

ORIGINAL INVESTIGATION

Apelin-13 reverses bupivacaine-induced cardiotoxicity: an experimental study



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Received 4 September 2023; accepted 26 March 2024

Available online 5 April 2024

KEYWORDS

Apelin;
Bupivacaine;
Cardiotoxicity;
Protein kinase C;
Sodium channel

Abstract

Introduction: Cardiac arrest or arrhythmia caused by bupivacaine may be refractory to treatment. Apelin has been reported to directly increase the frequency of spontaneous activation and the propagation of action potentials, ultimately promoting cardiac contractility. This study aimed to investigate the effects of apelin-13 in reversing cardiac suppression induced by bupivacaine in rats.

Methods: A rat model of cardiac suppression was established by a 3-min continuous intravenous infusion of bupivacaine at the rate of $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, and serial doses of apelin-13 (50 , 150 and $450 \mu\text{g} \cdot \text{kg}^{-1}$) were administered to rescue cardiac suppression to identify its dose-response relationship. We used F13A, an inhibitor of Angiotensin Receptor-Like 1 (APJ), and Protein Kinase C (PKC) inhibitor chelerythrine to reverse the effects of apelin-13. Moreover, the protein expressions of PKC, Nav1.5, and APJ in ventricular tissues were measured using Western blotting and immunofluorescence assay.

Results: Compared to the control rats, the rats subjected to continuous intravenous administration of bupivacaine had impaired hemodynamic stability. Administration of apelin-13, in a dose-dependent manner, significantly improved hemodynamic parameters in rats with bupivacaine-induced cardiac suppression ($p < 0.05$), and apelin-13 treatment also significantly upregulated the protein expressions of p-PKC and Nav1.5 ($p < 0.05$), these effects were abrogated by F13A or chelerythrine ($p < 0.05$).

Conclusion: Exogenous apelin-13, at least in part, activates the PKC signaling pathway through the apelin/APJ system to improve cardiac function in a rat model of bupivacaine-induced cardiac suppression.

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Introduction

Bupivacaine, a long-acting and low-cost amide local anesthetic, is frequently used for conducting regional anesthesia; however bupivacaine can cause profound cardiac toxicities such as arrhythmia, hypotension and even cardiac arrest, as it may be accidentally injected or excessively absorbed into circulation, where the inhibition of the cardiac voltage-gated sodium channel is considered as the essential factor contributing to bupivacaine-induced cardiotoxicity.¹ Therefore, mitigating its cardiotoxicity is of vital concern to clinical anesthesiologists.

Apelin, the ligand of the Angiotensin Receptor-Like 1 (APJ), directly increases the frequency of spontaneous activation and the propagation of action potentials, and ultimately promotes cardiac contractility.^{2,3} Its inotropic effect may be associated with an increase in the sodium current at more negative potentials, with implications in the transduction mechanisms that can lead to Protein Kinase C (PKC) activation,⁴ something that, in turn, opens a window allowing to explore such a connection and how it could interfere with the deflagrated inherent mechanisms which lead to bupivacaine-induced toxicity. Therefore, apelin seems to be a promising target for therapeutic intervention in bupivacaine-induced cardiotoxicity.

Here, in a rat model of bupivacaine-induced cardiac suppression, we tested the hypothesis that apelin-13, the most abundant member of the apelin family, can mitigate bupivacaine-induced cardiotoxicity, and that PKC activation may be involved in its protective mechanism. With the aim of studying such condition, we chose to look for what were the implications of cardiotoxicity in the hemodynamics parameters, electrocardiographic waves, protein expression levels and in the immunofluorescence assay pattern.

Methods

Animals

The experimental protocols were approved by the Animal Ethics Committee of Wenzhou Medical University (No. wydw2016-0267, date of approval: May 25th, 2016), and the study was conducted in accordance with ARRIVE guidelines. The specific pathogen-free, 6–8 weeks old, 200–250 g, male Sprague-Dawley rats were purchased from the SLAC Laboratory Animal Company. After 1 week of adaptive feeding with free access to diet and water intake, the rats were randomized according to a random number table for further experiments.

Three different investigators were involved as follows for each animal: a first investigator, who was the only person aware of the treatment group allocation, performed the anesthesia and pharmacological interventions based on the randomization table. A second investigator was responsible for the hemodynamic and electrocardiographic (ECG) recordings, and the third investigator performed the Western blotting and immunofluorescence assay.

