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A key role of TRPC channels in the regulation of electromechanical activity of the developing heart

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Aims It is well established that dysfunction of voltage-dependent ion channels results in arrhythmias and conduction disturbances in the foetal and adult heart. However, the involvement of voltage-insensitive cationic TRPC (transient receptor potential canonical) channels remains unclear. We assessed the hypothesis that TRPC channels play a crucial role in the spontaneous activity of the developing heart.

Methods and results TRPC isoforms were investigated in isolated hearts obtained from 4-day-old chick embryos. Using RT-PCR, western blotting and co-immunoprecipitation, we report for the first time that TRPC1, 3, 4, 5, 6, and 7 isoforms are expressed at the mRNA and protein levels and that they can form a macromolecular complex with the α1C subunit of the L-type voltage-gated calcium channel (Cav1.2) in atria and ventricle. Using *ex vivo* electrocardiograms, electrograms of isolated atria and ventricle and ventricular mechanograms, we found that inhibition of TRPC channels by SKF-96365 leads to negative chrono-, dromo-, and inotropic effects, prolongs the QT interval, and provokes first- and second-degree atrioventricular blocks. Pyr3, a specific antagonist of TRPC3, affected essentially atrioventricular conduction. On the other hand, specific blockade of the L-type calcium channel with nifedipine rapidly stopped ventricular contractile activity without affecting rhythmic electrical activity.

Conclusions These results give new insights into the key role that TRPC channels, via interaction with the Cav1.2 channel, play in regulation of cardiac pacemaking, conduction, ventricular activity, and contractility during cardiogenesis.

Keywords TRPC channels • Cav1.2 channel • Electromechanical activity • Developing heart

1. Introduction

Intracellular calcium signalling plays an important role in the cardiac physiology and development.[1] In the adult mammalian myocardium, depolarizing current resulting in a relatively small calcium influx across the sarcolemma contributes to the initiation of action potential in atrial pacemaker cells or to the plateau phase in ventricular myocytes. This entry is followed by a further calcium release from the sarcoplasmic reticulum (SR) via a mechanism termed Ca^{2+}-induced Ca^{2+} release, which leads to contraction, the so-called excitation–contraction (EC) coupling.[2] However, in the mammalian embryo/foetus, calcium appears to come exclusively through sarcolemmal calcium influx because of immature SR.[3]

In contrast, in the embryonic chick heart, all the components necessary for a functioning SR and EC coupling are expressed early such as ryanodine receptor (RyR2), SR Ca^{2+}-ATPase (SERCA2), L- and T-type voltage-dependent calcium channels, and Na^+/Ca^{2+} exchanger.[4–8] Additionally, Brotto and Creazzo have estimated in the embryonic chick heart that 70% of the calcium available for the transient comes through sarcolemmal calcium channels, while the remainder is from SR calcium release.[7]

The traditional view of the electrical and contractile activities of the developing and adult heart is the result of cooperative activity of series of sarcolemmal voltage-dependent channels generating spontaneous action potentials and subsequently muscle contraction (as reviewed in DiFrancesco[9]). It becomes clear that cyclical release of Ca^{2+} from SR stores occurs in pacemaker cells and that IP_3R- or RyR-mediated calcium release has a novel role in regulating cardiac pacemaker activity during early development.[10,11] Preliminary reports suggest that another pathway involving TRPC (transient receptor potential canonical)-dependent calcium entry related to SR Ca^{2+} depletion and to receptor-coupled phospholipase C (PLC) activation contributes to pacemaker activity in adult toad and mouse pacemaker node.[12,13]

The membrane subfamily of TRPC channels, composed of seven members (TRPC1-7), is thought to assemble as homo- or hetero-tetramers to form non-selective cation channels permeable to Na^+

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and Ca^{2+}. TRPC channels contribute in a widespread number of non-excitable and excitable cells to non-voltage-dependent cation entry with many physiological roles or pathological consequences. They are activated (i) in response to G-protein receptor stimulation through PLC signalling by IP_3 or by diacylglycerol referred to receptor-operated channels,[14,15] (ii) by depletion of internal calcium stores referred to SOCs (store-operated channels),[16] and (iii) in response to increased membrane stretch termed stretch-activated channels.[17] In addition to conventional SOCs, calcium entry called excitation-coupled Ca^{2+} entry (ECCE) through dihydropyridines receptor (DHPR)/TRPC channels initiated by membrane depolarization has been reported in skeletal muscle.[18,19]

The identification of TRPC channels was a new starting point in the search for their function in skeletal muscle (as reviewed in Sabourin *et al.*[20]) and their involvement in cardiovascular function and heart diseases.

All TRPC isoforms, excepted TRPC5, are expressed in the adult mouse heart[21,22] and a tight control of calcium homeostasis has been attributed to SOCs in neonatal and adult cardiomyocytes.[23,24] Indeed, uncontrolled TRPC-dependent calcium entry is involved in the development of cardiac hypertrophy and failure (as reviewed in Eder and Molkentin[25]), dilated cardiomyopathy, muscular dystrophy, and arrhythmogenesis.[26–28]

In light of the growing evidence linking TRPC channels to normal function of adult cardiomyocytes, we first examined TRPC genes and proteins expression during cardiogenesis. We assessed the crucial role played by TRPC channels in the spontaneous activity of the 4-day-old embryonic chick heart model, free of extrinsic innervation, and neurohumoral influences. The present study shows for the first time that TRPC channels play a key role in regulation of pacemaking, conduction, and contractility in the developing heart.

