The voltage-sensitive dye di-4-ANEPPS slows conduction velocity in isolated guinea pig hearts

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BACKGROUND Voltage-sensitive dyes are important tools for mapping electrical activity in the heart. However, little is known about the effects of voltage-sensitive dyes on cardiac electrophysiology.

OBJECTIVE To test the hypothesis that the voltage-sensitive dye di-4-ANEPPS modulates cardiac impulse propagation.

METHODS Electrical and optical mapping experiments were performed in isolated Langendorff perfused guinea pig hearts. The effect of di-4-ANEPPS on conduction velocity and anisotropy of propagation was quantified. HeLa cells expressing connexin 43 were used to evaluate the effect of di-4-ANEPPS on gap junctional conductance.

RESULTS In electrical mapping experiments, di-4-ANEPPS (7.5 μM) was found to decrease both longitudinal and transverse conduction velocities significantly compared with control. No change in the anisotropy of propagation was observed. Similar results were obtained in optical mapping experiments. In these experiments, the effect of di-4-ANEPPS was dose dependent. di-4-ANEPPS had no detectable effect on connexin 43–mediated gap junctional conductance in transfected HeLa cells.

CONCLUSION Our results demonstrate that the voltage-sensitive dye di-4-ANEPPS directly and dose-dependently modulates cardiac impulse propagation. The effect is not likely mediated by connexin 43 inhibition. Our results highlight an important caveat that should be taken into account when interpreting data obtained using di-4-ANEPPS in cardiac preparations.

KEYWORDS Voltage-sensitive dye; di-4-ANEPPS; Guinea pig; Conduction; Electrical mapping; Optical mapping

ABBREVIATIONS **BDM** = 2,3-butanedione monoxime; **CV** = conduction velocity; **Cx43** = connexin 43; **NKA** = sodium-potassium ATPase; **VSD** = voltage-sensitive dye

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Introduction

Voltage-sensitive dyes (VSDs) are important tools in cardiac electrophysiology. The use of VSDs allows for simultaneous recording of changes in membrane potential from hundreds to thousands of sites, thereby greatly surpassing the spatial resolution of standard microelectrode and unipolar electrode techniques. However, an important premise for the use of VSDs is that they do not interfere with the system and the parameters being measured. In contrast to the widespread use of VSDs in cardiac preparations, the number of studies investigating the electrophysiological effects of these dyes is limited.

Inarguably, the most widely used dye in cardiac electrophysiology is di-4-ANEPPS despite some adverse effects on cardiomyocytes and cardiac tissue. Schaffer et al[1] described photodynamic damage to isolated cardiomyocytes in the presence of di-4-ANEPPS. Prolongation of the action potential in isolated cardiomyocytes due to phototoxicity has been shown at concentrations as low as 0.3 μM of di-4-ANEPPS.[2] Interestingly, this phenomenon has not been reported in intact cardiac tissue. However, in the isolated rat heart, Nygren et al[3] showed that di-4-ANEPPS prolongs the PQ interval and transiently blocks atrioventricular conduction during dye loading. The VSD RH421 has been demonstrated to increase contractility in both isolated cardiomyocytes and Langendorff perfused hearts from rats.[4] In their report, Cheng et al[4] note that they observed similar effects during staining with di-4-ANEPPS in both isolated rabbit hearts and human atrial preparations. Similarly, di-4-ANEPPS has been reported to induce vasoconstriction in mice hearts.[5] Collectively, these studies suggest that VSDs may not be electrophysiologically inert.

In a previous study,[6] we observed that QRS duration was significantly prolonged by di-4-ANEPPS. Similarly, Liang et al[7] reported that the total activation time was greater in the presence of di-4-ANEPPS. Taken together, these findings suggest that di-4-ANEPPS may modulate cardiac impulse propagation. Here, we test the hypothesis that di-4-ANEPPS slows cardiac impulse propagation.

We found that the application of di-4-ANEPPS to isolated guinea pig hearts results in reduced cardiac conduction velocity (CV) without affecting the anisotropy of conduc-

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tion. We also demonstrate that di-4-ANEPPS does not affect cell-to-cell coupling through gap junctions.

Methods

All animal procedures were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocols were approved by the Institutional Animal Care and Use Committee of the University of Utah (Protocol No. 09-09008).

Experimental preparation

Male guinea pigs (retired breeders, weight 800–1000 g, Charles River, Wilmington, MA) were anesthetized with 200 mg/kg of pentobarbital sodium (Nembutal, intraperitonial). Upon full anesthesia, the hearts were rapidly excised and perfused as Langendorff preparations by using oxygenated (100% O_2) Tyrode's solution consisting of 1.25 mM $CaCl_2$, 140 mM NaCl, 4.5 mM KCl, 5.5 mM dextrose, 0.7 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). Both atria were removed to avoid competitive stimulation during the epicardial pacing protocols. Experiments were performed at 36°C ± 1°C. The isolated hearts were submerged in the coronary perfusate to maintain temperature and were allowed to stabilize for 30–60 minutes prior to any interventions. All recordings were performed within 60 minutes following the stabilization period. To ensure steady-state CV conditions, pacing was initiated at least 20 seconds before any recordings were done. Hearts were not paced between protocols.