Animal preparation and monitoring

All rats were anesthetized with intraperitoneal administration of sodium pentobarbital 30 mg.kg⁻¹ and ketamine 50 mg.kg⁻¹, and then placed on a warming pad in supine position to keep body temperature at 36–37 °C. The rats were then ventilated with a rodent ventilator in a volume-controlled mode (tidal volume 6 mL.kg⁻¹, 80 stroke/min). The carotid artery was cannulated using a PE50 catheter and connected to a pressure transducer to monitor hemodynamic parameters. Rats with a baseline blood pressure > 130/100 mmHg or < 110/70 mmHg were excluded. Another PE50 catheter was then inserted into the femoral vein and connected to a micro infusion pump (3500, Graseby, UK). Two biopotential leads were anchored subcutaneously over the right pectoral muscle and on the lower left side of the chest wall to record ECG parameters using a computer-based electrical physiology system. Data were collected after a 15-min stabilization period.

Establishment of bupivacaine-induced cardiac suppression in rats

We infused 15 mg.kg⁻¹ bupivacaine via the right femoral vein with a micro infusion pump at 5 mg.kg⁻¹.min⁻¹ for 3 min.⁵ Bupivacaine-induced cardiac suppression was confirmed when the heart rate systolic Blood Pressure Product (RPP) was less than 30% of the baseline value.

Effect of apelin-13 in mitigating bupivacaine-induced cardiac suppression

Rats were randomized into five groups: Control group, Bupivacaine group (Bup group), bupivacaine + 50 µg.kg⁻¹ apelin-13 group (Bup+Apln50 group), bupivacaine + 150 µg.kg⁻¹ apelin-13 group (Bup+Apln150 group), and bupivacaine + 450 µg.kg⁻¹ apelin-13 group (Bup+Apln450 group), with eight rats in each group. The bupivacaine-induced cardiac suppression model was developed in the Bup group, while the rats in the Control group were continuously infused with the same volume of Normal Saline (NS) alone, and an extra 0.1 mL NS was given via the jugular vein immediately after the end of the infusion to both groups. We administered 50, 150, and 450 µg.kg⁻¹ apelin-13, dissolved in 0.1 mL NS, via the jugular vein immediately after the bupivacaine infusion in the Bup+Apln50, Bup+Apln150, and Bup+Apln450 groups, respectively.

Effects of F13A and chelerythrine on apelin-13 in mitigating bupivacaine-induced cardiac suppression in rats

Rats were divided into six groups: Bupivacaine group (Bup group), Bupivacaine + 150 µg.kg⁻¹ apelin-13 group (Bup+Apln150 group), Bupivacaine + F13A group (Bup+F13A group), Bupivacaine + chelerythrine group (Bup+Chel group), Bupivacaine + 150 µg.kg⁻¹ apelin-13 + F13A group (Bup+Apln150+F13A group), and Bupivacaine + 150 µg.kg⁻¹ apelin-13 + chelerythrine group (Bup+Apln150+Chel group), with eight rats in each group. We administered 150 µg.kg⁻¹ F13A and 5 mg.kg⁻¹ chelerythrine through the jugular vein 15 min before bupivacaine infusion in the Bup+F13A and Bup

+Chel groups, and 0.1 mL NS was given immediately after the end of bupivacaine infusion.⁶⁻⁸ The Bup+Apln150 group received intravenous administration of 150 $\mu\text{g}\cdot\text{kg}^{-1}$ apelin-13 immediately after the end of bupivacaine infusion, while the Bup+Apln150+F13A and Bup+Apln150+Chel groups were instead administered an additional 150 $\mu\text{g}\cdot\text{kg}^{-1}$ F13A and 5 $\text{mg}\cdot\text{kg}^{-1}$ chelerythrine 15 min before the bupivacaine infusion.

The initiation of the bupivacaine infusion was defined as time 0 min, and cardiac suppression was established at time 3 min. The hemodynamic and ECG parameters were recorded every five minutes for 30 min after bupivacaine infusion. Subsequently, the rats were sacrificed by anesthetic overdose, and their hearts were removed for protein measurements.

Western blotting

The tissues extracted from the left ventricular wall of eight rats in each group were lysed in 0.9 mL RIPA lysis buffer (No. 89900, ThermoFisher, China) and mixed with protease and phosphatase inhibitors for 15 min. The protein concentration was determined using a BCA protein assay kit (No. 23235, ThermoFisher, China). Each 1 mL protein mixture containing 15 μg protein and 200 μL 5 \times loading buffer was denatured by boiling at 100 °C for 5 min. Each sample was separated using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes by electrophoresis. The membranes were blocked in 5% non-fat dry milk for 2 h and incubated with specific primary antibodies against p-PKC (No. ab23513, Abcam, China), total PKC (No. ab32376, Abcam, China), Nav1.5 (No. ab187830, Abcam, China) and APJ (No. ab214369, Abcam, China) overnight (4 °C) and a secondary antibody for 2 h. The resulting band was established using an ECL detection kit with a Bio-Rad imager using GAPDH (Biorworld Technology, China) as an internal control.