2. Methods

2.1 Experimental model

Fertilized eggs from Lohman Brown hens were incubated during 96 h at 38°C and 95% relative humidity to obtain stage 24HH embryos (according to Hamburger and Hamilton, 1951) corresponding to embryonic Day 4 (ED4). Hearts were carefully excised from embryos and dissected to isolate the whole heart or to separate atria from ventricle with outflow tract in place to limit tissue injury.

2.2 Reagents and antibodies

Nifedipine [L-type calcium channel (LCC) inhibitor], SKF-96365 (1-[β-(3-(4-methoxyphenyl)propoxy)-4-methoxyphenethyl]-1H-imidazole hydrochloride, 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy] ethyl]imidazole named SKF, TRPC channels inhibitor), Pyr3 (inhibitor of TRPC3), and CPA (SR Ca^{2+}-ATPase inhibitor) were from Sigma-Aldrich (Sigma-Aldrich, Buchs, Switzerland). Rabbit polyclonal antibodies used against TRPC1, 3, 4, 5, 6, and Cav1.2 were from Alomone (Alomone labs, Jerusalem, Israel). Goat polyclonal anti-TRPC7 was from Everest Biotech (Everest Biotech, Oxfordshire, UK). The rabbit polyclonal antibody against GAPDH was from Abcam (Abcam, Cambridge, UK). Secondary antibodies for western blot were horseradish peroxidase-conjugated donkey anti-rabbit IgG (GE healthcare, Glattbrugg, Switzerland) and horseradish peroxidase-conjugated donkey anti-goat IgG (Santa cruz Biotechnology, Inc., Heidelberg, Germany). Purified Goat and Rabbit IgG were from Sigma-Aldrich.

2.3 Western blot

Embryonic hearts were dissected into atria, ventricle, and outflow tract. In brief, samples were denatured, 30 μg protein was loaded per lane, separated on SDS–polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked and probed overnight with antibodies against TRPC1, 3, 4, 5, 6, 7, and Cav1.2 or GAPDH. A control fusion protein was used as control antigen. After washes, the membranes were incubated with the secondary anti-rabbit or anti-goat IgG. Immunoreactive bands were detected with enhanced chemiluminescent procedure. See Supplementary material online for detailed methods.

2.4 Co-immunoprecipitation

After lysis, 2 μg of antibody was added to a volume of lysates containing 300 μg of atrial or ventricular proteins diluted with NET solubilization buffer and incubated at 4°C overnight with constant mixing. Then, the protein–antibody complex was incubated with Protein A Sepharose. The immune complexes were collected by centrifugation and washed in NET. After denaturing, samples were subjected to SDS–PAGE. See Supplementary material online for detailed methods.

2.5 PCR amplification

Messenger RNA isolation from embryonic heart tissue was performed using the RNeasy Plus Mini kit. RT-PCR was conducted using SuperScript III one-step RT-PCR with platinum *Taq* in a Biometra TRIO-thermoblock. See Supplementary material online for detailed methods.

2.6 *Ex vivo* mounting of the heart

Spontaneously beating hearts or isolated atria and ventricle were placed in the culture compartment of a stainless steel airtight chamber maintained at 37.5°C as described elsewhere in detail.[29] See Supplementary material online for detailed methods.

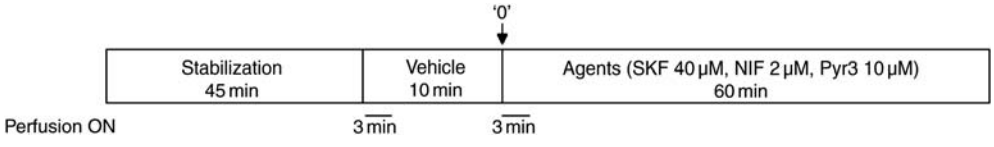
2.7 Recording of electrical and contractile activities

ECG, atrial and ventricular electrograms (EG), and apical shortening of the ventricle were recorded simultaneously and continuously throughout *ex vivo* experiments as previously described.[29] See Supplementary material online for detailed methods.

2.8 Experimental protocol

Once mounted in the chamber, the hearts were first stabilized during 45 min under normoxia at 37.5°C. Then, the medium was replaced by the vehicle using remote-controlled push–pull syringes, in order to assess a possible unspecific effect of the vehicle perfusion on cardiac activity. In the control group, only the medium/vehicle was introduced. Time '0' is the time point just before introduction of the pharmacological agents. ECG, EG, and ventricular shortening were continuously recorded throughout all experiments.

The experimental protocol is represented below.



2.9 Statistical analysis

All values are reported as mean ± SEM. The non-parametric Mann–Whitney test was used for dose–response curves of SKF and calculation of QTc. The significance of any difference between two groups was assessed with one-way repeated measures ANOVA completed by Tukey's *post hoc* test for ECG, EG, and contractile experiments. The statistical significance was defined by a value of $P < 0.05$ ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

3. Results

3.1 Expression of TRPC genes and proteins in the embryonic heart

We used RT-PCR to examine mRNA expression of the seven known mammalian TRPC isoforms in the embryonic chick heart (ED4). We used TRPC subunit PCR products that were initially validated and confirmed by sequencing in embryonic (ED8) chick retina RNA, a tissue known to express all TRPC isoforms except TRPC2, which is expressed only in mice.[30] TRPC1, 3, 4, 5, 6, and 7 transcripts were detected in the whole heart as well as in retina (*Figure 1A*).

