Size and Ionic Currents of Unexcitable Cells Coupled to Cardiomyocytes Distinctly Modulate Cardiac Action Potential Shape and Pacemaking Activity in Micropatterned Cell Pairs

Luke C. McSpadden, PhD; Hung Nguyen, BS; Nenad Bursac, PhD

Background—Cardiac cell therapies can yield electric coupling of unexcitable donor cells to host cardiomyocytes with functional consequences that remain unexplored.

Methods and Results—We micropatterned cell pairs consisting of a neonatal rat ventricular myocyte (NRVM) coupled to an engineered human embryonic kidney 293 (HEK293) cell expressing either connexin-43 (Cx43 HEK) or inward rectifier potassium channel 2.1 (Kir2.1) and Cx43 (Kir2.1+Cx43 HEK). The NRVM–HEK contact length was fixed yielding a coupling strength of 68.9±9.7 nS, whereas HEK size was systematically varied. With increase in Cx43 HEK size, NRVM maximal diastolic potential was reduced from −71.7±0.6 mV in single NRVMs to −35.1±1.3 mV in pairs with an HEK:NRVM cell surface area ratio of 1.7±0.1, whereas the action potential upstroke ([dV]<sub>m</sub>/dt)<sub>max</sub>) and duration decreased to 1.6±0.7% and increased to 177±32% in single NRVM values, respectively (n=21 cell pairs). Pacemaking occurred in all NRVM–Cx43 HEK pairs with cell surface area ratios of 1.1 to 1.9. In contrast, NRVMs, coupled with Kir2.1+Cx43 HEKs of increasing size, had similar maximal diastolic potentials, exhibited no spontaneous activity, and showed a gradual decrease in action potential duration (n=23). Furthermore, coupling single NRVMs to a dynamic clamp model of HEK cell ionic current reproduced the cardiac maximal diastolic potentials and pacemaking rates recorded in cell pairs, whereas reproducing changes in ([dV]<sub>m</sub>/dt)<sub>max</sub> and action potential duration required coupling to an HEK model that also included cell membrane capacitance.

Conclusions—Size and ionic currents of unexcitable cells electrically coupled to cardiomyocytes distinctly affect cardiac action potential shape and initiation with important implications for the safety of cardiac cell and gene therapies. (Circ Arrhythm Electrophysiol. 2012;5:821-830.)

Key Words: cardiac cell therapy • gap junctions • ion channels • micropatterning • passive cell

Unexcitable stem and somatic cells genetically engineered to express specific ion channels (eg, hyperpolarization-activated cyclic nucleotide-gated channel 1, hyperpolarization-activated cyclic nucleotide-gated channel 2, Kv1.3, or inward rectifier potassium channel 2.1 [Kir2.1]) have been proposed as a means to alter the cardiac electrophysiological substrate and prevent reentrant arrhythmias and pacemaking disorders. These cells exhibit a large variety of sizes with membrane capacitances ranging from <20 pF for fibroblasts to 55 pF for mesenchymal stem cells (MSCs). Furthermore, distinct sets of endogenous ion channels in these cells yield diverse input resistances at resting ranging from 380 to 25 GΩ and resting potentials ranging from −75 to −20 mV. Although it is well recognized that electric coupling between cardiomyocytes and endogenous or exogenously added unexcitable cells can profoundly alter cardiac action potential (AP) shape and conduction, systematic studies of these interactions in vivo are hampered by the complex geometry of the heart, limited access to interacting cells, and low reproducibility of experimental conditions. Similarly, traditional in vitro systems involve coculture of cells with random geometry, distribution, and the number of homo- and heterotypic contacts rendering the reproducibility and quantification of results difficult. Many of these difficulties can be overcome with the use of cell micropatterning techniques to precisely control the size, geometry, and contact length of the interacting cells.

Clinical Perspective on p 830

We previously studied impulse conduction in neonatal rat cardiac monolayers covered with different types of unexcitable cells, including human embryonic kidney (HEK293) cells engineered to express connexin-43 (Cx43), and found that, even at the highest coverage densities,
these cells only modestly depolarized cardiomyocytes and did not induce pacemaking activity, despite slowing cardiac conduction by as much as 5 times. Similarly, coupling of cardiomyocytes with fibroblasts in computer models did not cause significant cardiac depolarization or pacemaking. Other studies have, however, reported that covering cardiomyocyte monolayers at a moderate density with myofibroblasts expressing Cx43 not only slowed cardiac conduction but also induced pacemaking activity and significant cell depolarization from –78 to –50 mV. Additionally, human MSCs, HeLa cells, and HEK293 cells transfected to express hyperpolarization-activated cyclic nucleotide-gated channel 2 current moderately depolarized single adult canine ventricular myocytes from –75 to –65 mV and still induced pacemaking activity. Although together single adult canine ventricular myocytes from –75 to –65 mV and still induced pacemaking activity. 9 Although together these studies showed that coupling of unexcitable cells to cardiomyocytes can yield diverse functional outcomes, the mechanisms by which specific properties of unexcitable cells determine these outcomes remain largely unknown.

In our previous study, large numbers of micropatterned cell pairs with reproducible shape, size, and region of cell–cell contact were used to quantify the frequency of structural coupling between a neonatal rat ventricular myocyte (NRVM) and different nonmyocytes. In the current study, we modified this in vitro assay to precisely vary the relative size of the non-myocyte versus cardiomyocyte, while keeping contact length (and thus coupling strength) between the 2 cells constant. We used this system to dissect the roles that unexcitable cell size, resting potential, and ionic versus capacitive currents play in affecting cardiomyocyte AP shape and pacemaking behavior. The results of this study shed new light on the roles of heterocellular interactions in cardiac electrophysiology with important implications for current and future cell and gene therapies.

Methods
Microcontact printing of fibronectin was used to create large numbers of individual heterotypic cell pairs consisting of an NRVM coupled to a monoclonally derived HEK293 cell engineered to express either Cx43 HEKs or Kir2.1 and Cx43 (Kir2.1+Cx43 HEKs; Figure 1). The ratio of 2 cell surface areas in the pair was systematically varied over a wide range of values (otherwise unachievable using conventional cell culture techniques), while cell–cell contact length was kept constant. Whole-cell current or voltage clamp recordings were performed in single NRVMs or HEK293s, coupled NRVM–HEK pairs, or NRVMs connected through real-time dynamic clamp software to different HEK cell models (online-only Data Supplement Figure I). Electrophysiological recordings were analyzed to determine the dependence of cardiac maximal diastolic potential (MDP), maximum rate of AP rise ([dV/dt]max), AP duration at 80% repolarization (APD80), and pacemaking rate on the ratio of estimated HEK cell membrane surface area to NRVM membrane surface area (HEK:NRVM cell surface area ratio). An expanded Methods section is available in the online-only Data Supplement.

