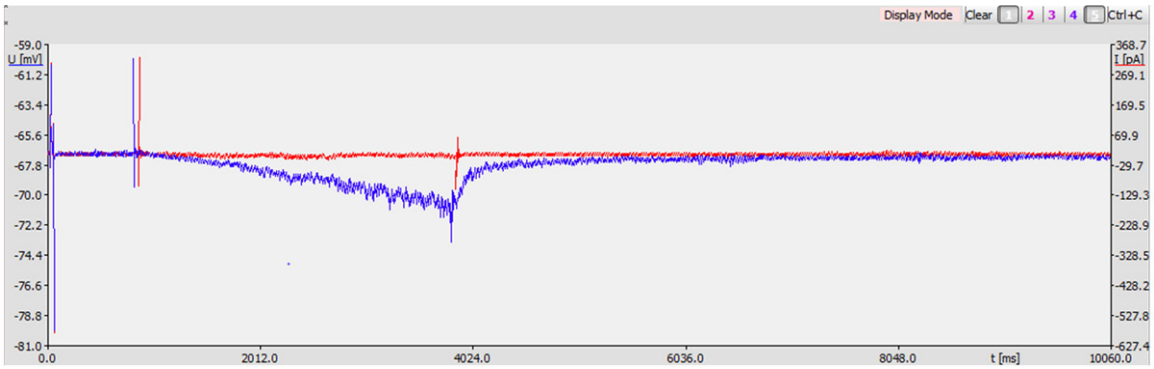


Figure 2. Application of the spermidine-gold complex (blue trace; 100 mM + 50 nM; in 60 mM K buffer with 100 µM ACh) to HL1 cell leads to inward currents with a slower activation relative to the ACh induced inward current (red trace). As stated in the results section, this effect may result from inhibition of IKACh by the complex (arrow). The remaining acetylcholine causes the reversal (n=4).

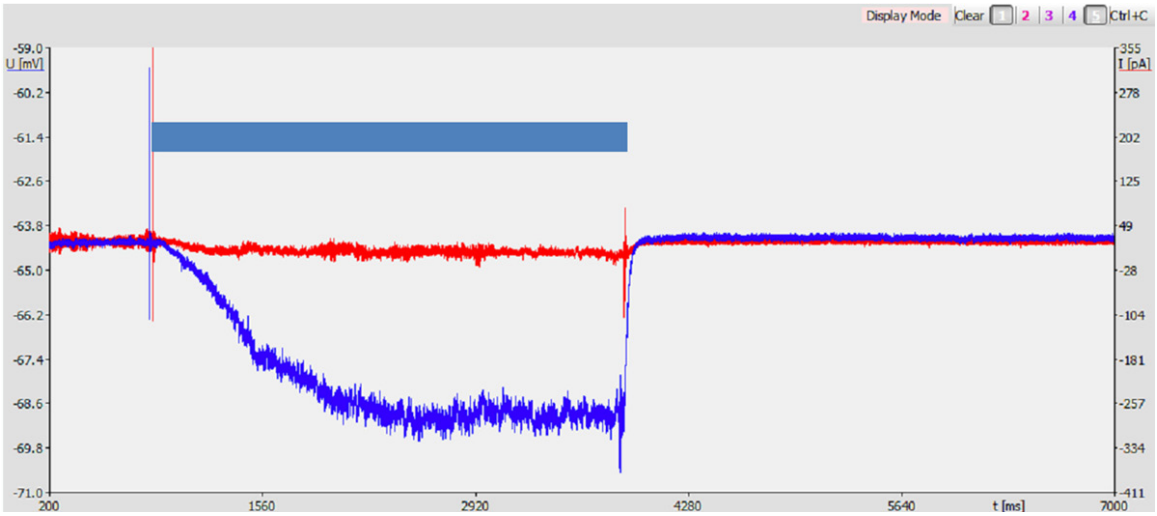


Figure 3. Application of 100 µM ACh in 60 mM K buffer leads to inward currents in HL-1 cells (blue trace). When only 50 nM gold nanoparticle were applied, the Ach-activated current did not change (n=4). Red trace: baseline.

usage, the extracellular was heated up to room temperature. For an extracellular K concentration of 57 mM NaCl was replaced by the equivalent amount of KCl. The intracellular solution (IC) was adjusted to the hERG ion channel electrophysiology and used to fill the patch pipettes. The IC contained in (mM): 100 K-Gluconate, 20 KCl, 1 CaCl, 1 $MgCl_2$, 10 HEPES, 11 EGTA-KOH, 4 ATP-Mg, 3 Phosphocreatine-Na -OH, 9 Sucrose. The intracellular solution was adjusted to pH 7.2 (± pH 0.1) and osmolality 295 (± 5 mOsmol/l), and then stored in 10 ml aliquots at -20°C. The intracellular solution was thawed before usage and applied for up to 4 hours. Measurements were performed on the in-house developed automated patch clamp device, the TM CytoPatch Instrument. In contrast to conventional patch clamp recordings, recordings on the TM CytoPatch Instrument are achieved on a planar microfluidic quarz chip. This chip is embedded into a silicon package with several microfluidic channels that can be used to deliver cells and solutions to the chip. The chip itself harbors two concentric openings. The first one is the ultimate patch pipette, which has a resistance of 7-9 M ohm. The other one, the so-called Cytocentering Channel, serves for catching a single cell out of the cell suspension. During the cell catch process, an appropriate pressure protocol applied via the Cytocentering channel ensures correct positioning of the cell directly on the patch pipette. Then, suction is applied via the pipette to establish the giga-seal formation and break-