Antibodies raised against TRPC1, 3, 4, 5, 6, and 7 were used to evaluate by western blot the expression of these proteins in the embryonic heart. TRPC1, 3, 4, 5, 6, and 7 proteins were expressed in atria, ventricle and outflow tract (*Figure 1B*). The densitometric analysis of the TRPCs normalized to GAPDH showed that each TRPC isoform was expressed at the same level in atria and ventricle (see Supplementary material online, *Figure S1*). For each TRPC isoform, a fusion protein was used as control antigen. Anti-TRPCs antibodies were preincubated with their relevant antigens for negative control (see Supplementary material online, *Figure S2A*). Moreover, immunoprecipitation for each isoform was performed showing a band corresponding to respective

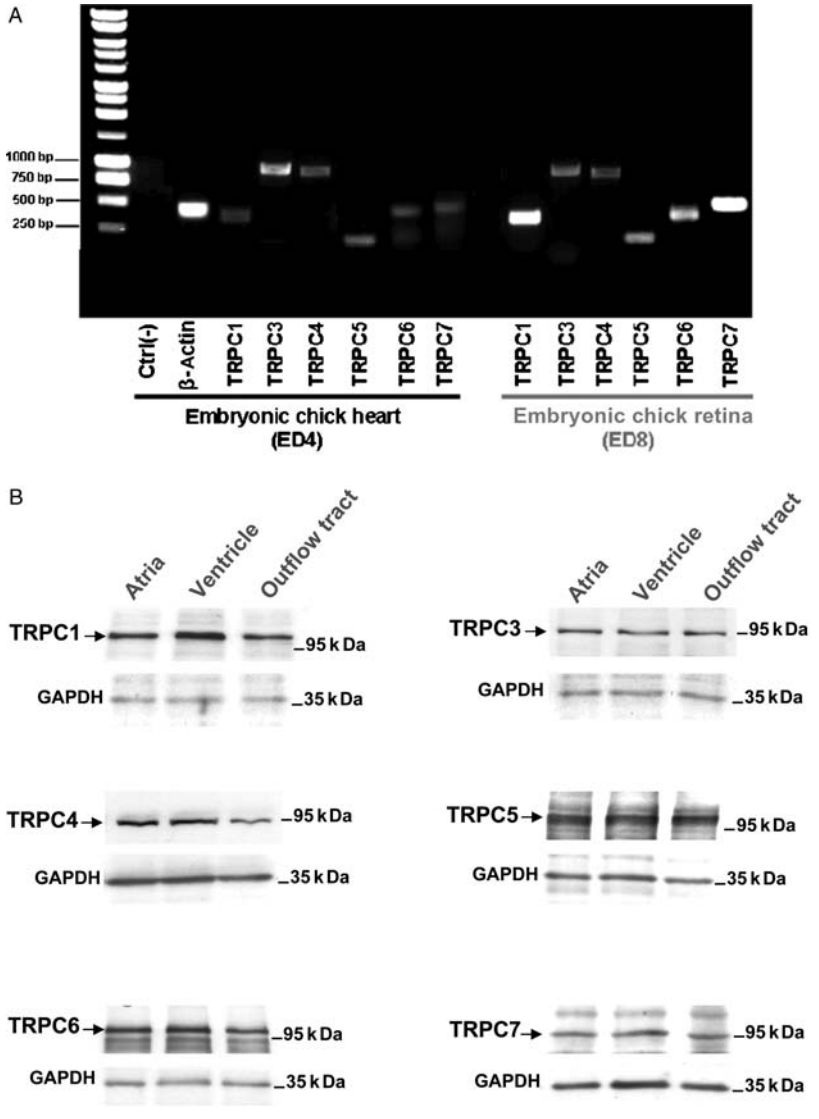


Figure 1 Genes and proteins expression of TRPC isoforms in the embryonic chick heart (ED4) and retina (ED8). (*A*) TRPC1, TRPC3-7 mRNAs were identified by RT-PCR. PCR products of the predicted sizes for the different TRPC species were between 150 and 950 bp. β-Actin was amplified as a positive control. The negative control [ctrl(−)] contained water instead of DNA. (*B*) TRPC1, TRPC3-7 proteins are all expressed in atria, ventricle, and outflow tract ($n = 3–4$). GAPDH was used as a control of equal loading of proteins.

TRPC isoforms at the same molecular weight as in total lysates (see Supplementary material online, *Figure S2B*).

3.2 Dose–response for SKF-96365 (SKF), the traditional inhibitor of TRPC channels

SKF is widely used as a blocker of TRPC channels (as reviewed in Putney[31]). Measurement of beating rates and detection of manifest arrhythmias were performed by visual observation in plastic wells 20 or 40 min after addition of SKF (*Figure 2A–C*). *Figure 2A* shows that concentrations higher than 5 μM of SKF induced arrhythmias (atrioventricular blocks and cardioplegia) in whole hearts with an IC_{50} of 10.3 μM as determined using the Hill equation (see Supplementary material online, *Figure S3A*). It should be noticed that ∼15% of control untreated hearts showed spontaneous arrhythmias but none was arrested in this group.

The intrinsic beating rate of the untreated isolated atria and ventricle was 168 ± 6 b.p.m. ($n = 29$) and 65 ± 6 b.p.m. ($n = 29$), respectively, and remained stable during at least 1 h. In non-arrested atria and ventricle, the atrial and ventricular spontaneous beating rate were progressively reduced and increased by SKF, respectively, in a dose-dependent manner (*Figure 2B*). The dose-dependent and time-dependent effects of SKF on activity of isolated atria and ventricle are presented in *Figure 2C* and *D*, respectively. Atria and ventricle showed a differential sensitivity to TRPC inhibition since SKF at 40 μM arrested 81% of the ventricles and only 36% of the atria after 20 min (*Figure 2C*). The IC_{50} for SKF determined in atria and ventricles from the dose–response curves obtained after 40 min was 25.3 and 8.7 μM, respectively (see Supplementary material online, *Figure S3B*). *Figure 2D* shows that SKF at 40 μM stopped ventricle more rapidly than atria, whereas neither control atria nor ventricles were arrested.