Results
Single Cell Electrophysiological Properties
Whole-cell membrane currents were measured in single micropatterned cells with systematically varied surface area (online-only Data Supplement Figures II and III). As cell surface area increased from 1000–1550 to 2100–2650 µm², input resistance in NRVMs, Cx43 HEK cells, and Kir2.1+Cx43 HEK cells decreased from 1.92±0.36, 4.52±0.53, and 0.649±0.22 GΩ to 0.433±0.17, 0.951±0.34, and 0.107±0.017 GΩ, respectively (Figure 2B). Simultaneously, with the increase in cell surface area, membrane capacitance of all cells increased linearly (slope, 1.3 µF/cm²; Figure 2B), while resting membrane potential remained unaffected (Figure 2D), and was, on average, –71.7±0.6, –26.5±1.9, and –72.7±0.8 mV in NRVMs, Cx43 HEK cells, and Kir2.1+Cx43 HEK cells, respectively. As expected, inward and outward steady-state current magnitudes

Figure 1. Microfabrication of heterotypic cell pairs. Microcontact printing techniques were used to stamp polydimethylsiloxane (PDMS)-coated coverslips with a large number of two-rectangle fibronectin patterns. Seeding of neonatal rat ventricular myocytes (NRVMs) at low density yielded a significant number of patterns where a single NRVM was spread on only 1 of the rectangles. Human embryonic kidney 293 (HEK293) cells transfected to express connexin-43 (Cx43) and red fluorescent protein (RFP) mCherry (Cx43 HEKs) or inward rectifier potassium channel 2.1 (Kir2.1), Cx43, and green fluorescent protein (GFP; Kir2.1+Cx43 HEKs) were added after 5 days to NRVM cultures and allowed to attach and form heterotypic pairs. Although micropatterned cell surface areas were varied in different pairs, cell contact length was kept constant. Electrophysiological experiments were performed after 24 hours of coculture. UV indicates ultraviolet.
in different cells increased with cell surface area (Figure 3A–3C). Interestingly, larger cells also exhibited larger membrane current density (Figure 3D–3F). No significant difference in any electrophysiological parameter was found in single NRVMs versus those cultured in the vicinity of HEK cells, suggesting an absence of paracrine effects of HEK cells on NRVM electric properties, similar to our previous findings.16,17

**Figure 2.** Dependence of resting membrane properties on cell size. A, Input resistance at rest in connexin-43 (Cx43) human embryonic kidney cells (HEKs; n=23 cells), inward rectifier potassium channel 2.1 (Kir2.1)+Cx43 HEKs (n=19 cells), and neonatal rat ventricular myocytes (NRVMs; n=10 cells) significantly decreased with increase in cell membrane surface area. B, Membrane capacitance of all 3 cell types increased linearly with cell surface area allowing data for all cells to be combined in a single plot. Different x-axis values resulted from data rebinning. C, Membrane time constant at rest also decreased with increased cell size. D, In all studied cell types, resting potential remained unaffected by changes in cell size.

**Formation of Heterotypic Cell Pairs**

After optimization of NRVM and HEK seeding densities, heterotypic NRVM–HEK pairs formed on ≈1% of available fibronectin islands. The HEK:NRVM cell surface area ratio in micropatterned cell pairs ranged from 0.4 to 2.0 (Figure 4A), with individual cell surface areas that were in the range of those studied in single micropatterned cells.

---

**Figure 3.** Dependence of current–voltage relationships on cell size. Steady-state current–voltage relationships were constructed from whole-cell patch clamp recordings for cells with total cell surface area between 1000 and 1550 (black), 1550 and 2100 (light gray), 2100 and 2650 (dark gray), and 2650 and 3200 µm² (white) in (A) connexin-43 human embryonic kidney cells (Cx43 HEKs; n=23 cells), (B) inward rectifier potassium channel 2.1 (Kir2.1)+Cx43 HEKs (n=19 cells), and (C) neonatal rat ventricular myocytes (NRVMs; n=10 cells; no 2650–3200 µm² bin). Normalized current per membrane capacitance in (D) Cx43 HEKs, (E) Kir2.1+Cx43 HEKs, and (F) NRVMs increased with increase in cell size. Data points are connected by straight lines for improved clarity.
A 20-μm long, 1-μm wide micropatterned gap between two rectangular fibronectin islands was used to confine the site of contact between NRVMs and HEK cells to the region of the gap, resulting in a relatively reproducible cell contact length of 19.6±1.3 μm.

Gap Junction Coupling in Cell Pairs

The ability of NRVMs and HEKs to structurally and functionally couple through gap junctions was investigated by immunostaining (Figure 4B and 4C) and dual whole-cell patch clamp recordings (Figure 4D and 4E). Cx43 gap junctions formed at the cell border between NRVMs and HEKs (Figure 4B) and were also internalized within both cell types, consistent with our previous studies.15 In contrast, pairs formed between NRVMs and wild-type HEK cells showed no detectable Cx43 staining (Figure 4C).16 Applying a 20-mV voltage gradient across the cell–cell contact through the HEK patch electrode created a junctional current measured through the NRVM electrode (Figure 4E).

Because of reproducible cell contact length, coupling conductance $G_j$ of 68.9±9.7 nS was reproducibly measured in different pairs and did not differ between NRVM–Cx43 HEK (67.1±13.0 nS) and NRVM–Kir2.1+Cx43 HEK pairs (71.3±17.6 nS). This was also expected because Kir2.1+Cx43 HEK line was derived from a monoclonal Cx43 HEK line. In the presence of gap junctional uncoupler (200 μM carbenoxolone), no fluorescence recovery after photobleaching occurred in the cell pairs demonstrating gap junctional origin of the measured $G_j$ (online-only Data Supplement Figure IV).

Changes in NRVM AP Shape Because of Coupling With a Cx43 HEK Cell and Dynamic Clamp Models

Electric coupling of NRVMs to Cx43 HEK cells of different size yielded apparent qualitative changes in cardiac AP shape including depolarization of the resting membrane potential, decrease of AP upstroke, and increase in APD (Figure 5A and 5B). Quantitatively, we found that MDP of NRVMs exhibited a sigmoidal dependence on the HEK:NRVM cell surface area ratio, depolarizing from –71.7±0.6 mV in single NRVMs to –37.8±0.7 mV in pairs with a cell surface area ratio of 1.71±0.09 (Figure 5C). This dependence was fitted with a Boltzmann function with a half-maximum cell surface area ratio $x_{1/2}=1.40±0.06$ and slope $b=0.18±0.05$. When single NRVMs were connected to dynamic clamp models containing either ionic current alone or combined ionic current and capacitance of different size Cx43 HEK cells (online-only Data Supplement Figure V), the resulting dependence of cardiac MDP on the HEK:NRVM ratio (Figure 5C) and fit parameters (not shown) did not statistically differ from those measured in cell pairs.
Concurrent with MDP depolarization, the maximum AP upstroke ($dV_m/dt_{max}$) of NRVMs decreased in a sigmoidal fashion from 138.3 ± 10.2 V/s in single NRVMs to 2.2 ± 1.0 V/s in NRVM–Cx43 HEK cell pairs with a cell surface area ratio of 1.71 ± 0.09. The dependence of normalized ($dV_m/dt_{max}$) on the HEK:NRVM cell surface area ratio was fitted with a Boltzmann function with $x_{1/2}=0.57±0.05$ and $b=-0.15±0.05$ (Figure 5D). When single NRVMs were connected to dynamic clamp models containing only ionic current of Cx43 HEK cells, the sigmoidal dependence of ($dV_m/dt_{max}$) on the HEK:NRVM cell surface area ratio was significantly right shifted ($x_{1/2}=0.73±0.05$). This shift was absent when the combined dynamic clamp models containing both ionic currents and corresponding membrane cell capacitances were connected to NRVMs (Figure 5D).

