

Various subtypes of phosphodiesterase inhibitors differentially regulate pulmonary vein and sinoatrial node electrical activities

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Abstract. Phosphodiesterase (PDE)3-5 are expressed in cardiac tissue and play critical roles in the pathogenesis of heart failure and atrial fibrillation. PDE inhibitors are widely used in the clinic, but their effects on the electrical activity of the heart are not well understood. The aim of the present study was to examine the effects of various PDE inhibitors on spontaneous cardiac activity and compare those effects between sinoatrial nodes (SANs) and pulmonary veins (PVs). Conventional microelectrodes were used to record action potentials in isolated rabbit SAN and PV tissue preparations, before and after administration of different concentrations (0.1, 1 and 10 μ M) of milrinone (PDE3 inhibitor), rolipram (PDE4 inhibitor) and sildenafil (PDE5 inhibitor), with or without the application of isoproterenol (cAMP and PKA activator), KT5823 (PKG inhibitor) or H89 (PKA inhibitor). Milrinone (1 and 10 μ M) increased the spontaneous activity in PVs by 10.6 \pm 4.9 and 16.7 \pm 5.3% and in SANs by 9.3 \pm 4.3 and

20.7 \pm 4.6%, respectively. In addition, milrinone (1 and 10 μ M) induced the occurrence of triggered activity (0/8 vs. 5/8; $P<0.005$) in PVs. Rolipram increased PV spontaneous activity by 7.5 \pm 1.3-9.5 \pm 4.0%, although this was not significant, and did not alter SAN spontaneous activity. Sildenafil reduced spontaneous activity in PVs to a greater extent than that seen in SANs. Both KT5823 and H89 suppressed milrinone-increased PV spontaneous activity. In the presence of isoproterenol, milrinone did not alter isoproterenol-induced PV arrhythmogenesis, suggesting that the effects of PDE3 are mediated by the protein kinase G and protein kinase A signaling pathways. In conclusion, inhibitors of different PDE subtypes exert diverse electrophysiological effects on PV and SAN activities.

Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia that causes cardiac dysfunction and strokes (1). Heart failure (HF) is characterized by chamber dilatation, which may promote the occurrence of AF through mechano-electrical feedback and electrical or structural remodeling (2-6). Different 3',5'-cyclic nucleotide phosphodiesterase (PDE) isozymes exert distinctive biological functions, therefore, pharmacological inhibition of these PDEs might offer novel therapeutic strategies through their abilities to modulate cardiovascular diseases, including HF (7,8). In particular, PDE3-5 were reported to be expressed in cardiomyocytes and might play a role in both HF and AF (7,8). The PDE3 inhibitor, milrinone, exerts positive inotropic, vasodilating and minimal chronotropic effects, which are expected to improve HF (9). However, milrinone has potentially fatal adverse effects, including ventricular arrhythmias, which limit its use (10-14). PDE4 is expressed by human atrial myocytes, and its D isoform (PDE4D) has been linked to stroke risk in a number of genome-wide association

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studies (15,16). In a human study, PDE4 activity decreased by almost 50% in AF compared with patients with SR (17). Furthermore, cardiac PDE4D expression was also reduced in humans with atrial fibrillation, and a clinical trial revealed a slight increase in incidence of atrial arrhythmia in the PDE4 inhibitor (rofluminalast)-treated group, suggesting that a decrease in PDE4 activity may be linked to the development of AF. The PDE5 inhibitor, sildenafil, blocks the L-type calcium current (I_{Ca-L}) and the human ether-a-go-go related gene potassium channel in ventricular myocytes (18,19). Higher concentrations of sildenafil (0.2 $\mu\text{g/ml}$), with a nitric oxide (NO) donor, increase ventricular tachycardia or ventricular fibrillation, suggesting a potential risk of atrial arrhythmogenesis with the use of PDE5 inhibitors (20).

Pulmonary veins (PVs) form complex histological components with vascular structures and cardiomyocytes (21). PVs serve critical roles in AF initiation and maintenance through their distinctive electrical and structural properties (22–25). Furthermore, sinoatrial node (SAN) electrical activity can modulate PV arrhythmogenesis by increasing PVs spontaneous activity, and while PVs spontaneous activity exceeds SAN activity, atrial arrhythmia may occur (26). In addition to direct effects on cardiomyocytes, inhibitors of different PDE subtypes significantly regulate vascular activity, as well as cardiac contractility and pacemaker activity (7). Accordingly, it was hypothesized that PDE3–5 inhibitors may differentially regulate PV and SAN electrical activities, thereby contributing to the pathogenesis of AF. The present study aimed to investigate the effects and mechanisms of inhibitors of the three PDE subtypes on cardiac spontaneous activity in SANs and PVs.