3.3 Interaction between TRPC channels and α1C subunit of the L-type calcium channel (LCC)

Both TRPC channels and LCC were partially inhibited by SKF (10 μM) and nifedipine (NIF 50 nM), respectively, at concentrations which, when administrated alone, did not alter significantly atrial rate. *Figure 3A* shows that atrial firing rate decreased by 62% after 20 min when SKF was combined with NIF compared with treatment with NIF or SKF alone suggesting a synergetic effect. It should be mentioned that the combined treatment not only reduced atrial beating rate, but also markedly increased incidence of arrhythmias and cardioplegia (*Figure 3B*), strongly suggesting a functional interrelation between these two types of channels.

Furthermore, the formation of a complex between TRPC channels and the α1C subunit of LCC was explored in the developing heart because it has been established that TRPC3 channel is required for full gain EC coupling via their association with DHPR and RyR in adult skeletal muscle.[19]

Figure 3C represents immunoblots revealed by Cav1.2 antibody in atria and ventricle. A band of ∼240 kDa, corresponding to α1C subunit of LCC, was detected in the anti-TRPC1, anti-TRPC3, anti-TRPC4, anti-TRPC5, anti-TRPC6, and anti-TRPC7 precipitates. Conversely, immunoblots revealed by TRPC1 and TRPC3-7 antibodies detected bands of ∼110, 95, 100, 110, 120, and 95 kDa

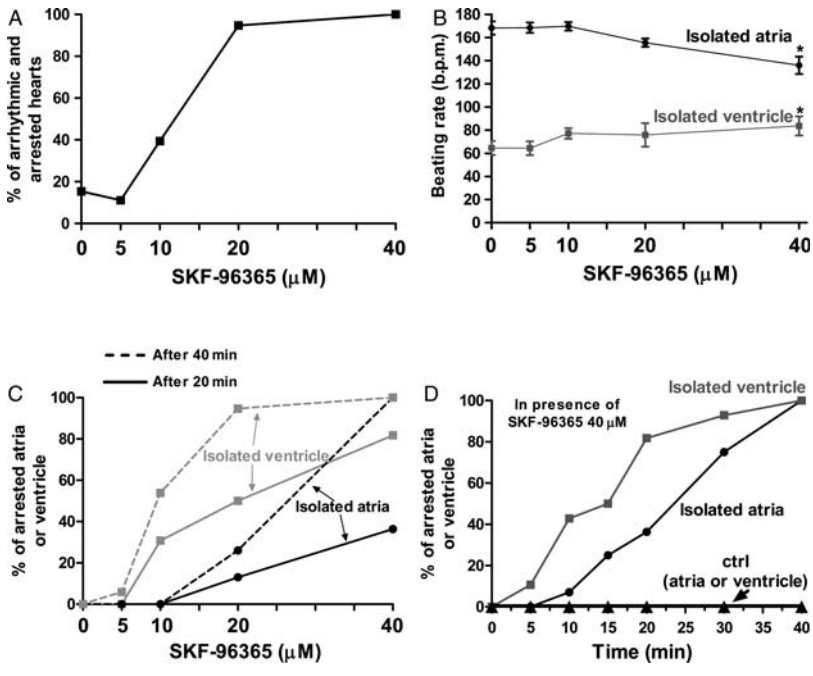


Figure 2 Effect of TRPC channels inhibition on cardiac function. (*A*) Dose–response curve of SKF in whole hearts after 20 min ($n = 19–76$). (*B*) Dose–response curves of isolated atria and ventricles that were non-arrested by SKF after 20 min. 0 μM, control with the vehicle; b.p.m., beats per min; $^*P < 0.05$ vs. 0 μM ($n = 6–29$). (*C*) Dose–response curves of isolated atria and ventricles treated 20 min (solid lines) or 40 min (dotted lines) with SKF ($n = 17–33$). (*D*) Time-dependent effect of SKF 40 μM on atrial and ventricular activities; ctrl, controls of atria and ventricle in vehicle ($n = 17–33$).