As a consequence of coupling with Cx43 HEK cells, the NRVM APD$_{80}$ was significantly prolonged, increasing from 130.1±7.9 ms in single NRVMs to 230.5±24.1 ms in pairs with an HEK:NRVM cell surface area ratio of 1.71±0.09 (Figure 5E). The Boltzmann function slope parameter fit to the normalized APD$_{80}$ in cell pairs ($b=0.21±0.11$) was significantly different from the ionic current model ($b=-0.12±0.07$) but not different from that found in NRVMs connected to the combined model. Other fit parameters were not significantly different between cell pairs and dynamic clamp models.

In addition to changes in AP shape, we investigated the dependence of NRVM pacemaking rate on the HEK:NRVM cell surface area ratio. From combined assessment by patch clamp measurements and video recordings, we observed pacemaking activity in only 2 of 38 (5.3%) single NRVMs compared to 25 of 50 (50%) in NRVM–Cx43 HEK cell pairs with all 25 spontaneously active pairs having an HEK:NRVM cell surface area ratio between 1.08 and 1.94 (online-only Data Supplement Figure VI). In these spontaneously active cell pairs, the average pacemaking rate depended on the HEK:NRVM cell surface area ratio in a biphasic manner with a peak beating rate of 1.29±0.13 Hz, measured in NRVM–Cx43 HEK cell pairs with an HEK:NRVM cell surface area ratio of 1.26±0.04 (Figure 5F). The use of either the Cx43 HEK ionic current model or the combined model reproduced the dependence of pacemaking rate on the HEK:NRVM cell surface area ratio with no significant difference in the fourth order polynomial fit parameters between the two models and cell pairs. The gap junction blocker carbenoxolone (100 μM) stopped pacemaking in 10/10 spontaneously active cell pairs with different HEK:NRVM cell surface area ratios (not shown).

Figure 5. Dependence of neonatal rat ventricular myocyte (NRVM) action potential (AP) parameters on coupling to connexin-43 human embryonic kidney (Cx43 HEK) cells of different size. A, NRVM resting potential and AP shape in NRVM–Cx43 HEK cell pairs are altered as a function of relative HEK size (HEK:NRVM cell surface area ratio). B, Close-up of the upstroke of the AP shown in A, C, Maximal cardiac diastolic potential, (D) normalized ($dV_m/dt_{max}$), (E) normalized AP duration at 80% repolarization (APD$_{80}$), and (F) average pacemaking rate in NRVM–Cx43 HEK cell pairs (n=21 pairs; solid circles), single NRVMs (n=38 cells; solid diamonds), NRVMs connected to the Cx43 HEK current model (n=12 cells; diamonds), or NRVMs connected to the Cx43 HEK combined model with $G_j=68.9$ nS (n=7 cells; upward triangles), $G_j=10$ nS (n=6 cells; squares), $G_j=1$ nS (n=10 cells; circles), or $G_j=0.1$ nS (n=10 cells; downward triangles) plotted against the HEK:NRVM cell surface area ratio.
Changes in NRVM AP Shape Because of Coupling With Kir2.1+Cx43 HEK Cells and Dynamic Clamp Models

Next, we investigated how the NRVM AP shape was altered because of coupling with Kir2.1+Cx43 HEK cells of different size (Figure 6A and 6B). MDP in NRVMs coupled to Kir2.1+Cx43 HEK cells or to corresponding dynamic clamp models (online-only Data Supplement Figure VII) remained unchanged compared to that of single NRVMs and equaled $-70.1\pm0.8$ mV when averaged over all studied NRVM–Kir2.1+Cx43 HEK pairs (Figure 6C). Despite no change in MDP, $(dV_m/dt)_{max}$ of NRVMs was significantly decreased down to $7.2\pm1.6$ V/s in pairs with an HEK:NRVM cell surface area ratio of $1.90\pm0.05$. This sigmoidal dependence of $(dV_m/dt)_{max}$ on the HEK:NRVM cell surface area ratio was fit with a Boltzmann function with $x_{1/2}=1.04\pm0.01$ and $b=-0.18\pm0.01$. Interestingly, when NRVMs were coupled to Kir2.1+Cx43 HEK ionic current models, the resulting $(dV_m/dt)_{max}$ increased with an increase in the HEK:NRVM cell surface area ratio. The use of the combined model was required to obtain the same dependence as that measured in cell pairs (Figure 6D).

Simultaneously, APD$_{80}$ of NRVMs was significantly shortened with an increase in the HEK:NRVM cell surface area ratio, equalling $97.3\pm7.6$ ms in pairs with a cell surface area ratio of $1.90\pm0.05$ (Figure 6E). Coupling the NRVMs with Kir2.1+Cx43 HEK ionic current models yielded a sigmoidal dependence of APD$_{80}$ on the HEK:NRVM cell surface area ratio with Boltzmann fit parameters that significantly differed from those obtained for NRVM–Kir2.1+Cx43 HEK cell pairs ($x_{1/2}=0.92\pm0.03$ versus $1.31\pm0.13$; $b=-0.16\pm0.03$ versus $-0.39\pm0.12$). In contrast, the use of combined dynamic clamp models replicated the results obtained in the cell pairs. Finally, no pacemaking activity was observed in any NRVM coupled to a Kir2.1+Cx43 HEK cell or connected to either the ionic current or the combined Kir2.1+Cx43 HEK cell model (Figure 6F; online-only Data Supplement Figure VI).

Changes in NRVM AP Shape Because of Coupling With Dynamic Clamp Capacitance Model

To dissect the specific roles that unexcitable cell capacitance plays in the alteration of cardiac AP shape, NRVMs were coupled to the pure capacitance model (online-only Data Supplement Figure I). The resulting capacitive loading significantly altered NRVM AP shape (online-only Data Supplement Figure VIII) without changing its MDP (Figure 7A). Despite no change in MDP, $(dV_m/dt)_{max}$ of NRVMs decreased in a sigmoidal fashion with increase.
in loading capacitance corresponding to an increase in the HEK:NRVM cell surface area ratio. The resulting $x_{1/2} = 0.75 \pm 0.06$ in this case was intermediate to and significantly different from those obtained for NRVM–Cx43 HEK and NRVM–Kir2.1+Cx43 HEK cell pairs (Figure 7B). In addition, applying capacitance models corresponding to increased HEK:NRVM cell surface area ratio significantly prolonged cardiac APD$_{80}$ (Figure 7C) and yielded a sigmoidal dependence of APD$_{80}$ on the HEK:NRVM cell surface area ratio with significantly different fit parameters of $x_{1/2} = 0.45 \pm 0.08$ and $b = 0.12 \pm 0.05$. Finally, this pure capacitive loading of NRVMs using dynamic clamp software did not induce any pacemaking activity.

