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Brief Communication

Phenotype-dependent role of the L-type calcium current in embryonic stem cell derived cardiomyocytes

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Abstract: Although the L-type Ca^{2+} current ($I_{Ca,L}$) plays an important role in cardiac contractility and pacemaking, its role in embryonic stem-cell derived cardiomyocytes (ESC-CMs) has not yet been explored in detail. We used patch-clamp techniques to characterize $I_{Ca,L}$, action potential properties, and nifedipine (an $I_{Ca,L}$ blocker) sensitivity on spontaneously contracting embryoid bodies (EBs) or isolated ESC-CMs. Cellular preparations exhibited differential sensitivity to nifedipine, with substantial variation in the dose required to abolish automaticity. Isolated ESC-CMs expressing nodal-like action potentials were highly sensitive to nifedipine; 1 nM significantly decreased firing rate, diastolic depolarization rate (DDR), and upstroke velocity, and 10 nM completely abolished spontaneous activity. In contrast, ESC-CMs expressing atrial-like action potentials were relatively nifedipine-resistant, requiring 10 μM to arrest automaticity; 1 μM significantly decreased upstroke velocity while the firing rate and DDR were unaffected. Nodal-like cells exhibited a more negative voltage for half-maximal I_{Ca} activation (-30 ± 1 mV vs. -20 ± 3 mV; $p<0.05$) and slower inactivation (71 ± 10 ms vs. 43 ± 3 ms; $p<0.05$) than atrial-like cells. Our data indicate that $I_{Ca,L}$ differentially regulates automaticity and chronotropy in nodal-like ESC-CMs, and primarily links excitation to contraction in atrial-like ESC-CMs by contributing to the upstroke phase of the action potential.

Keywords: Pacemaker cells, cardiac electrophysiology, cardiomyocyte, automaticity

Introduction

Spontaneously beating embryonic stem cell-derived cardiomyocytes (ESC-CMs) express cardiac specific markers, and display functional properties characteristic of embryonic cardiac muscle such as diastolic depolarization and spontaneous excitability [1, 2]. The diastolic depolarization phase of the action potential (AP) is initiated by a net inward current generated by the interaction of different ionic membrane currents and intracellular Ca^{2+} signaling pathways. Potential contributors include the funny current (I_f), the L-type calcium current ($I_{Ca,L}$), the T-type calcium current ($I_{Ca,T}$), and release of intracellular calcium from ryanodine-sensitive stores [3]. The functional importance of these different participants in the generation of the diastolic depolarization in the adult heart is still a matter of considerable debate.

In the embryonic heart, calcium influx through voltage-gated L-type Ca^{2+} channels plays a critical role in the initiation of cardiac excitability and in the excitation-contraction (E-C) coupling [4]. Thus $I_{Ca,L}$ is detectable as early as embryonic day (E) 9.5 [5], which closely approximates the temporal onset of regular heart contractions [6] and the current density has been shown to increase with maturity [5]. Two principal forms of L-type Ca^{2+} channel α_1 subunits, $Ca_v1.2$ (α_{1C}) and $Ca_v1.3$ (α_{1D}), have been detected in the embryonic heart [7]. $Ca_v1.2$, the predominant form in the myocardium, is an important contributor to the upstroke phase of the cardiac pacemaker action potential, and $Ca_v1.3$, which activates at more negative membrane potentials, has been shown to participate in the diastolic depolarization phase and modulate automaticity [8, 9]. Deletion of the $Ca_v1.2$ gene (*CACNA1C*) results in embryonic death before E14.5 in mice [10] whereas deletion of the $Ca_v1.3$ gene (*CACNA1D*) does not significantly affect embryonic development in mice but leads to sinoatrial (SA) node bradycardia in

postnatal animals [11]. In humans, the loss of $Ca_v1.3$ function results in deafness and pronounced SA node bradycardia [12].

Although previous studies have demonstrated the presence of $I_{Ca,L}$ in ESC-CMs [2, 13, 14], none has explored the specific contribution of $I_{Ca,L}$ to the automaticity of different cardiac phenotypes. Given the critical role that $I_{Ca,L}$ plays in initiating cardiac excitability and in excitation-contraction (E-C) coupling, defining the contribution of this current to the automaticity of ESC-CM is important to the understanding of the functional properties of this cellular model. This issue is of particular relevance to the application of ESC-CMs in cell-based therapy to treat the infarcted heart [15]. The purpose of this study was to characterize the contribution of $I_{Ca,L}$ to the automaticity of spontaneously beating nodal-like and atrial-like mouse ESC-CMs.