Materials and methods

Rabbit PV and atrial tissue preparations. The present study was approved of the Institutional Animal Care and Use Committee (approval no. IACUC-19-124) of the National Defense Medical Center, Taipei, Taiwan and conformed to the institutional Guide for the Care and Use of Laboratory Animals and the 'Guide for the Care and Use of Laboratory Animals' published by the United States National Institutes of Health (8 ed. Washington DC, 2011). Male New Zealand white rabbits ($n=47$; weight, 2.0–3.0 kg; age, 6–8 months) were used in the present study. All of the rabbits had access to food and water *ad libitum*, and were maintained in a temperature and humidity-controlled environment (20–22°C; 50–70% humidity) with a 12 h light/dark cycle, and were raised in stainless steel cages. After rabbits were euthanized using intramuscular injections of a mixture of Zoletil 50 (10 mg/kg) and xylazine (5 mg/kg) with an overdose of isoflurane (5% in oxygen) from a precision vaporizer as previously described (27,28). Hearts were rapidly removed and placed in perfusion fluid after a midline thoracotomy. To dissect the PV, the heart was opened by an incision along the mitral valve annulus, extending from the coronary sinus to the septum, in Tyrode's solution comprised of 137 mM sodium chloride, 4 mM potassium chloride, 15 mM sodium bicarbonate, 0.5 mM monosodium phosphate, 0.5 mM magnesium chloride, 2.7 mM calcium chloride and 11 mM dextrose. The PV was separated from the atrium at the level

of the left atrial-PV junction and separated from the lungs at the ending of the PV myocardial sleeve. The SAN was isolated from the right atrium. The adventitia or epicardial side of the preparation faced upwards. One end of the preparation, consisting of the PV and atrial-PV junction, was pinned with needles to the bottom of a tissue bath. The distal-PV was connected to a FT03C force transducer (Grass Instruments Co.) by silk threads. The PV tissue strips were superfused at a constant rate (3 ml/min) with Tyrode's solution and were saturated with a gas mixture of 97% O_2 /3% CO_2 . The temperature was maintained at 37°C and the preparations were allowed to equilibrate for 1 h before the electrophysiological assessment.

Electrophysiological and pharmacological studies.

Transmembrane action potentials (APs) of the PVs were recorded by machine-pulled glass capillary microelectrodes filled with 3 mol/l KCl, which were connected to a Duo 773 electrometer (World Precision Instruments, Ltd.) under a tension of 1.47 mN (150 mg). Electrical and mechanical events (contractile force and diastolic tension) were simultaneously displayed on a 4072 oscilloscope (Gould) and a TA11 recorder (Gould). Using a data acquisition system, signals were recorded with direct coupling and a filter with a 10-kHz low-pass cut-off frequency. Signals were recorded digitally with a 16-bit accuracy, at a rate of 125 kHz. Electrical stimulation was provided using a Grass S88 stimulator through a SIU5B stimulus isolation unit (Grass Instruments Co.). Different concentrations of a PDE3 inhibitor (milrinone; 0.1, 1 and 10 μM ; Sigma-Aldrich; Merck KGaA), PDE4 inhibitor (rolipram; 0.1, 1 and 10 μM ; Sigma-Aldrich; Merck KGaA) or PDE5 inhibitor (sildenafil; 0.1, 1 and 10 μM ; Sigma-Aldrich; Merck KGaA) were sequentially superfused to test for pharmacological responses. For each concentration, PV and SAN preparations were treated for at least 30 min. The electrical activity in isolated rabbit PVs was recorded before and after the application of 10 μM milrinone with or without isoproterenol (1 μM ; Sigma-Aldrich; Merck KGaA), KT5823 (1 μM ; Tocris Bioscience) [a potent selective inhibitor of cyclic guanosine monophosphate (cGMP)-dependent protein kinase G (PKG)] or H89 (a protein kinase A [PKA] inhibitor; 10 μM ; Sigma-Aldrich; Merck KGaA) (29).

Spontaneous activity was defined as the constant occurrence of spontaneous APs in the absence of any electrical stimuli. Early afterdepolarizations (EADs) were defined as the interruption of the smooth contour of phase 2 or 3 of the APs. Delayed afterdepolarizations (DADs) were defined as the presence of a spontaneous hump-shaped depolarization of the impulse after full repolarization had occurred. Burst firing was defined as the occurrence of an accelerated spontaneous potential (faster than the basal rate) with sudden onset and termination.

Statistical analysis. All continuous variables are expressed as the mean \pm SEM. Repeated-measures ANOVA followed by Duncan's post hoc test was used to compare the difference before and after drug administration. Electrophysiological and mechanical characteristics were compared between different groups by a Wilcoxon rank-sum test or an unpaired t-test, depending on the outcome of the normality test. Nominal

