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Maintenance of intercellular coupling by the antiarrhythmic peptide rotigaptide suppresses arrhythmogenic discordant alternans

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Kjølbye AL, Dikshteyn M, Eloff BC, Deschênes I, Rosenbaum DS. Maintenance of intercellular coupling by the antiarrhythmic peptide rotigaptide suppresses arrhythmogenic discordant alternans. Am J Physiol Heart Circ Physiol 294: H41–H49, 2008. First published November 2, 2007; doi:10.1152/ajpheart.01089.2006.—Discordant action potential alternans creates large gradients of refractivity, which are thought to be the mechanisms linking T-wave alternans to cardiac arrhythmogenesis. Since intercellular coupling acts to maintain synchronization of repolarization between cells, we hypothesized that intercellular uncoupling, such as during ischemia, would initiate discordant alternans and that restoration of intercellular coupling by the gap junction opener rotigaptide may provide a novel approach for suppressing arrhythmogenic discordant alternans. Optical mapping was used to record action potentials from ventricular epicardium of Langendorff-perfused guinea pig hearts. Threshold for spatially synchronized (i.e., concordant) alternans and discordant alternans was determined by increasing heart rate step-wise during J baseline, 2) treatment with rotigaptide or vehicle, and 3) global low-flow ischemia + rotigaptide or vehicle. Ischemia reduced the threshold for discordant alternans in both groups from 362 ± 8 to 305 ± 9 beats/min (P < 0.01) and for discordant alternans from 423 ± 6 to 381 ± 7 beats/min (P < 0.01). Interestingly, rotigaptide also increased the threshold for discordant alternans relative to vehicle both before (438 ± 7 vs. 407 ± 8 beats/min, P < 0.05) and during (394 ± 7 vs. 364 ± 9 beats/min, P < 0.05) ischemia. Rotigaptide increased conduction velocity and prevented conduction slowing and dispersion of repolarization during ischemia. Confocal immunofluorescence revealed that total connexin43 quantity and cellular distribution were unchanged before or after low-flow ischemia, with and without rotigaptide. However, connexin43 dephosphorylation in response to low-flow ischemia was significantly prevented by rotigaptide (15.9 ± 7.0 vs. 0.3 ± 6.4%, P < 0.001). These data suggest that intercellular uncoupling plays an important role in the transition from discordant to discordant alternans. By suppressing discordant alternans, repolarization gradients, and connexin43 dephosphorylation, rotigaptide may protect against ischemia-induced arrhythmias. Drugs that selectively open gap junctions offer a novel strategy for antiarrhythmic therapy.

T-WAVE ALTERNANS (TWA) is a change in the amplitude of the electrocardiographic T wave that occurs on an every-other-beat basis. TWA has been closely linked to the development of arrhythmias under a wide variety of clinical (31, 32) and experimental (10, 19, 20, 24) conditions, including myocardial ischemia (10, 19), suggesting that alternans may represent a common feature of arrhythmias caused by many different diseases. The underlying electrophysiological mechanism responsible for TWA is still not fully elucidated. However, we have previously demonstrated that TWA arises from alternation of repolarization at the cellular level, i.e., action potential duration (APD) alternans (25).

APD alternans does not occur uniformly throughout the heart. Some regions of the heart may alternate in a long-short-long pattern, while other regions alternate in a short-long-short pattern. This is referred to as spatially discordant alternans. Using optical mapping of isolated guinea pig hearts, Pastore et al. (25) previously demonstrated that discordant alternans creates large gradients of refractoriness of sufficient magnitude to cause unidirectional conduction block and reentry. Thus, discordant alternans was key to a mechanism linking TWA to cardiac arrhythmogenesis (25). In a subsequent study in isolated guinea pig hearts, Pastore and Rosenbaum (26) further investigated the mechanism responsible for discordant alternans by creating an insulating structural barrier on the epicardial surface thereby uncoupling neighboring cells. The structural barrier greatly increased the propensity for discordant alternans, suggesting that during normal conditions intercellular coupling electrotonically attenuates differences in ionic properties that may lead to spatially discordant alternans (26). Therefore, it seems that the development of discordant alternans is favored by conditions of cellular uncoupling such as ischemia, where neighboring cells can more easily manifest their underlying ionic differences because they are no longer under electrotonic influence of one another.