Electrical mapping procedure

An electrode array consisting of 64 electrodes arranged in an 8-by-8 grid with horizontal and vertical interelectrode spacing of 2 mm was placed on the anterior epicardium of the left ventricle. Unipolar electrograms were recorded from the array against a common reference wire placed in the bath solution. Data were acquired at a sampling rate of 4 kHz with 12-bit resolution. The data were low- and high-pass filtered at 0.03 and 500 Hz, respectively. Hearts were paced at a basic cycle length of 300 ms from the center of the array by using one of the central electrodes as a unipolar pacing electrode.

Optical mapping procedure

The details of the optical mapping system have been described previously.[8] Optical signals were sampled at 1 kHz. The interpixel resolution was 0.329 mm in the x direction (44 pixels) and 0.358 mm in the y direction (30 pixels). To reduce motion artifacts, 7.5 mM 2,3-butanedione monoxime (BDM) was added to the perfusate. A unipolar pacing wire was placed on the left ventricle epicardium in the center of the field of view, and the hearts were paced at a basic cycle length of 300 ms.

Pharmacological interventions

For both electrical and optical mapping experiments, the voltage-sensitive dye di-4-ANEPPS (Invitrogen, Carlsbad, CA) was dissolved in 99% ethanol and diluted in 100 mL of Tyrode's solution to the final concentration (1.9 or 7.5 μM). The dye solution was added to the hearts as a bolus through direct coronary perfusion. All recordings in the presence of di-4-ANEPPS were performed 20–30 minutes after the addition of the dye, and the experimental preparation was kept in the dark to avoid any potential phototoxic effects. The dye vehicle (ethanol) by itself did not affect the measurements in control experiments (data not shown).

The gap junction uncoupler carbenoxolone (Sigma-Aldrich, St Louis, MO) and the sodium-channel blocker flecainide (USP, Rockville, MD) were prepared as stock solutions in milliQ water. The stock solutions were diluted in Tyrode's solution to obtain their final concentrations (carbenoxolone 50 μM; flecainide 1 μM). For interventions with carbenoxolone and flecainide, recordings were done 15–20 minutes after continuous perfusion with drug solution was started.

Cell culture procedures

HeLa cells transfected with rat connexin 43 (Cx43) cDNA containing a C-terminal hexa-histidine tag have been characterized previously.[9] Transfected cells were maintained in plastic petri dishes under complete DMEM/HIGH GLUCOSE (HyClone, SH30243, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (HyClone, SH30070, Thermo Fisher Scientific, Waltham, MA), 1% antibiotics (CellGro, 30-004-Cl, Corning Mediatech, Manassas, VA), and 1% nonessential amino acids (M7145, Sigma-Aldrich, St Louis, MO). For electrophysiological recordings, cultures were dissociated into single cells by trypsinization (Trypsin-Versene; 17-161E, Lonza, Walkersville, MD) and seeded on 13-mm glass coverslips in complete DMEM. Cells were allowed to divide into cell pairs and became available for recordings after 10–12 hours.

Dual whole-cell voltage clamp

The dual whole-cell voltage clamp technique was applied to cell pairs to measure junctional conductance (g_j). Patch solution: 130 mM $CsCl_2$, 0.5 mM $CaCl_2$, 2 mM Na_2ATP, 3 mM MgATP, 10 mM EGTA, and 10 mM HEPES (pH 7.2). Recording solution: 130 mM NaCl, 7 mM $CsCl_2$, 2.0 mM $CaCl_2$, 0.6 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). The junctional conductance was determined by measuring transjunction currents (I_j) in one of the cells (held at constant zero voltage), while 200-ms voltage pulses of −10 mV were applied to its contiguous partner through a set of HEKA9 dual amplifiers (Heka Instruments, Lambrecht/Pfalz, Germany). The voltage pulse was alternatively applied to each cell at 1 Hz. At the end of all experiments, cells were challenged with a gap junction channel blocker (2 mM halothane) to determine that the conductance was due only to gap junction channels. Results from cell pairs linked by cytoplasmic bridges were rejected.

Calculation of conduction velocities

For unipolar electrograms, local activation times were assigned on the basis of maximum negative deflection of the

QRS. The activation time t = 0, coinciding with the beginning of the pacing artifact, was assigned to the pacing site. For optical signals, 4–6 consecutive action potentials were aligned on the basis of the pacing artifact and averaged to increase the signal-to-noise ratio. Local activation times were then assigned to the averaged signals on the basis of maximum first derivative of the action potential upstroke.

For both electrical and optical mapping experiments, the following procedure was used to estimate conduction velocities: Activation time maps were used to identify the longitudinal and transverse axes of propagation. Along each axis of propagation, a planar surface was then fitted to the space-time coordinates of activation by using a least squares optimization procedure. The CV and angle of propagation were obtained from the fitted parameters as described by Fitzgerald et al.[10] The anisotropy of propagation was calculated as the ratio between longitudinal and transverse CV. The estimation of conduction velocities was performed by using custom software written in Python (www.python.org) using the SciPy (www.scipy.org) library.