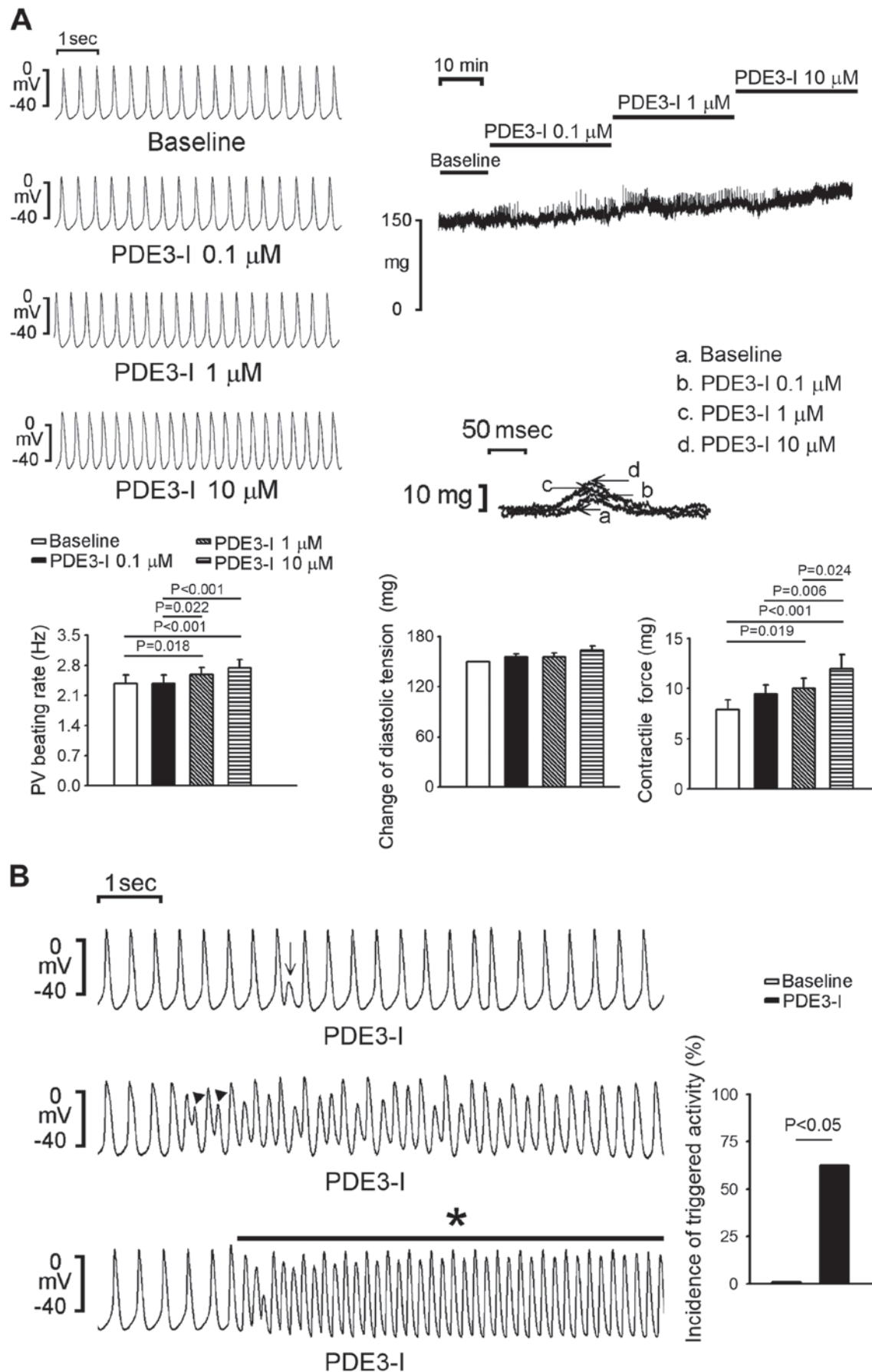


Figure 1. Effects of the PDE3 inhibitor (PDE3-I), milrinone, on the electrical activity of isolated PVs. (A) Representative data of the beating rate, diastolic tension and contractility of PVs after the application of different concentrations (0.1, 1 and 10 μ M) of the PDE3-I (n=8). (B) Representative data of the triggered activity of delayed afterdepolarizations (arrow), early afterdepolarizations (arrowheads) and burst firings (asterisk), induced by PDE3-I. PDE3, phosphodiesterase 3; PVs, pulmonary veins.

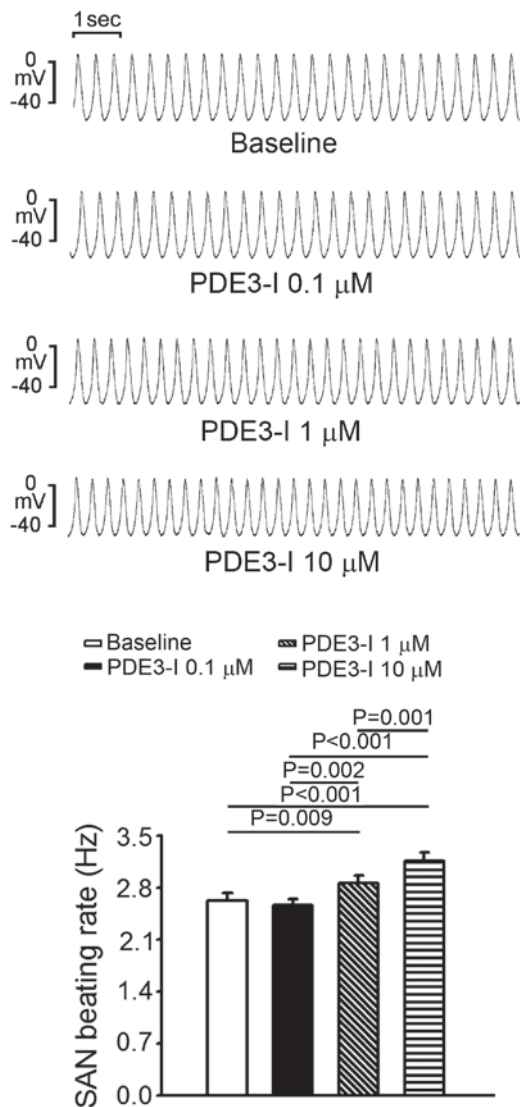


Figure 2. Effects of the PDE3 inhibitor (PDE3-I), milrinone, on the electrical activity of isolated SANs. Representative data of the beating rate after the application of different concentrations (0.1, 1 and 10 μ M) of the PDE3-I on SANs (n=7). PDE3, phosphodiesterase 3; SAN, sinoatrial node.

variables were compared by a χ^2 analysis with Fisher's exact test. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SigmaPlot software (version 12.0; Systat Software, Inc.).