Rotigaptide is a novel antiarrhythmic peptide analog that enhances gap junction intercellular conductance between ventricular myocytes without changing membrane conductance (36). Previously, Eloff et al. (11) showed that rotigaptide is able to prevent conduction slowing occurring during acidosis in the isolated guinea pig heart. In accordance with an effect on gap junction intercellular conductance, rotigaptide has been shown to reduce dispersion of APD in isolated rabbit (9, 17) and guinea pig hearts (11). Interestingly, rotigaptide has also been shown to reduce the inducibility of ventricular tachycardia during acute ischemia in dogs, supporting an antiarrhythmic effect associated with the effect on cell-to-cell coupling (36).

Since intercellular coupling acts to maintain synchronization of repolarization between cells, we hypothesized that intercellular uncoupling during ischemia facilitates discordant alternans and that it is possible to suppress arrhythmogenic discordant alternans with rotigaptide. The results of this study suggest that pharmacological modification of gap junction conductance is a promising novel approach in antiarrhythmic drug development.

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METHODS

Experimental preparation. All experimental protocols were approved by the Institutional Animal Care and Use Committee. Male guinea pigs (600–880 g) were anesthetized with 30 mg/kg ip pentobarbital sodium (Abbott Laboratories), tracheotomized, and ventilated on room air using a Harvard rodent ventilator (tidal volume: 5 ml, frequency: 60 per min). The aorta was exposed and cannulated as described previously (17). Hearts were perfused in the Langendorff mode at 31.9 ± 0.1°C with an oxygenated (100% O2), filtered (45 μm), and modified Tyrode’s solution containing (in mmol/l) the following: 1.25 CaCl2, 140 NaCl, 4.5 KCl, 5.5 dextrose, 0.7 MgCl2, and 5 HEPES, titrated to a pH of 7.4 with NaOH. Perfusion pressure was maintained at ~50 mmHg during baseline conditions by adjusting flow accordingly. The hearts were positioned so that the recording area was centered over a 14.2 × 14.2 mm region of the left ventricular epicardium as previously described (1, 13, 26). Gentle pressure was applied to the posterior surface of the heart with a movable piston to stabilize the heart against the imaging window. Motion artifacts were suppressed with diacetyl monoxide (5 mmol/l, Sigma) added to the Tyrode’s solution. The hearts were loaded with the voltage-sensitive dye 1-[4-[(di-n-butylamino)-6-naphthyl]vinyl]pyridinium (di-4-ANEPPS; 15 mmol/l, Molecular Probes) by perfusion for 10 min before the experiment. Action potentials were recorded optically from 256 sites on the anterior surface of the ventricle (0.89-mm interpixel resolution) as described in detail previously (1, 12, 22, 26). Cardiac rhythm was monitored by volume conducted ECG using three silver disc electrodes fixed to the chamber in positions corresponding to limb leads I, II, and III.

Study design. Animals were randomized to two groups: a rotigaptide-treated (n = 11) or a vehicle-treated (n = 9) group. In both groups, hearts were initially allowed to equilibrate for 15 min at baseline pacing 150 beats/min before the initiation of the first incremental pacing protocol (see Stimulation protocol) designed to measure the threshold heart rate for alternans (25). At the end of the first pacing protocol (Baseline), either 50 nM rotigaptide or vehicle was added to the perfusate. After a 15-min treatment period at baseline pacing, the incremental pacing protocol was repeated (Treatment). At the end of the second pacing protocol, coronary flow was reduced by 75% to induce global low-flow ischemia and a third pacing protocol was performed after 20 min of ischemia (Treatment + Ischemia).

Stimulation protocol. The hearts were paced at twice diastolic threshold (2 ms duration) from bipolar Teflon coated (except at the tip) platinum hook electrodes (A-M Systems, Carlsburg, WA, 0.003-in. diameter) inserted into the left ventricular apex (1.5-mm interelectrode spacing) through a 27-G butterfly cannula. To induce APD alternans, an incremental pacing protocol was applied in which pacing rate was increased stepwise from the basic pacing rate of 150 beats/min until discordant alternans was evident as previously described (25, 26). Pacing was maintained for 1 min at each pacing rate to obtain steady-state conditions. A 10-s recording was made at the end of each period. Effective refractory period (ERP) was determined before the beginning of each pacing protocol by a standard extrastimulus protocol.