Materials and methods

All reagents and materials were obtained from Sigma-Aldrich Canada (Oakville, ON) unless otherwise specified.

Culture of mouse ESC and differentiation into cardiomyocytes

R1 mouse embryonic stem cells (ESCs) [16] were cultured and differentiated into cardiomyocytes using the hanging drop method [2, 17]. On day 1 of differentiation, hanging drops of ESCs were grown and cultured for 2 days. On day 3, embryoid bodies (EBs) formed in hanging drops were grown further for 4 days. On day 7, EBs were plated onto 0.1% gelatin-coated culture plates. Spontaneous beating was observed 2 to 3 days after plating. EBs grown for 9 to 11 days post plating were considered to be at late developmental stage. Spontaneously beating ESC-CMs isolated from late stage EBs have been shown to express different cardiac action potential morphologies and all the ionic currents associated with cardiac pacemaking and E-C coupling [18]. Thus, late stage ESC-CMs were considered suitable to study the role of $I_{Ca,L}$ on initiation of cardiac excitability and E-C coupling in different ESC-CM phenotypes.

Preparation of single cardiomyocytes

Single cardiomyocytes were dissociated from spontaneously beating EBs as previously described [17]. Isolated cells were plated on poly D-lysine-coated glass bottom culture dishes (No.1.5, MatTek Corporation, Ashland, MA) and cultured overnight. Single spontaneous beating cells could be observed the following day. Only cells that contracted spontaneously and synchronously at a regular rhythm for 20 minutes prior to commencing recording were used for this study.

Measurement of spontaneous beating rate of EBs incubated with nifedipine

Spontaneously beating EBs were treated, at room temperature (RT), with increasing doses of nifedipine (Sigma) in Tyrode's solution by sequential application of 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM until spontaneous contractions were abolished. The incubation time for each dose was 10 minutes. The detailed procedure is described in Supplementary Materials.

Electrophysiology studies

Whole cell amphotericin-perforated (200 μg/ml) current and voltage clamp techniques were used to record spontaneous action potentials and ionic currents (MultiClamp 700A, Axon Instruments, Union City, CA) from isolated single cells. Solution composition and detailed procedures are described in Supplementary Materials. To record spontaneous electrical activities in the current clamp mode, cells were treated with serial doses of nifedipine, identical to those used in EB studies, until spontaneous activity was abolished.

When subsequently switched to voltage clamp mode, the external solution was switched to the standard external solution with 20 mM tetraethylammonium chloride, 2 mM 4 aminopyridine, and 30 μM tetrodotoxin (TTX). To measure the current-voltage (I-V) dependence of the nifedipine-sensitive $I_{Ca,L}$, currents were elicited by depolarizing the cell to different test potentials (between -50 and +50 mV in increments of 10 mV) from a holding potential of -60 mV. This holding potential has been shown to inactivate $I_{Ca,T}$ while inducing minimal inactivation of $Ca_v1.3$ $I_{Ca,L}$ within the diastolic depolarization range [9]. The protocol was repeated in the presence of 500 nM nifedipine, which has been shown to have no effect on $I_{Ca,T}$ [19]. $I_{Ca,L}$ was measured as the nifedipine-sensitive current and normalized to cell capacitance. All experiments were performed at RT.