Results

Effects of the PDE3 inhibitor on the electrical activities of isolated PVs and SANs. The PDE3 inhibitor (milrinone; 1 and 10 μ M) significantly increased PV spontaneous activity by $10.6 \pm 4.9\%$ ($P = 0.018$) and $16.7 \pm 5.3\%$ ($P < 0.001$) and contractility by $31.7 \pm 9.8\%$ ($P = 0.019$) and $58.8 \pm 19\%$ ($P < 0.001$) compared with the baseline, respectively, but had no significant effect on the diastolic tension of PVs (Fig. 1A). Milrinone also significantly increased PV diastolic tension by $8.9 \pm 3.7\%$ ($P = 0.002$) at 10 μ M. Moreover, milrinone ($\geq 1 \mu$ M) induced triggered activity including EAD, or DAD or burst firing in PVs (0/8 vs. 5/8; $P < 0.05$; Fig. 1B). Similarly, the PDE3 inhibitor increased SAN activity by $9.3 \pm 4.3\%$

and $20.7 \pm 4.6\%$ at 1 and 10 μ M compared with the baseline, respectively (Fig. 2). However, milrinone did not induce burst firing in SANs.

Application of KT5823 (1 μ M), a selective PKG inhibitor, abolished milrinone-accelerated PV electrical activity (Fig. 3A). In addition, H89 (10 μ M), a PKA inhibitor, also suppressed milrinone-accelerated PV electrical activity (Fig. 3B). Isoproterenol increased PV spontaneous activity, diastolic tension (Fig. 3B) and the occurrence of burst firing compared with the baseline (4/5 vs. 0/5; $P < 0.05$; Fig. 3C). However, in the presence of isoproterenol, milrinone (10 μ M) did not significantly alter the PV spontaneous activity or diastolic tension ($P > 0.05$) compared with the baseline. Taken together, PDE3 inhibition may regulate PV electrical activity through adrenergic activity and activation of the PKG and PKA signaling pathways.

Effects of the PDE4 inhibitor on the electrical activities of isolated PVs and SANs. The PDE4 inhibitor (rolipram; 0.1, 1 and 10 μ M) increased PV spontaneous activity by 7.5 ± 1.3 , 8.2 ± 3.1 and $9.5 \pm 4\%$, PV diastolic tension by 1.6 ± 4.7 , 6.8 ± 5.9 and $7.4 \pm 5.8\%$, and PV contractility by 11.7 ± 3.8 , 23.8 ± 3.1 and $32.3 \pm 7.5\%$ compared with the baseline, respectively (Fig. 4A). Rolipram (0.1, 1 and 10 μ M) did not significantly alter SAN activity compared with the baseline (Fig. 4B), and there was only one triggered beat in rolipram-treated PVs and none in rolipram-treated SANs (1/6 vs. 0/6; $P > 0.05$).

Effects of the PDE5 inhibitor on the electrical activities of isolated PVs and SANs. The PDE5 inhibitor, sildenafil, significantly reduced PV spontaneous activity by $13.5 \pm 5.5\%$ at 1 μ M and $25.9 \pm 9\%$ at 10 μ M compared with the baseline ($P < 0.05$; Fig. 5A). Sildenafil (0.1, 1 and 10 μ M) significantly reduced PV contractility by $16.6 \pm 4.6\%$ ($P = 0.013$), $27.5 \pm 7.0\%$ ($P < 0.001$) and $31.2 \pm 6.0\%$ ($P < 0.001$), respectively, but increased PV diastolic tension by $10.3 \pm 4.1\%$ ($P = 0.035$), $15.5 \pm 4.9\%$ ($P = 0.004$) and $17.8 \pm 6.7\%$ ($P = 0.002$), respectively, compared with the baseline, respectively. Moreover, the isoproterenol-induced accelerated PV spontaneous activity was attenuated by sildenafil (Fig. 5B). In addition, sildenafil suppressed isoproterenol-induced PV burst firing from 66.7 to 0% ($P < 0.001$; n=9; Fig. 5B). Sildenafil (0.1, 1 and 10 μ M) reduced SAN activity compared with the baseline, however, at 10 μ M sildenafil reduced SAN activity by 9.7%, which was less than the effect of sildenafil on PVs (Fig. 6).

