Gap junctions (GJs) are fundamental to a stable heartbeat. The cardiomyocytes of the mammalian heart are connected by large numbers of GJs, providing the basis for conduction of action potentials from one cell to another.1-3 The main GJ protein present in mammalian ventricular muscle is connexin43 (Cx)43.4,5 In 1991, studies of infarcted ventricular myocardium in humans6 and dogs7 provided the first indication that Cx43 GJ distribution is profoundly altered following ventricular injury.6,13,16,17

We reported previously on a 25-aa peptide comprised of a cell-permeabilization sequence linked to the last 9 amino acids of the Cx43 carboxyl terminus (CT).18 This Cx43-CT mimetic peptide, now referred to as aCT1,19 was shown to bind to the second postsynaptic density/disk-large/ZO-1 (PDZ2) domain of Zonula occludens (ZO)-1,18 thereby inhibiting a protein-protein interaction occurring between ZO-1 and Cx43,18,20-22 In association with inhibiting this interaction, aCT1 effected remodeling of GJs in HeLa cells expressing Cx43 and cultured neonatal rat ventricular myocytes.18 Specifically, it was determined that aCT1 treatment prompted increases in the size of membrane-localized GJs, increases in the p1 and p2 phosphorylation bands of Cx43 on Western blots and redistribution of Cx43 from nonjunctional to GJ-associated pools of the protein.18 Consistent with these results in vitro, it was subsequently found that aCT1 increased the
size of Cx43 GJs between epidermal cells proximal to skin wounds in vivo.\textsuperscript{23}

Because \textalpha CT1 inhibited remodeling of GJ size and distribution of Cx43 in vitro, we sought to establish whether \textalpha CT1 had similar effects on GJ remodeling in the myocardium in vivo. It was of specific interest to determine whether \textalpha CT1 would inhibit remodeling of GJs in the arrhythmia-prone tissues bordering ventricular injuries. To test this hypothesis, a cryoinjury model was developed to generate a uniformly sized injury and injury border zone (IBZ) in the left ventricle of mouse hearts.\textsuperscript{24} The cryoinjury protocol enabled avoidance of the variability in wound size and shape that often confounds interpretation of other mouse models of experimental injury, such as coronary artery ligation. Localized treatment of left ventricular (LV) injuries with \textalpha CT1 resulted in decreased propensity to develop arrhythmia in response to programmed stimulation and increased conduction velocity. These physiological changes were concomitant with stabilization of Cx43 at IDs in the IBZ, and with increases in phosphorylation at a serine at amino acid position 368 (S368) of Cx43 – a protein kinase C consensus site.\textsuperscript{25} Consistent with this observation in vivo, assays in vitro, indicated that \textalpha CT1 was sufficient to prompt dose-dependent increases in protein kinase (PK)C-\textepsilon-mediated phosphorylation of Cx43 at S368.

Methods
An expanded Methods section, which includes details regarding methods and reagents, is available in the Online Data Supplement at http://circres.ahajournals.org.

LV Cryoinjury and Treatment
Twelve- to 24-week female CD1 mice were given a left thoracotomy at the fourth intercostal space to expose the anterior free wall of the LV. A cryoinjury was generated by epicardial exposure to 5 seconds of contact with a liquid-N\textsubscript{2} chilled 3 mm circular flat-tip probe. The injury was immediately covered with an adherent methylcellulose patch containing either \textalpha CT1 (100 \textmu M/L), reverse peptide (Rev) (100 \textmu M/L), vehicle (Veh) (1X PBS), or Dif. The thoracotomy was closed, and the mice were allowed to recover before any further study.

Triphenyltetrazolium Chloride Staining Forty-Eight Hours After Injury
Cryoinjured mouse hearts were harvested 48 hours after cryoinjury, stained in 1% triphenyltetrazolium chloride (TTC), washed in 1X PBS, and fixed overnight at 4°C. The epicardial area and transmural depth of injuries were imaged and measured as described in supplement.

Microscopy
Immunohistochemistry and analyses were performed as described in the supplement. Isolated adult feline cardiomyocytes were stained with Fluorescein-conjugated streptavidin, and imaged for peptide uptake. Hearts were harvested 24 hours, 48 hours, and 1-week, then bisected along the base-apex midline of the injury with half embedded in paraffin and half processed for frozen sectioning. Paraffin-embedded sections from the middle of injuries 48 hours after injury were stained with hematoxylin/eosin or antibodies against Cx43 and atrial myosin light chain-2 (Mi2ca) to visualize the IBZ. Frozen sections from 24-hour and 1-week hearts were used for analysis of ID localization, and Cx43/ZO-1 and Cx43-pS368/total-Cx43 colocalization. Sections were stained with TRITC-wheat germ agglutinin (WGA), TO-PRO-3 nuclear stain, and antibodies to Cx43, ZO-1, and Cx43-pS368.