Statistical analysis

In the electrical mapping and in the dual patch-clamp experiments, all measurements were performed in a paired fashion and comparisons of data were performed by using Student's paired t test. For the optical mapping experiments, unpaired t tests were used. For parameters for which more than 1 t test was performed (longitudinal CV, transverse CV, and anisotropic ratio), the false discovery rate procedure was used to adjust for multiple comparisons.[11,12] A false discovery rate of 0.05 was used to calculate the critical significance levels. For a given comparison to be considered statistically significant, the *P* value had to be lower than the corresponding critical significance level (d) set by the false discovery rate procedure. Individual *P* values and the corresponding critical significance levels are reported in Table 1. Where a *P* value is given in the text or figures, an asterisk is used to indicate whether the *P* value is below the critical significance level and hence considered significant. When only a single comparison was performed on a parameter, a *P* value below .05 was considered statistically significant. All summary data are reported as mean ± standard deviation.

Results

Validation of CV measurements

To validate the electrical mapping system, experiments were performed to test the stability of the CV measurements as well as the ability to detect conduction slowing with and without a change in anisotropy. To test the stability of the measurements in the time window relevant to the experiments where pharmacological interventions took place, a time-control experiment was performed. Following the stabilization period, CV was measured every 30 minutes over a period of 60 minutes (t = 0, 30, and 60 minutes) without any pharmacological interventions. Representative activation time isochrone maps at t = 0 minute and t = 60 minutes are shown in Figure 1A. Both maps show the characteristic elliptical spread of excitation associated with epicardial stimulation. Only minimal changes occurred in the shape and localization of the isochrones over the 60-minute duration of the experiment. Correspondingly, both longitudinal CV (61 ± 3 cm/s at t = 0 minute; 60 ± 3 cm/s [*P* = .33] at t = 30 minutes; 61 ± 3 cm/s [*P* = .68] at t = 60 minutes; n = 6) and transverse CV (25 ± 1 cm/s at t =

Table 1 False discovery rate procedure: *P* values and corresponding critical significance levels (d)

i	Method	Comparison	*P*	d	Significance
Longitudinal CV					
6	Electrical mapping	Ctrl (t = 0 min) vs Ctrl (t = 60 min)	.677	0.050	Not significant
5	Electrical mapping	Ctrl (t = 0 min) vs Ctrl (t = 30 min)	.326	0.042	Not significant
4	Electrical mapping	Ctrl vs di-4-ANEPPS	.005	0.033	Significant
3	Electrical mapping	Ctrl vs flecainide	.004	0.025	Significant
2	Optical mapping	1.9 μM vs 15 μM	.002	0.017	Significant
1	Electrical mapping	Ctrl vs carbenoxolone	<.001	0.008	Significant
Transverse CV					
6	Electrical mapping	Ctrl (t = 0 min) vs Ctrl (t = 60 min)	.536	0.050	Not significant
5	Electrical mapping	Ctrl (t = 0 min) vs Ctrl (t = 30 min)	.360	0.042	Not significant
4	Optical mapping	1.9 μM vs 15 μM	.019	0.033	Significant
3	Electrical mapping	Ctrl vs flecainide	.002	0.025	Significant
2	Electrical mapping	Ctrl vs di-4-ANEPPS	<.001	0.017	Significant
1	Electrical mapping	Ctrl vs carbenoxolone	<.001	0.008	Significant
Anisotropic ratio					
6	Electrical mapping	Ctrl vs flecainide	.931	0.050	Not significant
5	Optical mapping	1.9 μM vs 15 μM	.912	0.042	Not significant
4	Electrical mapping	Ctrl (t = 0 min) vs Ctrl (t = 30 min)	.813	0.033	Not significant
3	Electrical mapping	Ctrl (t = 0 min) vs Ctrl (t = 60 min)	.524	0.025	Not significant
2	Electrical mapping	Ctrl vs di-4-ANEPPS	.056	0.017	Not significant
1	Electrical mapping	Ctrl vs carbenoxolone	.006	0.008	Significant

Comparisons are ordered by decreasing magnitude of the individual *P* values and are deemed significant if *P* <d. Critical significance levels (d) were calculated on the basis of a false discovery rate of f = 0.05 by using the formula d = i/6 × f.[11,12]

AR = anisotropic ratio; Ctrl = control; CV = conduction velocity; L = longitudinal; T = transverse.