**Discussion**

In this study, we used novel in vitro assays consisting of NRVMs coupled to real or modeled unexcitable cells to dissect the effects of unexcitable cell size and ionic currents on cardiac AP shape and the occurrence of pacemaking activity. Specifically, we created a reproducible and well-controlled experimental setting by employing (1) cell micropatterning techniques to systematically vary the relative sizes of HEK cells and NRVMs, while keeping their coupling strength constant, (2) genetic engineering of HEK cells to enable expression of gap junctions and specific ion channels in a monoclonal fashion, and (3) dynamic clamp models of engineered HEK cells to dissect the relative roles of unexcitable cell capacitance and ionic currents in shaping the AP of NRVMs. Using this system, we showed for the first time that coupling of cardiomyocytes to unexcitable cells of increasing size (ie, increased capacitive loading) strongly depresses cardiac AP upstroke and prolongs APD. Specific ionic currents of unexcitable cells further modulate the AP upstroke and APD and determine cardiac MDP and pacemaking activity.

**Choice of Unexcitable Cell Type**

We chose HEK cells as the unexcitable cell type for the described studies because they are permissive to the stable monoclonal expression of exogenous ion channel and gap junction proteins and were able to occupy a large range of micropatterned cell surface areas. Two monoclonal cell lines were selected: (1) Cx43 HEKs, because their depolarized resting potential of –26.5 mV falls in the range of those reported for bone marrow–derived MSCs$^6$ and skeletal myoblasts$^{19}$ used in cell therapies for infarct repair, as well as cardiac fibroblasts$^8$ and myofibroblasts$^5$ that recently have been proposed to electrically couple with cardiomyocytes,$^{19,20}$ and (2) Kir2.1+Cx43 HEKs because their resting membrane potential of –72.7 mV is similar to that of genetically modified cells proposed for cell therapies for tachyarrhythmias.$^4,7$

**Dependence of HEK Cell Electrophysiological Properties on Cell Size**

Varying the size of individual HEK cells or NRVMs caused no significant change in their resting membrane potentials, as the cell capacitance increased linearly with patterned cell surface area with a slope of $1.3 \mu F/cm^2$ that was comparable to reported specific membrane capacitance values of $\sim 1.1 \mu F/cm^2$ for HEK cells.$^{21}$ The measured values for Cx43 HEK input resistance at rest were consistent with those previously reported for unpatterned HEK cells ($1–5 \ G\Omega$).$^{22}$ Membrane ionic currents in HEK cells increased with cell capacitance, but curiously, despite the use of monoclonal cells, this increase was nonlinear, as the larger cells showed increased membrane current densities (Figure 3D and 3E), similar to what has been previously reported in rabbit sinoatrial node cells.$^{23}$ This nonlinear change in membrane currents with cell size required that the dynamic clamp models of engineered HEK cells incorporate exactly measured current–voltage relationships instead of simply injecting currents with amplitudes proportional to the modeled cell capacitance.
Relevance of Strong Heterocellular Coupling to Cardiac Cell Therapies

Forced expression of Cx43 in HEK cells in conjunction with controlled length of their contact with NRVMs yielded strong heterocellular gap junctional coupling with a reproducible conductance of ≈69 nS that was significantly larger than the resting input conductances of micropatterned HEK cells and cardiomyocytes (0.22–19.6 nS; Figure 2A). The observed coupling strength was in the range of strong coupling values previously reported between pairs of neonatal or adult cardiac myocytes or unexcitable cells engineered to overexpress Cx43.24–27 Dynamic clamp experiments further showed that the measured electrophysiological effects for $G_j=69$ nS remained unchanged when $G_j$ was reduced to 10 nS but were significantly attenuated when $G_j$ was further reduced to 1 and 0.1 nS (Figure 5C–5F). This finding is in agreement with modeling studies of cardiomyocyte loading by fibroblasts, which have suggested that for $G_j>8$ nS, the loading effects of fibroblasts on cardiac conduction velocity, APD, and ($dV_m/dt_{\text{max}}$) remain unchanged (saturated).11 Although the exact $G_j$ when this saturation effect occurs will depend on the unexcitable cell and cardiomyocyte input resistances, we speculate that our findings could be directly relevant for therapies that use unexcitable cells able to strongly couple with host cardiomyocytes, such as MSCs ($G_j=9$ nS$^2$), or cells genetically engineered to overexpress connexin proteins.24

Effect of Unexcitable Cells on Cardiac MDP

Increasing HEK cell size reduced the HEK input resistance relative to that of NRVMs yielding an MDP depolarization in NRVMs coupled to Cx43 HEK cells with a relatively positive resting potential or no change of MDP in NRVMs coupled to Kir2.1+Cx43 HEK cells with a resting potential similar to that of NRVMs. Specifically, depolarization of cardiac MDP resulted from the presence of increased inward currents (at $V_m<-26.5$ mV) in larger Cx3 HEK cells (Figure 3A). These results were fully reproduced in dynamic clamp studies using HEK models with ion currents alone, signifying the dominant role that steady-state current–voltage ($I–V$) relationships in unexcitable cells strongly coupled to cardiomyocytes play in setting the cardiac MDP, not only in quiescent but also in pacemaking cells (at least for the resulting pacemaking rates with cycle lengths significantly longer than the cell membrane time constants; Figures 2C and 5F).

Effect of Unexcitable Cells on Cardiac AP Upstroke

On the contrary, cardiac AP parameters that depended on relatively fast changes in $V_m$ (ie, [$dV_m/dt_{\text{max}}$ and APD/$V_m$) showed significant dependence on the HEK capacitive current. Specifically, increasing the size of Cx43 HEK cells slowed ($dV_m/dt_{\text{max}}$) by the additive contribution of both HEK ionic current and capacitance (Figures 5D and 7B). Although HEK ionic currents contributed to upstroke slowing by increasing cardiac MDP and, as a result, inactivating sodium channels, the HEK capacitance further reduced ($dV_m/dt_{\text{max}}$) by diverting a fraction of the depolarizing charge away from the NRVM. In NRVMs connected to Kir2.1+Cx43 HEK cells, in which no change in cardiac MDP was observed, increased capacitive load was the only source of upstroke slowing (Figures 6D and 7B). This significant contribution of capacitive loading to decrease in ($dV_m/dt_{\text{max}}$) is consistent with our previous studies where a large decrease in conduction velocity in NRVM monolayers loaded with Cx43 HEK cells was accompanied by only a moderate depolarization of cardiac resting potential.16

Taken together, these results indicate that implantation of any unexcitable cells of sufficient size able to strongly couple to host cardiomyocytes, including those that match cardiac resting potential (eg, by forced expression of potassium currents), would be expected to significantly reduce cardiac ($dV_m/dt_{\text{max}}$), locally slow conduction, and potentially generate a proarrhythmic substrate. Genetic engineering of small unexcitable cells (that do not impose significant capacitive load) along with the use of strong promoters to express large ionic currents would give the least nonspecific effects caused by endogenous membrane properties of the implanted unexcitable cells. In addition, the forced stable expression of both inward and outward currents in unexcitable cells (as done in our recent study)28 will be needed to prevent the negative effects on AP upstroke and permit safe and efficient cell therapies.