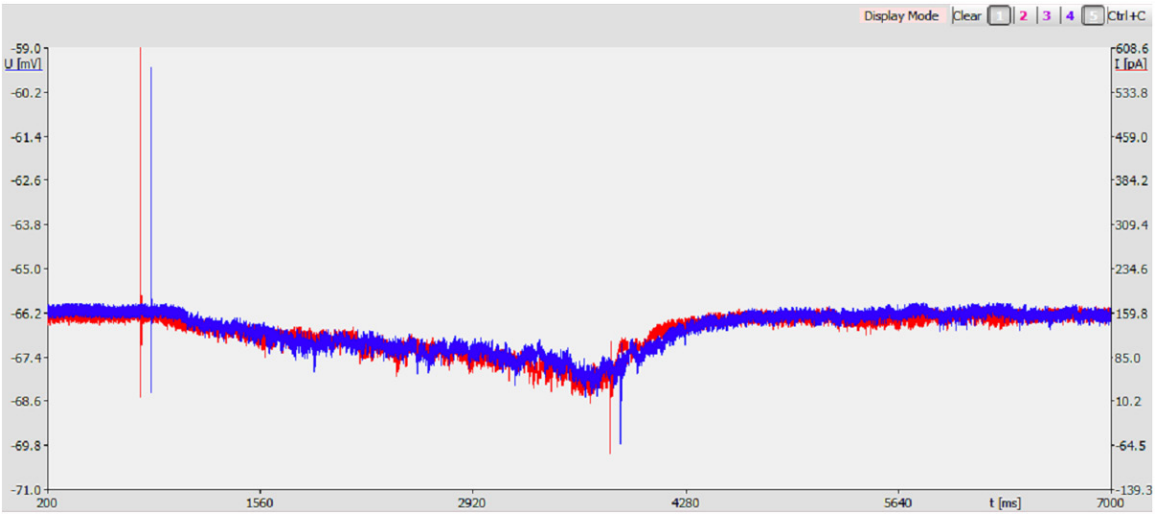


Figure 4. Co-application of 100 mM spermidine with 100 μM ACh induces an inward current in HL-1 cells (red trace). Application of the spermidine-gold complex does not change the current response (blue trace) (n=4).

through into whole-cell. In the whole-cell configuration, cells are continuously perfused with extracellular buffer. Agonists of ligand gated ion channels can be directly applied to the patched cell with wash in times of less than 100 ms and washed out completely. After obtaining a gigaseal and a stable whole-cell configuration cells were continuously perfused with extracellular buffer and kept at a holding potential of -70 mV. To evoke Ach induced inward positive currents 100 μM ACh in 60 mM K EC was rapidly applied for 4 s followed by a wash-out of EC. Spermidine or the undeca-gold spermidine complex was coapplied with the ACh (or without ACh in high K EC if indicated). Cells were always clamped at -70 mV during these applications. All experiments were performed at room temperature. The software files generated during the recordings were stored on computer hard disks. Patch TM clamp data were analysed using the CytoPatch software.

In vitro tissue (rabbit heart) study

Eighteen female New Zealand White rabbits weighing from 2.0 to 3 kg were used for the experiments. After intravenous anesthesia with phenobarbital and anticoagulation therapy with 1 mL heparin (=500 I.U. Liquemine) thoracotomy was performed and the heart was quickly explanted. Rabbits was stunned and then euthanized via cardiectomy, hearts was quickly removed, and the aorta was cannulated to a Langendorff apparatus to perfuse the coronary arteries in a retrograde manner with non-recirculating modified low K^+ Krebs-Henseleit buffer composed of (mM): NaCl (118.2), KCl (1.3), $MgSO_4$ (1.17), KH_2PO_4 (1.18), d-glucose (11.0), $CaCl_2$ (2.0), $NaHCO_3$ (24.8), and pyruvate (2.0) in deionized reagent grade water, adjusted to a pH of 7.4. The solution was balanced with a mixture of 95% O_2 to 37°C. A Master Flex peristaltic pump was used to perfuse the buffer into the Langendorff apparatus at a flow rate of 30-44 mL/min. A latex balloon was inserted via the left atrium and positioned in the left ventricle. The fluid filled latex balloon was connected with rigid tubing to a pressure transducer to permit for measurement of left ventricular developed pressure. The intraventricular balloon was expanded with distilled water to achieve an initial baseline left ventricular end-diastolic pressure of 5 mm Hg. Another pressure transducer is placed before the aortic cannula to measure the coronary perfusion pressure (CPP). CPP was corrected to 40-80 mm Hg only during equilibration by adjusting the peristaltic perfusion pump flow rate. Once the pump was set, the flow rate remained constant for the duration of the experiment. To monitor ECG, the heart will be maintained suspended in a glass chamber and bathed with 37°C low K^+ Krebs-Henseleit buffer. The glass chamber has multiple ECG electrodes arranged on the wall in a simulated 'Einthoven' configuration. Epicardial electrograms at the right atrial appendages were recorded with bipolar electrodes. Stimulating electrodes were also placed at the