Data analysis. Data analysis was performed using custom software designed for the analysis of optically recorded action potentials. Action potential alternation was calculated as the time interval between local depolarization and repolarization, determined using predefined criteria, as described previously (11, 22, 26). Briefly, automated algorithms were used to ensure objectivity and consistency in determining depolarization and repolarization times for each action potential. All computer-assigned depolarization and repolarization times were reviewed by the investigators. Repolarization time was defined as the maximum positive curvature (maximum positive second derivative) during repolarization and corresponds to ~95% repolarization. APD was calculated as the time interval between local depolarization and repolarization. APD alternans was defined as a difference in APD on two consecutive beats of ≥10 ms.

Concordant alternans was defined as APD alternation in phase spatially (i.e., long-short-long in all alternating sites), whereas discordant alternans was defined as alternation out of phase spatially (i.e., long-short-long in some channels and short-long-short in at least one other channel). The threshold for alternans was defined as the slowest pacing heart rate at which alternans was detected. The threshold for concordant and discordant alternans was determined during each of the three perfusion periods: 1) Baseline, 2) Treatment, and 3) Treatment + Ischemia.

To evaluate the role of conduction velocity (CV) in the transition from concordant to discordant alternans, CV was measured in each period during pacing at 375 beats/min based on the threshold for discordant alternans of the vehicle-treated group during ischemia (364 ± 9 beats/min = 166 ± 4 ms, see RESULTS). CV was calculated based on activation times (time for dVdt max) using an average of velocity vectors along the conduction path from relative to fiber orientation as described previously (11, 12).

In addition, maximum spatial dispersion of repolarization was calculated in each period during pacing at 375 beats/min from the SD of repolarization times measured from zones exhibiting the largest gradient of repolarization.

Cx43 expression levels. Western immunoblotting was performed on ventricular tissue samples as previously described (15). Briefly, in 10 additional animals, left ventricular homogenates were sampled from Langendorff perfused hearts during treatment and treatment plus ischemia in which 5 animals were randomized to vehicle treatment and 5 to rotigaptide treatment. To allow for paired analysis, each animal served as its own control (i.e., tissue samples were taken from different vascular territories before and post ischemia perfusion). Phosphatase inhibitors (Sigma) were added to the lysis buffer for the purpose of maintaining phosphorylation state. A commercially available polyclonal rabbit antibody to Cx43 (Zymed), which reacts with Cx43 independent of phosphorylation state, was used. In addition, an immunoblot for calsequestrin was used concurrently for the purpose of maintaining phosphorylation state. A commercially available polyclonal rabbit antibody to Cx43 (Zymed), which reacts with Cx43 independent of phosphorylation state, was used. In addition, an immunoblot for calsequestrin was used concurrently for the purpose of normalizing protein loading between samples. Lanes were loading with 17 μg of total protein, and values were averaged between columns to account for pipetting errors. Cx43 protein content was analyzed using ImageQuant TL (Molecular Dynamics). The signal intensity from bands at ~43 kDa corresponding to Cx43 protein was summed to calculate total Cx43 protein content, whereas the higher molecular mass bands were attributed to phosphorylated Cx43 as described previously (5).

To assess junctional Cx43 signal, immunofluorescence analysis was performed as previously described (4, 27). Briefly, formalin-fixed and paraffin-embedded tissue layers were sectioned at a thickness of 5 μm and mounted on gelatin-coated slides. Sections were deparaffinized, placed in citrate buffer, and boiled in a microwave oven for 10 min. The sections were incubated overnight with anti-Cx43 antibodies (Zymed, diluted 1:400) and then incubated with CY3-conjugated goat anti rabbit IgG (Zymed diluted 1:800) before being examined by laser scanning Confocal microscopy (×40 oil immersion lens,airy 1 pinhole). The degree of confocality was kept constant (depth of focus ~602 m) for each experiment to minimize overlap of Cx43 label. Each sample was analyzed from five fields to obtain an average Cx43 quantity within a sample. To eliminate artificial quantification of Cx43 protein, samples were discarded when the imaging plane was not parallel to the long axis of the fiber, as judged by the length-to-width ratios of myocytes <4 (27). Relative Cx43 quantity was defined as the proportion of total myocardial tissue area occupied by Cx43 immunofluorescent signal, as described previously (8).
RESULTS