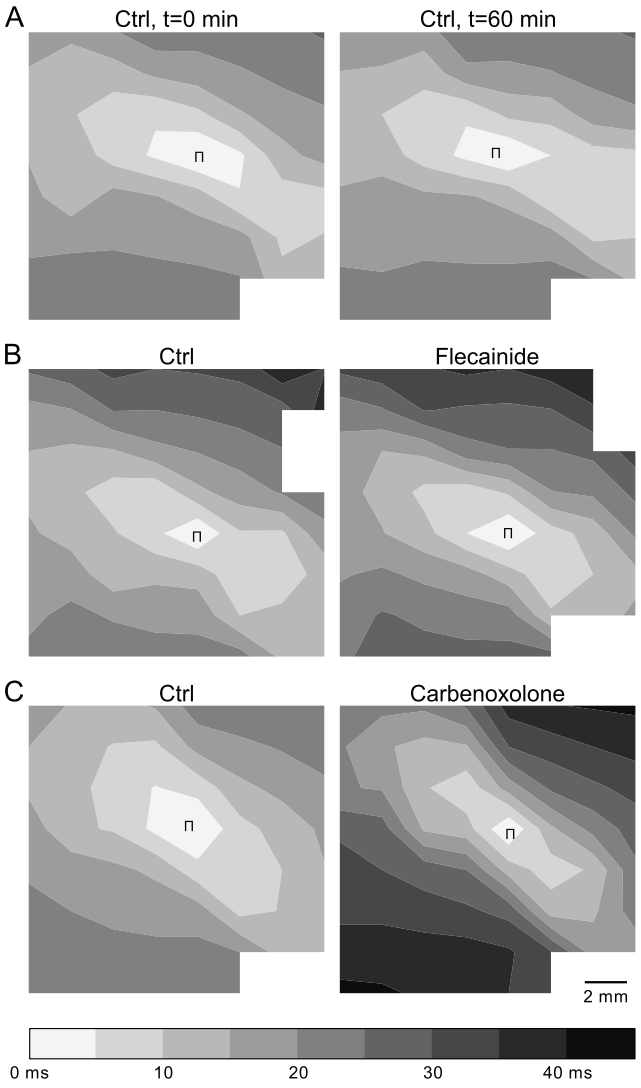

Figure 1 Representative control experiments. Representative activation time isochrone maps recorded electrically during 3 different control (Ctrl) experiments are shown: **A:** Isochrone maps recorded at t = 0 and t = 60 minutes without any pharmacological interventions, **B:** isochrone maps before (Ctrl) and during perfusion with the sodium-channel blocker flecainide, and **C:** isochrone maps before (Ctrl) and during perfusion with the gap junction blocker carbenoxolone. All experiments were performed in a paired fashion. Π indicates the pacing site.

0 minute; 24 ± 1 cm/s [P = .36] at t = 30 minutes, 24 ± 1 cm/s [P = .54] at t = 60 minutes; n = 6) remained constant. The anisotropy of propagation was 2.5 ± 0.2, 2.5 ± 0.2 (P = .81), and 2.5 ± 0.2 (P = .52) at t = 0, 30, and 60 minutes, respectively (n = 6). The sodium-channel blocker flecainide was used to verify conduction slowing without a change in anisotropy. In Figure 1B, representative activation time isochrone maps under control conditions and during perfusion with flecainide are shown. With flecainide, the isochrone spacing was reduced in both longitudinal and transverse directions of propagation, indicating slower conduction than under control conditions. Also, the overall shape of the elliptical spread of activation was not changed, indicating that the anisotropy of propagation was not affected. The slowing of conduction was statistically significant for both longitudinal (control [Ctrl] 62 ± 4 cm/s; flecainide 51 ± 3 cm/s; P = .004*; n = 5) and transverse (Ctrl 27 ± 3 cm/s; flecainide 22 ± 2 cm/s; P = .002*; n = 5) directions. Anisotropy was unchanged (Ctrl 2.4 ± 0.3; flecainide 2.4 ± 0.2; P = .93; n = 5). To validate the detection of anisotropic conduction slowing, the gap junction uncoupler carbenoxolone was used. Representative activation time maps before and during carbenoxolone perfusion are shown in Figure 1C. Carbenoxolone perfusion resulted in closer spacing of the isochrones in both directions of propagation. Importantly, the shape of the elliptical spread of activation was changed by carbenoxolone, indicating an anisotropic slowing of conduction. The summary data show that carbenoxolone significantly slowed conduction longitudinally (Ctrl 62 ± 5 cm/s; carbenoxolone 49 ± 5 cm/s; P <.001*; n = 5) and transversely (Ctrl 26 ± 1 cm/s; carbenoxolone 17 ± 1 cm/s; P <.001*; n = 5) and caused an increase in the anisotropy of propagation (Ctrl 2.4 ± 0.2; carbenoxolone 3.0 ± 0.4; P = 0.006*; n = 5). The time-control, flecainide, and carbenoxolone control experiments are summarized in Figure 2.

Effect of di-4-ANEPPS on CV

To test the hypothesis that di-4-ANEPPS slows cardiac impulse propagation, hearts were electrically mapped and CV was first measured under control conditions and then again after staining the hearts with a 7.5 μM bolus dose of di-4-ANEPPS. In Figure 3A, representative activation time isochrone maps recorded from the same heart under these 2 conditions are shown. After staining with di-4-ANEPPS, the isochrones are more closely spaced in both directions of propagation. Data from 7 experiments are summarized in Figure 3B, demonstrating that both longitudinal (Ctrl 63 ± 3 cm/s; di-4-ANEPPS 58 ± 4 cm/s; P = .005*; n = 7) and transverse CV (Ctrl 27 ± 3 cm/s; di-4-ANEPPS 23 ± 3 cm/s; P <.001*; n = 7) were indeed significantly reduced after staining with di-4-ANEPPS. The anisotropy of propagation was not changed (Ctrl 2.4 ± 0.2 cm/s; di-4-ANEPPS 2.6 ± 0.3 cm/s; P = 0.056; n = 7).