Western Blotting
Western blot analysis was undertaken on hearts 2, 4, 6, and 48 hours after injury and exposure to \textalpha CT1. Samples were processed as described in supplement. Peptide eluted from methylcellulose patches was detected in injured and remote myocardium on blots against biotin and Cx43. For assessment of Cx43-pS368 and total Cx43, samples were immunoblotted with rabbit pS368 Cx43 antibody and reprobed for total Cx43.

Electrophysiological Studies
Hearts were harvested, washed, and the aorta was cannulated for modified Langendorff procedure 7 to 9 days after injury. The atrioventricular (AV) node was ablated. Then, hearts were subjected to premature and overdrive pacing protocols with simultaneous recording. Scores for severity of arrhythmia were assigned by 3 investigators blind to treatment as outlined in supplemental Table I.

Optical Mapping Studies
Hearts were harvested and prepared as above, except the AV node was left intact so that activation under sinus rhythm could be studied. Hearts were stained with Di-4-ANEPPS, treated with Cytochalasin-D and optically mapped as described in the Online Data Supplement.

In Vitro Kinase Assay
PKC-\textepsilon phosphorylation of GST-Cx43-CT substrate at serine 368 PKC was evaluated under PKC assay conditions as detailed in supplement. Reactions were Western blotted for pS368 Cx43 and reprobed for total Cx43 as above. Assay was repeated 4 times.

Data Collection and Analysis
All data were collected blind to whether animals received treatment. Chi Sq was used to determine significance (\(P<0.05\)) of arrhythmia frequency comparisons. \(\geq11\) animals were tested in each treatment/control group in arrhythmia frequency tests. For determination of significance (\(P<0.05\)) of differences in arrhythmia severity a Kruskal–Wallis test with post testing was used. For other comparisons, significance (\(P<0.05\)) was determined by one-way, 2-way or repeated-measures ANOVA and post testing. The ANOVA model used for each data set is provided in figure legends. Data from 3 to 9 animals (figure legends provide animal numbers for specific experiments) were pooled for each treatment/control group, tested.

Non-standard Abbreviations and Acronyms

| Abbreviation | Acronym
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>carboxyl terminal</td>
</tr>
<tr>
<td>Cx43</td>
<td>connexin43</td>
</tr>
<tr>
<td>Cx43-pS368 or pS368</td>
<td>Cx43 phospho-isoform, p-serine 368</td>
</tr>
<tr>
<td>GJ</td>
<td>gap junction</td>
</tr>
<tr>
<td>IBZ</td>
<td>injury border zone</td>
</tr>
<tr>
<td>ID</td>
<td>intercalated disk</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricular</td>
</tr>
<tr>
<td>PDZ2</td>
<td>second postsynaptic density-95/disk-large/ZO-1</td>
</tr>
<tr>
<td>PK</td>
<td>protein kinase</td>
</tr>
<tr>
<td>Rev</td>
<td>reverse control peptide</td>
</tr>
<tr>
<td>S368</td>
<td>Cx43 amino acid serine 368</td>
</tr>
<tr>
<td>TTC</td>
<td>triphenyltetrazolium chloride</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula occludens-1</td>
</tr>
<tr>
<td>Veh</td>
<td>vehicle</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
</tr>
</tbody>
</table>
Results

Standardized LV Cryoinjury Model

Commonly used arterial ligation models for generating ventricular wounds can result in injuries with significant variability in size and shape. We developed a method that produced an injury of consistent size and shape (roughly hemispheric) on mouse LV (Figure 1A and 1B).²⁴ Our method was based on one described by van den Bos et al, who used a liquid-N₂-cooled cryoprobe.²⁶ We modified this protocol to include probe prechilling and nontransmural injury, as opposed to the more severe transmural injury they produced. Nontransmural injuries provided extended uniform regions of IBZ - a tissue of particular interest in our study. By varying exposure time to a liquid-N₂-cooled circular probe, it was determined that a 5-second probe application to the LV provided a repeatable acute cryolesion spanning ≈60% of the free wall, as assessed by TTC staining (Figure 1A and 1B).

Forty-eight hours after LV cryoinjury, hematoxylin/eosin histochemistry (Figure 1C) and Cx43 immunolabeling (Figure 1C, inset) showed a discrete border between necrotic tissue and viable myocardium. In hearts 8-weeks after cryoinjury, a narrow, but definite sector of IBZ could be observed (Figure 1D). This 10 to 20 cardiomyocyte-wide IBZ adjacent to the injury was also marked by increased immunolabeling for ventricular myosin light chain-2 and atrial myosin-light chain 2 (Mlc2a) (Figure 1D). Similar to reports on Cx43 at infarcts,⁵,¹³,¹⁶,¹⁷ Cx43 showed disorganized/lateralized patterns of distribution in the IBZ (Figure 1D, inset).