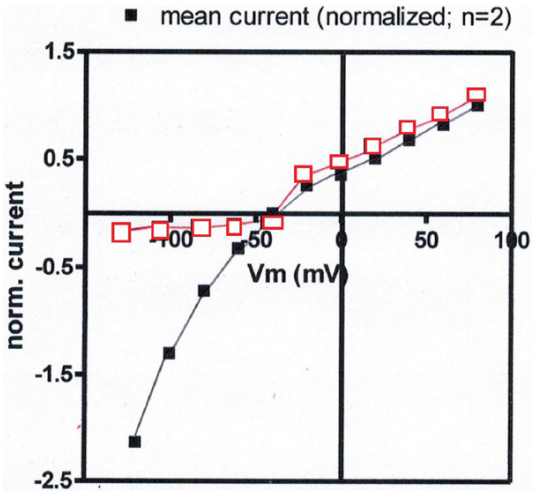


Figure 5. Cholinergic potentiation of hyperpolarizing inward rectification (black dot) was reversed after addition of the spermidine-gold nanoparticle complex to a CCh-containing solution. The red area indicates the tertiapin-sensitive shift of the I-V relationship. Extracellular [K^+] was 60 mM. No blocking data were obtained between -60 mV and +40 mV because of the low current amplitude.

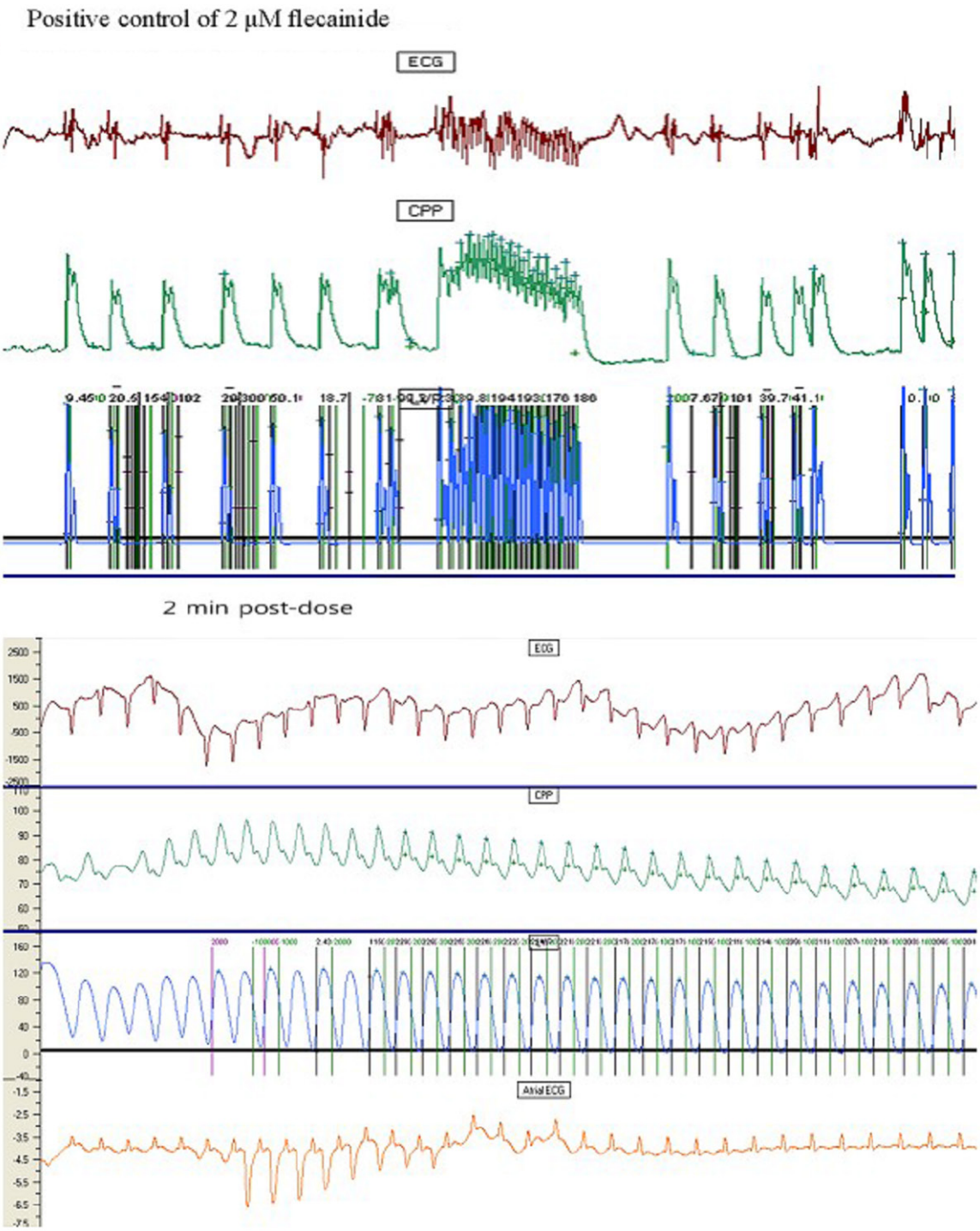
right atrial appendage. Recordings were obtained with NOTOCORD-Hem software platform. Hearts were allowed to stabilize for 10-15, and baseline AERP was measured. AERP was measured as the longest coupling interval of an extra stimulation which did not cause any atrial excitation after eight consecutive pacing pulses were given at cycle lengths (CL) of 250 ms. AF was induced with rapid pacing protocol (15 seconds episodes; 6000 bpm at 10 V) in the presence of acetylcholine [19]. AF induction was attempted until AF is sustained for at least 5 min, if AF is not induced after 25 attempts the experiment was terminated. There was a period of approximately 15 seconds between successive AF inductions. After 5 minutes of sustained AF, acetylcholine will be washed out for 3 minutes and the hearts was exposed to spermidine/undecagold solution and the duration until AF is reversed was recorded. After AF was reversed AERP measurements was obtained. For statistical data analysis, the Student's t test was used [20, 21].