Most experiments conducted with di-4-ANEPPS are optical mapping experiments in which the spread of excitation is recorded optically as relative changes in dye fluorescence. Obviously, it is not possible to record optical signals of transmembrane potential changes in the absence of a voltage-sensitive dye. Therefore, to investigate whether the effect of di-4-ANEPPS on conduction would also be apparent in the setting of optical mapping, CV was determined on the basis of optical recordings in hearts stained with different doses of di-4-ANEPPS. In Figure 4A, representative activation time isochrone maps recorded optically in hearts stained with 1.9 μM or 7.5 μM di-4-ANEPPS are shown. On comparing the 2 maps, it can be seen that the isochrones are more closely spaced at the higher dose of di-4-ANEPPS, indicating that conduction is slower than in the heart stained with the lower dose. In Figure 4B, data from 4 hearts stained with 1.9 μM and 4 hearts stained with 7.5 μM di-4-ANEPPS are summarized. The summary data demonstrate

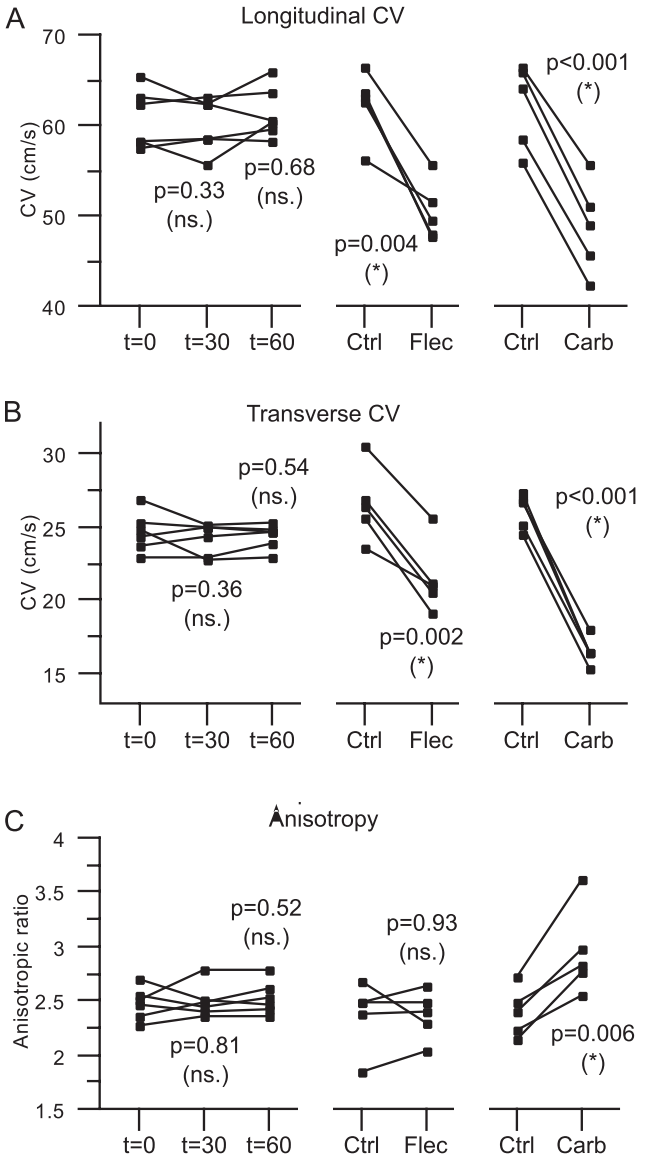

Figure 2 Summary of control (Ctrl) experiments. Summary data of longitudinal CV **(A)**, transverse CV **(B)**, and the anisotropy of propagation **(C)** measured during the 3 control experiments are shown as indicated. Data from individual paired experiments are connected by solid lines. *P* values for paired *t* tests are shown for each measure. Carb = carbenoxolone; CV = conduction velocity; Flec = flecainide; ns = not significant. Asterisk indicates that the *P* value is below the critical significance level.

that longitudinal (1.9 μM, 68 $\pm$ 5 cm/s, n = 4; 7.5 μM, 56 $\pm$ 2 cm/s, n = 4; P = .002*) and transverse (1.9 μM, 25 $\pm$ 2 cm/s, n = 4; 7.5 μM, 21 $\pm$ 2 cm/s, n = 4; P = .019*) CV is significantly slower at 7.5 μM than at 1.9 μM di-4-ANEPPS. Anisotropy was similar between the 2 doses (1.9 μM, 2.7 $\pm$ 0.3 cm/s, n = 4; 7.5 μM, 2.7 $\pm$ 0.2 cm/s, n = 4; P = .91).

Effect of di-4-ANEPPS on gap junctional coupling

No significant changes in the anisotropy of propagation were observed in the mapping experiments, suggesting that di-4-ANEPPS did not slow propagation through changes in gap junction communication. To further verify that di-4-ANEPPS did not modulate gap junctions, Cx43 was expressed in HeLa cells and gap junctional communication between cell pairs was evaluated in the absence or presence of di-4-ANEPPS by using a dual whole-cell voltage clamp protocol. Representative current recordings from such an experimental protocol are shown in Figure 4A. The currents (I_1 and I_2) were elicited by applying -10 mV (200-ms duration) voltage pulses (V_1 and V_2; see inset in Figure 5A) to each cell in the pair in an alternating manner. After control recordings, di-4-ANEPPS was applied in a concentration of 7.5 μM for 6 minutes as indicated by the black bar above the current traces. During the application of di-4-ANEPPS, gap junctional conductance was not significantly changed (n = 5). The effect of di-4-ANEPPS on gap junctional conductance is summarized in Figure 5B.

