Experimental Study

Antiarrhythmic Peptide AAP10 Prevents Arrhythmias Induced by Protein Kinase C Activation in Rabbit Left Ventricular Wedges

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Summary

As the mechanisms underlying PKC activation induced arrhythmias are not yet fully verified, we investigated the role of gap junctions in arrhythmias induced by PKC activation.

Arterially-perfused rabbit left ventricular preparations were randomly assigned to perfusion with phorbol ester (PMA) or in combination with AAP10. Transmural ECG as well as action potentials from both endocardium and epicardium were simultaneously recorded throughout all experiments. Changes in connexin43 (Cx43) and nonphosphorylated Cx43 on S368 were measured by Western blot analysis.

In the PMA group, the QT interval was shortened, the interval from the peak to the end of the electrocardiographic T wave (Tp-e) and induced nonsustained ventricular tachycardia (VT) were increased, and the expressions of Cx43 and nonphosphorylated Cx43 on S368 were decreased compared with the control group. Compared with the PMA group, without significant changes in the QT interval and the expression of nonphosphorylated connexin43 on S368, Tp-e and induced VT decreased and the expression of Cx43 increased in the AAP10 group.

AAP10 can prevent PMA-induced rabbit ventricular arrhythmias by attenuating the increase of Tp-e and the decrease of expression of Cx43. These data suggest that increasing gap junction coupling prevents arrhythmias induced by protein kinase C activation. (Int Heart J 2015; 56: 234-238)

Key words: Gap junction, Phorbol ester, QT interval, Dispersion of repolarization

Previous studies have shown that the gap junction plays an important role in cardiac arrhythmias;1 the electrical coupling between myocytes mainly depends on gap junctions which are composed of gap junction protein termed connexin43 (Cx43). Phorbol ester (phorbol-12-myristate-13-acetate, PMA) is a common protein kinase C (PKC) agonist, which has been shown to down-regulate the coupling between myocytes.2-4 The PKC family consists of at least 10 isozymes, the most significant PKC family members for cardiac function belong to the subgroups PKC-α and PKC-β. Up to now, less attention was paid to the relationship between the proarrhythmic effects of PKC activation and its potential effects on cardiac gap junctions or ion channels. In fact, PKC is downstream of G-protein-coupled signaling pathways that activate several effectors, such as ion channels in the heart.

The antiarrhythmic peptide AAP10 (H2N-Gly-Ala-Gly-4Hyp-Pro-Tyr-CONH2) has been proven to enhance the coupling between myocytes,5,6 but whether AAP10 can prevent PMA-induced arrhythmias is still unknown. As the mechanisms underlying PKC activation induced arrhythmias are not yet fully verified, the present study used rabbit left ventricular wedges perfused with PMA or in combination with AAP10 to investigate the effects of PMA on rabbit ventricular arrhythmias and the effects of AAP10 on cardiac arrhythmias induced by PMA.

Methods

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Tongji Medical College.

Arterially perfused rabbit left ventricular wedge preparations: Thirty New Zealand White rabbits weighing 1.8 to 2.2 kg were anaesthetized with sodium pentobarbital (35 to 40 mg/kg i.v.) and anticoagulated with heparin. The heart was excised and submerged in cold (4°C) cardioplegic solution consisting of Tyrode’s solution containing 8.5 mmol/L [K+]o and anticoagulated with heparin. The heart was excised and submerged in cold (4°C) cardioplegic solution consisting of Tyrode’s solution containing 8.5 mmol/L [K+]o. The left circumflex branch of the coronary artery was cannulated and perfused with the same cardioplegic solution. Unperfused areas of the left ventricle, easily identified by their reddish appearance due to the existence of unflushed erythrocytes, were removed. The cannulated preparation was then placed in a small heated tissue bath and arterially perfused with Tyrode’s solution con-
taining the following composition (in mmol/L): 129 NaCl, 4 KCl, 0.9 NaH₂PO₄, 20 NaHCO₃, 1.8 CaCl₂, 0.5 MgSO₄, and 5.5 glucose, buffered with 95% O₂ and 5% CO₂ (35.7 ± 0.2°C). The perfusate was delivered to the artery by constant flow with a roller pump. Perfusion pressure was monitored continuously with a pressure transducer and maintained at 35 to 45 mmHg by adjusting the flow rate.