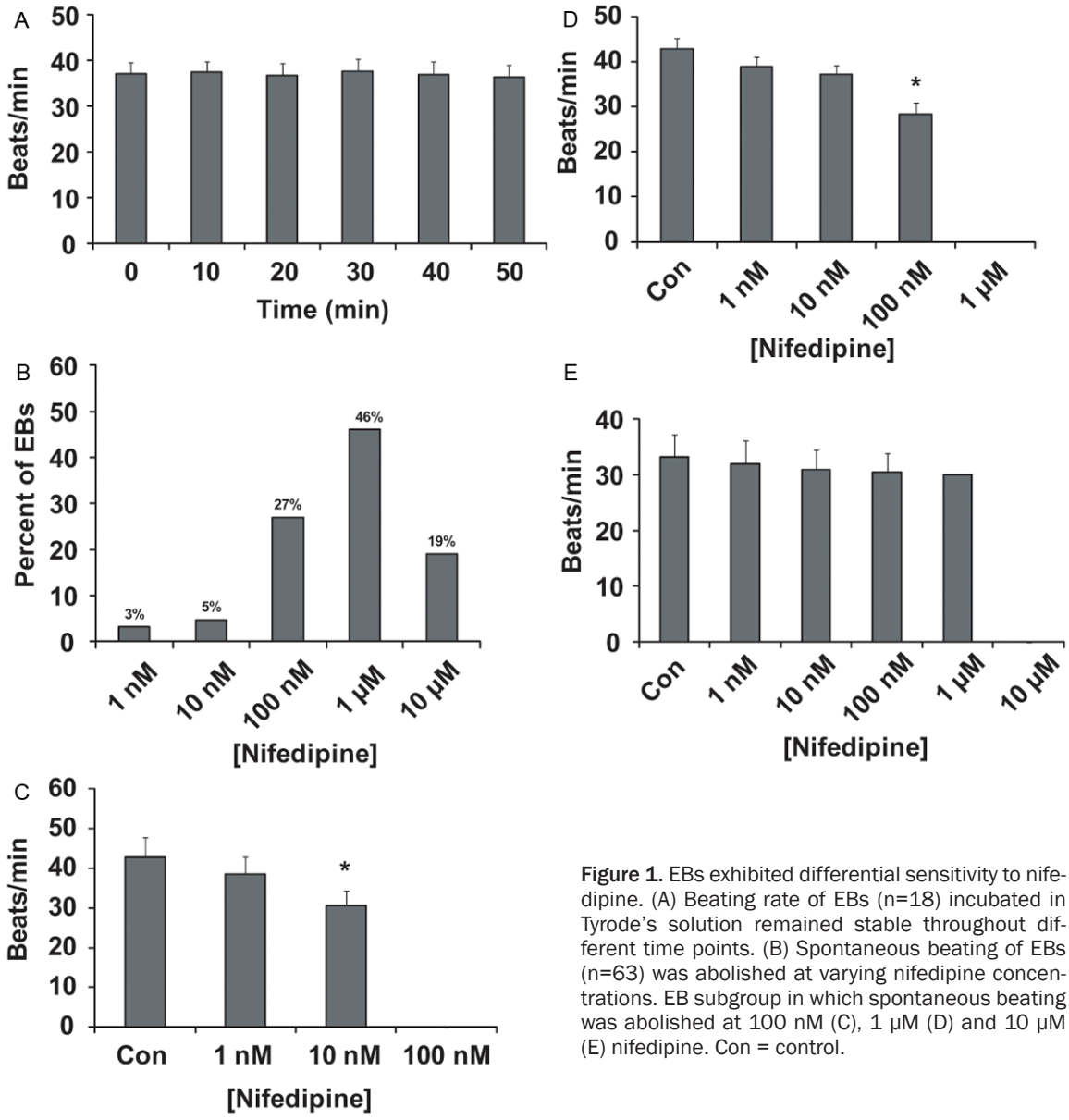

Figure 1. EBs exhibited differential sensitivity to nifedipine. (A) Beating rate of EBs (n=18) incubated in Tyrode's solution remained stable throughout different time points. (B) Spontaneous beating of EBs (n=63) was abolished at varying nifedipine concentrations. EB subgroup in which spontaneous beating was abolished at 100 nM (C), 1 μM (D) and 10 μM (E) nifedipine. Con = control.

Statistical analysis

Data are presented as mean ± SEM unless otherwise stated. Statistical significance was assessed by paired Student's *t* test or repeated measure one way ANOVA. A value of $p<0.05$ was considered statistically significant. Analysis was performed by employing Prism 4 software (ver. 4.03, GraphPad Software Inc., San Diego, CA).

Results

Differential nifedipine sensitivity of EBs

To characterize the dependency of automaticity on $I_{Ca,L}$, spontaneously beating EBs were incubated with serial doses of the L-type Ca^{2+} channel blocker, nifedipine, until the spontaneous contractions were abolished. Under control conditions, EBs (n=18) exhibited stable beating rates over a time course of 50 minutes (**Figure 1A**). Nifedipine treatment abolished spontaneous contraction in all preparations (n=63), but the required concentration varied amongst EBs (**Figure 1B**).