Results

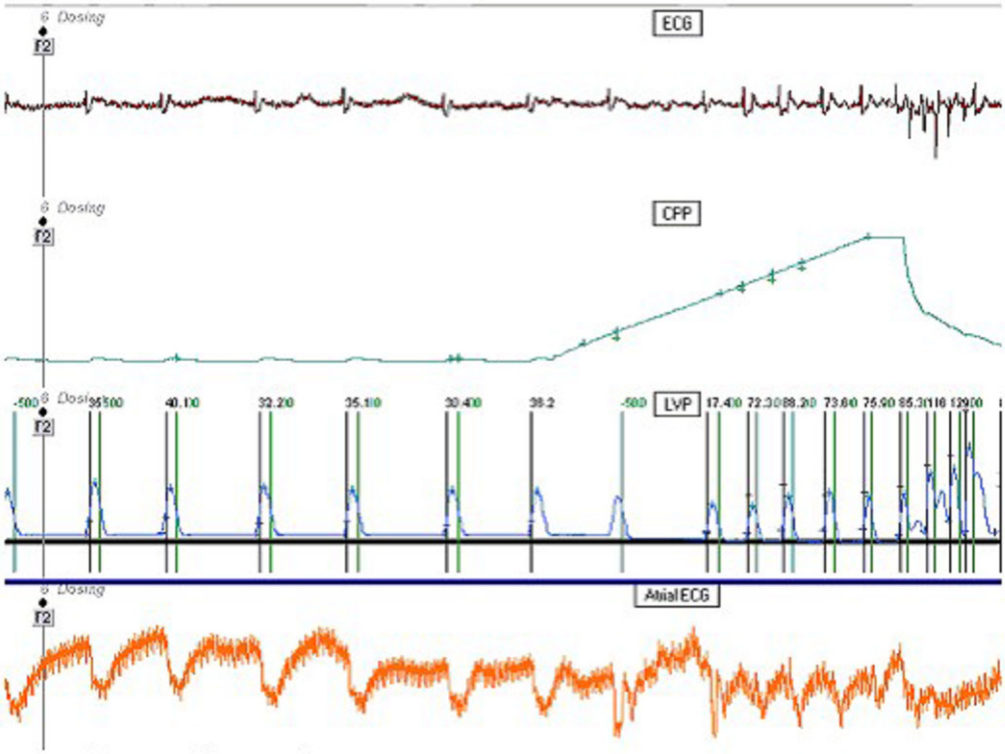
Electrophysiology

The spermidine-gold nanoparticle complex can block the KAch channel completely through lipid bilayer penetration of cardiomyocyte. When the human atrial myocyte cells were voltage clamped at -100 mV, a bath application of 30 μM Ach produced a current. With internal application of 100 mM spermidine and 50 nM of the nanoparticle complex, the amplitude of the current was decreased by 75.4% ± 4.4% (n=5). This inhibition was reversible and was easily eliminated by a 10 min washout (n=3). In this instance, the remaining current was 26.1% ± 2.1% of the control current. Addition of Ach (30 uM) reversed the blockade and restored a current to 67.9% ± 2.9% of the control. Upon internal application of the complex, the amplitude of the current decreased to near the baseline, because of complete occlusion of the channel pores. Upon external application of the complex, the Ach-activated current was blocked by 48.8% ± 3.1% with 82.7% ± 3.1% reversal. Therefore, the complex can penetrate the cell membrane by passive diffusion and decreased the amplitude of the potassium current from within the cell (**Figure 1**).