Effect of Unexcitable Cells on Cardiac APD

Increase in unexcitable cell capacitance not only yielded slowing of AP upstroke, but also significantly prolonged cardiac AP repolarization (Figure 7C) because the discharge of HEK and NRVM capacitances took longer than the discharge of NRVM capacitance alone. In the cell pairs, the APD increase because of pure capacitive loading of NRVMs was opposed by the flow of outward currents at $V_m>–26.5$ mV in Cx43 HEKs (Figures 3A and 5E), aiding early cardiac repolarization (Figure 5A), or at $V_m>–72.7$ mV in Kir2.1+Cx43 HEK cells (Figure 3B), aiding both early and late repolarization (Figure 6A). Collectively, these results show that the net APD prolongation or shortening in cardiomyocytes coupled to unexcitable cells will be determined by the specific unexcitable cell current–voltage relationship, whereas unexcitable cell capacitance (size) will only act to prolong the APD. Consistent with previous modeling studies of fibroblasts coupled to cardiomyocytes17,12 and dynamic clamp studies of a cardiomyocyte loaded by a resistor–capacitor circuit with battery,20 the unexcitable cells with hyperpolarized resting potential (relative to that of NRVMs) shortened cardiac APD, whereas those with relatively depolarized resting potential prolonged cardiac APD. Besides the resting potential, our studies establish unexcitable cell size (capacitance) and expression of specific ion currents as additional independent and potent modulators of cardiac APD.

Effect of Unexcitable Cells on Cardiac Pacemaking

Spontaneous activity in NRVMs coupled to Cx43 HEK cells was induced for a range of HEK cell sizes (HEK:NRVM cell surface area ratio of 1.08–1.94) with pacemaking rates that depended in a biphasic fashion on the amplitude of HEK ion currents and did not depend on HEK cell capacitance (Figure 5F). Specifically, increased inward currents in larger HEK cells (for $V_m<-26.5$ mV; Figure 3A) yielded NRVM depolarization that not only decreased the threshold for activation of inward currents (first sodium and then L-type calcium) to promote...
pacing, but also inactivated these currents to ultimately prevent any excitation. Significantly, pacemaking in NRVM–Cx43 HEK pairs was induced and modulated solely by the relative difference in the sizes of the 2 cells (and endogenous HEK currents) without a need for the additional expression of any exogenous pacemaker currents in HEK cells. Conceivably, this pacemaking activity was driven by the inflow of current from Cx43 HEK cells into NRVMs through gap junction channels and may have been additionally contributed by the activity of the cardiac sodium–calcium exchanger, similar to modeling studies in cardiomyocytes with reduced $I_{\text{K1}}$.

**Limitations**

Cells used for cardiac therapies, such as MSCs, often have larger sizes compared to HEK cells (55.2 pF capacitance in MSCs compared to the maximum HEK capacitance studied here of 31.0 pF). However, size and $I_{\text{K1}}$ expression in adult cardiomyocytes are also larger than in the neonatal myocytes studied here, so the net loading effects in adult myocardium may be attenuated compared to those found in this study. In the adult setting, the most readily observed effect of coupling to unexcitable cells is expected to be the decrease in cardiac $(\Delta V/\Delta t)_{\text{max}}$ since this change occurred at the smallest HEK:NRVM cell surface area ratios and independently of the unexcitable cell MDP. Decreased $(\Delta V/\Delta t)_{\text{max}}$ would be further expected to yield conduction slowing with potential for arrhythmogenic outcomes.

In addition to findings from our simplified in vitro assay, the electrophysiological effects of host–donor coupling in situ will also depend on other factors, including the number of unexcitable cells coupled per myocyte, spatial distribution of this coupling, and position of engrafted unexcitable cells relative to the direction of propagation. Different tissue engineering strategies may be used in the future to systematically study functional roles of these more complex heterocellular interactions and design safer and more efficient cell and gene therapies.

**Acknowledgments**

We thank R. Kirkton for assistance with plasmid construction, cell transfection, and fluorescence recovery after photobleaching analysis, D. Christini and the real-time experiment interface (www.rtxi.org) for dynamic clamp software, and A. Krol for NRVM isolation.

**Sources of Funding**

Dr McSpadden receives funding from the American Heart Association Predoctoral Fellowship and Dr Bursac from the National Institutes of Health, The National Heart, Lung, and Blood Institute grants HL104326 and HL106203.

**Disclosures**

None.

**References**

Recent studies have suggested the application of genetically engineered unexcitable stem and somatic cells for the treatment of cardiac arrhythmias. The potential clinical safety and effectiveness of this approach are yet to be explored, but initial results in animal models have been encouraging. The cell types proposed for these therapies exhibit a wide range of passive electric properties in terms of membrane capacitance, input resistance, resting potential, and cell–cell coupling strength. Although it is well recognized that electric coupling between cardiomyocytes and unexcitable cells can alter cardiomyocyte electrophysiological properties, traditional in vitro and in vivo systems lack sufficient flexibility and reproducibility to allow systematic and quantitative studies of these phenomena. The current study applies cell micropatterning and genetic engineering techniques to independently control geometry, electric properties, and coupling strength of cardiomyocytes and unexcitable cells in individual cell pairs. Studies in these cell pairs reveal that electric coupling between cardiomyocytes and unexcitable cells of increasing size strongly depresses cardiac action potential upstroke and prolongs action potential duration, while specific ionic currents of unexcitable cells further modulate the action potential upstroke and duration and determine cardiac maximal diastolic potential and pacemaking activity. Furthermore, cardiac action potential upstroke is found to be the most sensitive to coupling of cardiomyocytes with large unexcitable cells. The implantation of small, well-coupled unexcitable cells engineered to express large ionic currents is expected to yield the safest and most effective cardiac therapies.
Size and Ionic Currents of Unexcitable Cells Coupled to Cardiomyocytes Distinctly Modulate Cardiac Action Potential Shape and Pacemaking Activity in Micropatterned Cell Pairs