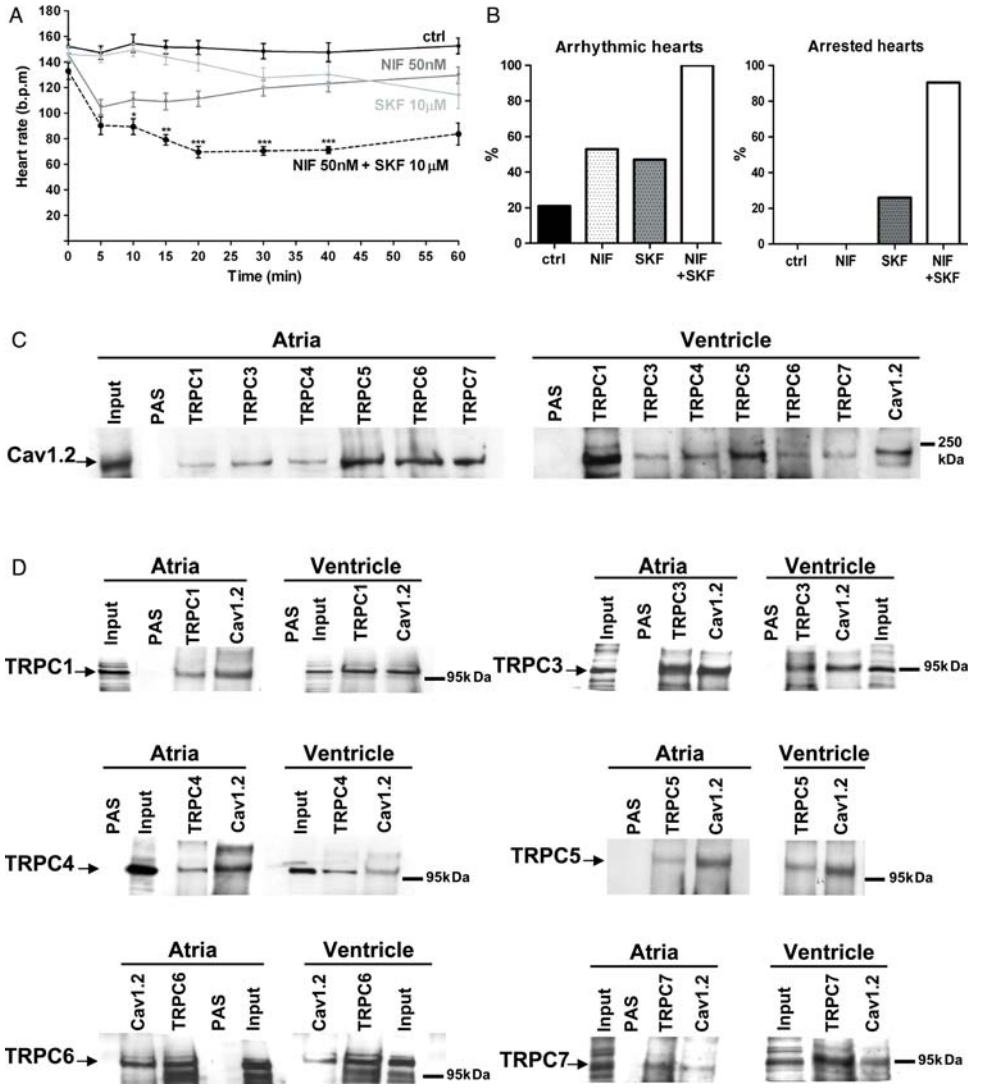


Figure 3 (*A*–*B*) Synergetic effects of SKF and NIF on arrhythmias. (*A*) Time dependency of beating rate of hearts treated with SKF (10 μM) or nifedipine (NIF 50 nM) alone or combined. b.p.m., beats per min ($n = 12–34$). (*B*) Histogram showing the synergetic and detrimental effect of SKF combined with NIF after 60 min (% of arrhythmic or arrested hearts). (*C*–*D*) All isoforms of TRPC channels formed a macromolecular complex with Cav1.2 in the embryonic heart. (*C*) Lysates (input lane) from atria and ventricles were incubated with antibodies against TRPC1 and TRPC3-7. Western blots of the immunoprecipitated proteins were probed with antibody against Cav1.2; PAS, Protein A Sepharose. (*D*) Lysates from atria and ventricle were incubated with antibodies against TRPC1, TRPC3-7, and Cav1.2. Western blots of the immunoprecipitated proteins were probed with antibodies against all TRPC proteins.

corresponding to the molecular weight of TRPC1, 3, 4, 5, 6, and 7, respectively, with anti-Cav1.2 precipitates (*Figure 3D*). These results clearly show that endogenous TRPC1 and TRPC3-7 may form multiple complexes with the endogenous α1C subunit of Cav1.2 channel in atria and ventricle.

3.4 Involvement of TRPC and L-type calcium channels (LCC) in cardiac electrical activity

We explored the contribution of TRPC channels to electrical activity of the developing heart. The effect of SKF, Pyr3, a specific blocker of TRPC3,[32] or NIF was determined on atrial and ventricular beating rate, atrioventricular conduction (PR interval), ventricular activity (QTp duration), and intra-ventricular conduction (QRS complex width). We focused on the 15 min time point at which SKF had the most pronounced effect on cardiac activity. Additionally, various types of arrhythmias were identified. It should be noticed that the corresponding vehicle had no effect on the electrical parameters of the whole heart, isolated atria, or ventricle for at least 60 min (not shown). The important changes in ECG and EG morphology induced by SKF, Pyr3, and NIF are illustrated in *Figure 4* and the altered functional parameters reported in *Table 1*.

In six out of seven experiments, inhibition of TRPC channels by SKF induced rapidly and transiently (during the first 15 min) second-degree atrioventricular blocks, essentially in the form of Mobitz type I (Wenckebach phenomenon) characterized by a progressive