Discussion

HF is a common risk factor for AF (2-4). Milrinone potentiates the effect of cyclic adenosine monophosphate (cAMP) and enhances the relaxation of the left ventricle by increasing Ca^{2+} -ATPase activity in the cardiac sarcoplasmic reticulum, by increasing calcium ion uptake (7). Although the PDE3 inhibitor, milrinone, improves HF through positive inotropic effects, milrinone also increases the risk of AF in patients with HF (13). In the present study, milrinone increased PV and SAN spontaneous activities to a similar extent. The accelerating effect of milrinone on PVs was abolished by the PKG inhibitor (KT5823; 1 μ M) and the PKA inhibitor (H89; 10 μ M), suggesting that the electrophysiological effects of

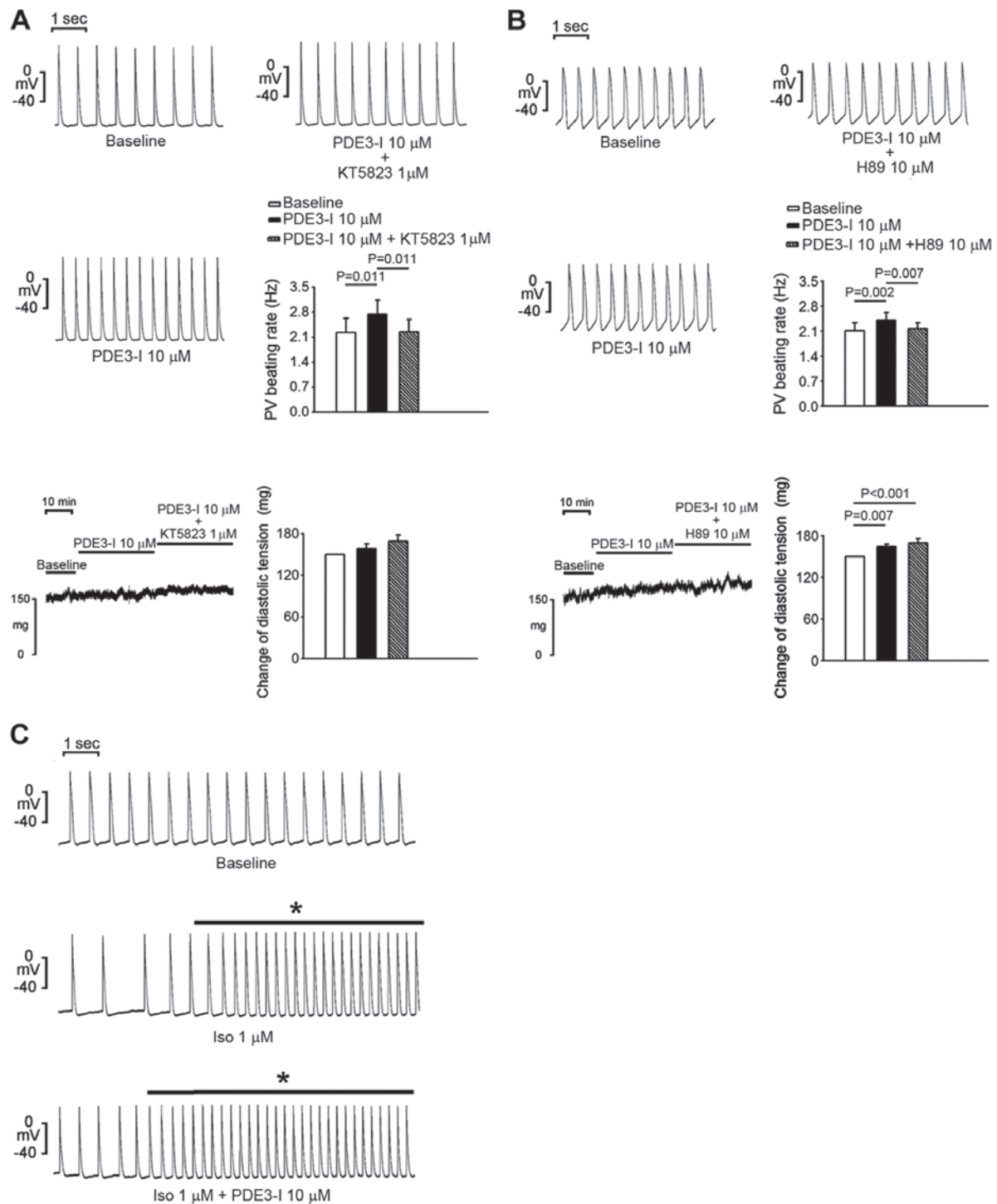


Figure 3. Effects of the protein kinase G inhibitor, KT5823, and the protein kinase A inhibitor, H89, on PDE3 inhibitor (PDE3-I)-modulated electrical activity of isolated PVs. (A) Representative data of the beating rate and diastolic tension of PVs after the application of 10 μ M PDE3-I, milrinone, and 1 μ M KT5823. (B) Representative data of the beating rate and diastolic tension of PVs after the application of 10 μ M PDE3-I and 10 μ M H89. (C) Examples of burst firing (asterisks) in PVs after the administration of 1 μ M isoproterenol and 10 μ M PDE3-I. PDE3, phosphodiesterase 3; PV, pulmonary vein; Iso, isoproterenol.

milrinone could involve the PKG and PKA signaling pathways. Additionally, the effect of milrinone on PV arrhythmogenesis in the presence of isoproterenol indicated that milrinone and isoproterenol might regulate PV electrical activity in a similar manner, via the activation of cAMP.