Rotigaptide does not affect coronary flow or perfusion pressure. The coronary flow required to maintain a perfusion pressure of ~50 mmHg during the baseline and treatment periods was similar in the two groups as was the drop in perfusion pressure caused by the reduction of coronary flow during global low-flow ischemia (Table 1).

Rotigaptide suppresses discordant but not concordant alternans. Twenty minutes of global low-flow ischemia promoted the development of concordant as well as discordant alternans. Figure 1 shows optically recorded action potentials recorded from two different sites on the epicardium during moderate (286 beats/min) and rapid (375 beats/min) pacing under baseline conditions and during global low-flow ischemia. During baseline conditions, no alternans was present at moderate pacing rates (Fig. 1A); however, when the pacing rate was increased, concordant alternans developed (Fig. 1B). After 20 min of global low-flow ischemia, concordant alternans was present even at moderate heart rates (Fig. 1C), while during ischemia the faster heart rate induced discordant alternans (Fig. 1D). Figure 2 summarizes the results on alternans threshold. Ischemia significantly enhanced susceptibility to alternans in both groups by reducing the threshold for concordant alternans from 362 ± 8 to 305 ± 9 beats/min (P < 0.01) and discordant alternans from 423 ± 6 to 381 ± 7 beats/min (P < 0.01). Interestingly, rotigaptide significantly suppressed the onset of discordant alternans as evidenced by an increased threshold for discordant alternans relative to vehicle both before (rotigaptide: 438 ± 7 vs. vehicle: 407 ± 8 ms, P = 0.011) and during global low-flow ischemia (rotigaptide: 394 ± 7 vs. vehicle: 364 ± 9 ms, P = 0.019; Fig. 2B).

Rotigaptide did not significantly affect the threshold for concordant alternans (Fig. 2A), further supporting a selective effect of rotigaptide on intercellular coupling between cells rather than an effect on ionic processes that determine alternans within individual cells.

Figure 3 compares iso-alt ernans contour plots from a vehicle-treated vs. a rotigaptide-treated heart. The phase of cellular alternans (long-short APD vs. short-long APD) is depicted by red and blue contours, while the magnitude of cellular alternans is depicted by the intensity of each color. It can be readily appreciated that the vehicle-treated heart demonstrated discordant alternans, whereas under identical pacing conditions, the rotigaptide-treated heart shows concordant alternans only suggesting that rotigaptide suppressed the development of discordant alternans.

Rotigaptide enhances conduction velocity. The effect on conduction velocity was analyzed in a subset of seven rotigaptide-treated hearts and eight vehicle-treated hearts. Figure 4 summarizes the effect of rotigaptide on conduction velocity before and during ischemia. To account for differences in baseline CV, all values were expressed as percent change from the level during baseline. As expected, in the absence of ischemia, CV was relatively stable over time in vehicle-treated hearts. In contrast, treatment with rotigaptide enhanced CV by almost 20% (P < 0.001) vs. baseline. Also, as expected, ischemia was associated with CV slowing. In vehicle-treated hearts, ischemia slowed conduction by 20%. Interestingly, pretreatment with rotigaptide completely prevented ischemia-induced conduction slowing (P < 0.05 vs. vehicle).