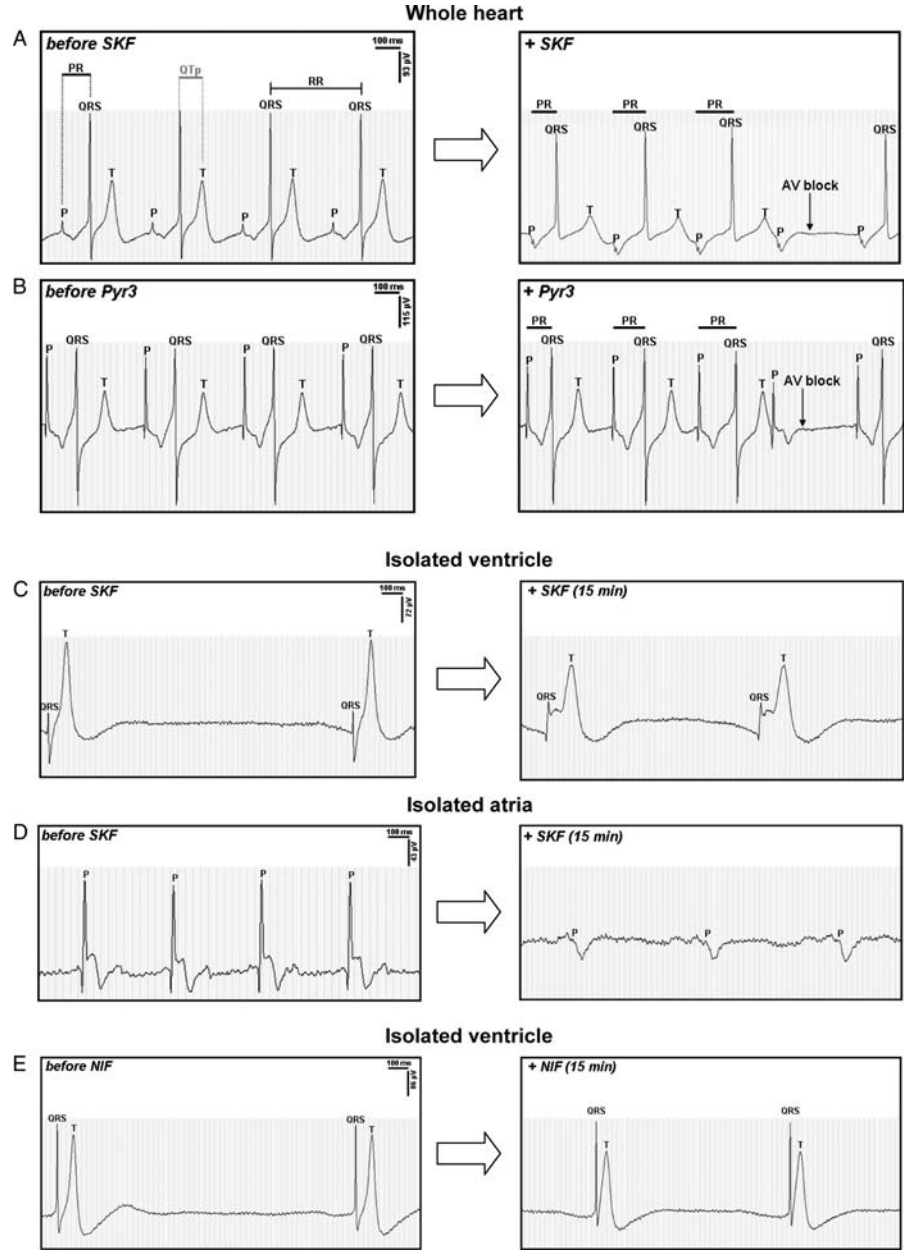


Figure 4 Representative ECG recordings showing the P, QRS and T components of the embryonic heart spontaneously beating *ex vivo* treated with SKF 40 μM (*A*) or Pyr3 10 μM (*B*). SKF and Pyr3 provoked the same type of arrhythmia: Mobitz type I second-degree atrioventricular block. Representative electrograms of isolated ventricle (*C*) and atria (*D*) before and after 15 min treatment with SKF 40 μM. (*E*) Ventricular electrogram before and after 15 min treatment with NIF 2 μM.

lengthening of the PR interval followed by a dropped QRS without alteration of the atrial rhythm (*Figure 4A*). This episode of Wenckebach phenomenon was followed by first-degree atrioventricular block characterized by a prolonged PR interval (+27%) and a markedly increase in QTp duration by 35% (*Table 1*). The temporal pattern of ECG morphology under control conditions and in the presence of SKF showed clearly the significant prolongation of PR and QTp intervals induced by TRPC inhibition throughout 60 min (see Supplementary material online, *Figure S4A* and *S4B*).

Pyr3 also induced immediately and transiently Wenckebach phenomenon in all experiments ($n = 4$; *Figure 4B*) but, in contrast to SKF, had no additional effect on the measured parameters.

The prolongation of QTp induced by inhibition of TRPC channels was confirmed in isolated ventricle (*Figure 4C*; *Table 1*). Moreover, SKF widened significantly the QRS complex after 15 min, suggesting a reduction in intra-ventricular conduction (*Table 1*).

Importantly, exposure of isolated ventricle to SKF in combination with NIF (2 μM) prevented the SKF-induced lengthening of QTp (*Table 1*), suggesting that LCC were overactivated. Moreover, SKF combined with NIF did not prevent the SKF-induced widening of QRS complex (*Table 1*). This last result suggests that the regulation of intra-ventricular conduction is dependent on TRPC channels.