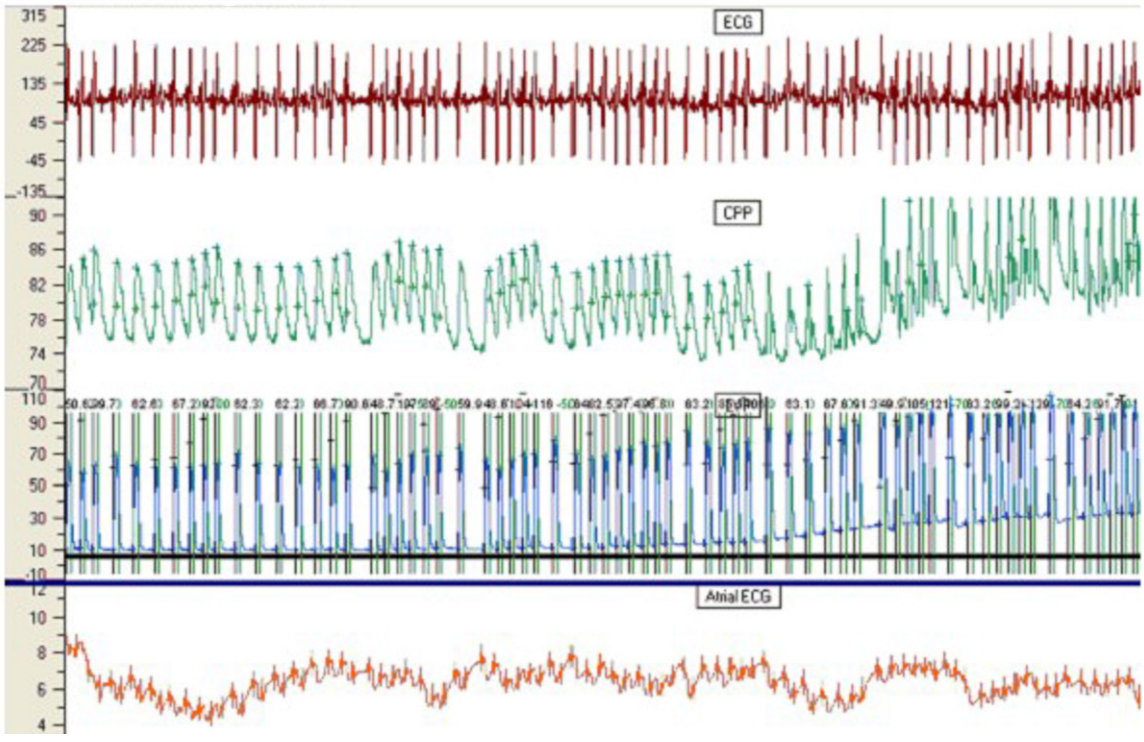
In HL-1 cells, application of 100 μM ACh (in 60 mM K buffer) led to whole-cell inward currents at a holding potential of -70 mV, on application of 100 mM spermidine complexed with 50 nM gold nanoparticle, The decreased current was mediated by blockade the IKACh ion channel (Kir3.1/Kir3.4) (n=4). Without washing, the presence of remaining acetylcholine causes reversal and expels the nanoparticles complexed with spermidine out of the channel pore (**Figure 2**). With the external application of only 50 nM, the Ach-activated current did not change (n=4, **Figure 3**). To investigate the origin of the change in current response 100 mM spermidine (in 60 mM K buffer) was applied (n=4), which led to an inward current with kinetics similar to those observed for co-application of ACh with the spermidine-gold complex (**Figure 4**). Subsequent application of the spermidine-gold complex (in 60 mM K buffer, 100 mM/50 nM) did not affect on the spermidine induced inward current. i.e.- spermidine induced the inward current but did not open the channel pore. Therefore, gold nanoparticles can not be introduced into inside ion pore. This supports the author's theory of that the only gold nanoparticles introduce into the channel pore, thus blocking the pore after spermidine penetrates the cell membrane. We evaluated the effect of spermidine-gold nanoparticle