**Electrophysiological recordings from wedge preparations:** The preparations were stimulated with bipolar silver electrodes insulated except at the tips and applied to the endocardial surface (S1). A transmural pseudo-ECG of the wedge was recorded with a pair of silver/silver chloride electrodes positioned in the bath at the opposite sites of the wedge and along the same vector as the transmembrane recordings. Transmembrane action potentials were recorded simultaneously from epicardium (Epi) and endocardium (Endo) using flat glass microelectrodes (DC resistance: 10 to 20 MΩ) filled with 2.7 mol/L KCl. The impalement of both microelectrodes was maintained approximately on the axis of the transmural ECG recording.

**Study protocols:** The ventricular wedge preparations were allowed to equilibrate in the tissue bath for 1 hour prior to electrical recordings. All drugs used in this study were dissolved in Tyrode’s solution and infused into the wedge preparation via the cannulated artery. PMA (Sigma, St Louis, MO, USA) was used to activate protein kinase C.³⁵³⁶ AAP10 (Chinese Peptide Co., Hangzhou, Zhejiang, China), a gap junction enhancer, was used to enhance gap junction coupling.³⁷ After baseline data acquisition, preparations were divided into 3 groups:

- Control group (n = 10): wedge preparations perfused with Tyrode’s solution;
- PMA group (n = 10): wedge preparations perfused with Tyrode’s solution plus PMA (0.1 umol/L);
- AAP10 group (n = 10): 15 minutes prior to PMA administration, wedge preparations were pretreated with AAP10 (0.5 umol/L).

The development of spontaneous and programmed electrical stimulation (PES)–induced VT was assessed in baseline conditions and in the presence of PMA or PMA in combination with AAP10. PES-induced arrhythmias were evaluated by use of a single extrastimulus (S2) applied to the endocardial surface of the wedge. The QT interval was defined as the time from the onset of the QRS to the point at which the final down-slope of the T wave crossed the isoelectric line. Tp-e was defined as the time from the peak of the T wave to the point at which the final down-slope of the T wave crossed the isoelectric line.

**Western blotting:** Ventricular preparations were removed from the tissue bath after finishing electrical stimulation and immediately frozen in liquid nitrogen. The frozen tissues were pulverized with a mortar and pestle that had been cooled in liquid nitrogen. Pulverized frozen heart samples were homogenized with a lysis buffer containing 30 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 1 µg/mL pepstatin, and 1 µg/mL leupeptin. Homogenates were cleared by centrifugation at 10,000 g for 30 minutes at 4°C. Protein (50 µg) was analyzed by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes; an immunoblot for GAPDH protein was used as a control for equal protein loading. Primary antibody incubations were performed overnight at 4°C using mouse polyclonal antibody to measure total Cx43 or monoclonal antibody to measure nonphosphorylated Cx43 on Ser368 site (both 1:1000 dilution; Zymed Laboratories Inc., San Francisco, CA, USA). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:1000 dilution; NovoGene Inc., Madison, WI, USA), treated with chemiluminescence reagent (ECL; Pierce Inc., Rockford, IL, USA), and exposed to X-ray film. Immunoreactivity was quantified by densitometric analysis with Image-Pro Plus 6.0 software (Media Cybernetics Inc., Silver Spring, MD, USA) and Cx43 quantity was defined as the band density corresponding to Cx43 protein normalized to GAPDH protein.

**Statistical analysis:** Statistical analysis was performed using Student’s t-test or one-way analysis of variance (ANOVA). Fisher’s exact test was used for comparing event incidence, such as the occurrence of VT. All values are reported as the mean ± SD unless otherwise noted. A P < 0.05 was considered statistically significant.