![Fig. 1. Ischemia enhances susceptibility to concordant and discordant alternans. A: optically recorded action potentials during 2 sequential beats (dark and light traces) are superimposed to illustrate cellular alternans at two different sites near the apex (A) and base (B) of the left ventricle. Under baseline conditions, APD alternans was absent; when the pacing rate was increased [(from 286 to 375 beats/min (bpm)), concordant alternans developed (B)]; global low-flow ischemia caused concordant alternans even at moderate pacing rates (286 beats/min; C); and when the pacing rate was increased to 375 beats/min during ischemia, discordant alternans developed (D).](image)

<table>
<thead>
<tr>
<th>Perfusion Pressure, mmHg</th>
<th>Baseline</th>
<th>Treatment</th>
<th>Treatment + Ischemia</th>
</tr>
</thead>
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<tr>
<td>Vehicle</td>
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<td>53±1</td>
<td>11±2</td>
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<tr>
<td>Rotigaptide</td>
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<td>54±0</td>
<td>13±1</td>
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<table>
<thead>
<tr>
<th>Coronary Flow, ml/min</th>
<th>Baseline</th>
<th>Treatment</th>
<th>Treatment + Ischemia</th>
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<tbody>
<tr>
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<tr>
<td>Rotigaptide</td>
<td>22.6±0.7</td>
<td>20.5±0.8</td>
<td>5.0±0.2</td>
</tr>
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Figure 5 shows representative isochrone maps of ventricular activation from a vehicle-treated (A) and a rotigaptide-treated (B) heart. As evident by the crowding of isochrone lines in Fig. 5A, global low-flow ischemia caused significant conduction slowing in the vehicle-treated heart, whereas pretreatment with rotigaptide (Fig. 5B) essentially prevented ischemia-induced conduction slowing. Since rotigaptide does not effect sodium current or cellular excitability (11), the preservation of CV provides further evidence that the effects of rotigaptide on discordant alternans were attributable to preservation of intercellular coupling.

Rotigaptide reduces dispersion of repolarization. ERP increased significantly during global low-flow ischemia in both groups (average: Baseline: 189 ± 4 ms vs. Ischemia: 207 ± 5 ms); however, there was no difference between groups. Also, there was no difference between groups in average APD.
During ischemia, average APD of the short beat (i.e., during alternans) shortened significantly (average: Baseline: 114 ± 2 ms vs. Ischemia: 101 ± 1 ms), whereas APD of the long beat remained relatively constant (average: Baseline: 122 ± 2 vs. Ischemia: 120 ± 4 ms).

Ischemia resulted in a dramatic increase in dispersion of repolarization times in the area where alternans most often arose. This increased dispersion, giving rise to large gradients of repolarization, was completely blocked by rotigaptide (Fig. 6).

**Rotigaptide inhibits ischemia-induced Cx43 dephosphorylation.** To explore a potential mechanism for the effect of rotigaptide during ischemia, additional hearts were subjected to the same experimental protocol as described in Study design and Stimulation protocol. Representative confocal immunofluorescence images revealed that total Cx43 protein expression and cellular distribution remained the same before and after global low-flow ischemia during vehicle and rotigaptide perfusion (Fig. 7A), suggesting that the ability of rotigaptide to preserve conduction during ischemia was likely attributable to an effect on gap junction conductance (i.e., function, rather than quantity or cellular distribution). To investigate the potential mechanism of rotigaptide’s effect on gap junction function, Western blot analysis was performed, which revealed an enhancement of dephosphorylated Cx43 during ischemia (Fig. 7B); i.e., ischemia resulted in a substantial increase in dephosphorylated Cx43, amounting to a ∼16% enhancement of dephosphorylated Cx43. These results suggest that low-flow ischemia caused changes in Cx43 phosphorylation consistent with the time course of ischemia-induced uncoupling (5, 18). Interestingly, rotigaptide prevented ischemia-induced dephosphorylation of Cx43, suggesting a possible molecular mecha-

**DISCUSSION**

In the present study, we sought to determine if discordant alternans could be suppressed by increasing gap junction intercellular conductance with rotigaptide. This hypothesis was motivated by our earlier observations that insulating structural barriers significantly increased the propensity for discordant alternans (26), thereby implicating a role for intercellular uncoupling in the mechanism of discordant alternans. This question is timely and novel because intercellular uncoupling from gap junction remodeling is often suggested to play a role of reentrant arrhythmias in ischemic heart disease (6, 33), and essentially all previous studies have investigated mechanisms in discordant alternans associated with the absence of myocardial disease. To investigate this we used the guinea pig model of pacing-induced alternans. Cellular uncoupling was introduced by global low-flow ischemia as a means for impairing gap junction intercellular conductance in a controlled manner. Previous studies (5, 18) have shown that uncoupling of gap junction intercellular conductance occurs after ∼15 min of ischemia. In the present study, we initiated our pacing protocol after 20 min of ischemia and maintained global low flow during the duration of the pacing protocol so that all hearts were subjected to global low-flow ischemia for 30–35 min.