Localized Delivery of αCT1 to Cryoinjured LV

Figure 2A illustrates the Cx43-CT peptide αCT1 (α-connexin carboxyl terminal 1). As previously reported,¹⁸ αCT1 has a 2-part design comprising an amino-terminal (NT) antennapedia cell-permeabilization domain linked to the CT-most 9 amino acids of Cx43 (RPRPDDLEI-CT). We have previously demonstrated that αCT1 inhibits Cx43/ZO-1 interaction via binding to the ZO-1 PDZ2 domain and not other ZO-1 PDZ domains.¹⁸ For a control, we used a peptide (reverse) (Figure 2A) consisting of the antennapedia permeabilization sequence and the Cx43-CT 9-aa sequence reversed (IELDDPRPR-CT). In previous work, we showed that this control peptide is inactive with respect to inhibiting Cx43/ZO-1 interaction.¹⁸ 30 µmol/L αCT1 was rapidly taken up into neonatal¹⁸ and adult ventricular cardiomyocytes (Figure 2B and 2C).

In a previous report, αCT1 was delivered to skin wounds in a 25% pluronic F127 gel.²³ We found that gel would not remain localized to the LV cryolesion during surgery. To achieve sustained localized delivery of peptides to injured LV for up to 48 hours, we reformulated αCT1 in a “dry” methylcellulose patch (patch) that adhered stably to cryoinjured tissue. Figure 2D through 2G shows a cryoinjured heart harvested 2 hours after application of a patch containing DiI. Whole mount images of the epicardial surface show injury location (Figure 2D) and the location of the applied patch (Figure 2E). A transverse cut at level of red line on 2D demonstrates local diffusion of DiI (red) into underlying LV in the region of injury (Figure 2E and 2G).

Samples were harvested from injured and remote ventricular myocardium at 2, 4, 6, and 48 hours following application of the patch containing αCT1 (100 µmol/L) to cryoinjured hearts. αCT1 was detected on Western blots from 2, 4, and 6-hour LV injury samples after application, but by 48 hours, αCT1 was minimally detectable (Figure 2H). No peptide was found in myocardial samples remote from cryoinjury at any point after treatment (Figure 2H).

αCT1 Decreases Cx43/ZO-1 Colocalization

Twenty-Four Hours After Injury

Analysis of ZO-1 interaction with Cx43 was performed on IBZ tissues 24 hours after injury. Initial attempts to dissect sufficient IBZ tissue from cryoinjures for immuno-precipitation of Cx43/ZO-1 were unsuccessful. However, in Hunter et al, a method of quantification of ZO-1 colocaliza-
tion with Cx43 GJs was shown to correlate with biochemical assays of Cx43/ZO-1 interaction.18

Colocalization analysis was undertaken in IBZ at 24 hours after injury in Veh (Figure 3A, 3B, and 3B/H11032), Rev (Figure 3C and 3C/H11032), and CT1-treated (Figure 3D and 3D/H11032) hearts. Cx43/ZO-1-association levels in the IBZ varied significantly (P<0.05) between CT1, Rev and Veh groups in a multiple comparison test by one-way ANOVA. Rev and Veh controls did not differ significantly, and when combined showed significantly higher levels of Cx43/ZO-1 colocalization (P<0.05) in comparison to the CT1 group in post testing (Figure 3E).

**αCT1 Increases GJ Intercalated Disk Localization in the IBZ**

We have previously reported in echocardiography studies of cryoinjured hearts that the αCT1 effect on LV dilation was most pronounced 1 week after cryoinjury.24 We examined the cellular distribution of Cx43 24 hours and 1 week after injury to determine whether there was a concomitant change in GJ localization at intercalated disks(ID) following cryoinjury and treatment. Figure 4A through 4F shows IBZ (Figure 4A through 4C) and ventricular tissue remote from injury (Figure 4D through 4F) in αCT1 (Figure 4A and 4D), Rev (Figure 4B and 4E), and Veh (Figure 4C and 4F) hearts 24 hours after injury. Cx43 is immunostained green, with cell membranes...
stained red by TRITC-WGA to assist discrimination of end-to-end contacts, IDs, between cardiomyocytes. Dashed lines mark the boundary between the cryoinjury necrotic area and the IBZ (in Figure 4A through 4C and 4G through 4I).

At 24 hours, differences in Cx43 distribution between treatment and controls were not easily visualized. Consistent with this, statistical comparisons of Cx43 levels at IDs at 24 hours in the IBZ or remote myocardium indicated no significant differences between the αCT1 group and the Rev or Veh groups (Figure 4M and 4N).