**RESULTS**

**Effects of PMA on the QT interval, Tp-e, QRS duration, and incidence of VT:** Administration of PMA at 0.1 µM caused a marked shortening of the QT interval and significant increases in Tp-e and Tp-e/QT. Figure 1 illustrates the effects of PMA on the ECG and action potential morphology of the endocardial and epicardial cells at a basic cycle length (BCL) of 1000 ms. PMA also led to a shortening of the action potential duration (APD), which was more prominent in the epicardium of the preparation as compared with the endocardium. This subsequently resulted in an augmentation of Tp-e (Table I and Figure 1). In the PMA group, the QT interval, in parallel to the changes in the APD of the endocardial cells, was shortened from 302 ± 21 ms to 260 ± 25 ms (P < 0.01 versus control), Tp-e increased from 51 ± 7 ms to 61 ± 13 ms (P < 0.01 versus control), and the Tp-e/QT increased from 0.17 ± 0.02 to 0.24 ± 0.05 (P < 0.01 versus control), but the QRS duration was not altered significantly (P > 0.05 versus control). A single extras-
timulus reproducibly induced nonsustained ventricular tachycardia (VT) in 7 out of 10 preparations in the PMA group ($P < 0.05$ versus control, Table I and Figure 2).

Effects of AAP10 on QT interval, Tp-e, and QRS duration: Prior to administration of PMA, perfusion of AAP10 (0.5 μmol/L) did not significantly alter the QT interval, Tp-e, or QRS duration, as compared with the measurement at baseline ($P > 0.25$ for all 3, Table II).

Effects of PMA and AAP10 on QT interval, Tp-e, QRS duration, and incidence of VT: In order to test whether AAP10 can prevent PMA-induced shortening of the QT interval, and increases in Tp-e and the incidence of VT, we added AAP10 to the solution administered with PMA. Interestingly, perfusion of AAP10 at a concentration of 0.5 μM did not significantly alter the QT interval (241 ± 22 ms versus 260 ± 25 ms, $P > 0.05$) or QRS duration (37.4 ± 2.1 ms versus 37.5 ± 2.4 ms, $P > 0.05$), but did lead to a decreased Tp-e (41 ± 6 ms versus 61 ± 13 ms, $P < 0.01$) and Tp-e/QT (0.17 ± 0.02 versus 0.24 ± 0.05, $P < 0.01$), as compared with the PMA group (Table I and Figure 1). Moreover, AAP10 significantly reduced PES-induced VT to 2 of 10 preparations ($P < 0.05$), as compared with the PMA group.

Changes in Cx43 and nonphosphorylated Cx43 on S368: A comparison of total Cx43 and nonphosphorylated Cx43 on S368 expression profiles among all groups is shown in Figure 3. The Western blot results of quantitative densitometric analysis of total Cx43 (T-Cx43) and nonphosphorylated Cx43 on S368 (NP-Cx43 S368) (B) signal (bottom) and representative immunoblot (top). Cx43 signals were quantified by densitometry and normalized to GAPDH. As compared with the control group, PMA led to a marked decrease of total Cx43 ($P < 0.05$) and nonphosphorylated Cx43 on S368 ($P < 0.01$). However, the reduction of total Cx43 caused by PMA was significantly improved in the AAP10 group ($P < 0.05$), although the expression of nonphosphorylated Cx43 on S368 did not change significantly ($P > 0.05$) compared with the PMA group. Vertical bars are mean ± SD.