Immunofluorescence assay

Heart tissues obtained from eight rats per group were embedded in OCT compound and frozen immediately in liquid nitrogen. We incubated 5 μm cardiac tissue sections with antibodies against p-PKC (1:100) and Nav1.5 (1:100) overnight at 4 °C. Then, membranes were incubated with secondary antibodies of FITC-conjugated goat anti-mouse IgG (1:200, No. 33207ES60, YEASEN, China) and TRITC-conjugated goat anti-rabbit IgG (1:200, No. 33109ES60, YEASEN, China) for 2 h at room temperature. After washing with phosphate-buffered saline, nuclei were stained with fluorescent dye 4', 6-diamidino-2-phenylindole dihydrochloride for 5 min. Fluorescence was observed using a fluorescent microscope system (DP72, Olympus, Japan).

Sample size and statistical analysis

The sample size of $n = 8$ in each group for each set of the experiment was determined based on the sample size used in our previous study.⁵ All statistical analyses were performed using SPSS version 13.0 software. The Shapiro-Wilk test was used for normal distribution test, and all continuous data that were normally distributed were expressed as mean

\pm Standard Deviation (SD). The overall differences in ECG and hemodynamic parameters within -15–0 min and 3–30 min period were respectively compared across time using two-way repeated measures analysis of variance (ANOVA). The paired *t*-test was used to compare repeatedly measured data among different timepoints within each group. Other outcomes were compared using one-way ANOVA followed by the Bonferroni post hoc method among groups. A value of $p < 0.05$ was considered significant.

Results

All animals completed the experiment, and eight rats in each group were included in the final analysis. The Shapiro-Wilk test revealed that all the variables were normally distributed.

Compared to data at -15 min and 0 min, the rats in the Bup group developed lower Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Heart Rate (HR), RPP, and prolonged QRS and QT segment durations at 3-min ($p < 0.05$, Supplemental Table 1 and 2 in Supplemental File 1), suggesting the successful establishment of bupivacaine-induced cardiac suppression. Compared to the Bup group, the rats improved SBP, HR, RPP, QRS, QT segment duration and shortened duration required for recovering to 70% of baseline RPP in the Bup+Apln150 and Bup+Apln450 group, and DBP was increased only in the Bup+Apln150 group ($p < 0.05$). Compared to the Bup+Apln50 group, the rats in the Bup+Apln150 and Bup+Apln450 groups exhibited higher SBP, DBP, HR, RPP, shortened duration of QRS segment and duration required for recovering to 70% of baseline RPP in the 3–30 min interval ($p < 0.05$). DBP was increased, and QRS and QT segment durations were shortened in the 3–30 min period in the Bup+Apln450 group compared with the Bup+Apln150 group ($p < 0.05$, Fig. 1).

Compared to the Bup+Apln group, the Bup+Apln+F13A and Bup+Apln+Chel groups showed lower SBP, DBP, HR, and RPP. Additionally, the duration required for recovering to 70% of baseline RPP and QRS and QT segment durations in the 3–30 min interval were prolonged in the Bup+Apln+F13A group ($p < 0.05$, Fig. 2).

Compared to the Bup group, protein expression of p-PKC and Nav1.5 in the ventricular tissues of the Bup+Apln150 and Bup+Apln450 groups were upregulated, with Nav1.5 being more upregulated in the Bup+Apln450 group than in the Bup+Apln150 group ($p < 0.05$, Fig. 3). There were no significant differences in ventricular protein expressions of total PKC and APJ among Bup, Bup+Apln50, Bup+Apln150, and Bup+Apln450 groups ($p > 0.05$).

The fluorescence of p-PKC and Nav1.5 predominately accumulated around plasma membranes in rat ventricular cardiomyocytes from the Control group but displayed a relatively dispersive distribution in the Bup group. The membranous accumulation of fluorescence of p-PKC and Nav1.5 was more prominent in the Bup+Apln50, Bup+Apln150, and Bup+Apln450 groups than in the Bup group (Fig. 4).

There were no differences in expression of p-PKC, Nav1.5, PKC, and APJ among Bup+F13A, Bup+Chel, and Bup groups ($p > 0.05$). Compared to the Bup+Apln group, ventricular protein expression of p-PKC and Nav1.5, but not total PKC and APJ, were lower in rats from the Bup+Apln+F13A and Bup+Apln+Chel groups ($p < 0.05$, Fig. 3). Under