By 1–week (Figure 4G through 4L), differences in GJ remodeling between treatment and control groups were more readily observed. Figure 4G through 4I shows magnified images from IBZ of αCT1 (Figure 4G), Rev (Figure 4H), and Veh-treated (Figure 4I) hearts 1 week after injury. Corresponding images of remote myocardium
from the same hearts are also shown (Figure 4J through 4L). Arrows in Figure 4G denote immunolabeled ID-associated Cx43 GJs in the IBZ of a αCT1-treated heart. Arrowheads in Figure 4H and 4I denote lateralized GJs in IBZs from controls. Pairwise comparison indicated that Cx43 localized to IDs was significantly decreased in both Rev and Veh IBZs relative to the αCT1 group (Figure 4M; \( p < 0.001 \)). The fraction of Cx43 at IDs in αCT1 group decreased by only 4% (\( P > 0.1 \)), between 24-hour and 1-week time points (Figure 4M), indicating maintenance of Cx43 distribution in IDs. By contrast, ID localization of Cx43 decreased approximately 55% in Rev and Veh control groups over the first week (Figure 4M; \( P < 0.00005, P < 0.00008 \)). No significant treatment- or time-dependent effects on Cx43 localization at IDs occurred in remote myocardium (Figure 4N), suggesting that αCT1 effects on GJ remodeling were localized to IBZ tissues.

αCT1 Inhibits Arrhythmias in the Cryoinjured Heart
IBZ GJ remodeling is associated with increased arrhythmogenicity following infarction.27 Accordingly, our next step was to determine whether there was a difference in arrhythmogenic propensity following treatment. As before, mice received standardized cryoinjury and patches, containing αCT1, Rev, and Veh. At \( \approx 1 \) week (7 to 9 days), cryoinjured and uninjured control hearts were isolated and kept viable by retrograde aortic perfusion. There was no notable difference in survival between the groups up to \( \approx 1 \) week after injury.

Once stable, isolated hearts were subjected to 2 induced-arrhythmia protocols: premature, \( s_2-s_3 \) pacing and overdrive pacing. Uninjured hearts demonstrated minimal propensity to develop induced arrhythmias, after stabilization in the bath. However, 75% of cryoinjured hearts developed arrhythmias when subject to either premature or overdrive pacing. Arrhythmias ranged in severity from ectopic beats to ventricular fibrillation (Figure 5A through 5E).

Figure 5A shows a recording from a cryoinjured heart stimulated using the premature pacing protocol with a \( s_1 \) interval of 200ms, \( s_2 \) of 80ms, and \( s_3 \) of 30ms. Each of the stimuli are followed by an action potential (AP) (blue arrows in Figure 5A). Figure 5B shows a recording of a cryoinjured heart when subject to either premature or overdrive pacing. Arrhythmias are reduced by αCT1 treatment of cryo-injured hearts. A through E, Representative tracings from premature ventricular pacing protocol on isolated perfused hearts illustrate no arrhythmia (A), 3 spontaneous PVCs (B), resolving tachycardia (C), sustained tachycardia (D), and fibrillation (E). Green numbers in Figures A through D label the \( s_1, s_2, \) and \( s_3 \) stimuli. Blue arrows in A denote the stimulated ventricular action potential. F, Number of hearts displaying arrhythmias (dark red and blue colors) that were unsustained (left bar graph) or sustained (right bar graph) in αCT1, Rev, and Veh groups following pacing. Lighter red and blue colors within bars indicate number of hearts within groups in which arrhythmia was not induced by pacing. G, Graphic representation of the median severity of arrhythmia for the 3 treatment groups (\( P < 0.02 \) αCT1/Rev, \( P < 0.02 \) αCT1/Veh); \( n = 11 \) (mice/group). Probability values for comparisons of frequency and severity of induced arrhythmia were generated from \( \chi^2 \) and Kruskal-Wallis tests and post tests, respectively.

We further classified the tachycardia by whether it was sustained or sustained irrespective of additional stimuli (Figure 5C and 5D), or if it progressed to fibrillation (Figure 5E). The premature pacing protocol, αCT1-treated hearts showed a significantly decreased number of sustained (or more pronounced) arrhythmias compared pairwise to either the Rev (\( P < 0.007 \)) or Veh control groups (\( P < 0.03 \)) (Figure 5F). All hearts were ranked for severity of arrhythmia, according to a 10-point scale (supplemental Table I). The severity of arrhythmia was significantly reduced in αCT1-treated hearts compared to either Rev (\( P < 0.02 \)) or Veh (\( P < 0.02 \)) groups (Figure 5G).

αCT1 Increases Rate of Depolarization of Cryoinjured Hearts
It has been shown that GJ remodeling can potentiate arrhythmias by decreasing conduction velocity through the IBZ.13 Previously described methods were used for optical mapping of electrical activation in cryoinjured hearts.28–32 Figure 6A is a light microscope image of a frame from the ultrafast digital camera taken before imaging of Di-4-ANEPPS fluorescence. Notice that the cryoinjury stains with decreased intensity compared to surrounding ventricle.

Figure 6B shows optically recorded action potentials (APs) corresponding to points 1 to 7 in Figure 6A, and Figure 6C
wave fronts caused by the cryoinjury complicated the identification of primary vectors necessary for calculation of longitudinal and transverse conduction velocities (Figure 6D through 6F). To address this, we took a simple approach of calculating the time required to depolarize given fractional areas of the ventricular epicardium.