A small number of EBs were arrested by 1 or 10 nM nifedipine. In 27% and 46% of preparations, application of 100 nM and 1 μM, respectively led to a cessation of automatic contraction (**Figure 1B**). In all of these groups, lower nifedipine doses significantly decreased the beating

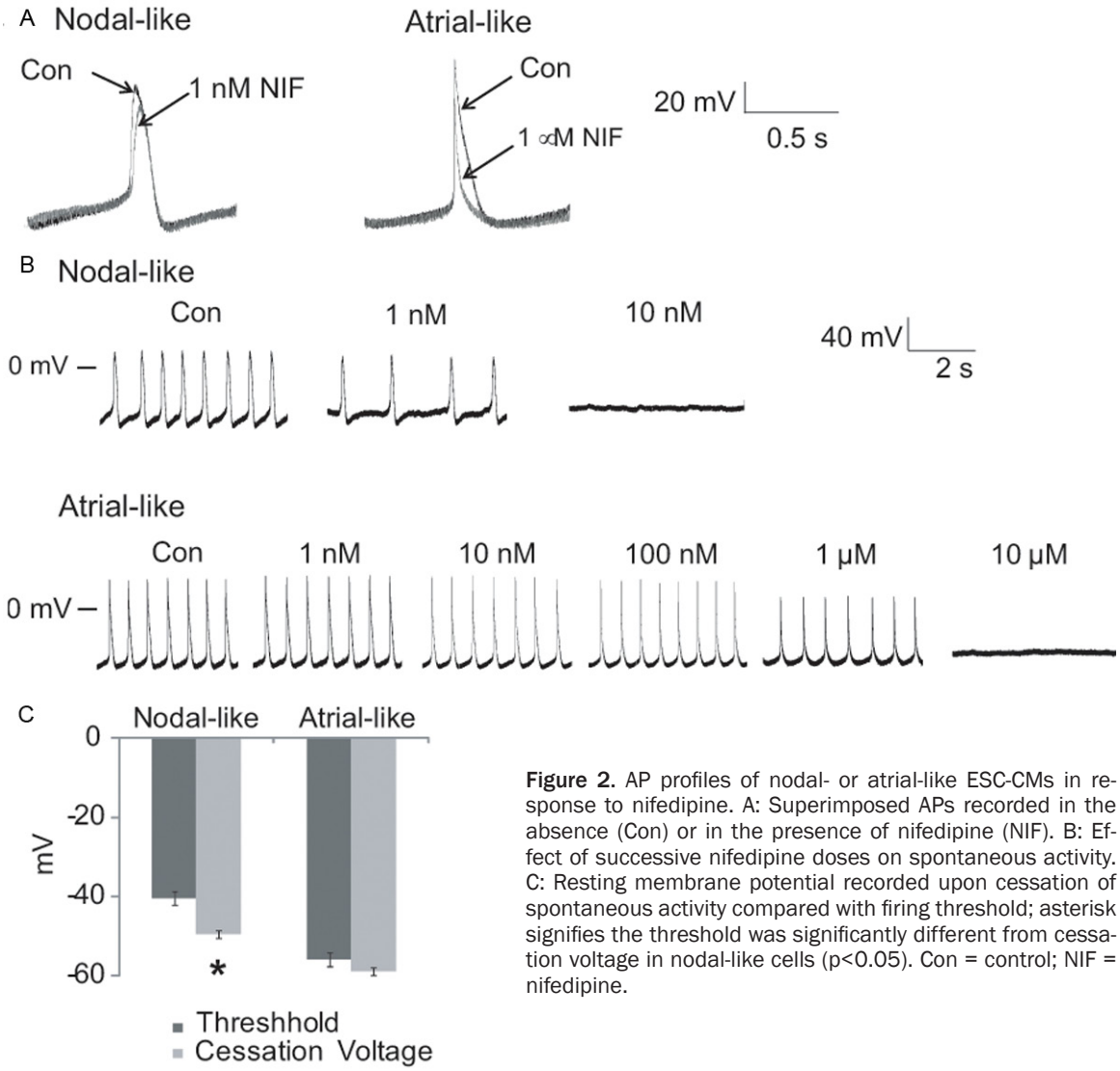

Figure 2. AP profiles of nodal- or atrial-like ESC-CMs in response to nifedipine. A: Superimposed APs recorded in the absence (Con) or in the presence of nifedipine (NIF). B: Effect of successive nifedipine doses on spontaneous activity. C: Resting membrane potential recorded upon cessation of spontaneous activity compared with firing threshold; asterisk signifies the threshold was significantly different from cessation voltage in nodal-like cells ($p<0.05$). Con = control; NIF = nifedipine.

frequency (**Figure 1C** and **1D**). A fraction (19%; **Figure 1B**) of EBs required 10 μM to abolish spontaneous beating and showed no significant changes in the beating rate at lower doses (**Figure 1E**). These results indicate that the EBs exhibited differential sensitivity to nifedipine, suggesting that the contribution of $I_{Ca,L}$ to automaticity differs among the sampled population.