As expected, global low-flow ischemia was associated with clear electrophysiological signs of ischemia as evidenced by decreased upstroke velocity and triangulation of the action potentials. Also, global low-flow ischemia significantly promoted the development of concordant as well as discordant alternans.

**Fig. 5.** Rotigaptide prevents conduction slowing during ischemia. Representative isochrone maps of ventricular activation during pacing at 375 beats/min from: a vehicle-treated heart (A) and a rotigaptide-treated heart (B). Note the relative crowding of isochrones during global low-flow ischemia in A but not in B.
alternans. This is in accordance with previous findings (10, 19) showing an increased level of alternans during ischemia. However, the effects of ischemia or intercellular uncoupling on the spatial organization of cellular alternans in general, and discordant alternans in particular, were previously unknown. As in the previous study of Pastore and Rosenbaum (26), in which a computer-driven argon laser was used to create regional uncoupling by a structural barrier, cellular uncoupling by global low-flow ischemia significantly increased the propensity for discordant alternans. Thus, in both studies discordant alternans occurred at lower heart rates during cellular uncoupling than during normal conditions. These results suggest that intercellular uncoupling on a macroscopic scale (i.e., barriers or scar) as well as a cellular scale (i.e., at gap junction level) can promote spatially discordant alternans.

Since rotigaptide targets cardiac gap junctions and not sarcolemmal ion channels, it is not surprising that rotigaptide did not affect susceptibility to discordant alternans. This finding supports the notion that discordant alternans arises at the level of the single cell due to heart rate dependent effects on sarcolemmal ion channels or intracellular calcium handling processes (28, 34). So far there has been no indication of an effect of rotigaptide on sarcolemmal ion channels or intracellular calcium handling processes, i.e., several studies have shown that rotigaptide does not affect APD or myocardial contractility (11, 17, 36). In addition, in a large screening of rotigaptide binding to a panel of more than 80 major ion channels and receptors, rotigaptide showed lack of binding to any of the ion channels or receptors tested (11). However, the effect of rotigaptide has not been tested on all relevant channels and receptors; therefore, we cannot completely rule out an effect on discordant alternans or that a possible effect could have been missed due to sample size (i.e., beta error).

In contrast to discordant alternans, rotigaptide significantly suppressed the onset of discordant alternans both during normal flow and during global low-flow ischemia. This supports the hypothesis that the transition from concordant to discordant alternans is affected by the degree of gap junction intercellular conductance as suggested by experimental (26) as well as computer studies (29).

Qu et al. (30) used a two-dimensional tissue model to study the effect of altered cellular coupling (uniform or random uncoupling) on conduction velocity, APD, and discordant alternans. Upon uniform or random reduction of cellular coupling across the tissue, a planar wave propagated much slower, which is consistent with global low-flow-ischemia-induced conduction slowing, while CV restitution remained unchanged. Importantly, Qu et al. demonstrated that uniform cellular uncoupling caused larger amplitude APD alternans, while randomly and severely uncoupling cells from their neighbors facilitated the onset of discordant alternans at longer cycle lengths and enhanced the amplitude of alternans, suggesting that not only must gap junctional conductance decrease but that cells must partially decouple from their neighbors to cause discordant alternans. As previously mentioned, hearts were subjected to at least 30 min of global low-flow ischemia in the present study, making it entirely plausible that some cells completely decoupled from their neighbors, thereby facilitating the onset of discordant alternans at longer cycle lengths. Therefore, the present model of global low-flow-ischemia-induced discordant alternans is in accordance with previous theoretical predictions. A limitation of optical mapping is that the assessment of electrical activity is restricted to the mapping field. Therefore, we certainly cannot rule out that discordant alternans may arise in areas outside of the mapping field, even with rotigaptide treatment.