Previous studies reported that milrinone might relax the pulmonary arterial and venous vascular beds in guinea pig and human lung tissues by activating the ATP-sensitive potassium

channel (30,31). However, the present study suggested that milrinone increased diastolic tension in PVs. The discrepancy may have been caused by the differences between distal, with simple venous structures, and proximal PVs, since the atrial-PV junction used in the present study contained the myocardial sleeve surrounding the PV vascular components. Milrinone may increase PV diastolic tension via positive inotropic effects on cardiomyocytes, as shown by increased PV contractility.

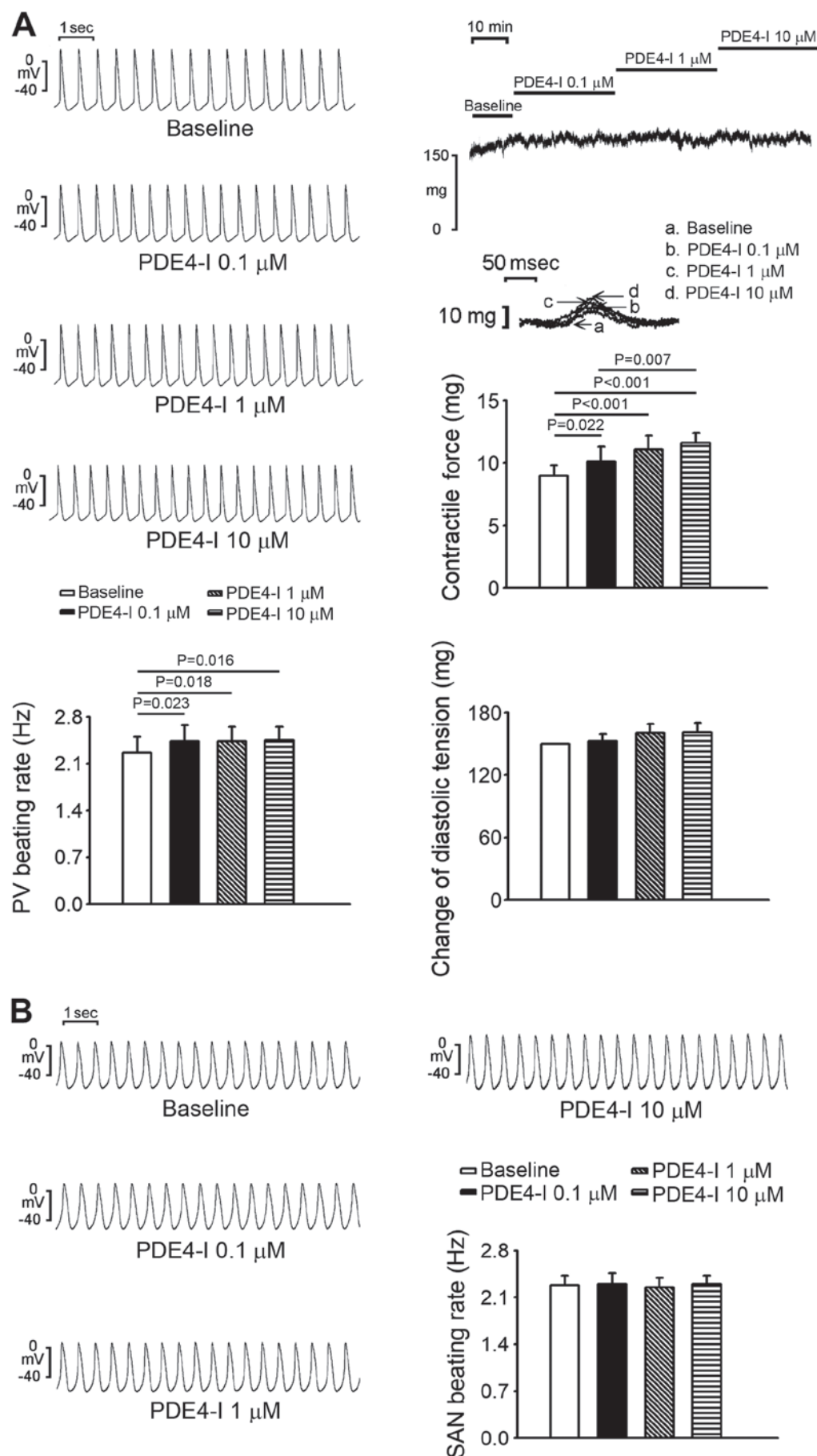


Figure 4. Effects of the PDE4 inhibitor (PDE4-I), rolipram, on the electrical activities of isolated PVs and SANs. (A) Representative data of the beating rate, diastolic tension and contractility of PVs after the application of different concentrations (0.1, 1 and 10 μ M) of the PDE4-I (n=6). (B) Representative data of the beating rate of SANs after the application of different concentrations (0.1, 1 and 10 μ M) of the PDE4-I (n=6). PDE4, phosphodiesterase 4; PV, pulmonary vein; SAN, sinoatrial node.