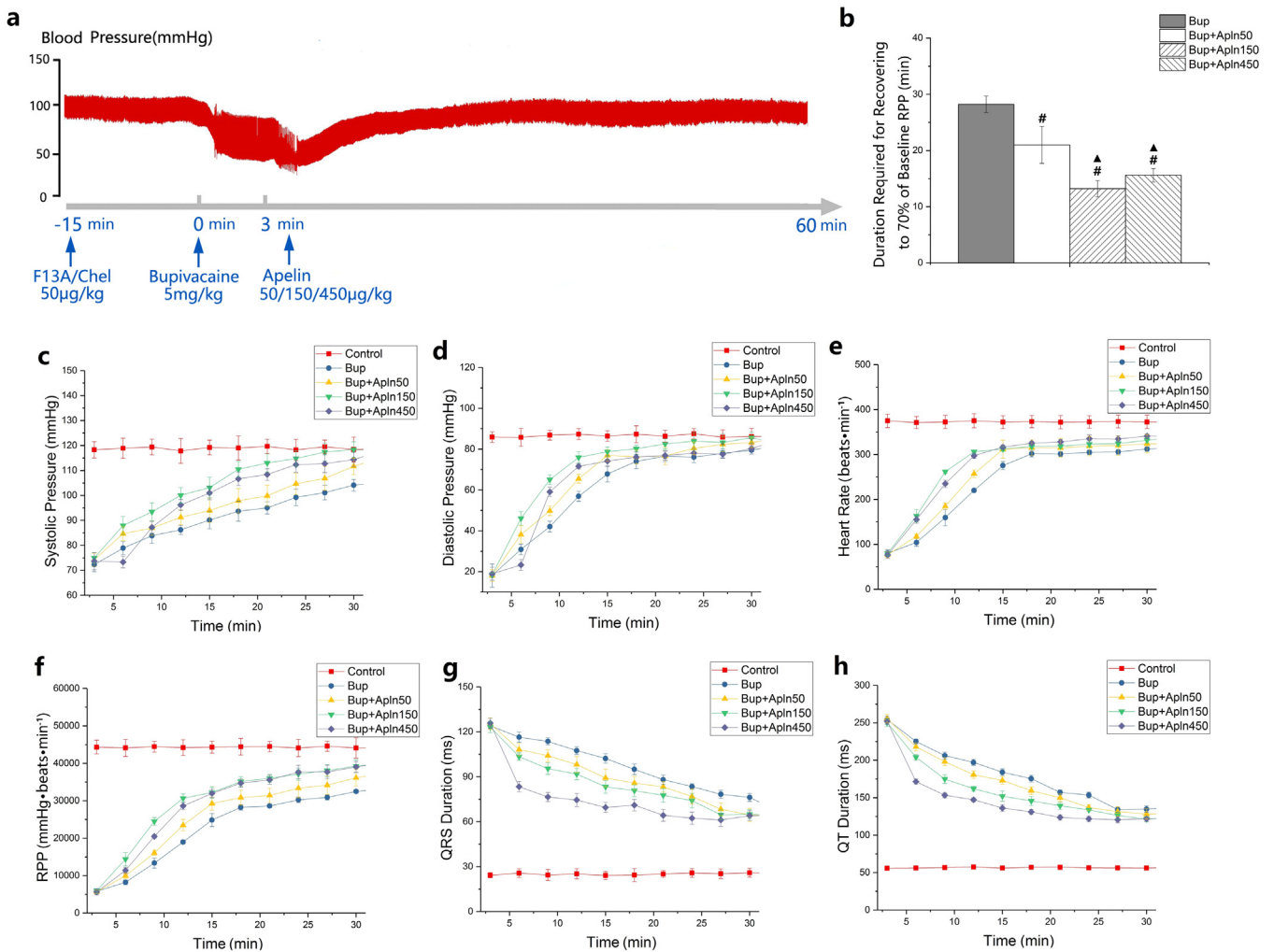


Figure 1 Effects of Apelin-13 on cardiac suppression induced by bupivacaine (3–30 min). The timeline of hemodynamics parameters (a), duration required for recovering to 70% of baseline RPP (b), systolic blood pressure (c), diastolic blood pressure (d), heart rate (e), RPP (f), QRS segment duration (g) and QT segment duration (h) of each group were plotted against time (3–30 min), and the initiation of bupivacaine infusion or normal saline was defined as time 0 min. Data were expressed as mean \pm SD ($n = 8$). Continuous ECG and hemodynamic parameters in rats surviving at 30 min were compared across time by two-way repeated measures ANOVA. RPP, heart rate systolic blood pressure product. # $p < 0.05$ vs. Bup group, $\blacktriangle p < 0.05$ vs. Bup+ApIn50 group.

immunocytochemistry assay, the membranous fluorescence of p-PKC and Nav1.5 in ventricular cardiomyocytes in the Bup+ApIn+F13A and Bup+ApIn+Chel groups was lower than in the Bup+ApIn group (Fig. 5).

Discussion

The results of this study showed that the administration of apelin-13 tends to reduce bupivacaine-induced cardiac suppression in a dose-dependent manner, and these supposedly protective effects tend to be modified by both APJ inhibitor F13A and PKC inhibitor chelerythrine.