There are at least two mechanisms that could explain why enhanced gap junction intercellular conductance would suppress discordant alternans: first, conduction slowing attributable to conduction velocity restitution has been strongly implicated as a mechanism for discordant repolarization alternans (29, 35). Under these circumstances, cardiomyocytes distant from the pacing site will experience a longer diastolic interval than cardiomyocytes close to the pacing sites. Therefore, in spite of short coupling intervals, cells distant from the pacing site may develop paradoxically longer action potentials giving rise to discordant alternans. Compounds, like rotigaptide, enhance conduction not by altering excitability (11; i.e., the
cellular property ascribed to conduction velocity restitution) but by promoting gap junction intercellular conductance. Therefore, through an entirely different mechanism, pharmacological targeting of gap junctions can prevent conduction delays, which have been implicated in the initiation of discordant repolarization alternans.

Another mechanism by which increased gap junction intercellular conductance may have prevented discordant alternans is by enhancing electrotonic current flow between neighboring cells during repolarization (i.e., synchronization effect). It is well recognized that enhanced coupling, independent of its effects on conduction, serves to synchronize repolarization by attenuating differences in APD driven by differences in ion channel composition between neighboring cells (23). For example, the maintenance of marked APD gradients between epicardial and mid-myocardial layers has been attributed to reduced expression of cardiac gap junctions in this region (27). Similarly, we have previously shown that electrically insulating two regions of ventricle strongly promotes the loss of synchronized repolarization associated with discordant APD alternans (26). Therefore, the ability of rotigaptide to suppress discordant alternans may be attributed to an effect on synchronization of action potentials during repolarization. As discussed in the following paragraphs, in the present study, we looked at the effect of rotigaptide on both of these potential mechanisms, i.e., conduction and synchronization of repolarization.

Rotigaptide essentially prevented the conduction velocity slowing during global low-flow ischemia. Conduction velocity is primarily regulated by Na\(^+\) current excitability, gap junction intercellular conductance, membrane resistance, and factors related to source-sink mismatch such as fiber structure and wavefront geometry (7). In our experimental conditions, fiber structure and wavefront geometry were maintained constant. In addition, we have previously shown that rotigaptide has no effect on Na\(^+\) current under acidotic and normal conditions (11). Moreover, rotigaptide has no effect on input conductance and holding current in isolated pairs of guinea pig cardiomyocytes (36) and does not affect resting membrane potential or action potential amplitude under normal and acidic conditions in guinea pigs (11) or in previously ischemic dog myocardium (36). This strongly suggests that rotigaptide does...
not change the electrical properties of the plasma membrane including the membrane resistance. Therefore, the likely explanation for the effect on conduction velocity by rotigaptide is through its effect on gap junction intercellular conductance. Xing et al. (36) have previously shown by double cell patch-clamp experiments that rotigaptide increases gap junction intercellular conductance by ~70% in pairs of guinea pig cardiomyocytes. In addition, Eloff et al. (11) have tested the effect of rotigaptide on CV during acidosis in isolated guinea pig hearts and showed that rotigaptide could inhibit acidosis-induced conduction slowing by ~60% with a peak effect after 16 min of acidosis, consistent with inhibition of uncoupling. Thus, the effect of rotigaptide on CV and discordant alternans in the present study is in accordance with our previous findings and is consistent with an effect of rotigaptide on gap junctions to maintain intercellular coupling.

In the present study, rotigaptide also significantly increased CV during “normal” conditions before the induction of global low-flow ischemia, whereas in previous experiments of Eloff et al. (11) in isolated guinea pig hearts subjected to acidosis, rotigaptide had no effect on CV during normal conditions in the absence of acidosis. This difference may be explained by differences in the pacing rate. In the previous study, CV was measured during baseline pacing at 150 beats/min, whereas in the present study CV was measured during rapid pacing at 375 beats/min. It is conceivable that at this rapid pacing rate the hearts were indeed under metabolic “stress.” Therefore, we cannot say with certainty that the drug effects occurred without metabolic deprivation.