Luke C. McSpadden, Hung Nguyen and Nenad Bursac

Circ Arrhythm Electrophysiol. 2012;5:821-830; originally published online June 7, 2012; doi: 10.1161/CIRCEP.111.969329

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circbp.ahajournals.org/content/5/4/821

Data Supplement (unedited) at:
http://circbp.ahajournals.org/content/suppl/2012/06/07/CIRCEP.111.969329.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Arrhythmia and Electrophysiology 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: Arrhythmia and Electrophysiology is online at:
http://circbp.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Size and Ionic Currents of Unexcitable Cells Coupled to Cardiomyocytes Distinctly Modulate Cardiac Action Potential Shape and Pacemaking Activity in Micropatterned Cell Pairs

Luke C. McSpadden, PhD, Hung Nguyen, BS, and Nenad Bursac, PhD
Department of Biomedical Engineering, Duke University, Durham, North Carolina
SUPPLEMENTAL METHODS

Cell Pair Micropatterning

Fibronectin micropatterns in the shape of single rectangular islands or pairs of rectangular islands were prepared using microcontact printing techniques as previously described\(^1\), \(^2\). Briefly, a silicon wafer was coated with a 3 µm thick layer of photoresist (SU-8 2; Microchem, Newton, MA), exposed to UV light through a photomask (chromium on soda-lime glass; Advance Reproductions, North Andover, MA), and washed with a developer to remove uncrosslinked photoresist. Poly(dimethylsiloxane) (PDMS) stamps were cast against the wafer, coated with fibronectin solution (15 µg/mL; Sigma, St. Louis, MO), and used to transfer the desired fibronectin patterns to 22-mm diameter glass coverslips coated with PDMS. Unstamped areas of the coverslips were coated with a solution of pluronic F-127 (0.2%; Invitrogen, Carlsbad, CA) to block cell adhesion in unwanted areas.

Dimensions of single rectangular islands printed on the photomask were varied from 10x20 µm\(^2\) to 30x54 µm\(^2\) in 2 µm increments. For pairs of islands, the dimensions of one island was held constant at 30x30 µm\(^2\) while the other was varied from 20x10 µm\(^2\) to 20x36 µm\(^2\) in 2 µm increments. The contact length between the islands was held constant at 20 µm. The patterns were arranged such that approximately 5000 island pairs fit within a single coverslip.

Neonatal rat ventricular myocytes culture

All animals were treated according to protocols approved by the Duke University Institutional Animal Care and Use Committee. Cardiac cells were isolated from the ventricles of 2 day old Sprague-Dawley rats by enzymatic digestion with trypsin and collagenase as previously described\(^1\), \(^3\). The isolated cell population was enriched in NRVMs using two one-hour differential preplating steps to remove faster attaching non-myocytes. The remaining cells were resuspended in DMEM/F-12 media (Gibco, Carlsbad, CA) supplemented with 10% calf serum and 10% horse serum.
Genetically engineered human embryonic kidney 293 cells

Selected ion channel and gap junction proteins were stably expressed in human embryonic kidney 293 (HEK) cells (ATCC, CRL-1573), as previously described\(^3,\ 4\). The rat connexin-43 gene (GJA1) was cloned from NRVMs using primers based upon the published rat connexin-43 sequence (PubMed NM_012567) and subcloned into a plasmid following the fluorescent protein mCherry\(^5\) and an IRES sequence. The human Kir2.1 gene was subcloned into a plasmid following the fluorescent protein eGFP and an IRES sequence. The plasmids were transformed into bacteria and 3 µg of isolated plasmid DNA was conjugated to GenJet (SignaGen Labs) reagent and used to transfect HEK cells.

For connexin-43 expressing cells, wild type HEK cells transfected with the connexin-43 plasmid were identified by mCherry expression and selected using puromycin. A stable monoclonal cell line (Cx43 HEK) was isolated from the polyclonal population. For cells coexpressing connexin-43 and Kir2.1, the Cx43 HEK monoclonal cell line was transfected with the Kir2.1 plasmid, identified by eGFP expression, and selected using G418. A stable monoclonal cell line (Kir2.1+Cx43 HEK) was isolated from the polyclonal population. All HEK cell lines were cultured in DMEM (Gibco) supplemented with 10% FBS and penicillin/streptomycin.

Preparation of micropatterned cell pairs

PDMS coated glass coverslips stamped with fibronectin cell-pair patterns were first plated with suspended NRVMs at a density of 10x10\(^3\) cells/cm\(^2\) (Figure 1). After two days of culture, the cardiac seeding media was replaced with maintenance media containing M199 (Gibco) supplemented with 2% horse serum, HEPES (10 mM), MEM nonessential amino acids, GlutaMAX (2 mM; Invitrogen), vitamin B\(_{12}\) (2 ug/mL), and penicillin-G (20 U/mL). Media was exchanged every other day.
After 5 days of NRVM culture on island pair patterns, HEK cells were resuspended in HEK culture media and added at a density of $2 \times 10^3$ cells/cm$^2$ to coverslips containing NRVMs. Patch clamp and immunostaining studies were performed after an additional 24 hours of co-culture. For single cell experiments, HEK cells or NRVMs were plated on PDMS coated glass coverslips stamped with single-cell fibronectin islands and assessed on culture day 6 or 1, respectively.

**Immunostaining**

Cells were fixed in methanol-acetone, permeabilized in a 1% solution of Triton-X in PBS, and blocked in a 5:1 mixture of 1% BSA and chicken serum$^{1-3}$. Primary antibodies (1 hour at room temperature in PBS) used were: anti-sarcomeric α-actinin (Sigma, mouse monoclonal) and anti-connexin-43 (Zymed, rabbit polyclonal). Secondary antibodies (Alexa Fluor 488, chicken anti-rabbit and Alexa Fluor 594, chicken anti-mouse) were applied in PBS for 1 hour at room temperature. Nuclei were counterstained with DAPI (Sigma). Images were acquired with a CCD camera (SensiCam QE, Cooke) attached to an inverted fluorescence microscope (Nikon TE2000).

**Cell surface area measurement**

Confocal microscopy was used to measure cell thickness and regardless of the patterned cell surface area or cell type the thickness was found to be $7.9 \pm 0.62 \, \mu m$. Immediately before electrophysiological recordings, an image was acquired of the single cell or cell pair to be analyzed. In all experiments, mCherry or eGFP fluorescence was used to distinguish HEK cells from NRVMs. We used custom MATLAB software to trace the cell border and determine the substrate area covered by the cells. The total cell surface area was calculated using the average cell thickness determined from confocal microscopy and assuming a cuboid cell shape.
Data were grouped into bins based on the cell surface area for single cells or the HEK to NRVM cell surface area ratio (HEK:NRVM cell surface area ratio) for cell pairs.

