SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell Monolayer

Our cell culture procedure to create cell monolayers has been previously described (6). Briefly, neonatal rat ventricular myocytes (NRVMs) were dissociated from 2-day old Sprague-Dawley rat hearts with the use of the enzymes, trypsin and collagenase. The resulting cell suspension was plated at high density onto 21 mm diameter plastic coverslips (10^6 myocytes per coverslip) to form monolayers that became confluent after 3-4 days of culture. Experiments were performed on days 6 to 8 after plating.

Experimental Conditions

Monolayers were optically mapped at three experimental conditions: physiological (35-37°C), room (24-26°C), or cold temperature (18-20°C). Several monolayers were mapped at two temperatures. The monolayer was continuously superfused with Tyrode's solution (in mmol/L: 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 0.33 NaH_2PO_4, 5 HEPES, 5 glucose). The solution was heated by a thermocouple-regulated feedback temperature controller (Warner Instruments, Hamden, CT) placed in series with tubing connected to the chamber for physiological temperature experiments. For cold temperature experiments, the solution was cooled by placing the inlet tubing in an ice bath. For all experiments, the chamber temperature was monitored by a thermocouple. Following a change in temperature, the monolayer was continuously superfused for 10 minutes to allow for steady-state conditions to be reached. The ATP-sensitive potassium

channel agonist, pinacidil (50 μM), was added to the Tyrode's solution in a subset of monolayers at room and physiological temperatures.

Optical Mapping

Optical mapping was performed using contact fluorescent imaging (CFI) as previously described (4, 9, 11). For voltage (or calcium) mapping, the cell monolayers were stained during (or before) the experiment with 10 μM di-4-ANEPPS (or 5 μM Rhod-2-AM), a fluorescent voltage (or calcium) -sensitive dye, and continually superfused with Tyrode's solution. The fluorescent dye signal was digitized at a 1 kHz sampling rate, processed by custom written MATLAB software, and converted into pseudo-colored maps of V_m or Ca_i.

For increased spatial resolution, calcium mapping was also performed using an Andor (South Windsor, CT) iXon+ 860 electron multiplying charged coupled device (EMCCD) camera (128 x 128 pixels). The mapping area was typically 20.5 mm x 20.5 mm, resulting in a pixel size of 160 μm. The excitation source consisted of a quartz-tungsten halogen lamp (Oriel, Newport Corporations, Irvine, CA), and the output light was directed via a light guide onto the cell monolayer. The fluorescent dye signal passed through an emission filter (bandpass 580 ± 5 nm, Omega Optical, Brattleboro, VT) and was projected on the camera chip by a demagnification lens (Cinegon f/1.4, Schneider Optics, Germany). The signal was digitized by the camera at a 490 Hz sampling rate, processed by custom written MATLAB software, and converted into pseudo-colored maps of Ca_i.

Signal Processing

The individual signals recorded during CFI mapping were temporally filtered using a 5 point median filter, baseline-corrected by subtraction of a fitted 3rd order polynomial, and range normalized. Voltage or calcium maps were created by interpolating to a 100 μm x 100 μm grid over the mapping area. Channels with poor signal were not used for the interpolation. The individual signals recorded during mapping with the EMCCD camera were spatially filtered using a 3 x 3 box filter, temporally filtered using a 5 point median filter, baseline-corrected by subtraction of a fitted 3rd order polynomial, and range normalized. Interpolation was not used to create calcium maps from the EMCCD recordings.

Data Analysis

The activation time at each recording site was computed as the time of the maximum first derivative of the action potential (dV_m/dt_{max}) or calcium transient upstroke (dCa/dt_{max}). Repolarization time was computed as the time of 80% recovery from the peak amplitude, and APD or calcium transient duration (CTD) was computed as the difference of repolarization and activation times. A global measurement for conduction velocity (CV) was computed by taking the distance of a path perpendicular to the direction of propagation, and dividing by the difference of activation times at the path endpoints. The path was taken at least 2 mm from the stimulus site to avoid errors due to direct activation near the stimulating electrode. Electrical wavelength was computed as $WL_E = CV \times APD$ and calcium wavelength was computed as $WL_C = CV \times CTD$.