High concentration of SKF (40 μM) reduced significantly the beating rate of whole heart and isolated atria at 15 min (*Figure 4D*;

Table 1 Functional parameters before (vehicle) and after 15 min of SKF 40 μM and NIF 2 μM alone or combined

		Vehicle	SKF	NIF	SKF +NIF
Pacemaker					
Beating rate (b.p.m.)	Whole heart	162 ± 13 (5)	106 ± 15 * (5)	ND	
	Isolated atria	190 ± 15 (3)	137 ± 11 * (3)	ND	
	Isolated ventricle	49 ± 3 (6)	64 ± 3 *** (6)	—	
		46 ± 6 (4)	—	58 ± 6 (4)	
Conduction					
PR interval (ms)	Whole heart	117 ± 9 (5)	148 ± 13 * (5)	ND	
Duration of electrical activation					
QTp duration (ms)	Whole heart	105 ± 5 (5)	142 ± 4 ** (5)	ND	
	Isolated ventricle	94 ± 6 (6)	141 ± 10 *** (6)	—	
		94 ± 7 (4)	—	59 ± 1 *** (4)	
		115 ± 4 (4)	142 ± 10 *** (4)	—	110 ± 5 (4)
QTc value (ms)	Whole heart	268 ± 7 (4)	308 ± 12 * (4)		
	Isolated ventricle	136 ± 10 (6)	240 ± 15 * (6)		
QRS width (ms)	Isolated ventricle	11.4 ± 0.9 (6)	19.2 ± 1.2 *** (6)	—	
		10.0 ± 1.3 (4)	—	11.1 ± 1.7 (4)	
		9.6 ± 1.2 (4)	17.9 ± 2.1 * (4)	—	20.3 ± 2.3* (4)

( ), number of hearts, isolated atria, or ventricles investigated.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. vehicle.

Table 1). On the contrary, SKF had a positive chronotropic effect on isolated ventricle (*Figure 4C*; *Table 1*).

The QT interval corrected for heart rate (QTc) was calculated with Bazett's formula. SKF increased significantly QTc of whole hearts (+15%) and isolated ventricles (+76%), indicating an increased risk of ventricular arrhythmias when TRPC channels are inhibited (*Table 1*).

SKF is usually identified as a selective blocker of TRPC channels but recently, two reports suggest that SKF can also inhibit L- and T-type calcium channels (TCC).[33,34] Thus, to validate the specific effect of SKF on TRPC channels, we performed the same experiments in isolated ventricle with LCC and TCC inhibitors, NIF, and mibefradil, respectively. As expected, inhibition of LCC with NIF 2 μM induced a progressive and significant shortening of QTp (*Figure 4E*) and had no effect on QRS width (*Table 1*). Additionally, mibefradil 2 μM transiently decreased QTp duration by 17% (before mibefradil, 107 ± 11 ms; +mibefradil (5 min), 89 ± 8 ms; $n = 5$; ***$P < 0.001$; see Supplementary material online, *Figure S5*) suggesting a minor role of TCC in spontaneous electrical activity of the embryonic chick heart. Thus, these results show that NIF, mibefradil, and SKF have distinct targets according to their distinct effects on QTp duration and QRS width. SKF inhibits essentially TRPC channels according to the results.

3.5 Involvement of TRPC and L-type calcium channels (LCC) in myocardial contractility

In order to investigate the relative contribution of LCC and TRPC channels to ventricular contractility, EG and shortening of isolated ventricle were recorded simultaneously in the presence of NIF or SKF as shown in *Figure 5A* and *B*. The specific contribution of TRPC3 to ventricular contractility was also explored in whole heart with the specific inhibitor, Pyr3 (*Figure 5C*). SKF (40 μM) and NIF (2 μM) had a negative inotropic effect (*Figure 5A*, *B*, and *D*), whereas Pyr3 (10 μM) had no effect on ventricular shortening (*Figure 5C* and *D*), suggesting that TRPC3 isoform is not involved in contractility.

NIF progressively prolonged the ventricular electromechanical delay (reflecting the EC coupling) after 5 min [before NIF, 16.0 ± 0.8 ms; +NIF (5 min), 25.1 ± 1.9 ms; $n = 3$; **$P < 0.01$] and finally suppressed ventricular shortening within 35 min (*Figure 5A* and *D*), whereas SKF reduced shortening by 31% after 5 min and 50% after 60 min (*Figure 5B* and *D*). Interestingly, contractile activity was abolished by NIF, whereas a spontaneous and regular electrical activity persisted (*Figure 5A*). Such a permanent electromechanical dissociation indicates that pacemaking mechanisms in the ventricular cardiomyocytes were not significantly affected by blockade of LCC, whereas the contraction mechanism is strongly dependent on LCC. Inhibition of TRPC channels by SKF altered neither ventricular electromechanical delay [before SKF, 18.1 ± 0.8 ms; $n = 6$; +SKF (5 min), 17.1 ± 0.9 ms; $n = 6$; +SKF (60 min), 17.9 ± 1.5 ms; $n = 3$] nor contraction to relaxation shortening velocities ratio [before SKF, 1.18 ± 0.05; $n = 6$; +SKF (5 min), 1.09 ± 0.05; $n = 6$; +SKF (60 min), 1.12 ± 0.07; $n = 5$].

4. Discussion

4.1 Involvement of TRPC channels in atrial pacemaking and atrioventricular conduction

We found for the first time that TRPC1, 3, 4, 5, 6, and 7 are expressed in atria, ventricle, and outflow tract of the developing chick heart, which suggests a functional role of these isoforms. Indeed, the traditional inhibitor of TRPC channels, SKF, slowed pacemaker firing rate in the whole heart or isolated atria, supporting a role for TRPC channels in atrial pacemaker function. These observations raise the possibility that inward current (Na^+ and Ca^{2+}) through TRPC channels can influence pacemaking rate as suggested in neurons, interstitial cell of Cajal, rat cardiomyocytes, and pacemaker cells.[12,22,35–37] These channels, as