The rate of ventricular depolarization was significantly faster in αCT1-treated hearts than either Rev (\(\beta P<0.05\)) or Veh (\(\alpha P<0.05\)) controls (Figure 6G). For example, the time required to depolarize 50% of the epicardium for the αCT1 group was interpolated from Figure 6G as 3.45 ms. By comparison, it took 4.94 ms and 5.17 ms to depolarize 50% of the ventricle in Rev and Veh control groups.

**αCT1 Causes an Acute Increase in Cx43-pS368 Levels in the IBZ**

Reports by others have indicated that phosphorylation of a serine at amino acid residue 368 (Cx43-pS368) of Cx43 is associated with maintenance of the connexin at IDs in hearts subjected to low-flow ischemia.\(^{33}\) We sought to determine whether Cx43-pS368 levels were also associated with increased Cx43/ID localization following αCT1 treatment.

Visual assessment of myocardium indicated Cx43-pS368 in immunolabeled sections at 24 hours after injury (Figure 7A through 7F; Online Figure I, A through I). The insets in Figure 7A through 7F shows representative GJs in IBZ and remote myocardium from each group. The white overlay on the green Cx43 signal in these insets represents overlap of Cx43-pS368 with a pan-Cx43 marker (total Cx43). The IBZ (Figure 7A through 7C) showed an elevation in Cx43-pS368 24 hours after injury compared to remote myocardium (Figure 7D through 7F; arrows indicate colocalization). The largest increase in IBZ Cx43-pS368 occurred in IBZs from αCT1-treated hearts (Figure 7A). The increased ratio of immunolabeled Cx43-pS368 to total Cx43 in αCT1-treated IBZs at 24 hours after injury was significant in ANOVA post testing (\(P<0.04\)) relative to Rev and Veh control groups combined (Online Figure I, J).

To further quantify this effect, we performed Western blot assays of Cx43-pS368 normalized to total Cx43 levels and GAPDH in the IBZ and remote ventricular myocardium at 4, 6, 24, and 48 hours and 1-week after cryoinjury and treatment (Figure 7G). Pairwise comparison indicated that normalized Cx43-pS368 levels were significantly lower in control versus αCT1-treated IBZs (\(P<0.03\)) over the 1-week time course (Figure 7H). Moreover, the increase in Cx43-pS368 in treatment versus control was significant at 6 hours after the cryoinjury (Figure 7H; \(P<0.04\)). It was concluded that a localized application of αCT1 delivered just after cryoinjury results in increased levels of IBZ Cx43-pS368 over a 7-day period compared to controls.

No significant treatment effect was observed on normalized Cx43-pS368 in ventricle remote from the injury between groups over the postinjury time course. However, remote myocardium did show a time-dependent decrease (\(P<0.00002\)) in Cx43-pS368 over the 7 days that was similar in both αCT1-treated and control groups (Figure 4H). This time-dependent effect was not seen (\(P=0.65\)) in IBZ tissues (Figure 4H), indicating that the cryo-injury may have a
regional effect on sustaining Cx43-pS368 level independent of treatment.

**αCT1 Increases PKC-ε-Mediated Phosphorylation of GST-Cx43 at S368**

As αCT1 increased levels Cx43-pS368 following injury in vivo, we next determined whether peptide affected Cx43 phosphorylation via modulation of PKC-ε, the kinase responsible for Cx43 phosphorylation at serine 368 (Figure 8). Standard reactions incorporating purified enzyme (human PKC-ε) and substrate (GST-Cx43-CT: amino acids 244 to 382) were undertaken in vitro in the presence of αCT1, isolated ZO-1 PDZ domains and control peptides.

Assays of Western-blotted Cx43-pS368 to total Cx43 indicated that αCT1 caused a dose-dependent increase in phosphorylation of Cx43-CT by PKC-ε, relative to baseline.