Ca_i (APD) alternans maps were computed by taking the difference of Ca_i transient amplitudes (APDs) on successive beats at each recording site. Cycle length (CL) alternans maps were computed by taking the difference of the CLs, computed from activation times, on successive cycles. Local CV was measured as previously described (1). Specifically, at each recording site, activation times within a 3 mm radius were fit to a plane, and the inverse of the gradient of activation times in the *x*- and *y*-directions gave the *x*- and *y*-components of CV. CV alternans maps were computed by taking the difference of local CVs on successive beats at each recording site. Ca_i, APD, CL, and CV alternans maps computed from CFI recordings were then interpolated over a grid with 100 μm step size. The color scale on the maps is such that regions with Ca_i alternans with amplitude less than 10% or APD and CL alternans with amplitude less than 5 ms are in white.

Supplemental Results

Conduction velocity and cycle length alternans

Spatially discordant alternans can occur due to a broad CV restitution relationship, as has been previously shown both theoretically (5, 8, 10) and experimentally (3, 5, 7). If this is the case, then alternation in CL and CV should also be present. Theoretically, the location of the APD and CV nodal lines should coincide with each other, and the CL nodal line should fall at the APD (or CV) antinodal line (region of maximum alternans between two nodal lines) (10). The spatial relationship between the APD and Ca_i nodal lines will depend on calcium-voltage coupling and electrotonic effects (5). A representative example of spatially discordant Ca_i alternans is shown in Figure S1A. In this case, a single CL nodal line formed near the region of maximum Ca_i alternans, between the two Ca_i nodal lines (Figure S1B), and two CV nodal lines formed in spatially locations similar to the location of the Ca_i nodal lines (Figure S1C).

APD restitution

We measured APD restitution at physiological and lowered temperatures for *NA* and *A* monolayers (Figure S2A). APD restitution slopes at physiological and room temperature were similar, whereas reducing temperature to cold temperature steepened the restitution curve and increased the maximum value of restitution slope (Figure S2B), consistent with a previous study of isolated papillary muscle (2). However, comparing restitution for *NA* and *A* monolayers at both room and cold temperatures, we found that the incidence of APD alternans did not correlate well with APD restitution slope. At room temperature, slope values were small (< 0.5) and in fact slightly larger for *NA* monolayers, compared with *A* monolayers, although not significantly so (magenta and blue curves). At cold temperature, slope values were larger but still < 1. *A*

monolayers had much larger restitution slope at longer DIs but similar values at shorter DIs occurring near MCL (green and cyan curves). Following the addition of pinacidil, APD was not significantly shortened at 500 ms pacing but was more so at 333, 250, and 200 ms pacing, leading to steepening of the APD restitution curve, although the maximum slope value remained < 1 (Figure S2, black curves).

Key parameters linked to incidence of Ca_i and APD alternans

The values for the groups shown in Figure 3 A, B (right) are summarized in Tables S1 (at physiological temperature), S2 (at room temperature), and S3 (at cold temperature).

The effects of pinacidil and the values for groups shown in Figure 5 A, B (right) are summarized in Table S4. Note that PCL_C and PCL_D values are given for *NA* and A_C monolayers in the middle of the table, because spatially concordant and discordant alternans were induced in several *NA* and A_C monolayers following the addition of pinacidil (see Figure 5). In Figure 5 (right), in the Con columns, PCL_C, PCL_D, and MCL are defined as in Figure 3. In the Pin columns, MCL is averaged from all monolayers in the group, AVW from all *A* monolayers, and AVW_D from all A_D monolayers. For example, in Figure 5A (left), out of the 11 Con *NA* monolayers, 2 remained *NA*, 5 became A_C, and 4 became A_D following the addition of pinacidil (arrows). In Figure 5A (right), for the *NA* group (on the *x*-axis) in the Pin column, MCL was computed from the average of all 11 monolayers following the addition of pinacidil. The size of AVW was computed from the average of all 9 *A* monolayers, and the size of AVW_D was computed from the 4 A_D monolayers. Because of this method of presentation, the transition from 1:1 capture to spatially concordant alternans (and the transition from concordant to discordant alternans) does not necessarily reflect the average value for PCL_C (and PCL_D) from *A* (and A_D)

monolayers. However, the effects of pinacidil were more pronounced on the size of AVW and AVW_D, compared with the values of PCL_C and PCL_D, and therefore, we decided to present the data in this manner.