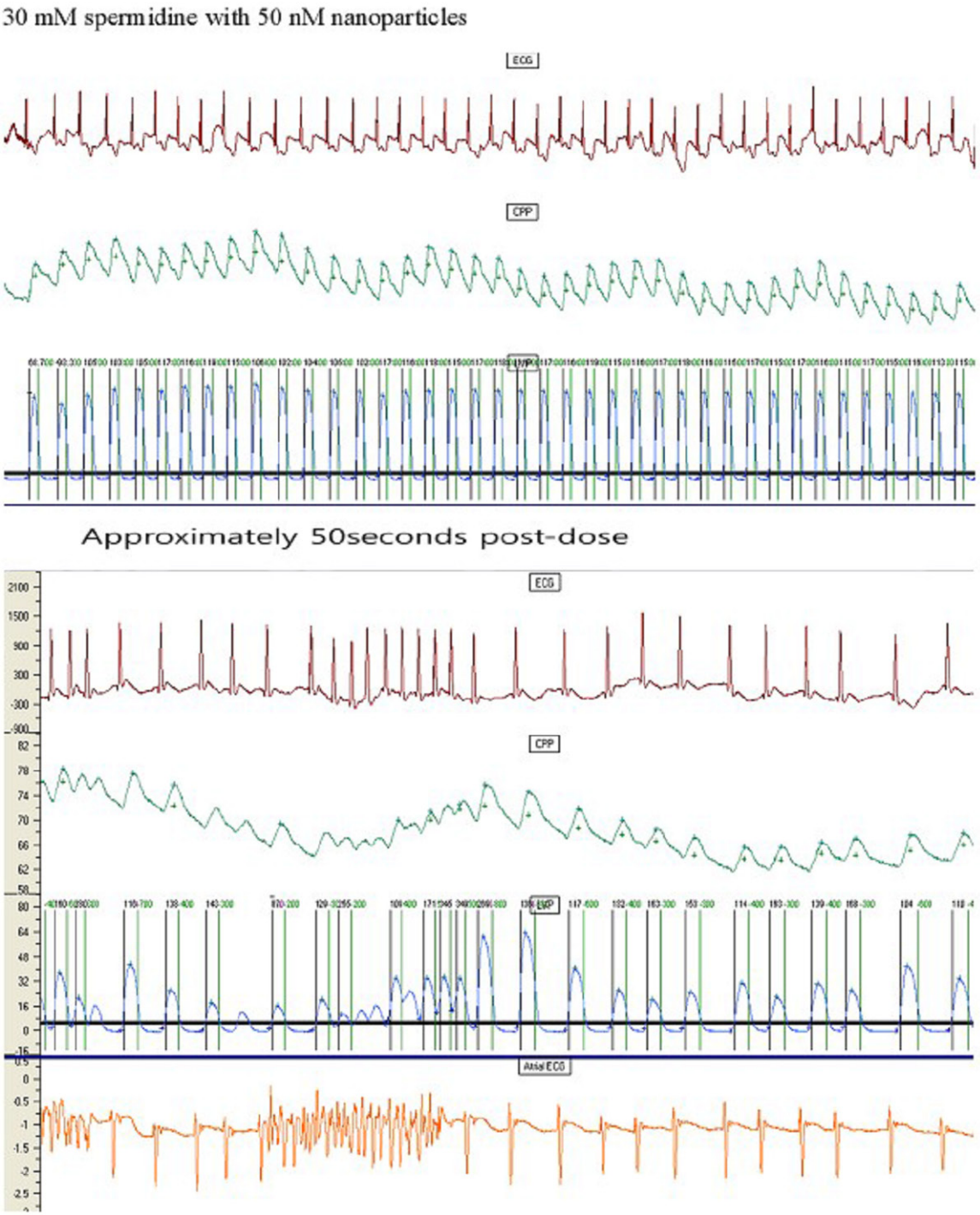

Positive control of 2 μM flecainide
ECG
CPP
2 min post-dose
ECG
CPP
Atrial ECG

100 mM spermidine with 50 nM nanoparticles



19 seconds post-dose




30 mM spermidine with 50 nM nanoparticles
ECG
CPP
Approximately 50seconds post-dose
ECG
CPP
Atrial ECG