Figure 7. Cx43-pS368 is increased in the IBZ by αCT1 treatment. A through C, Representative montaged microscopic fields from IBZ of αCT1, Rev, and Veh group hearts stained for total Cx43 (green), Cx43-pS368 (red), and nuclei (DAPI). Asterisks in B and C denote intersection of images from adjacent microscopic fields. High-magnification insets (bottom right, A through C) illustrate regions labeled for total Cx43 that colabel with antibody to Cx43-pS368. Lower-magnification insets (A’-C[prime]) from the above images illustrate single channel signal for Cx43 (green) and Cx43-pS368 (red). Dashed lines in A through C and A’ through C’ denote IBZ/injury transition. D through F, Corresponding images of remote myocardium from hearts with high-magnification insets and arrows denoting Cx43-pS368/Cx43 colabeling. G, Western blot showing a representative set of samples probed for Cx43-pS368 and total Cx43 at 0 (uninjured), 4, 6, 24, 48, 168 hours after cryoinjury and treatment with αCT1 or control. H, Graphic representation of the average ratio of Cx43-pS368 to total Cx43 for all time points in each treatment group compared with uninjured hearts for IBZ and remote myocardium. P<0.03, αCT1/control in IBZ overall; P<0.04, αCT1/control 6 hour IBZ. No significant treatment (ns) effect occurred in remote myocardium; n=3 mice/group per time point. Probability values for comparisons within IBZ and remote ventricular regions are post tests generated from 2-way ANOVAs (treatment×time). Scale bar, 20 μm.
PKC-\(\text{CT1}\) in reaction mixtures (from left to right) containing both substrate and enzyme, as well as increasing concentrations of GST-PDZ2, and control peptides. A, Blots of Cx43-pS368 (top) and total Cx43 (bottom) in mixtures (from left to right), including a no-kinase control that includes substrate (GST-Cx43-CT; amino acids 244 to 382) but no PKC-\(\epsilon\) enzyme (−PKC-\(\epsilon\)) and then blot lanes of reaction solutions containing both substrate and enzyme, as well as increasing concentrations of αCT1 (ie, at 5, 10, 20, 50, 100, and 200 \(\mu\)mol/L). B, Blots of Cx43-pS368 (top) and total Cx43 (bottom) in reaction mixtures (from left to right) containing PKC-\(\epsilon\) and GST-Cx43-CT, as well as lanes for increasing concentrations of GST-PDZ2 (ie, at 0, 27, 54, 108, 270, and 540 \(\mu\)mol/L). Right-most lane in B is −PKC-\(\epsilon\). C, Blots of Cx43-pS368 (top) and total Cx43 (bottom) in reaction mixtures (from left to right) containing both PKC-\(\epsilon\) and GST-Cx43-CT, 100 \(\mu\)mol/L inactive reverse control peptide (rev) (first and sixth lanes), 100 \(\mu\)mol/L αCT1 (second and seventh lanes), and 100 \(\mu\)mol/L αCT1 plus 100 \(\mu\)mol/L PDZ2 in lane 5. Fourth and middle lanes in C are −PKC-\(\epsilon\). Each assay was repeated 3 or more times.

(Figure 8A; −PKC-\(\epsilon\)). By contrast, increasing concentrations of PDZ2 had no effect on PKC-\(\epsilon\)-mediated phosphorylation of GST-Cx43-CT (Figure 8B). The reverse control peptide also did not increase PKC-\(\epsilon\) phosphorylation of GST-Cx43-CT (Figure 8C). Interestingly, although PDZ2 had no direct effect, addition of PDZ2 (but not PDZ1 or PDZ3) with αCT1-containing reactions caused a reduction in Cx43-pS368 below that of reverse control and αCT1 minus control levels (Figure 8C). Thus, αCT1 alone appeared sufficient to increase PKC-\(\epsilon\) activity in vitro, but this activity could be abrogated by PDZ2.

**Discussion**

Here, we show that treatment of a standardized cryoinjury of the mouse LV with a peptide (αCT1) incorporating the last 9 amino acids of the Cx43-CT decreased stimulated arrhythmias such as ventricular tachycardia or fibrillation. Associated with these effects on cardiac electrical function, the peptide reduced colocalization between ZO-1 and Cx43, and gap junction (GJ) lateralization in arrhythmia-prone tissues of the IBZ. However, αCT1 effects on GJ remodeling may not have resulted solely from affects on ZO-1/Cx43 interaction. Instead, a novel target for αCT1 was identified: PKC-\(\epsilon\). αCT1 was sufficient to increase PKC-\(\epsilon\)-mediated phosphorylation of a consensus target on the Cx43-CT - the serine residue at amino acid position 368 (S368). Consistent with the in vitro results, levels of phosphorylated S368(pS368) in IBZ were elevated significantly above controls within hours of exposure to αCT1. Although ZO-1 was not necessary for enhanced PKC-\(\epsilon\) activity, PDZ2 inhibited αCT1 enhancement of pS368 levels, indicating potential regulatory interplay between PKC-\(\epsilon\) and ZO-1 at the Cx43-CT.

Our observation that αCT1 enhancement of Cx43-pS368 in the IBZ was associated with stabilization of Cx43 at the ID parallels observations made by others in ischemia models. Ek-Vitorin et al reported that in response to transient ischemia, Cx43-pS368 remained stabilized at IDs, even as total Cx43 underwent remodeling to lateral myocyte surfaces. Kardami and colleagues showed that ischemic preconditioning (IPC) or FGF-2 treatment increased PKC-\(\epsilon\)-mediated Cx43 phosphorylation of serines at positions 262 and 368, and inhibited remodeling of Cx43 away from IDs in response to an ischemic insult. This dual S368 and S262 modification was referred to as “the P*Cx43 state.” They concluded that the “P*Cx43 state” was necessary for preconditioning, as mutant mice incompetent to undergo these Cx43 phosphorylations did not develop cardioprotection in response to IPC or FGF-2.