Supplemental References

1. **Bayly PV, KenKnight BH, Rogers JM, Hillsley RE, Ideker RE, and Smith WM.** Estimation of conduction velocity vector fields from epicardial mapping data. *IEEE Trans Biomed Eng* 45: 563-571, 1998.

2. **Bjornstad H, Tande PM, Lathrop DA, and Refsum H.** Effects of temperature on cycle length dependent changes and restitution of action potential duration in guinea pig ventricular muscle. *Cardiovasc Res* 27: 946-950, 1993.

3. **de Diego C, Pai RK, Dave AS, Lynch A, Thu M, Chen F, Xie LH, Weiss JN, and Valderrabano M.** Spatially discordant alternans in cardiomyocyte monolayers. *Am J Physiol Heart Circ Physiol* 294: H1417-1425, 2008.

4. **Entcheva E, Lu SN, Troppman RH, Sharma V, and Tung L.** Contact fluorescence imaging of reentry in monolayers of cultured neonatal rat ventricular myocytes. *J Cardiovasc Electrophysiol* 11: 665-676, 2000.

5. **Hayashi H, Shiferaw Y, Sato D, Nihei M, Lin SF, Chen PS, Garfinkel A, Weiss JN, and Qu Z.** Dynamic origin of spatially discordant alternans in cardiac tissue. *Biophys J* 92: 448-460, 2007.

6. **Iravanian S, Nabutovsky Y, Kong CR, Saha S, Bursac N, and Tung L.** Functional reentry in cultured monolayers of neonatal rat cardiac cells. *Am J Physiol Heart Circ Physiol* 285: H449-456, 2003.

7. **Mironov S, Jalife J, and Tolkacheva EG.** Role of conduction velocity restitution and short-term memory in the development of action potential duration alternans in isolated rabbit hearts. *Circulation* 118: 17-25, 2008.

8. **Qu Z, Garfinkel A, Chen PS, and Weiss JN.** Mechanisms of discordant alternans and induction of reentry in simulated cardiac tissue. *Circulation* 102: 1664-1670, 2000.

9. **Tung L and Zhang Y.** Optical imaging of arrhythmias in tissue culture. *J Electrocardiol* 39: S2-6, 2006.

10. **Watanabe MA, Fenton FH, Evans SJ, Hastings HM, and Karma A.** Mechanisms for discordant alternans. *J Cardiovasc Electrophysiol* 12: 196-206, 2001.

11. **Weinberg S, Lipke EA, and Tung L.** In vitro electrophysiological mapping of stem cells. In: *Stem Cells for Myocardial Regeneration: Methods and Protocols, Methods in Molecular Biology*, edited by Lee RJ: Humana Press, 2009.

Supplemental Figure Legends

Figure S1. Calcium (Ca_i) conduction velocity, and cycle length alternans. A. Ca_i alternans maps showing spatially discordant alternans. The monolayer was paced near the left edge, and two nodal lines formed. Scale in fraction of normalized fluorescence (0-1). B. Cycle length (CL) alternans. Scale in ms. C. Conduction velocity (CV) alternans. The color scale was set to amplify and show more clearly the alternation of the discordant regions, which resulted in saturation of the amplitude of CV alternans in the region near the pacing site. Scale in cm/s.

Figure S2. APD restitution at different temperature conditions. A. APD restitution and B. Restitution slope in NRVM monolayers. APD is plotted as a function of the diastolic interval (DI), defined to be pacing cycle length minus APD. *A* is the subset of monolayers having alternans. *NA* is the subset of monolayers lacking alternans. RT + Pinacidil *A* (black curve) refers to the subset of *A* monolayers in which pinacidil was added at RT. Restitution curves were fit to a monoexponential relationship, and slope was computed analytically from the fit. *n* is the number of monolayers used to compute APD restitution.