Arrhythmias rather than asystole appear to be more common with inadvertent intravenous administration of bupivacaine.⁹ We established a rat model of cardiac suppression with continuous administration of bupivacaine for a relatively prolonged duration, and the total dose of bupivacaine was identical to the one we used to induce cardiac arrest in a

previous study.⁵ Here, the bupivacaine administration protocol in this study caused profound cardiac suppression in a short period, which was considered to be more compatible with the clinical scenarios. The QRS and QT segment duration on ECG, which represents the process of depolarization of the ventricle and the entire process of ventricular depolarization and repolarization, respectively, are closely related to sodium current. In our study, prolonged QRS and QT segments were demonstrated along with impaired hemodynamic stability, which supported the notion that sodium channel block serves as the predominate mechanism in bupivacaine-induced cardiotoxicity. It is probable that, within certain transduction mechanisms related to cellular sodium channel impairment triggered by bupivacaine-induced toxicity, the pathway induced by apelin-13 may interrupt the degeneration process. This interruption could result in a mitigated deleterious effect on the depolarization and repolarization patterns of the ECG, consequently ameliorating the contractile force and, consequently, influencing hemodynamic variables.

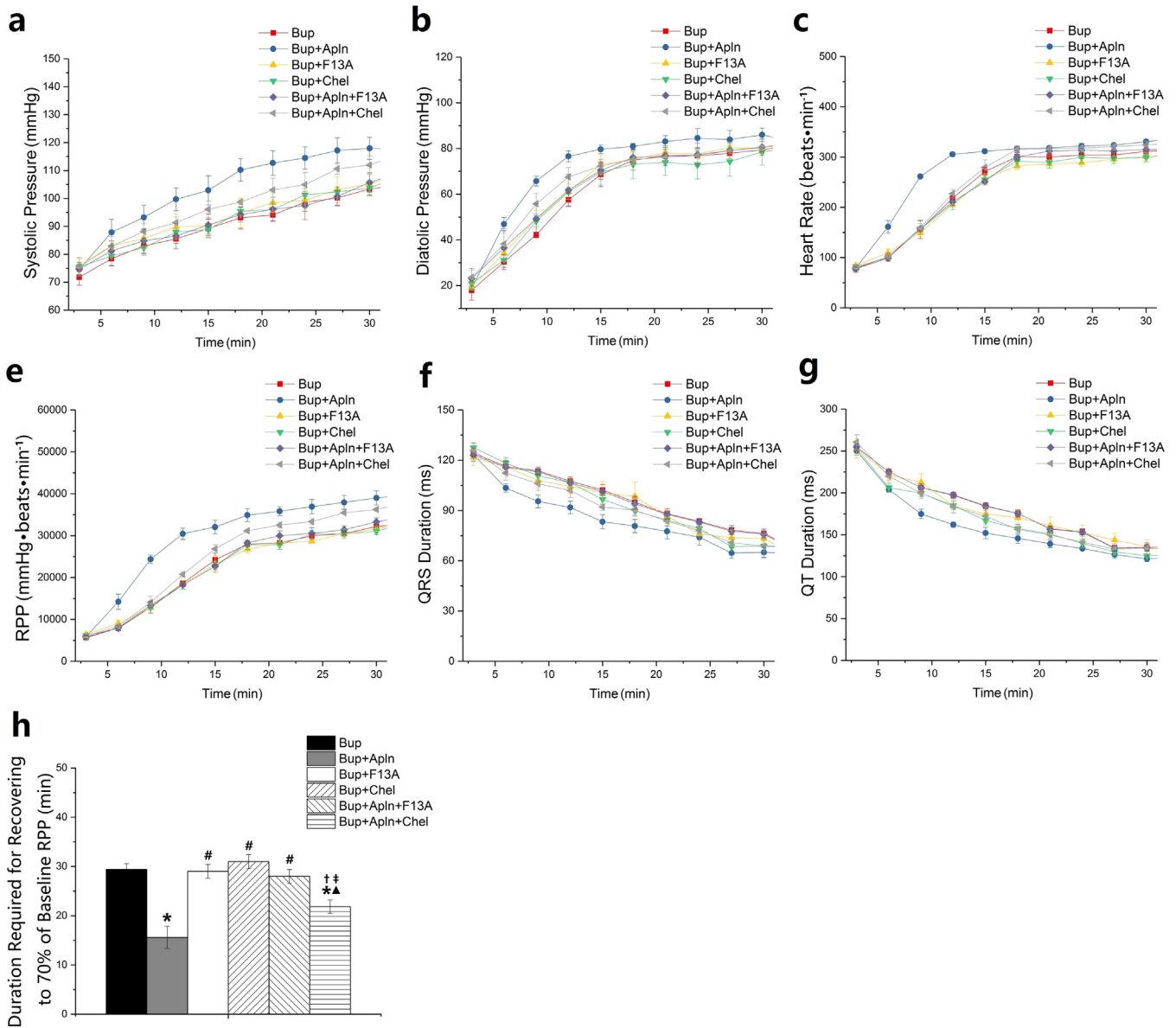


Figure 2 F13A and chelerythrine abolished the protective effects of apelin-13 on cardiac function in bupivacaine-treated rats (3–30 min). Systolic blood pressure (a), diastolic blood pressure (b), heart rate (c), RPP (d), QRS segment duration (e), QT segment duration (f) and duration required for recovering to 70% of baseline RPP (g) of each group were plotted against time (3–30 min). Data were expressed