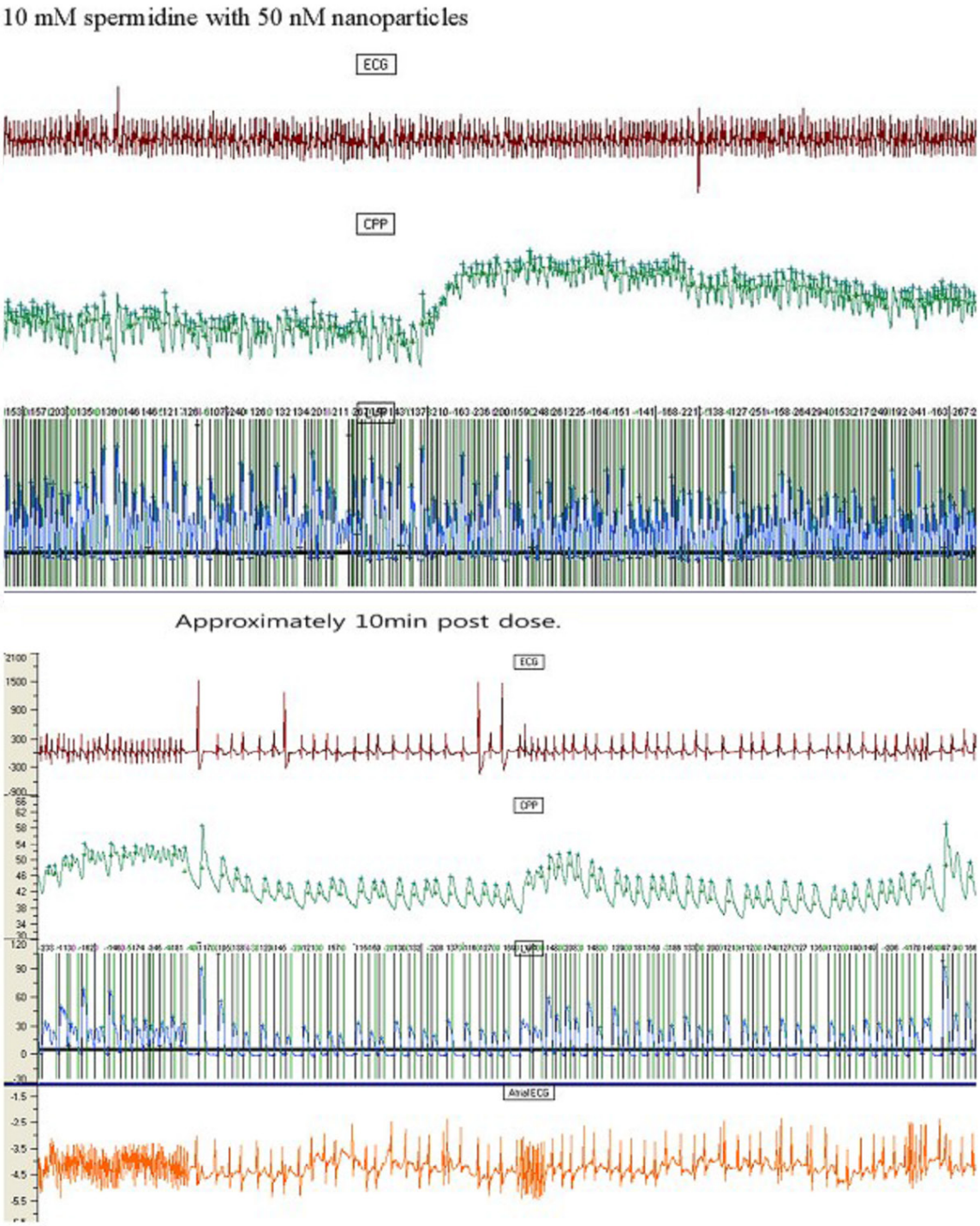


Figure 6. Recordings were obtained from multiple EKG electrodes on the glass chamber. In addition, epicardial electrograms at the right atrial appendages were also recorded. After 5 min of sustained AF, acetylcholine was washed out for 3 min. The positive control of 2 μM flecainide was injected and AF was transited to ventricular fibrillation (VF) after 2 min. The complex containing 100 mM spermidine with 50 nM nanoparticles was then injected and AF ceased after 19 s. Heart function was normal following the termination of AF. Upon injection of the complex containing 30 mM spermidine with 50 nM nanoparticles, AF termination occured after 50 s. When the complex containing 10 mM spermidine with 50 nM nanoparticles was injected, AF termination required 10 min.

complex on HL1 cells at various holding potentials (n=4). At voltage steps from -120 to +80 mV, in all HL1 cells, Ach application (65 mM Kex) resulted in a moderate inward rectification

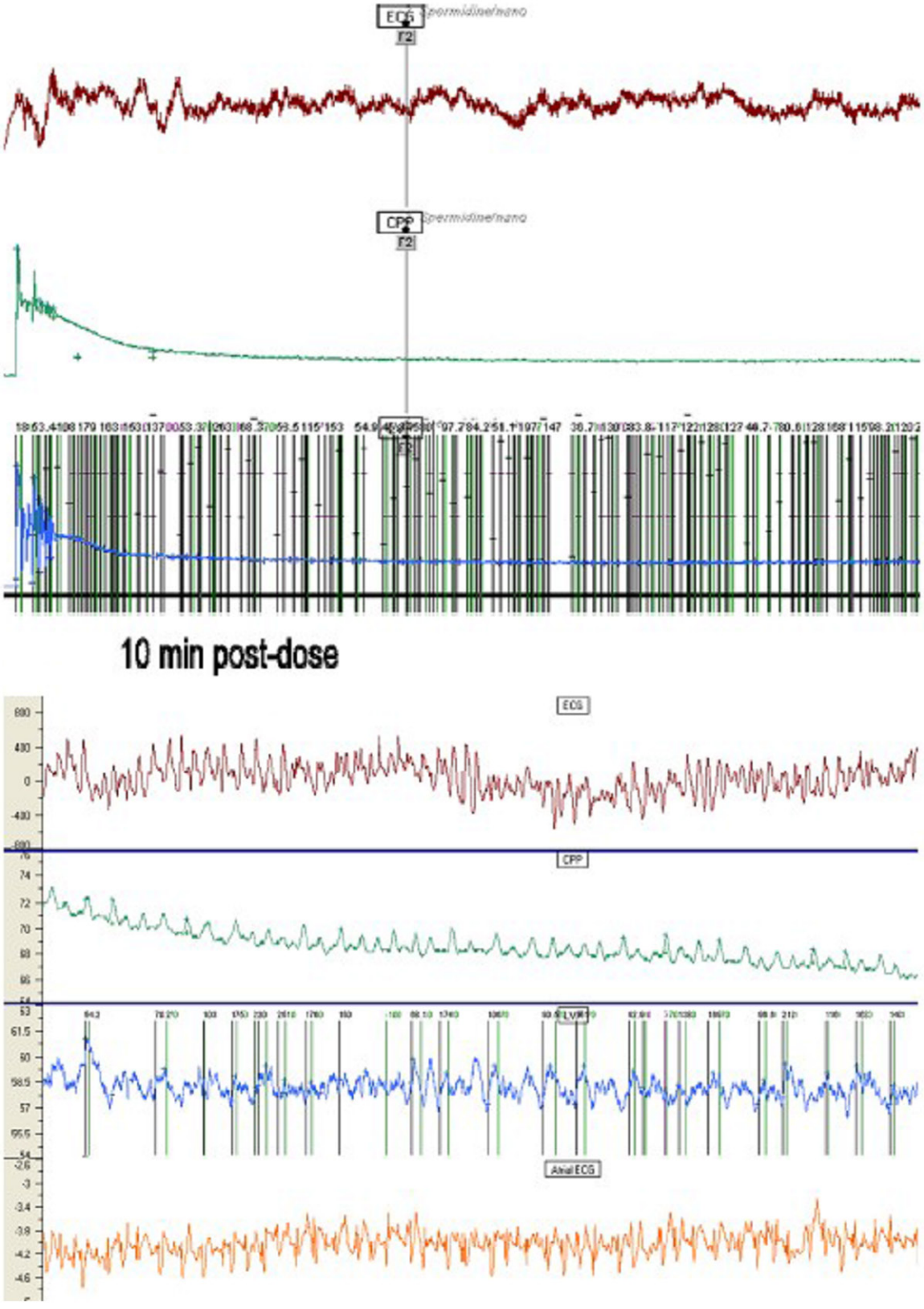


Figure 7. When the complex containing 100 mM spermidine with 100 nM nanoparticles was injected, AF transited to VF after 5 min which is called "proarrhythmea".