**Whole-cell recordings**

Coverslips with adherent cells were transferred to an experimental chamber perfused with Tyrode’s solution containing (in mmol/L): 135 NaCl, 5.4 KCl, 1.8 CaCl₂, 5 HEPES, 5 glucose, 1 MgCl₂, and 0.33 sodium phosphate. Patch pipettes were fabricated using a microelectrode puller (Sutter Instrument, Novato, CA) and fire-polished to generate electrodes with tip resistances of 1-3 MΩ when filled with (in mmol/L): 140 KCl, 10 NaCl, 1 CaCl₂, 10 EGTA, 10 HEPES, 2 MgCl₂, and 5 MgATP and adjusted to pH 7.2 with KOH and 270-280 mOsm with glucose. Whole-cell voltage- and current-clamp measurements were performed after correction for liquid junction potentials with a Multiclamp 700B amplifier (Axon Instruments, Sunnyvale, CA), digitized at 20 kHz with a PCI-6221 acquisition card (National Instruments), and controlled by the WinWCP software package (Dr. John Dempster, University of Strathclyde). The patch seal resistance was routinely greater than 20 GΩ.

**Steady-state current recordings in single cells**

Currents from single micropatterned HEK cells or NRVMs were recorded in whole-cell voltage clamp mode at room temperature, filtered using a low-pass Bessel filter with 8 kHz cutoff frequency, and saved to a PC. The membrane voltage of individual cells was stepped from a holding potential of -40 mV to a test potential of -120 mV to 40 mV in 3 s long, 10 mV steps. The average current during the final 100 ms of each voltage step was used to construct steady-state current-voltage (I-V) relationships. Input resistance was determined from the slope of a 6th order polynomial fit to the I-V curve at the resting potential. Resting potential was obtained by briefly switching to current clamp mode after membrane breakthrough.
Action potential recordings in single NRVMs and NRVM-HEK pairs

Membrane voltage in micropatterned NRVMs was recorded in whole-cell current clamp mode at 35°C, filtered using a low-pass Bessel filter with 10 kHz cutoff frequency, and saved to a PC. In cells with no pacemaking activity, action potentials were elicited by applying a 10 ms current pulse at 1.1x threshold amplitude. In spontaneously active cells, action potentials were recorded without the application of stimulus current.

Dual whole-cell recordings

Gap junction conductance (Gj) in NRVM–HEK pairs was measured using dual whole-cell voltage clamp recordings. The NRVM was held at 0 mV while the HEK cell was stepped from -40 mV to +40 mV in 20 mV increments and held for 4 seconds at each step. The effect of HEK cell membrane resistance was minimized by analyzing only those HEK holding voltages (-40 mV and -20 mV for Cx43 HEK; +40 mV and +20 mV for Kir2.1+Cx43 HEK) that corresponded to high HEK membrane resistance (>1 GΩ for all cell sizes). Steady-state gap junction conductance was determined from the measured junctional current and the applied voltage difference after correcting for series resistance (3.62±0.4 MΩ) and membrane resistance as described by Veenstra and Brink6.

Fluorescence recovery after photobleaching

Functional intercellular coupling was investigated using fluorescence recovery after photobleaching (FRAP), as described previously3. Briefly, micropatterned NRVM-Cx43/HEK cell pairs were loaded with the fluorescent dye, Calcein AM (Molecular Probes, 0.5 µM diluted in DMEM, 20 minutes at 37°C), washed with PBS, exchanged to complete growth media, and imaged/bleached with an upright confocal microscope (Zeiss LSM 510). Individual Cx43/HEK cells within the micropatterned pairs were recognized by their mCherry fluorescence and photobleached with a 488 nm Argon laser to remove Calcein fluorescence. Calcein
fluorescence recovery in the bleached cell was monitored by acquiring an image every 15 seconds for 2 minutes after bleaching. Custom software (MATLAB) was used to analyze the time course of fluorescence recovery after first normalizing the recorded fluorescence to the values immediately before and after bleaching as well as correcting for the gradual bleaching of the entire field during image acquisitions. To verify that dye transfer during fluorescence recovery occurred through gap junctional coupling (rather than possible cell-fusion events), the FRAP data was compared before and after the addition of the gap junction inhibitor Carbenoxolone (200 µM, Sigma).

**Dynamic clamp setup**

Dynamic clamp experiments were performed to dissect how membrane capacitance and ionic currents of HEK cells coupled to NRVMs affect cardiac action potential shape and pacemaking. We used the free, open source Real Time eXperimental Interface 7 (RTXI, rtxi.org) software package to enable real-time monitoring of NRVM membrane potential and injection of current through a single patch pipette. The RTXI software interfaced with a PCIE-6251 acquisition card (National Instruments) with drivers from the Comedi project (comedi.org). The membrane voltage signals acquired from a single micropatterned NRVM in whole-cell current clamp mode were digitized and feedback current was injected back into the NRVM at a real-time rate of 20 kHz. Custom written RTXI plugins modeled the coupling of an unexcitable cell of varying size through a gap junction to the micropatterned NRVM.

**Unexcitable cell models and dynamic clamp recordings**

We utilized three unexcitable cell models in this study: a steady-state ionic current model, a capacitance model, and a combined capacitance and current model. The ionic current model (Online Figure IA) represented an idealized unexcitable cell contributing only the total membrane ionic current and no capacitance. Conversely, the capacitance model (Online Figure
IB) represented an idealized cell contributing only capacitive and no membrane ionic currents. The combined model (Online Figure IC) represented a more realistic unexcitable cell with both capacitive and ionic currents present. In each case, a gap junction with conductance $G_j$ connected the model unexcitable cell to the micropatterned NRVM. Connecting the NRVM to combined HEK model cell represented the closest approximation to the experimental cell pair setting.

The unexcitable cell–gap junction combination was represented by two separate modules in RTXI. The gap junction module was governed by the equation $I_{gj} = G_j(V_{NRVM} - V_u)$ where $V_u$ was the unexcitable cell membrane potential, $V_{NRVM}$ was the NRVM membrane potential measured through the patch pipette, and $I_{gj}$ was the gap junction current injected into the NRVM. The unexcitable cell module was governed by the equation $C_m \frac{dV_u}{dt} = I_{gj} - I_{ion}$.

Forward Euler time stepping with $dt = 25 \mu s$ was used to numerically solve the differential equation relating membrane current and voltage of the model cell. In the ionic current model $C_m$ was set to 0 and in the capacitive model $I_{ion}$ was set to 0.