**Table I.** Comparison of QT Interval, Tp-e, Tp-e/QT, QRS Duration, and Incidence of VT Among All Groups

<table>
<thead>
<tr>
<th></th>
<th>QT/ms</th>
<th>Tp-e/ms</th>
<th>Tp-e/QT</th>
<th>QRS/ms</th>
<th>VT%</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>302 ± 21</td>
<td>51 ± 7</td>
<td>0.17 ± 0.02</td>
<td>36.6 ± 2.0</td>
<td>0%</td>
</tr>
<tr>
<td>PMA</td>
<td>260 ± 25&quot;</td>
<td>61 ± 13&quot;</td>
<td>0.24 ± 0.05&quot;</td>
<td>37.5 ± 2.4&quot;</td>
<td>70%***</td>
</tr>
<tr>
<td>AAP10</td>
<td>241 ± 22&quot;</td>
<td>41 ± 6&quot;</td>
<td>0.17 ± 0.03&quot;</td>
<td>37.4 ± 2.1&quot;</td>
<td>20%&quot;***</td>
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$P < 0.01,$ $P > 0.05,$ $P < 0.05$ versus control; $P < 0.05,$ $P < 0.01,$ $P < 0.05$ versus PMA. Basic cycle length (BCL) = 1000 ms. Values are given as mean ± SD.

**Table II.** Effects of AAP10 on QT Interval, Tp-e, and QRS Duration

<table>
<thead>
<tr>
<th></th>
<th>QT/ms</th>
<th>Tp-e/ms</th>
<th>Tp-e/QT</th>
<th>QRS/ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL</td>
<td>301 ± 19</td>
<td>50 ± 6</td>
<td>0.17 ± 0.02</td>
<td>37.6 ± 2.1</td>
</tr>
<tr>
<td>PRE</td>
<td>302 ± 20&quot;</td>
<td>51 ± 6&quot;</td>
<td>0.17 ± 0.02&quot;</td>
<td>36.9 ± 1.8&quot;</td>
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$P > 0.05$ versus BL. BL indicates baseline; and PRE, pretreatment. Basic cycle length (BCL) = 1000 ms. Values are given as mean ± SD.

**Discussion**

Numerous studies have demonstrated that the coupling of cardiac gap junction is related closely to transmural dispersion of repolarization (TDR), $^{6,8-10}$ and the uncoupling of gap junction will lead to an amplification of TDR, which is considered as a substrate for cardiac arrhythmogenesis. The present study investigated the underlying mechanisms for PMA-induced arrhythmias and the antiarrhythmic effect of AAP10 on PMA.
induced arrhythmias.

Tp-e has been shown to be correlated to TDR. However, in studies of various animal species, it has been found that not only the QT interval and Tp-e increase linearly with increased body mass, but also that Tp-e/QT remains relatively constant within a range of values from 0.17 to 0.23. Furthermore, although the Tp-e of those patients suffering short QT syndrome with high risk of ventricular tachycardia was not significantly different from that of normal controls, the Tp-e/QT was significantly elevated as compared to controls. These findings clearly suggest that the Tp-e/QT ratio is a better index of arrhythmogenesis under such conditions. Gupta, et al reported that Tp-e/QT serves as a more sensitive index of arrhythmogenesis since it eliminates the confounding effects of variability of heart rate and interindividual variation of the QT interval, and provides an estimate of dispersion of repolarization relative to the total duration of repolarization.

In the present study, PMA significantly shortened the rabbit ventricular QT interval, increased Tp-e and Tp-e/QT, and facilitated the inducibility of VT. In regard to the effects of PKC activation on myocardocyte action potential duration (APD), Puglisi, et al found that PMA can lead to a decreased outward potassium current and an increased Na\(^+\) Ca\(^+-\) exchange inward current, resulting in a prolongation of APD. Woo and Lee thought that activation of PKC first excited and later depressed L-Ca\(^+\) channels, and finally reduced the inward current of action potential repolarization. However, these effects of PKC stimulation on cardiac electrophysiology were mainly linked to data obtained from isolated myocytes. In our study, PMA led to a marked shortening of the rabbit ventricular QT interval, indicating that PKC activation could shorten cardiac repolarization in whole heart. Since Tp-e and Tp-e/QT increased in the present study, we believe that PMA can lead to an amplification of the dispersion of repolarization and an increase of inducibility of VT.