A number of other chemical agents have been reported to have effects on myocardial Cx43-S368 phosphorylation. Miura and coworkers showed that the δ-opioid ligand induced PKC-\(\epsilon\)-mediated phosphorylation of Cx43 at S368, and that activation of the δ-opioid receptor is an IPC-like adjunct mechanism of infarct size limitation. Joziwak and Dhein determined that a short peptide (AAP10) prevented ischemia-induced Cx43 dephosphorylation and remodeling of Cx43 GJs away from myocyte IDs. In a parallel to the results with αCT1 as reported here, AAP10 was found to attenuate ischemia-induced slowing of activation. A 6 amino acid peptide related to AAP10 called rotigaptide has been reported to suppress dephosphorylation of Cx43-pS368 and also a second serine at position 297 on Cx43. Moreover, similar to αCT1, AAP10 and rotigaptide have been shown to...
improve cardiac function in animal models, significantly extending time to ischemia-induced asystole and demonstrating antiarrhythmic effects. It is presently unclear if rotigaptide is efficacious as an antiarrhythmic agent in humans. Phase IIa clinical trials involving intravenous infusion of rotigaptide in myocardial infarction patients were begun in 2005, but terminated before efficacy end-points. The provision of a sustained, localized concentration of αCT1 to the injury and IBZ via the adherent methylcellulose patch was necessary for the efficacy of our approach. In light of this experience with αCT1, a revised clinical protocol, involving focal delivery of rotigaptide directly to infarcts, may be worth considering.

AAP10 and rotigaptide promote maintenance of PKC-dependent phosphorylation of Cx43 by inhibiting dephosphorylation. This suggests a difference in mechanism to αCT1, which prompts increased levels of pS368 via upregulation of PKC-ε catalytic activity. Also unlike αCT1, Kiljbye et al found that rotigaptide had no effect on GJ remodeling from IDs following ischemic insult. These workers reported the antiarrhythmic mechanism of rotigaptide was that it suppressed transition from concordant to discordant alternans—the antiarrhythmic mechanism of rotigaptide was that it efficiently end-points.41 The provision of a sustained, localized concentration of αCT1 to the injury and IBZ via the adherent methylcellulose patch was necessary for the efficacy of our approach. In light of this experience with αCT1, a revised clinical protocol, involving focal delivery of rotigaptide directly to infarcts, may be worth considering.

AAP10 and rotigaptide promote maintenance of PKC-dependent phosphorylation of Cx43 by inhibiting dephosphorylation.43 This suggests a difference in mechanism to αCT1, which prompts increased levels of pS368 via upregulation of PKC-ε catalytic activity. Also unlike αCT1, Kiljbye et al found that rotigaptide had no effect on GJ remodeling from IDs following ischemic insult.42 These workers reported the antiarrhythmic mechanism of rotigaptide was that it suppressed transition from concordant to discordant alternans following ischemia, perhaps via promoting GJ coupling—a conclusion supported by electrophysiological studies of communication between myocyte pairs.43 Other workers have reported that Cx43-S368 phosphorylation is associated with reduced cell-to-cell electric conductance by GJ channels.44 Consistent with these data, Burt, Lampe and colleagues determined that S368 phosphorylation prompts a 2-fold reduction in electric conductance of unitary Cx43 channels.33 It was also shown that this decrease in single channel electric coupling was associated with a paradoxical increase in selective permeability of GJs. The authors concluded, that in addition to antiarrhythmic benefits, the formation of distinct tissue compartments defined by Cx43 phospho-status might have implications for repair following myocardial infarction. The localized increase in pS368 in the IBZ and improvement in cardiac function that we observe in response to αCT1 may be consistent with this hypothesis.

αCT1 appears to induce a preconditioned-like state in injured myocardial tissues via local enhancement of PKC-ε phosphorylation of Cx43-S368. The PKC-ε consensus domain on Cx43 that includes S368 also incorporates sequences at the CT of Cx43 involved in ZO-1 binding.45,46 Physical interaction between Cx43 and PKC-ε has been confirmed in communoprecipitations from myocardial tissues and is increased by IPC and pharmacophores activating PKC-ε. These studies raise the prospect that αCT1 induction of a preconditioned-like state could involve modulating normal processes of cooperativity or competition between Cx43-interacting proteins. Consistent with this, we found that ZO-1 PDZ2 inhibited αCT1 enhancement of pS368 by PKC-ε. It also remains to be determined whether αCT1 enhances the actions of PKC-ε by allosteric interaction with PKC-ε or by making the Cx43 CT more available for S368 phosphorylation. The CT of PKC-ε displays significant homology with the Cx43-CT. This similarity includes a class II PDZ-binding domain (x-Φ-x-Φ, where Φ indicates hydrophobic amino acids and x indicates any amino acid) Asp-Leu-Met-Pro, a similarity that to the best of our knowledge has not been remarked on previously in the literature. The PKC-ε CT is involved in intramolecular interactions, which are necessary for catalytic competence.47 It remains to be shown that PKC-ε binds ZO-1 or other PDZ-containing proteins via its CT. Indeed, we could find no evidence over the range of concentration tested that PKC-ε was effectuated by PDZ2. Interestingly though, the combined presence of αCT1 and PDZ2 consistently decreased pS368 levels below that even of vehicle control and the control peptide. Further work will be required to determine the mechanism of this combinatorial effect that results in a loss of ability of PKC-ε to phosphorylate Cx43 at S368.