Nodal-like and atrial-like action potential phenotypes

Single cardiomyocytes dissociated from spontaneously beating EBs maintained a regular spontaneous activity and showed heterogeneity in the AP morphology. Nodal-like (**Figure 2A**, left panel, Con) and atrial-like (**Figure 2A**, right panel, Con) APs were observed, and the former was characterized by a significantly slower upstroke. AP parameters are described in **Table 1**; the range of observations for each parameter is listed in Supplementary Materials.

To characterize the contribution of $I_{Ca,L}$ to automaticity, spontaneously beating ESC-CMs were perfused with serial doses of nifedipine, identical to those used in EB studies. Nodal-like ESC-CMs were highly sensitive to nifedipine. In 6 of 7 cells examined, treatment of 10 nM arrested spontaneous activity (**Figure 2B**, top), while 1 nM led to a significant decline in AP frequency, DDR, upstroke velocity, overshoot, action potential amplitude (APA), and a prolongation of cycle length (**Figure 2A**, left; **Table 1**). Other AP

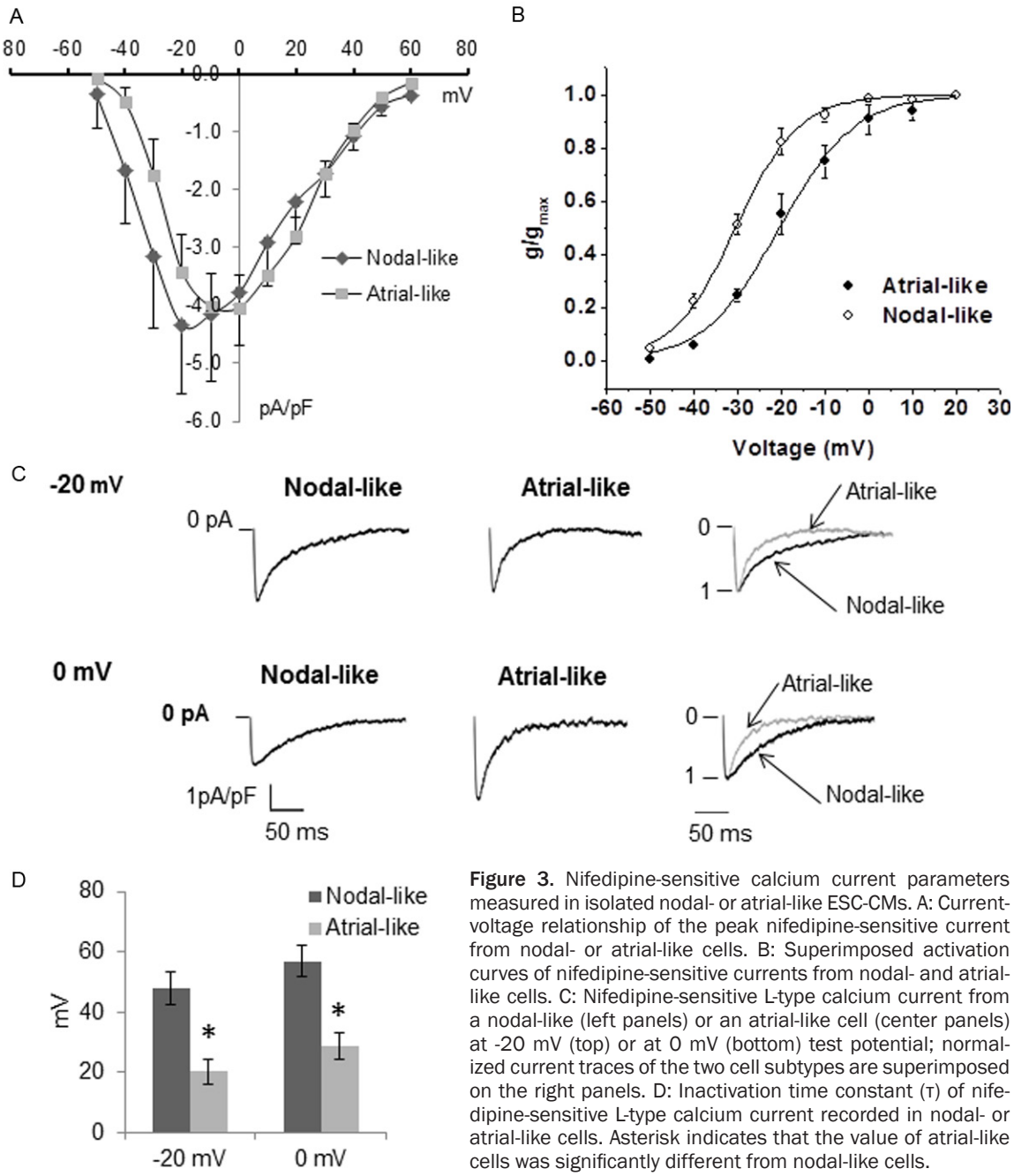

Figure 3. Nifedipine-sensitive calcium current parameters measured in isolated nodal- or atrial-like ESC-CMs. A: Current-voltage relationship of the peak nifedipine-sensitive current from nodal- or atrial-like cells. B: Superimposed activation curves of nifedipine-sensitive currents from nodal- and atrial-like cells. C: Nifedipine-sensitive L-type calcium current from a nodal-like (left panels) or an atrial-like cell (center panels) at -20 mV (top) or at 0 mV (bottom) test potential; normalized current traces of the two cell subtypes are superimposed on the right panels. D: Inactivation time constant (τ) of nifedipine-sensitive L-type calcium current recorded in nodal- or atrial-like cells. Asterisk indicates that the value of atrial-like cells was significantly different from nodal-like cells.

parameters, including maximum diastolic potential (MDP), threshold, and action potential duration at 50% repolarization (APD_{50}), were not significantly affected (**Table 1**). In 1 of 7 cells, even the lowest dose tested (1 nM) abolished automaticity. The average resting membrane potential recorded after the cessation of spontaneous activity was -50 ± 1 mV, which was significantly more hyperpolarized than the average firing threshold at -40 ± 1 mV ($p<0.05$; **Figure 2B**, top; **Figure 2C**).