Discussion

The novel finding of this study is that the voltage-sensitive dye di-4-ANEPPS significantly modulates cardiac impulse propagation in isolated guinea pig hearts. Specifically, by using electrical mapping with unipolar electrodes we have demonstrated that propagation in both the longitudinal and transverse directions is significantly reduced in hearts stained with di-4-ANEPPS. Furthermore, we have demonstrated that the effect of di-4-ANEPPS on cardiac conduction is dose dependent and can be detected in optically mapped hearts.

Cardiac conduction is dependent on several factors including membrane excitability and gap junctional coupling.[13,14] Changes in gap junctional coupling affect transverse conduction preferentially, causing a change in the anisotropy of propagation.[15,16] We did not observe any change in the anisotropy of propagation after staining with di-4-ANEPPS although the P value (P = .056, compared with a critical significance level of d = 0.017) related to the comparison could be considered trending toward increased anisotropy. As di-4-ANEPPS slowed conduction less than carbenoxolone, where, as expected, a change in anisotropy was detected, it is possible that the degree of conduction slowing with di-4-ANEPPS was too small to detect a potential change in anisotropy. However, we did not observe any direct effect of di-4-ANEPPS on the conductance of heterologously expressed Cx43. Together, these experiments strongly suggest that di-4-ANEPPS does not significantly affect cell-to-cell coupling and thereby anisotropy in the intact heart. Instead, it is possible that di-4-ANEPPS alters membrane excitability. The main ionic current playing a role in cardiac conduction is the cardiac sodium current (I_{Na}). According to Hardy et al,[2] di-4-ANEPPS does not have any significant effect on hNav1.5, the molecular correlate of I_{Na}, in concentrations up to 16 μM. However, there may be differences between heterologously expressed hNav1.5 and cardiac I_{Na}. Alternatively, it has been suggested that a number of styryl dyes, including di-4-ANEPPS and RH421, interact directly with the sodium-potassium ATPase (NKA).[17] It was suggested that the polar head group of the dyes interacted electrostatically with NKA. A

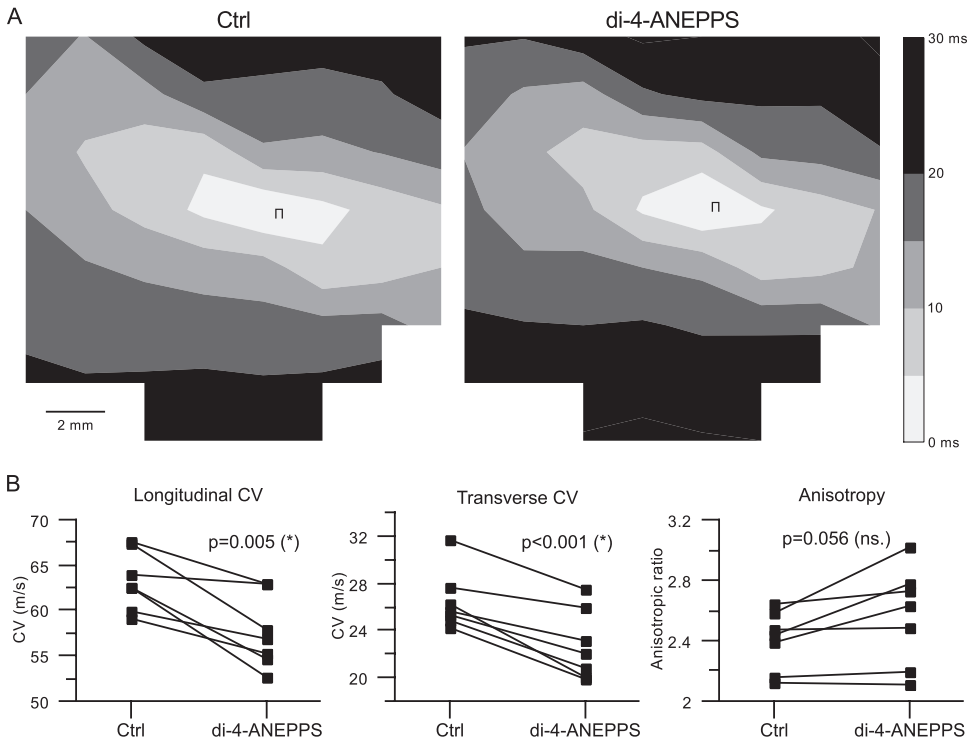

Figure 3 Effect of di-4-ANEPPS on conduction measured by electrical mapping.**A:** Representative activation time isochrone maps recorded electrically from the same heart are shown before (Ctrl) and after staining with 7.5 μM di-4-ANEPPS. Π indicates the pacing site. **B:** Summary data of CV measured in the longitudinal and transverse directions of propagation and the anisotropy as indicated. Data from individual paired experiments are connected by solid lines. *P* values for paired t tests are shown for each measure. CV = conduction velocity. **P* value is below the critical significance level.

well-characterized inhibitor of NKA is ouabain. Interestingly, the features of NKA inhibition by ouabain are qualitatively similar to the reported effects of VSDs in cardiac preparations. Ouabain application results in increased cardiac contractility.[18,19] RH421 and di-4-ANEPPS also increase contractility.[4] Ouabain has also been reported to slow cardiac impulse propagation in terms of both reduced CV[18,20] and QRS prolongation.[21] Previously, we have reported QRS prolongation following di-4-ANEPPS staining[6] and here we report a reduction in CV. It is possible that di-4-ANEPPS inhibits NKA and thereby increases intracellular sodium levels, which, in turn, results in reduced membrane excitability and reduced CV. Clearly, future studies are needed to address this issue.