A caveat here is that cryoinjury has limited clinical relevance. In the clinic, cryoablation is regularly used in the left atrium and regions with proximity to the atrio-ventricular node. The strength of our cryo-model lies in its ability to provide a consistent IBZ that is not complicated by topological heterogeneities associated with ischemia reperfusion or ligation injuries. We suggest that our model may provide an approach for identifying molecular targets in injured myocardium that cause arrhythmia and LV dysfunction. It is encouraging that αCT1 reduces inducible arrhythmia following cryoinjury in mouse hearts. Tests of inducible arrhythmia have been widely used as a prognostic indicator for morbidity and mortality in patients who have recently experienced from a myocardial infarction.48 This being said, in future studies it would be useful to investigate propensity to develop spontaneous arrhythmia and long-term survival. The findings with the standardized cryoinjury model will also need to be verified in ischemic injury before definite conclusions can be drawn on possibilities for ameliorating infarction-induced arrhythmia. To this end, studies of αCT1 in myocardial infarction in large animal models should be informative.

Acknowledgments
We thank Jane Jourdan for outstanding technical assistance and skills. The generous support of Dr Phil Saul, MD (Chief, Pediatric Cardiology, Medical University of South Carolina) is acknowledged with gratitude.

Sources of Funding
This work was supported by American Heart Association Predoctoral Fellowship 051283U (to M.P.O.); NIH training grant T32 HL007260 (to M.P.O.); NIH grants F30 HL095320 (to J.A.P.), HL56728 (to R.G.G.), and HL082802 (to R.G.G.); and American Heart Association Grant-in-Aid 87651 (to R.G.G.).

Disclosures
R.G.G. is a member of the Scientific Advisory Board of FirstString Research Inc, a biotechnology startup company spun off from the Gourdie laboratory (Medical University of South Carolina) that has taken αCT1 to clinical trials for indications in skin wound healing. R.G.G. has modest equity (<5% of total) in FirstString Research Inc.

References


Novelty and Significance

What Is Known?

- Pathological changes to gap junctions between myocytes occur in a thin layer of heart muscle next to myocardial infarcts called the infarct border zone.
- These pathological changes to gap junctions are thought to be a causal factor in fatal arrhythmias and sudden cardiac death.
- Serine 368 phosphorylation of connexin (Cx43) has been associated with gap junctions becoming more resistant to arrhythmia-causing changes in organization.

What New Information Does This Article Contribute?

- A short peptide based on the Cx43 carboxyl terminus increased S368 phosphorylation in the border zone when administered to an injured heart.
- Associated with treatment with the Cx43 mimetic peptide, pathological changes to gap junction organization were inhibited.
- Treated injured hearts electrically activated more efficiently and were more resistant to developing arrhythmias.
- Evidence is provided that the Cx43 peptide works by enhancing the action of the protein kinase (PKC-ε) responsible for S368 phosphorylation.

- Technical innovations include a protocol for generating a nontransmural cryoinjury and an adherent methylcellulose membrane for peptide delivery in vivo.

Gap junctions form electric connections between heart muscle cells. Disorganized or remodeled gap junctional connections are a hallmark of myocytes bordering myocardial infarcts. This pathological remodeling of gap junctions is thought to be a cause of fatal arrhythmias. Here, we report that treatment of injuries to mouse hearts with a short peptide based on the gap junction protein Cx43 inhibited remodeling. Moreover, the peptide treatment reduced the propensity of hearts to be electrically provoked into an arrhythmic state. The inhibition of arrhythmia and remodeling in treated injuries was associated with increases in a serine phosphorylation of Cx43. Evidence is provided that the mechanism of the mimetic peptide is via direct interaction either with the protein kinase responsible for the phosphorylation or with the Cx43 substrate of the enzyme. This study provides the basis for a novel pharmacological approach to preventing fatal arrhythmias following heart attack.
A Peptide Mimetic of the Connexin43 Carboxyl Terminus Reduces Gap Junction Remodeling and Induced Arrhythmia Following Ventricular Injury
Michael P. O'Quinn, Joseph A. Palatinus, Brett S. Harris, Kenneth W. Hewett and Robert G. Gourdie

Circ Res. 2011;108:704-715; originally published online January 27, 2011;
doi: 10.1161/CIRCRESAHA.110.235747

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/108/6/704

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/01/27/CIRCRESAHA.110.235747.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/