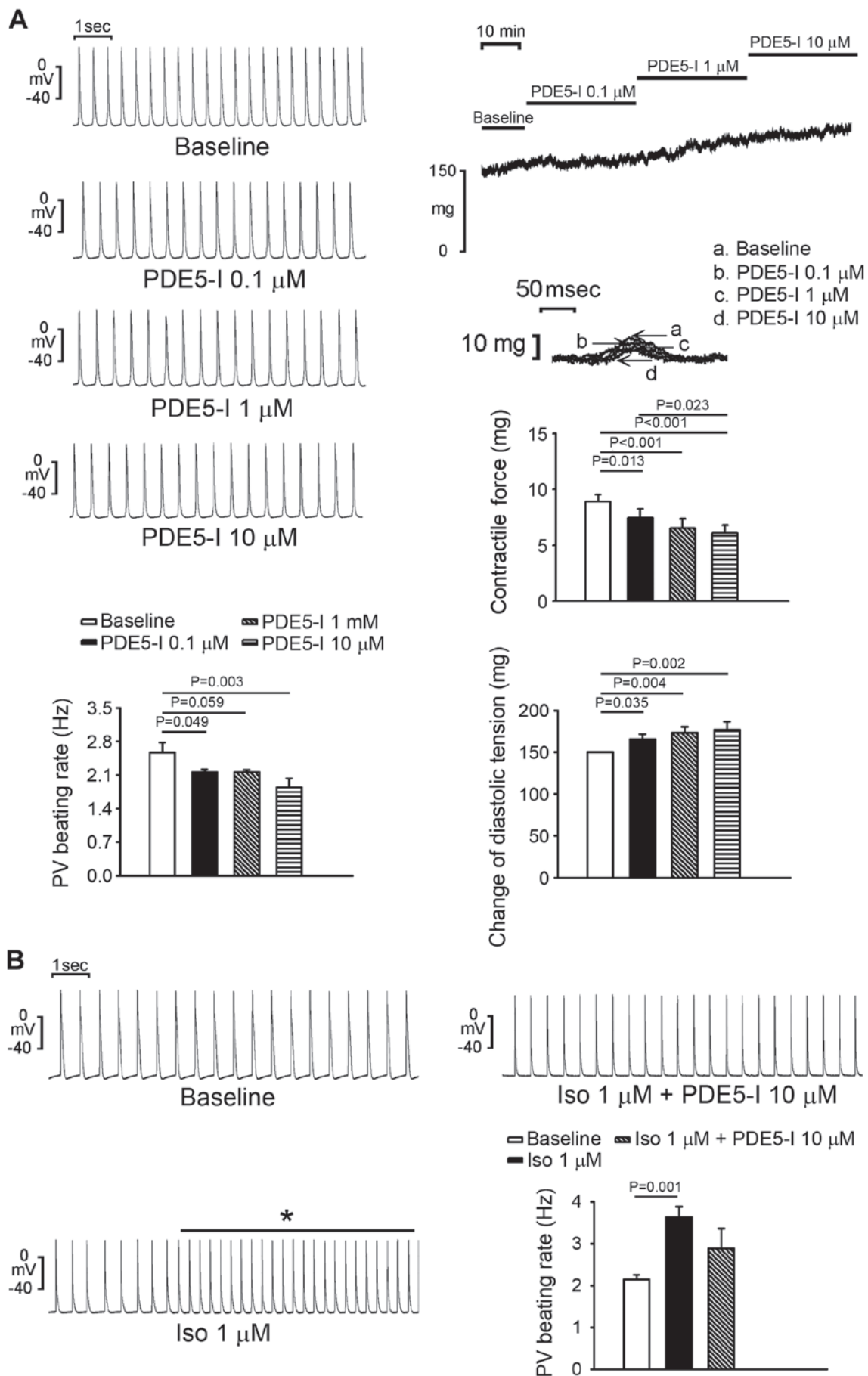


Figure 5. Effects of the PDE5 inhibitor (PDE5-I), sildenafil, on the electrical activity of isolated PVs. (A) Representative data of the beating rate, diastolic tension and contractility of PVs after the application of different concentrations (0.1, 1 and 10 μ M) of the PDE5-I (n=7). (B) Examples of burst firing (asterisks) in PVs after the application of 1 μ M isoproterenol and 10 μ M PDE5-I (1 μ M; n=9). PDE5, phosphodiesterase 5; PV, pulmonary vein; Iso, isoproterenol.