At the same time, we detected the changes in Cx43 and nonphosphorylated Cx43 on S368 after administration of PMA. Interestingly, PMA dramatically reduced the expression of total Cx43 and nonphosphorylated Cx43 on S368. Studies have shown that PMA can affect Cx43 half-life, cause internalization of Cx43, and lead to a decrease in gap junction assembly. Lampe, et al found that PMA can decrease gap junction coupling via phosphorylation of Cx43 on S368 sites. Therefore, we conclude that the gap junction coupling might be down-regulated via decreasing the expression of total Cx43 and nonphosphorylated Cx43 on S368. However, as PMA did not alter the QRS duration, it is difficult to conclude the conduction velocity was slowed down in the presence of PMA. The most likely explanation is that the conduction velocity is not only determined by the coupling level but also the excitability of cardiac myocytes, and how PMA affects the excitability of myocytes is still unclear. Since the repolarization currents also affect TDR, we can only conclude that gap junction uncoupling is partially involved in the amplification of the dispersion of repolarization in the presence of PMA.

Furthermore, in order to further test whether the gap junction is involved in PMA-induced arrhythmogenesis, we added the gap junction enhancer AAP10 to the solution. Interestingly, AAP10 significantly decreased Tp-e and Tp-e/QT, and reduced the inducibility of VT without changing the QT interval. More importantly, AAP10 attenuated the decrease of total Cx43 induced by PMA without altering the phosphorylation state of Cx43 on S368. In our study, AAP10 decreased Tp-e and Tp-e/QT without affecting the QT interval, indicating that AAP10 decreased the dispersion of repolarization, which contributed to the antiarrhythmic effect. Previous studies have shown that AAP10 has no effect on ion channels without altering the action potential duration (APD) of single heart cells, and consistent with this finding, our study showed that perfusion of AAP10 did not significantly change QT interval, Tp-e, or QRS duration, as compared with the measurement at baseline. Previous studies have suggested that when gap junction coupling was increased, the enhanced intercellular coupling resulted in a greater electrotonic effect that lowered the APD gradient, and then TDR was decreased. Consistently, our study also showed AAP10 prevented PMA-induced amplification of dispersion of repolarization. Previous studies have shown that AAP10 can prevent run-down of gap junctions and increase them over time. With regard to the mechanism concerning how AAP10 regulates gap junctions, recent studies have suggested that AAP10 can increase the expression of Cx43, and our study also showed that AAP10 prevented the decrease of Cx43 expression induced by PMA. Since we did not observe a change in phosphorylation state on S368 after the addition of AAP10, our study indicates that AAP10 increases gap junction coupling mainly through preventing a PMA-induced decrease of Cx43 expression.

**Study limitations and conclusion:** As VT was seldom induced by PMA at a baseline of 2000 ms, the present experiment was conducted at 1000 ms, at which VT was readily induced. We did not study the effects of PMA and AAP10 on ionic channels, so we are not able to exactly describe the mechanisms underlying PKC-induced shortening of repolarization. However, we do not believe the pathological shortening of the QT interval is due to the uncoupling of gap junctions. In addition, the transmural differences in the pathological shortening of APD (Figure 1), which led to an increased Tp-e, was responsible for the PMA-induced arrhythmias. Since we did not measure gap junction function directly, we can only conclude that the gap junction is at least partially involved in the generation of the dispersion of repolarization.

In summary, the present study suggests that increasing gap junction coupling prevents arrhythmias induced by protein kinase C activation. Moreover, cardiac gap junctions might be involved in PMA-induced ventricular arrhythmias and gap junction coupling might be down-regulated. Furthermore, increasing gap junction coupling by AAP10 can prevent PMA-induced ventricular arrhythmias via attenuating the dispersion of repolarization and decreasing the expression of Cx43. These findings indicate that amplifying dispersion of repolarization and the uncoupling of gap junctions might play a significant role in some malignant arrhythmias caused by pathological shortening of the QT interval, and that increasing gap junction coupling might be a promising therapy to treat such arrhythmias.

**References**