We derived the model parameter values from whole-cell patch clamp experiments performed on single cells. The steady-state I-V curves constructed for Cx43 HEK cells (Figure 3A) and Kir2.1+Cx43 HEK cells (Figure 3B) of different size provided the I-V relationships for the model ionic currents ($I_{ion}$). Models utilizing the Cx43 HEK I-V relationships were referred to as Cx43 HEK current models while models utilizing the Kir2.1+Cx43 HEK I-V relationships were referred to as Kir2.1+Cx43 HEK current models. A sixth order polynomial fit to each of the eight I-V curves (two cell types, four cell sizes each) gave the membrane currents as a continuous function of voltage. For all models, gap junction conductance ($G_j$) was set at 68.9 nS, i.e., the average value measured in heterotypic NRVM–HEK pairs using dual whole-cell patch clamp recordings (Figure 4).
The model cell size was varied by adjusting the capacitance and/or the membrane I-V curve according to the relationships established between these parameters and cell size (Figure 2B & Figure 3A&B). Prior to dynamic clamp experiments, the micropatterned NRVM cell surface area was measured by tracing the cell border in a bright-field image. This allowed the calculation of HEK:NRVM cell surface area ratio for each HEK model cell we connected to the NRVM. By patching NRVMs of different sizes connected to model cells of different sizes, we varied HEK:NRVM cell surface area ratio over the same range seen in experiments with NRVM-HEK cell pairs, approximately 0.4–2.0. Action potentials in NRVMs were elicited by stimulating with a 10 ms current pulse at 1.1x threshold. After the recording with no model connected, various HEK models were connected and action potentials were recorded as model type and parameters were varied. If connecting the NRVM to a HEK cell model induced pacemaking activity, action potentials were recorded without the application of stimulus current.

**Analysis of action potential recordings**

The recordings were analyzed with custom MATLAB software to determine maximal diastolic potential (MDP), maximum action potential upstroke velocity \((dV_m/dt)_{max}\), action potential duration at 80% repolarization \((APD_{80})\), and, for pacemaking cells, spontaneous beating rate. Maximum upstroke velocity and APD measured in NRVMs with models connected were normalized to values from the same NRVM with no connected model. For comparison, the same parameters measured in cell pairs were normalized to the average single NRVM values.

**Video recordings of cardiomyocyte beating**

To more efficiently characterize the pacemaking activity in cell pairs with specific HEK:NRVM cell surface area ratios, a CCD camera (Pixelfly; Cooke Corporation, Romulus, MI) was used to capture videos (22.2 frames per second) of spontaneously beating NRVMs. Average beating rate was determined from the number of beats in a 30 second recording.
Statistics

Reported sample sizes reflect the number of independent single cells or cell pairs from which data was collected. Data were divided into bins based on cell surface area or HEK:NRVM cell surface area ratio and were expressed as mean ± standard error.

For comparing single cell electrophysiological properties between cell types, differences between bins were analyzed using an ANOVA followed by Tukey’s multiple comparison test. The capacitance vs. cell surface area relationship was fit with linear regression.

For cell pairs, electrophysiological measurements were plotted as a function of HEK:NRVM cell surface area ratio and in selected cases fit with a fourth order polynomial or with a Boltzmann function of the form \( y = y_0 + \frac{y_\infty - y_0}{1 + \exp\left(-\frac{x-x_{1/2}}{b}\right)} \) to determine the cell surface area ratio \( x_{1/2} \) at which the change in the measured parameter was half of the total change, the parameter value at infinite cell surface area ratio \( y_\infty \), and the slope \( b \).

Differences between fit parameters were analyzed with a t-test. Fitting was conducted with the Matlab Signal Processing Toolbox and statistical tests were carried out with the Matlab Statistics Toolbox. For all statistical tests, differences were considered significant when \( p < 0.05 \).
Online Figure I. HEK cell dynamic clamp models. NRVMs were connected via real-time interface software to unexcitable HEK cell models consisting of (A) membrane current alone, (B) capacitance alone, or (C) combined membrane current and capacitance. HEK cells of different sizes were modeled by scaling unexcitable membrane current \( (I_m) \) and cell capacitance \( (C_m) \) values based on the experimentally determined dependence of these parameters on cell size. Gap junction conductance \( (G_j) \) remained constant at 68.9 nS, the value measured in dual patch clamp experiments on cardiomyocyte–HEK cell pairs, except where noted otherwise.
Online Figure II. Single micropatterned cells with defined cell surface areas. (A-B) Examples of single HEKs (A) and NRVMs (B) of increasing patterned cell surface area used for patch clamp studies. Shown total cell surface areas were estimated by measuring average cell thickness by a confocal microscope and assuming a cuboid cell shape. These total cell surface areas were classified in the bins described in the results and shown in Figure 3.
Online Figure III. Representative whole-cell membrane currents. Displayed current traces were measured in a whole-cell voltage clamp mode in response to voltage steps from -120 mV to 30 mV in 10 mV increments in single micropatterned (A) Cx43 HEK cells, (B) Kir2.1+Cx43 HEK cells, and (C) NRVMs.
Online Figure IV. Analysis of functional gap junctional coupling by fluorescence recovery after photobleaching (FRAP). (A) Micropatterned cell pairs were loaded with Calcein dye (green). Cx43/HEK cells were photobleached using a 488 nm laser and monitored for Calcein fluorescence recovery during control conditions (top panels) and in the presence of the gap junctional inhibitor, Carbenoxolone (200 µM, bottom panels). (B) Average time courses of fluorescence recovery for each of the settings shown in (A).
Online Figure V. Representative action potentials from an NRVM connected to the Cx43 HEK current and combined models. Action potentials recorded from a single micropatterned NRVM (black), the same NRVM connected to the Cx43 HEK current model (dark grey), and the same NRVM connected to the Cx43 HEK combined model (light grey). Shown are the: (A) changes in NRVM resting potential and action potential shape and (B) close-ups of action potential upstrokes.
Online Figure VI. **Pacemaking in NRVM–Cx43 HEK cell pairs.** (A) Representative NRVM membrane voltage traces showing spontaneous activity (pacemaking) in some NRVM–Cx43 HEK cell pairs (red), but not in NRVM–Kir2.1+Cx43 HEK cell pairs (green). (B) Pacemaking was found in all NRVM–Cx43 HEK cell pairs (N = 50, red circles) with HEK:NRVM cell surface area ratio between 1.08 and 1.94. No pacemaking occurred in any NRVM–Kir2.1+Cx43 HEK cell pairs (N = 23, green circles), while only 5.3% of single NRVMs (N = 38, black diamond) exhibited pacemaking.
Online Figure VII. Representative action potentials from an NRVM connected to the Kir2.1+Cx43 HEK current and combined models. Action potentials recorded from a single micropatterned NRVM (black), the same NRVM connected to the Kir2.1+Cx43 HEK current model (dark grey), and the same NRVM connected to the Kir2.1+Cx43 HEK combined model (light grey). Shown are the: (A) changes in NRVM resting potential and action potential shape and (B) close-ups of action potential upstrokes.
Online Figure VIII. Representative action potentials from an NRVM connected to the HEK capacitance models. Action potentials recorded from a single micropatterned NRVM (black), the same NRVM connected to the capacitance model with HEK:NRVM cell surface area ratio 1.06 (dark grey), and the same NRVM connected to the capacitance model with HEK:NRVM cell surface area ratio 1.49 (light grey). Shown are the: (A) changes in NRVM resting potential and action potential shape and (B) close-ups of action potential upstrokes. Stimulus current threshold increased with increasing cell surface area ratio (not shown).
SUPPLEMENTAL REFERENCES