Table 1. AERP elongation was observed after treatment with complex containing the 10 mM spermidine and 50 nM nanoparticle (from 52 to 61.33 sec, P<0.01). AERP elongation was also observed after treatment with the complex containing 30 mM spermidine and 50 nM nanoparticle (from 46 to 52 sec, P<0.01)

30 mM spermidine + 50 nM Undeca-gold			
Time	Basic cycle length (msec)	Atrial threshold voltage	AERP
Base line	250	36	46
Post-dose after AF reverses	250	42	52
10 mM spermidine + 50 nM Undeca-gold			
Time	Basic cycle length (msec)	Atrial threshold voltage	AERP
Base line	250	34	52
Post-dose after AF reverses	250	36	61.33

of the current. The effect of the complex was investigated at different voltages. In all cases, the initial current onset in response to application of ACh alone was blocked by the complex (**Figure 5**). Due No blocking data were obtained between -60 mV and +40 mV because of low current amplitude.

In vitro tissue study

We also examined the in vitro effect of the spermidine-gold nanoparticle complex in explanted rabbit heart by EKG monitoring and atrial effective refractory period (AERP) measurement in the Langendorff apparatus. The complex provided complete resolution of AF and elongation of AERP. The effect of the complex, which is a selective KAch channel blocker, on atrial electrical remodeling was evaluated in a rabbit rapid atrial stimulation model. Acetylcholine was added, and burst pacing was then started to initiate AF. The burst pacing induced sustained AF for at least 5 min. The remaining AF (n=3) was used as a negative control. Treatment with 2 μM flecainide was used as a positive control (n=3) in which AF ceased after 2 min [22, 23]. After 5 min of sustained AF, the complex containing 100 mM spermidine and 50 nM nanoparticles was injected (n=3) and AF ceased after 19 sec (P<0.05). When the complex containing 30 mM spermidine and 50 nM nanoparticles was injected (n=3), AF ceased after 50 sec (P<0.05). When the complex containing 10 mM spermidine with 50 nM nanoparticles was injected (n=3), AF termination required 10 min (P<0.05), this finding suggests that a higher spermidine concentration facilitates cell penetration, thereby providing a shorter onset time (**Figure 6**). When the complex containing 100 mM spermidine with 100 nM nanoparticles was injected (n=3), AF was converted to ventricular fibrillation (VF) i.e.- "proarrhythmea occured" (**Figure 7**). It should be noted that a currently used class Ia, Ic, and III antiarrhythmic drugs are associated with side effects, such as life-threatening ventricular proarrhythmia. Blockade of potassium channels may prolong ventricular repolarization - and hence, the refractory period, thus - resulting in prolongation of the QT-interval [24]. The AERP was measured before and after AF reversal by the complex containing 30 mM spermidine with 50 nM nanoparticles. At the infusion of the complex, AERP was significantly lengthened after AF reversal (from 46 to 52, P<0.01). Upon infusion of 10 mM spermidine with 50 nM nanoparticles complex, the AERP was lengthened from 52 to 61.33, P<0.01. (**Table 1**) The heart function was normal following the termination of AF [25].

Discussion

When we applied the atrial lumen, the complex could penetrate the cardiomyocyte membrane and completely block I(KAch) inside the cell. This application can foster the complex as an anti-AF agent. Gold nanoparticles (0.8 nm) constitute a new class of ion channel inhibitors that directly block the human inward rectifying potassium channel- I(KAch) when accompanied into the ion pore by the antagonist spermidine. Blocking of the I(KAch) channel caused by the gold nanoparticle-spermidine complex likely occurs in the atrial cardiomyocyte. A reduced K^+ current delays repolarization with the complex in patients with AF [26].

The gold nanoparticle-spermidine complex can penetrate atrial cardiomyocyte cell membranes by simple diffusion. Spermidine molecules also facilitate the passage of the gold nanoparticles through the cell membrane. We treated the

complex in an extracellular solution for cell membrane penetration. Inside the cell, the complex can directly block the I(KAch) channels. The gold nanoparticles can be led by spermidine to the ion pores of the I(KAch) channels. The mechanism appears to be governed by the formation and dissolution of the gold-sulfur bonds between the gold nanoparticles and the cysteine loop of the I(KAch) channels.

The complex is a new class of 3 anti-arrhythmic agents which may potentially resolve AF [27]. We conducted EKG monitoring in a glass chamber and epicardium with AERP, which we measured after application of the complex to an artificial AF rabbit heart. A higher dose of the complex lead to paradoxically precipitated "Proarrhythmea" indicated that it is a side effect associated with the administration of an existing anti-arrhythmic drug. The complex is designed to be a selective I(KAch) blocker in contrast to multiple ion channels in class 1 and 3 anti-arrhythmic agents with less proarrhythmic potential [28].

Conclusion

We identified a new human inward rectifying potassium channel I(KAch) blocker (i.e., the gold nanoparticle spermidine complex). This direct blocking agent has high therapeutic potential with an anti-AF effect since it offers a near 75% blocking rate in a patch clamp study with complete resolution of AF in the rabbit heart.

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Disclosure of conflict of interest

None.

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