Previous studies have described electrophysiological effects of di-4-ANEPPS in isolated cardiomyocytes.[1,2] These effects were largely attributed to photodynamic toxicity. Phototoxicity has not been reported in isolated heart preparations possibly because of a greater reserve of antioxidant species in intact tissue. Instead, effects of VSDs on contractility[4,5] and on various measures of conduction[3,6,7] have been noted. Nevertheless, except for the study by Cheng et al,[4] no studies have been dedicated to the potential effects of di-4-ANEPPS (or other VSDs) in intact hearts. Our study is the first to directly demonstrate an effect of di-4-ANEPPS on cardiac CV. Our findings may have significant consequences for the interpretation of data obtained by using di-4-ANEPPS. For example, in optical mapping experiments using di-4-ANEPPS, pharmacological activation of I_{Kr} resulted in a significant reduction of cardiac CV.[6] Importantly, this finding could not be reproduced in the absence of di-4-ANEPPS, thereby demonstrating a direct confounding effect of the dye. Our data strongly suggest that conclusions based on data obtained with di-4-ANEPPS should be validated by a different experimental method, particularly if the experiments are designed to determine the effect of a drug. In addition, investigations into the mechanisms of conduction slowing during disease states may overestimate the degree of conduction slowing if conduction is quantified by using optical mapping. Similarly, scroll-wave stability and simpler reentrant circuits will likely be influenced by the speed of impulse propagation and caution should be exercised when these phenomena are visualized optically. However, as demonstrated by the results from our optical mapping experiments, the effect of di-4-ANEPPS on cardiac conduction is dose dependent. Thus, depending on the preparation in question, an optimal concentration, that minimizes potential confounding effects of the dye, may exist. Nevertheless, our study suggests that it is highly recommended that optical mapping experiments include appropriate controls for a possible confounding effect of the dye.

The unwanted effects of VSDs are not limited to cardiac preparations. In pituitary tumor cells, di-8-ANEPPS activates large-conductance calcium-activated potassium (BK) channels, resulting in a decrease in the firing frequency of action potentials.[22] Similarly, another VSD diBAC4(3) has also been shown to activate large-