as mean \pm SD ($n = 8$). Continuous ECG and hemodynamic parameters in rats surviving at 30 min were compared across time by two-way repeated measures ANOVA. RPP, heart rate systolic blood pressure product. * $p < 0.05$ vs. Bup group, # $p < 0.05$ vs. Bup+Apln group, \blacktriangle $p < 0.05$ vs. Bup+F13A group, \dagger $p < 0.05$ vs. Bup+Chel group, \ddagger $p < 0.05$ vs. Bup+Apln+F13A.

Apelin, a ligand of the G protein-coupled receptor Angiotensin Receptor-Like 1 (APJ), is the most potent endogenous positive inotropic substance identified so far, yielding above 70% of the increase force observed with isoproterenol.⁸ Apelin is unable to modulate L-type calcium current or voltage-activated potassium currents of ventricular myocytes. In contrast, apelin-13 has the beneficial effects of increasing sodium current amplitude and the “window” current of ventricle cardiomyocytes as well as shifting the mid-activation potential and facilitating channel opening at negative voltage. Thus, the increased intracellular sodium promotes the propagation of the action potential, contributing to the inotropic effects of apelin-13.⁴ Our results showed that, along

with improving hemodynamics, apelin-13 in varying doses may significantly allow recovery of the QRS and the QT segments that are both closely related to sodium current, thereby implying that apelin-13 modulated the sodium current in rats subjected to bupivacaine administration.

Apelin, through the APJ system, increases Sodium-Hydrogen (NHE) activity, leading to intracellular alkalinization, and an increased myofilament sensitivity for calcium.² Also, via a PKC-dependent mechanism, apelin was reported to increase the sarcoplasmic reticulum Ca²⁺-ATPase and cause an increase in systolic [Ca²⁺]_i, but a decrease in diastolic [Ca²⁺]_i.⁹ Changes in iNa amplitude and kinetics may modulate intracellular Na⁺ concentrations and the turnover of