In contrast, atrial-like ESC-CMs (n=6) were relatively nifedipine resistant, requiring 10 μM to abolish the automatic rhythm (**Figure 2B**, bottom). At 1 μM nifedipine, the upstroke velocity, overshoot, APA, and APD_{50} were significantly reduced ($p<0.05$; **Figure 2A**, right; **Table 1**), but unlike nodal-like cells, the AP frequency, cycle length, DDR were not significantly affected. At doses lower than 1 μM the AP rhythm remained regular. The resting membrane potential recorded upon cessation of spontaneous activity was

Table 1. AP parameters of ESC-CMs exposed to successive doses of nifedipine

[Nif][1]		AP/min[1]	Cycle Length (ms)	DDR[1] (mV/ms)	MDP[1] (mV)	Thr[1] (mV)	Upstroke Velocity (V/s)	Over-shoot (mV)	APA[1] (mV)	APD_{50}[1] (ms)
Nodal-like (n=7[2])	Con[1]	65 ± 17	1.2 ± 0.3	49 ± 16	-69 ± 2	-40 ± 1	1.6 ± 0.2	16 ± 1	78 ± 6	77 ± 6
	1 nM	*47 ± 19	*2.5 ± 0.8	*32 ± 14	-68 ± 3	-39 ± 2	*1.1 ± 0.2	*10 ± 2	*70 ± 7	70 ± 6
	10 nM	0[3]	-[4]	-	-	-	-	-	-	-
Atrial-like (n=6)	Con	84 ± 11	0.8 ±0.1	49 ± 5	-75 ± 1	-57 ± 2	21 ± 4	30 ± 5	106 ± 5	43 ± 7
	1 nM	79 ± 10	0.9 ±0.2	40 ± 5	-75 ± 1	-56 ± 1	22 ± 4	30 ± 5	108 ± 6	37 ± 6
	10 nM	81 ± 10	0.8 ±0.1	40 ± 5	-74 ± 1	-55 ± 2	20 ± 4	29 ± 5	104 ± 6	32 ± 5[£]
	100 nM	81 ± 10	0.8 ±0.1	40 ± 6	-74 ± 1	-53 ± 2	18 ± 3	25 ± 5	98 ± 7	25 ± 5[£]
	1 μM	69 ± 11	0.9 ±0.1	37 ± 6	§-69 ± 2	§-49 ± 2	£15 ± 5	§15 ± 7	§85 ± 9	23 ± 5[£]
	10 μM	0	-	-	-	-	-	-	-	-

[1][Nif] = nifedipine concentration applied; AP/min = action potentials/min; DDR = diastolic depolarization rate; MDP = maximum diastolic potential; Thr =threshold; APA = action potential amplitude; APD_{50} = action potential at 50% repolarization; Con = control. [2]One of 7 cells arrested spontaneous beating at 1 nM. [3]Zero indicates spontaneously beating was arrested by indicated [Nif]. [4]Value is not available due to cessation of spontaneous beating. *Value is significantly different from Con. §Value is significantly different from other [Nifedipine], except for 100 nM. £Value is significantly different from Con and 1 nM.