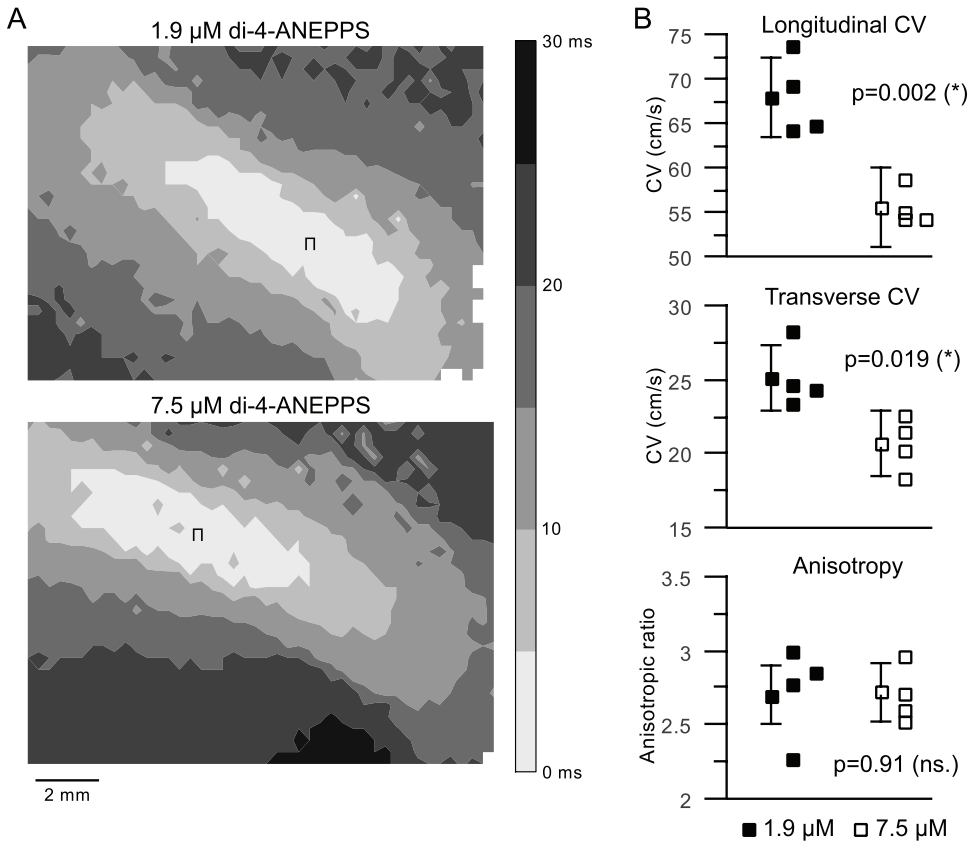

Figure 4 Effect of di-4-ANEPPS on conduction measured by optical mapping.**A:** Representative activation time isochrone maps recorded optically from the left ventricle after staining the hearts with 1.9 μM or 7.5 μM di-4-ANEPPS as indicated. Π indicates the pacing site. **B:** Summary data of CV measured in the longitudinal and transverse directions of propagation and the anisotropy as indicated. Values for individual experiments are shown next to mean and standard deviation values. *P* values for unpaired t tests are shown for each measure. CV = conduction velocity. *indicates that the *P* value is below the critical significance level.

conductance calcium-activated potassium (BK) channels.[23] Mennerick et al[24] found that a number of VSDs from the ANEP and oxonol families of dyes potentiated $GABA_A$ receptor function. In that study, di-4-ANEPPS was shown to potentiate $GABA_A$ receptors to the same level as barbiturates and to suppress network activity in cultures of hippocampal neurones. Although the underlying mechanism is different, it appears that the common denominator for the adverse effects of VSDs is a reduction in excitability: slowing of cardiac conduction (this

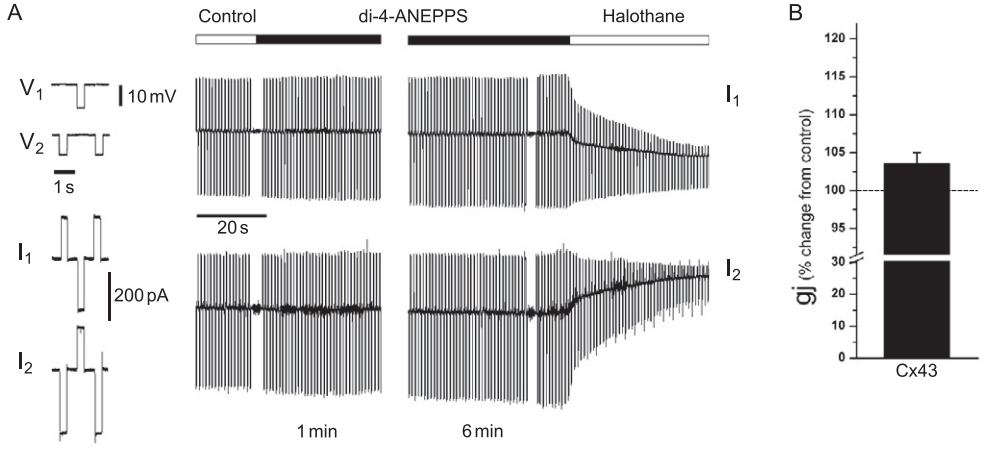

Figure 5 Effect of di-4-ANEPPS on Cx43 conductance.**A:** Representative current recordings from a HeLa cell pair (I_1 and I_2) expressing Cx43. The currents were elicited by alternating voltage pulses of −10 mV applied to each of the 2 cells. The voltage protocols (V_1 and V_2) and resulting representative current traces (I_1 and I_2) are shown in the inset. Currents were recorded under control conditions, in the presence of 7.5 μM di-4-ANEPPS and in the presence of halothane as indicated by the bar above the traces. **B:** Quantification of the change in Cx43 conductance in response to di-4-ANEPPS.

study), decreased firing frequency of pituitary tumor cells,[22] and suppression of hippocampal network activity.[24]

Study limitations

To investigate the effect of di-4-ANEPPS on gap junctional coupling, we used cloned rat Cx43 expressed in HeLa cells. The effect of di-4-ANEPPS during prolonged exposures may be different on guinea pig gap junctions, in particular if protein secondary modification occurs (eg, phosphorylation) and modifies channel gating or remodeling.[25,26] However, as discussed above, the finding that di-4-ANEPPS does not significantly change anisotropy further suggests that gap junction block does not significantly contribute to the underlying mechanism of conduction slowing.

BDM was present as a motion uncoupler in the optical experiments. It is possible that BDM played a role in the dose dependency of di-4-ANEPPS. However, the CV values obtained in the optical mapping experiments (7.5 μM di-4-ANEPPS, 7.5 mM BDM) are comparable to the CV values obtained from the electrical mapping experiments (7.5 μM di-4-ANEPPS, no BDM), suggesting that BDM did not likely affect the results.

We did not investigate the effect of di-4-ANEPPS in other species. The effect of di-4-ANEPPS may be less (or possibly more) pronounced in other species. Also, we investigated only di-4-ANEPPS as this is the most commonly used VSD in cardiac preparations. A number of other VSDs are available, and these need to be validated individually.

Conclusion

In conclusion, we have demonstrated that the voltage-sensitive dye di-4-ANEPPS directly slows cardiac impulse propagation without affecting anisotropy. The effect is not likely mediated by the inhibition of Cx43 conductance. Although the underlying mechanism is not known, our results highlight an important caveat that should be taken into account when interpreting data obtained by using di-4-ANEPPS in cardiac preparations.

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