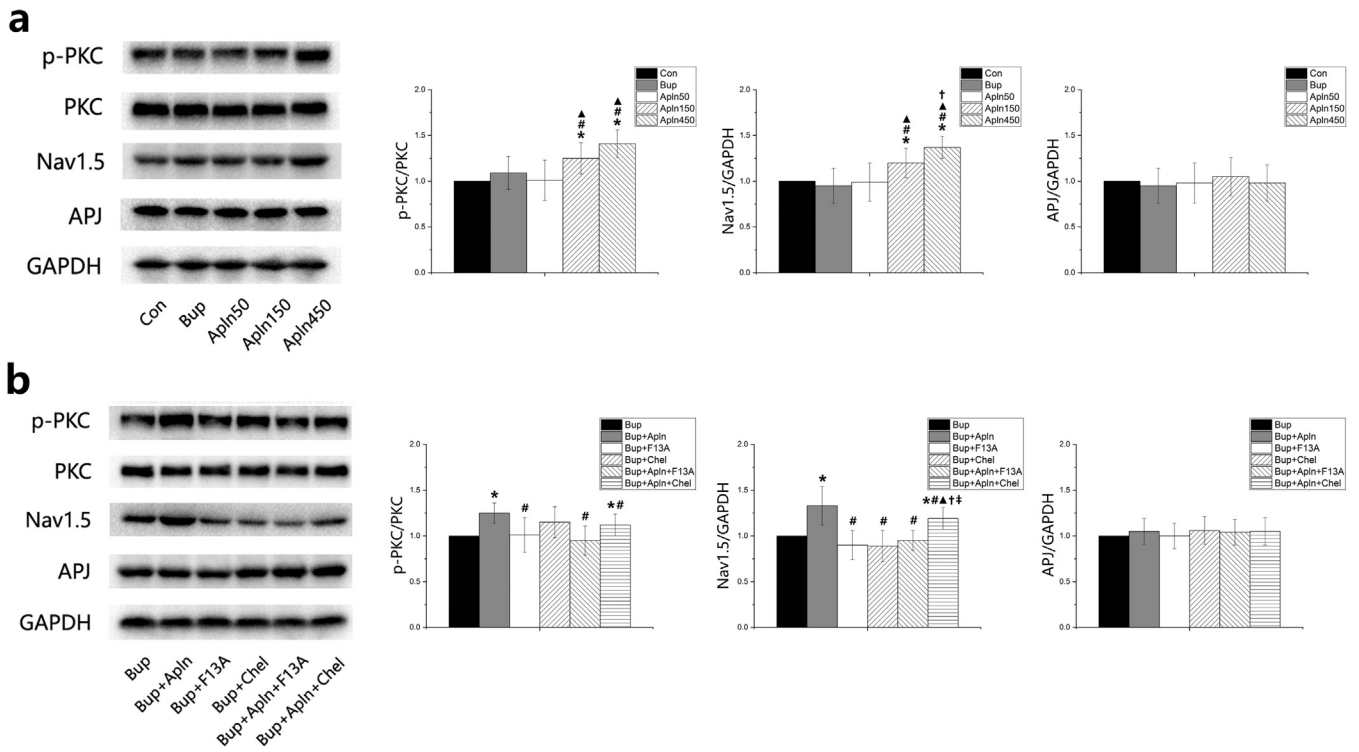


Figure 3 Apelin-13 increased p-PKC and Nav1.5 protein expressions as measured by Western Blotting. The protein expressions of p-PKC, PKC, Nav1.5 and APJ by Western blotting: (a) The dose-dependent manner of apelin-13 in increasing the ventricular expressions of p-PKC/PKC and Nav1.5 in bupivacaine-treated rats; (b) F13A and chelerythrine abolished the effects of apelin-13 on p-PKC and Nav1.5 protein expressions in bupivacaine-treated rats. Data were expressed as mean \pm SD (n = 8). The relative protein expression in ventricular tissue was compared among groups by two-way repeated measures ANOVA. PKC, Protein Kinase C. * $p < 0.05$ vs. Control group, # $p < 0.05$ vs. Bup group, \blacktriangle $p < 0.05$ vs. Bup+Apln50 group, \dagger $p < 0.05$ vs. Bup+Apln150 group.

sodium-calcium exchangers thereby increasing intracellular calcium. Thus, modulation of sodium current by Apelin could play an essential role in its inotropic effect.⁴ Mechanically, the APJ receptor when coupled to the G protein family member Gq protein can activate PKC, which is involved in cardiac

excitability and electrical remodeling in a variety of cardiovascular diseases. Activation of PKC, but not PKA, is involved in the modulation of sodium current by apelin-13, since chelerythrine, the specific inhibitor of PKC, abolishes the increase in sodium current by apelin-13.⁴ Here, our results

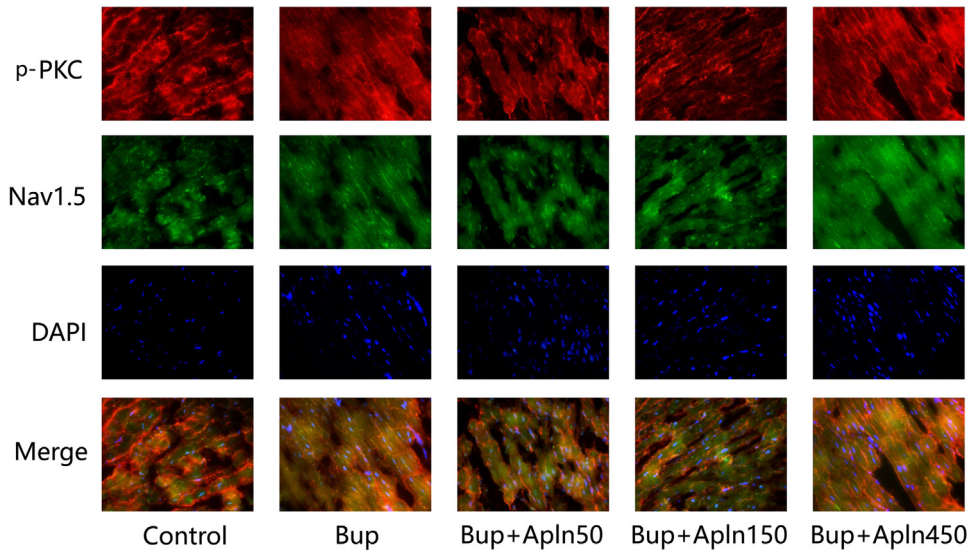


Figure 4 Apelin-13 increased p-PKC and Nav1.5 protein expression in bupivacaine-treated rats as measured by immunocytochemistry assay. PKC, Protein Kinase C.