-58 ± 1 mV, which was similar to the firing threshold under control condition (-56 ± 2 mV; **Figure 2B**, bottom; **Figure 2C**).

Functional properties of $I_{Ca,L}$ in nodal-like and atrial-like ESC-CMs

To investigate further the role of $I_{Ca,L}$ in ESC-CMs, nifedipine-sensitive calcium currents were measured in each cell type. The I-V relationship of the nifedipine-sensitive current is illustrated in **Figure 3A**. Nodal-like cells (n=4) had an average current density of 4.4 ± 1.3 pA/pF that peaked at -20 mV while atrial-like cells (n=4) had an average current density of 4.0 ± 0.6 pA/pF that peaked at -10 or 0 mV. **Figure 3C** shows representative nifedipine-sensitive current traces at -20 mV or 0 mV test potential for nodal-like (left panels) and atrial-like cells (center panels).

Boltzmann fitting to the activation curve indicated a detectable nifedipine-sensitive current at -50 mV in both cell types, suggesting that the activation threshold was negative to -50 mV. The activation curve in **Figure 3B** shows that the voltage at half-maximum activation ($V_{0.5act}$) in the nodal-like cells (-30 ± 1 mV) was significantly more hyperpolarized than in atrial-like cells (-20 ± 3 mV; P<0.05). The slope of activation (*k*) was similar in nodal-like (7 ± 1) and atrial-like (8 ± 1) ESC-CMs.

To compare $I_{Ca,L}$ inactivation kinetics between the two phenotypes, the inactivation phase of the nifedipine-sensitive current was fit with a single-exponential equation. As shown in the superimposed normalized nifedipine sensitive currents in **Figure 3C** (right panels), $I_{Ca,L}$ inactivation was slower in nodal-like than atrial-like ESC-CMs at -20 mV or at 0 mV test potential. Consistent with this observation is the fact that the inactivation time constants (τ) recorded in nodal-like cells (48 ± 5 ms at -20 mV and 57 ± 6 ms at 0 mV) were significantly longer than those recorded in atrial-like cells (20 ± 3 ms at -20 mV; and 29 ± 3 ms at 0 mV; p<0.05; **Figure 3D**).

Discussion

Nifedipine sensitivity is cardiac phenotype dependent

Our studies in EBs and isolated ESC-CMs showed that $I_{Ca,L}$ is essential for maintaining automaticity since nifedipine treatment arrested spontaneous beating in all preparations. However, sensitivity to the blocker was directly dependent on the cardiac phenotype. The automatic rhythm of nodal-like ESC-CMs was consistently abolished by 1 or 10 nM nifedipine. A previous study showed that nifedipine at such low concentrations does not affect other ion channels present in cardiomyocytes [8]. Therefore, the reduction in action potential frequency and DDR, and the prolongation in cycle length suggest that I_{Ca} plays a central role in regulating automaticity and chronotropy of nodal-like ESC-CMs. The membrane potential recorded upon cessation of spontaneous activity in the pres-

ence of nifedipine remained constant at 10 mV below the firing threshold, suggesting that the Ca^{2+} channel blockade prevented cells from reaching threshold potentials.

In contrast, the spontaneous activity of atrial-like cells was relatively nifedipine-resistant, requiring 10 μM to abolish automaticity. At sub-maximal concentrations, the blocker had no significant effect on the firing rate, cycle length, or DDR. The lack of $I_{Ca,L}$ contribution to diastolic depolarization may be attributed to these cells having a more hyperpolarized AP threshold (-57 ± 2 mV) than the $I_{Ca,L}$ activation threshold; $V_{0.5act}$ values in atrial-like cells (-20 ± 3 mV) were significantly more positive than those recorded in nodal-like cells (-30 ± 1 mV; $p<0.05$).

Additionally, spontaneous activity consistently ceased at membrane potentials near the firing threshold, suggesting that the $I_{Ca,L}$ blockade did not prohibit atrial-like cells from reaching their firing threshold. Nifedipine at sub-maximal concentrations did, however, significantly reduce the upstroke velocity, overshoot, APA, and APD_{50}. The greater upstroke velocity of atrial-like cells compared to nodal-like cells, suggests that $I_{Ca,L}$ is not the sole contributor to this phase. In agreement, all ESC-CMs tested in this study also expressed the sodium current (I_{Na}) that required 30 μM TTX for a full block (data not shown) indicating that $Na_V1.5$ likely contributes significantly to the AP upstroke. I_{Na} has also been reported in other studies on cardiac pacemakers [20, 21] including spontaneously beating mouse [2] and human ESC-CMs [22].