Figure S3. Alternans incidence and average AVW size. A. Alternans incidence *I* (Eqn. 6 in Appendix) is plotted as a function of AVW population mean μ. B. Average AVW size *W* (Eqn. 7 in Appendix) is plotted as a function of μ. C. Alternans incidence *I* is plotted as a function of the average AVW size *W*, both computed parametrically as functions of μ. AVW population standard deviation $\sigma = 10$.

Supplemental Tables

Table S1. Electrophysiological parameters at physiological temperature.

	Ca_i	APD
	NA	*NA*
N	23	15
PCL_C (ms)	(49)*	(95)*
PCL_D (ms)	(37)*	(94)*
MCL (ms)	136±4	140±9
APD / CTD (ms) †	203±7	138±7

Values are means ± SE. * Calculated value for PCL_C and PCL_D extrapolated from Eqn. 1. † APD and CTD were measured following steady-state pacing at 2 Hz.

Table S2. Electrophysiological parameters at room temperature.

	Ca_i			APD		
	NA	A_C	A_D	*NA*	A_C	A_D
N	21	19	20	16	2	3
PCL_C (ms)	-	201±6	207±5	-	210±10	213±7
PCL_D (ms)	-	-	161±5	-	-	203±9
MCL (ms)	196±9	166±7	***133±3***	206±7	190±10	**177±9**
AVW (ms)	-	35±5	*74±4*	-	20±0	37±6
AVW_D (ms)	-	-	28±4	-	-	27±13
APD / CTD (ms) †	192±10	175±6	174±5	153±6	144±4	147±4

Values are means ± SE. † APD and CTD were measured following steady-state pacing at 3 Hz. Bold values are significantly different compared with *NA* monolayers, and italic values are significantly different compared with A_C monolayers.

Table S3. Electrophysiological parameters at cold temperature.

	Ca_i			APD		
	NA *	A_C	A_D	*NA*	A_C	A_D
N	1	4	6	5	3	1
PCL_C (ms)	-	455±21	493±29	-	347±33	340
PCL_D (ms)	-	-	403±32	-	-	320
MCL (ms)	580	340±8	333±37	372±24	**280±31**	280
AVW (ms)	-	115±17	*160±13*	-	67±7	60
AVW_D (ms)	-	-	70±11	-	-	40
APD / CTD (ms) †	435	371±3	*352±10*	234±7	230±9	220

Values are means ± SE. * The one *No Alt* monolayer during calcium mapping had MCL of 580 ms, so CTD was measured following pacing at 1.67 Hz. † APD and CTD were measured following steady-state pacing at 2 Hz. Bold values are significantly different compared with *NA* monolayers, and italic values are significantly different compared with A_C monolayers.

Table S4. Electrophysiological parameters before (control) and during pinacidil at room temperature

	Ca_i			APD		
Control	*NA*	A_C	A_D	*NA*	A_C	A_D
N	11	7	1	8	2	1
PCL_C (ms)	-	199±13	140	-	210±10	200
PCL_D (ms)	-	-	100	-	-	190
MCL (ms)	211±8	179±15	100	214±10	190±10	190
AVW (ms)	-	20±9	40	-	20±20	10
AVW_D (ms)	-	-	0	-	-	0
Pinacidil						
PCL_C (ms)	150±9 (9)	171±12 (7)	100	158±7 (5)	180±20 (2)	190
PCL_D (ms)	136±7 (4)	143±12 (6)	-	130 (1)	-	-
MCL (ms)	138±7 (11)	115±8 (7)	90	133±6 (8)	150±30 (2)	140
AVW (ms)	18±7 (9)	56±7 (7)	10	26±7 (5)	30±10 (2)	50
AVW_D (ms)	20±6 (4)	33±7 (6)	-	0 (1)	-	-

Values are means ± SE. The number in parenthesis indicates the number of monolayers for which the parameter value was calculated.