Rotigaptide did not affect APD or ERP. This is in accordance with previous findings in vitro and in vivo (11, 17, 36, 36) and further supports a selective effect of rotigaptide on gap junction intercellular conductance. However, in spite of lack of a direct effect of rotigaptide on APD, rotigaptide prevented the increased dispersion of repolarization during global low-flow ischemia. The ability of rotigaptide to prevent increased action potential dispersion confirms previous findings with the peptide in isolated rabbit (17) and guinea pig (11) hearts as well as findings for the AAP analogs AAP10 (9) and HP-5 (16). Pastore et al. (25) have previously demonstrated that these large repolarization gradients may lead to conduction block causing reentry. Therefore, the ability of rotigaptide to prevent the formation of these gradients supports the hypothesis that increased gap junction intercellular conductance may be a novel antiarrhythmic approach. In further support of that, Xing et al. (36) showed that rotigaptide prevented the formation of unidirectional conduction block and reentrant ventricular tachycardia after acute myocardial infarction in vivo in open-chest dogs.

These data support the hypothesis that intercellular uncoupling plays an important role in the transition from concordant alternans to discordant alternans. Moreover, the ability of rotigaptide to suppress discordant alternans and prevent the formation of large repolarization gradients suggests that gap junctions may represent an attractive and novel target for antiarrhythmic therapy. To exploit this opportunity, it is important to understand the molecular mechanisms underlying the modulation of intercellular coupling by rotigaptide.

In the present study, we investigated the state of Cx43 phosphorylation during ischemic perfusion both with and without rotigaptide. Cx43 dephosphorylation has been implicated as an important mechanism of impaired junctional coupling during ischemia (5). To date, Cx43 dephosphorylation and its implication for gap junction gating have been unclear. Of the 16 different phosphorylation sites identified in the C-terminal domain of Cx43, Ser368 is at the center of the phosphorylation controversy. Reports (3, 21) linking a reduction in dye coupling of cultured fibroblasts with phosphorylation of Ser368 have been challenged by others reporting that dephosphorylation of Ser368 promotes electrical uncoupling during ischemia in isolated rat hearts (5). Recently, Axelsen et al. (2) performed a systematic analysis of serine phosphorylation sites in Cx43 revealing three new phosphorylation sites and, importantly, that pretreatment with rotigaptide prevented dephosphorylation of Ser297 and Ser368 during ischemia. The findings of the present study are consistent with the time course of Cx43 dephosphorylation and uncoupling (5, 14) described previously for ischemic conditions. Before ischemia induction, phosphorylation status remained stable over time as expected (data not shown). Moreover, the ability of rotigaptide to suppress ischemia-induced Cx43 dephosphorylation suggests a potential molecular basis for the effect of rotigaptide on conduction velocity, spatial synchronization of repolarization, and alternans. Importantly, confocal immunofluorescent evaluation of tissue revealed no changes in total Cx43 quantity or distribution patterns, which further underscores the role of Cx43 dephosphorylation as a mechanism of gap junctional uncoupling. We cannot exclude from our data that rotigaptide could have also influenced intracellular trafficking of Cx43 or could have additionally effected signaling pathways that modulate gap junctions. Regardless of its precise mechanism, this study provides the first evidence supporting the hypothesis that intercellular uncoupling and its modulation by rotigaptide can play a role in the mechanism of arrhythmogenic discordant alternans.

A limitation of this study is the lack of a functional index of coupling. An important surrogate for coupling is tissue resistance or effective space constant (1). A fundamental assumption of space constant measurements, however, is that membrane resistivity remains stable. In the presence of ongoing ischemia, this condition is not met and the space constant measurement cannot be made. In light of this limitation, conduction velocity is presented as a viable surrogate for coupling, particularly since rotigaptide was shown previously to have no effect on sodium currents under experimental conditions that simulate ischemia (11).

Finally, a myriad of factors can contribute to cellular uncoupling in the clinical setting of cardiac disease such as myocardial infarction, chronic heart failure, or hypertrophy. One such factor is enhanced fibrosis, which can act to promote electrical uncoupling. Therefore, the results of this study should be extrapolated with caution because rotigaptide may not be effective in disease states in which uncoupling can be caused by remodeling of the extracellular matrix, or scarring.

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**DISCLOSURES**

A. L. Kjølbye is a full-time employee at Zealand Pharma. D. S. Rosenbaum has served as a consultant to Zealand Pharma and Wyeth Pharmaceuticals.
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