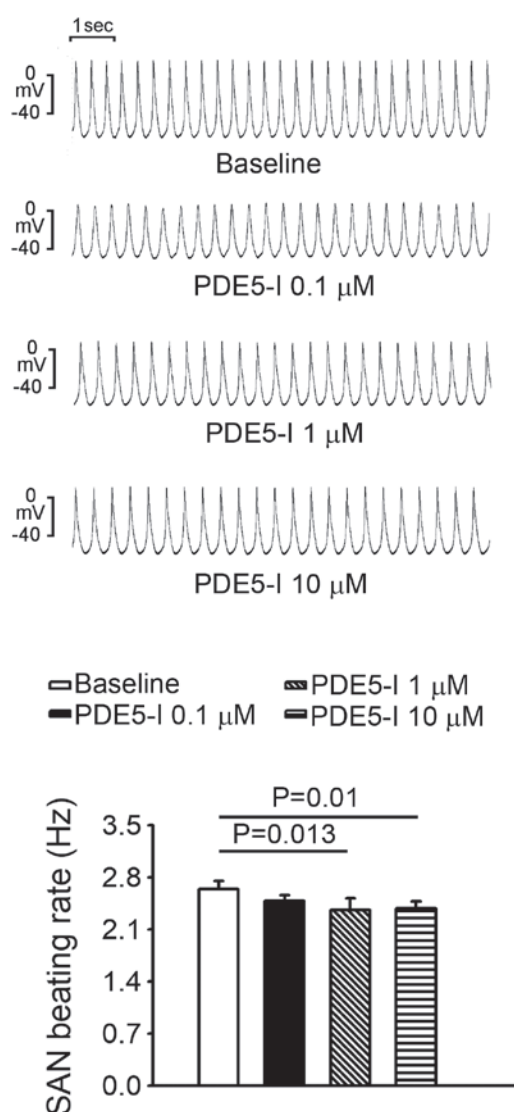


Figure 6. Effects of the PDE5 inhibitor (PDE5-I), sildenafil, on the electrical activity of isolated SANs. Representative data of the beating rate of SANs after the application of different concentrations (0.1, 1 and 10 μ M) of the PDE5-I (n=7). PDE5, phosphodiesterase 5; SAN, sinoatrial node.

PDE4 inhibition increased intracellular cAMP levels and the I_{Ca-L} in atrial myocytes, as well as increasing the frequency of spontaneous Ca^{2+} release (18), which may predispose individuals to AF. However, in the present study, the PDE4 inhibitor, rolipram, had no significant effect on PV and SAN spontaneous activities. The results from the present study were consistent with that of a previous study, which suggested that PDE4 inhibition did not significantly alter SAN electrical activity (32). Therefore, clinical observations of an increased risk of AF in patients receiving PDE4 inhibition therapy might be explained by AF substrate modifications, rather than the enhancement of the arrhythmogenesis that AF triggers (33).

PDE5 expression is strongly increased in HF (34-36). Previous studies reported that inhibition of PDE5 ameliorates cardiac dysfunction and sildenafil blocks I_{Ca-L} in ventricular myocytes in a dose-dependent manner (18). In the present study, sildenafil significantly reduced PV and SAN spontaneous activities. The stronger inhibitory effect of sildenafil

on PV activity compared with SAN activity might reduce the risk of AF. The rate reduction effect of sildenafil on PVs may be partially attributed to the NO synthase-mediated signaling pathways, since NO has been shown to play an important role in PV arrhythmogenesis (37). Sildenafil increased the PV diastolic tension, but decreased PV contractility, which may be accounted for by the electrophysiological effects of sildenafil via mechanoelectrical feedback. Contrastingly to PDE3 inhibitors, which exhibit a high affinity for both cAMP and cGMP, sildenafil, as a cGMP-specific PDE5 blocker (7), abolished isoproterenol-induced increases in PV activity. This result was in line with a previous report, which indicated that sildenafil can attenuate β -receptor agonist-induced cAMP generation and accelerate the beating rate of PVs (38). Similar to the present study, PDE5 inhibition was previously reported to have a negative chronotropic effect on mice SANs (26). Accordingly, sildenafil may potentially protect individuals from AF by reducing PV arrhythmogenesis.

Acute infusion of the kinase inhibitors, including KT5823 and H89, was performed in the present study to investigate the effect of milrinone on PVs. The suppression of the PDE3 accelerating effect on PVs following acute infusion of the kinase inhibitors suggested a role for the cGMP and PKA signaling pathways in PDE3 inhibitor mediated PV arrhythmogenesis. However, it is not clear whether the PDE inhibitors may have further unknown effects or whether the kinase inhibitors exert their effects at the cellular level. Since only the acute effects of the kinase inhibitors were evaluated in the present study, the protein expression of components of the cGMP and PKA signaling pathways in PVs at such a short exposure time is highly unlikely to be affected or to be shown by western blot analysis. Therefore, this requires further investigation.

In conclusion, different subtypes of PDE inhibitors regulate PV and SAN electrical activities in distinct manners and may contribute to susceptibility to atrial arrhythmogenesis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YKL and CCC contributed to the experimental design, performed the *in vitro* experiments, analyzed the experimental results and wrote the paper. YKL and JHH and gave various contributions in the statistical analysis, and interpretation of the results and discussion. YAC contributed to the *in vitro* experiments and provided technical assistance in the study. SAC and YJC contributed to the experimental design, analysis of the results and final revision of the paper for publication. YYL and YCC conceived and designed the study, and reviewed the paper prior to submission.

Ethics approval and consent to participate

The present study was approved of the Institutional Animal Care and Use Committee by the local review board (approval no. IACUC-19-124) of the National Defense Medical Center, Taipei, Taiwan and conformed to the institutional Guide for the Care and Use of Laboratory Animals and the 'Guide for the Care and Use of Laboratory Animals' published by the United States National Institutes of Health (8 ed. Washington DC, 2011).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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