As in isolated ESC-CMs, EBs showed variable sensitivity to nifedipine. Previous work shows that the individual EB is not composed of a heterogeneous mix of cardiomyocytes, but rather populated by one predominating cardiac cell type [1]. Differences in nifedipine sensitivity of EBs could be linked to the predominant expression of a specific cardiac phenotype within the individual preparation. Based on our findings, EBs expressing mainly nodal-like cells would be more susceptible to a nifedipine block than those populated with predominantly nifedipine-resistant atrial-like cells.

Variation in nifedipine sensitivity has also been observed in studies of pacemakers in the SA node where the spontaneous activity of pacemakers situated at the center of the SA node were more sensitive to nifedipine than pacemakers located at the periphery, which exhibited a more hyperpolarized MDP and rapid upstroke velocity [23]. These central SA nodal pacemakers share similarities with nodal-like ESC-CMs observed in this study. Alternatively, the variation in nifedipine sensitivity could be secondary to differences in membrane potentials between the two cardiac subtypes. Nifedipine has been shown to preferentially bind inactivated L-type Ca^{2+} channels with high affinity [24]. The MDP and firing threshold of nodal-like cells were significantly more depolarized than those observed in atrial-like cells, which may explain their higher sensitivity to nifedipine. However, spontaneously beating cells undergo cyclical depolarization and repolarization, which should provide nifedipine ample opportunity to bind inactivated channels during upstroke or repolarization.

Distinct roles of $I_{Ca,L}$ in nodal-like and atrial-like ESC-CMs

The differential effect of nifedipine on the automaticity of the two cardiac phenotypes suggests differences in the functional properties of L-type calcium channels. Although both cell types registered similar nifedipine-sensitive current densities, the I-V relationship, the voltage dependency of $I_{Ca,L}$ activation, and $V_{0.5act}$ were consistently left-shifted by about 10 mV in nodal-like ESC-CMs. This shifted I-V relationship shares similarities with the changes in $I_{Ca,L}$ properties recorded in SA nodal cells isolated from wild-type and $Ca_v1.3^{-/-}$ mice that predominately expresses the $Ca_v1.2$ channel [9]; abolition of the $Ca_v1.3$ gene positively shifted the peak current and the $V_{0.5act}$ of $I_{Ca,L}$. Similar results were obtained in heterologous systems expressing recombinant $Ca_v1.2$ and $Ca_v1.3$ channels [25].

In addition, $Ca_v1.3$ displayed significantly slower inactivation kinetics, which is thought to enable the low threshold channel to mediate long lasting Ca^{2+} influx during weak depolarization and be available in the voltage range spanning the diastolic depolarization [25]. Consistent with this observation, our findings of a significantly slower $I_{Ca,L}$ inactivation and a significantly more hyperpolarized $I_{Ca,L}$ $V_{0.5act}$ in nodal-like cells suggests that $I_{Ca,L}$ contributes to the DDR in these cells. In atrial-like cells, the

lack of effect on the firing rate and DDR by nifedipine suggest that other ionic currents such as I_f or $I_{Ca,T}$ play a major role in diastolic depolarization. In agreement, we have previously recorded the I_f current in this same stem cell line R1 and demonstrated that this current also plays a major role in controlling automaticity [17].

Conclusion

The present study reveals that $I_{Ca,L}$ contributed differentially to the automaticity of ESC-CM subtypes. A low threshold component $I_{Ca,L}$ regulated diastolic depolarization and chronotropy in nodal-like ESC-CMs; these parameters were highly sensitive to nifedipine. In contrast, the automatic activity of atrial-like ESC-CMs was relatively nifedipine-resistant; the high threshold component $I_{Ca,L}$ in these cells mainly contributed to the upstroke of the action potential, linking excitation to contraction. These findings may be of particular relevance to the use of cell-based therapy to repair injured cardiac tissues. Specifically, nifedipine is commonly prescribed as an anti-anginal and anti-hypertensive drug [26], and this could interfere with the activity of ESC-derived cardiomyocytes intended for pacemaking. On the other hand, very low doses of nifedipine could be used to eliminate undesired spontaneous activity of nodal-like cardiomyocytes present in ESC-CMs intended for myocardial repair.

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Disclosure of conflict of interest

None.

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