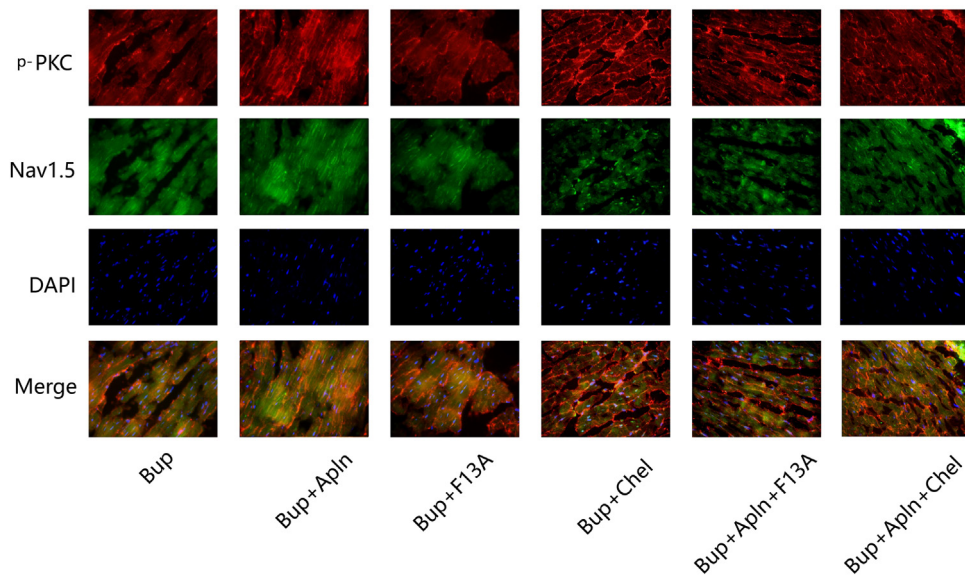


Figure 5 F13A and chelerythrine abolished the effects of apelin-13 on p-PKC and Nav1.5 protein expressions in bupivacaine-treated rats as measured by immunocytochemistry assay. PKC, Protein Kinase C.

indicate that PKC activation, as a downstream of the apelin/APJ system, may be involved in mitigating bupivacaine-induced cardiac suppression by apelin-13.

In our current study, $150 \mu\text{g}\cdot\text{kg}^{-1}$ apelin-13 exerted a similar effect as the dose of $450 \mu\text{g}\cdot\text{kg}^{-1}$ in attenuating bupivacaine-induced RPP reduction in rats; however, the larger dose caused transient SBP decline in the early stage. We assumed that the inotropic effect of apelin-13 prevailed over its vasodilatory effect when the dose was relatively low, but this was not so in rats receiving the higher dose of apelin-13. Several studies have been conducted to investigate the vasodilatory effect of apelin. The administration of apelin can lead to a rapid Nitric Oxide (NO)-dependent decrease in blood pressure and mean capillary filling pressure in rodent models,¹⁰ and a NO clamp may significantly attenuate vasodilation induced by apelin. In vitro, apelin causes NO-dependent vasodilation in human mesenteric arteries and vasoconstriction in endothelium-denuded human saphenous veins.^{11,12} The inotropic and vasodilatory effects of apelin-13 make it a promising candidate for heart failure therapy. There is evidence that short-term infusion of apelin-13 causes direct peripheral and coronary vasodilation, reduced cardiac preload and afterload, and increased cardiac output in patients with chronic heart failure.¹³

Several phosphorylation sites have been identified within the first intracellular linker loop of Nav1.5 consistent with direct regulation of Nav1.5 channel function by phosphorylation.¹⁴ Since apelin-13 functioned on cardiomyocytes for a relatively short period, we previously surmised that apelin-13 and/or PKC activation would not alter the synthesis and recruitment of new channels. However, in our current study, apelin-13 treatment surprisingly increased the synthesis and recruitment of sodium channel protein in a dose-dependent manner, as evidenced in the upregulated expression of Nav1.5 demonstrated by Western blot and immunofluorescence assays.

There are some limitations in our study that need to be addressed. First, apelin appears to be rapidly cleared from the circulation with a short plasma half-life of no longer than 8 min,^{8,15} but in this study we did not examine the benefit of the long-term administration of apelin-13. Second, using a specific inhibitor of PKC and APJ, we concluded that PKC might function as a vital protein following the binding of apelin-13 to APJ, but the exact mechanisms that may be involved in this process are still unclear and need further evaluation. Third, we did not measure NO expression or evaluate the inhibitory effect of the endothelial nitric oxide synthase inhibitor in apelin-13-treated rats; doing so may have assisted in the elucidation of potential mechanisms effecting a decrease in blood pressure. Finally, detailed in vitro data are needed to explore specific mechanisms.

Conclusion

Exogenous apelin-13, at least in part, activates the PKC signaling pathway through the apelin/APJ system to improve cardiac function in a rat model of bupivacaine-induced cardiac suppression.

Conflicts of interest

The authors declare no conflicts of interest.

Funding

This study was supported by the National Natural Science Foundation of China (Grant N° 81670219) and Science and Technology Bureau of Wenzhou City (N° Y2020161).

Data availability statement

Data are available from the authors upon reasonable request

Supplementary materials

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.bjane.2024.844501](https://doi.org/10.1016/j.bjane.2024.844501).

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