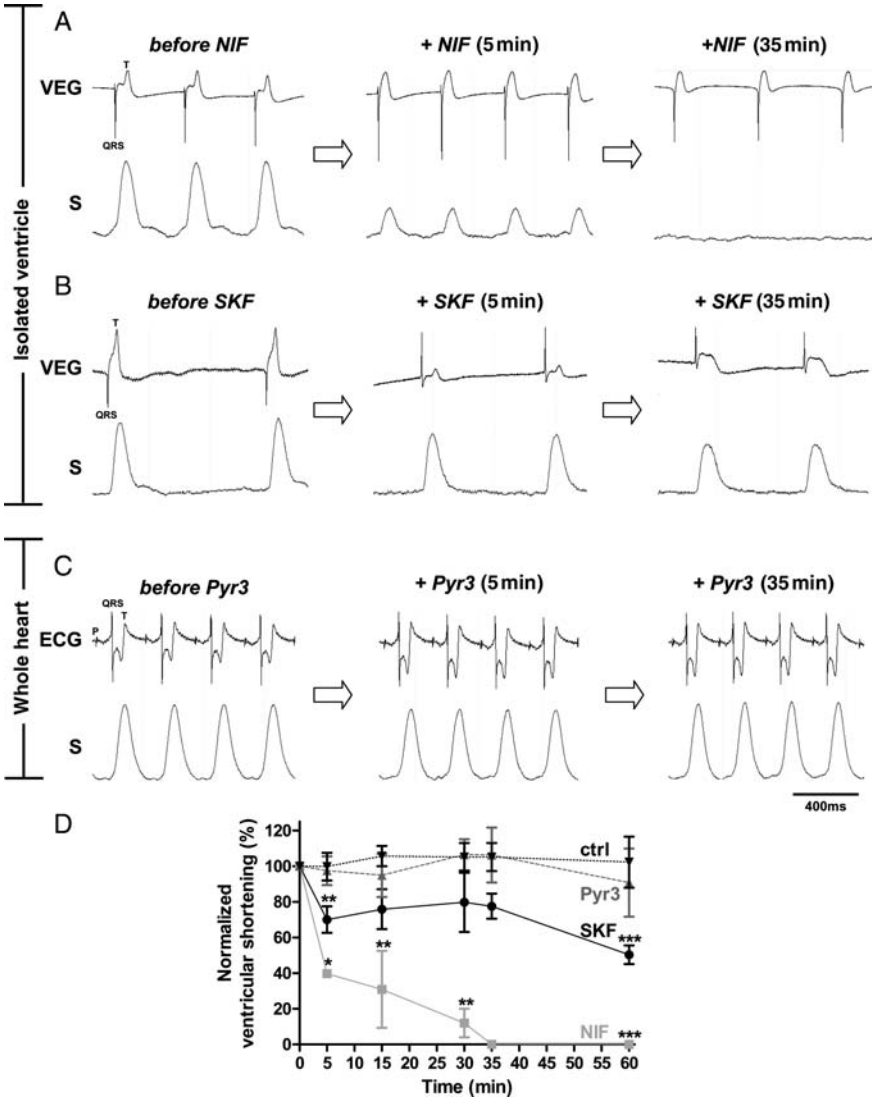


Figure 5 Representative simultaneous recordings of ventricular electrogram (VEG) and apical shortening (S) after 5 and 35 min exposure to NIF (*A*) and SKF (*B*) showing negative inotropic effect ($n = 3–6$). (*C*) The absence of the effect of Pyr3 10 μM on ventricular shortening ($n = 4$). (*D*) Time-dependent effects of NIF 2 μM, Pyr3 10 μM, and SKF 40 μM on ventricular shortening ($n = 3–6$). The shortening is expressed as per cent of the respective control value; ctrl, vehicle.

SOCs, may also be modulated by fluctuations of Ca^{2+} SR stores during each cardiac cycle. Indeed, depletion of Ca^{2+} SR stores either by activation of RyR with ryanodine 10 nM[5] or by inhibition of the SR Ca^{2+}-ATPase with CPA (see Supplementary material online, *Figure S6*) had a positive chronotropic effect in our model. Moreover, partial inhibition of TRPC channels with SKF 5 μM prevented the CPA-induced tachycardia, which strongly supports the concept that TRPC channels act as SOCs (*Figure 6A*).

The interesting finding that all TRPC channels formed multiple complexes with the α1C subunit of Cav1.2 channel can be explained by the fact that distinct TRPC subunits can associate to form heterotetramers, increasing the probability that all TRPC isoforms interacted with Cav1.2 channel. Thus, the voltage-independent TRPC channel may form a functional unit with Cav1.2 channel, this complex being sensitive to membrane depolarization (*Figure 6A*) in a manner comparable with ECCE observed in skeletal muscle.[18] Furthermore, our findings that inhibition of TRPC channels prolonged PR interval and induced mainly Wenckebach phenomenon clearly indicate that these channels play a crucial role in atrioventricular conduction. This suggests that cell membrane excitability and/or gap junction communication could be strongly altered in the embryonic myocardium in the case of TRPC dysfunction. Supporting the hypothesis of a role of TRPC channels in conduction, recent studies demonstrate a functional link between Cx43 protein and TRPC1 in S1P-differentiating myoblasts and a relationship between Cx43 and TRPC3 function in ovine uterine artery endothelial cells.[38,39] Our findings that the specific inhibition of TRPC3 by Pyr3, as well as inhibition of all TRPC channels by SKF, induced essentially Wenckebach phenomenon strongly suggest that TRPC channels and especially TRPC3 can be involved in conduction disturbances. Hence, these channels may represent potential pharmacological targets in order to reduce arrhythmias arising from propagation disturbances. Interestingly, the rhythm and conduction disturbances induced by

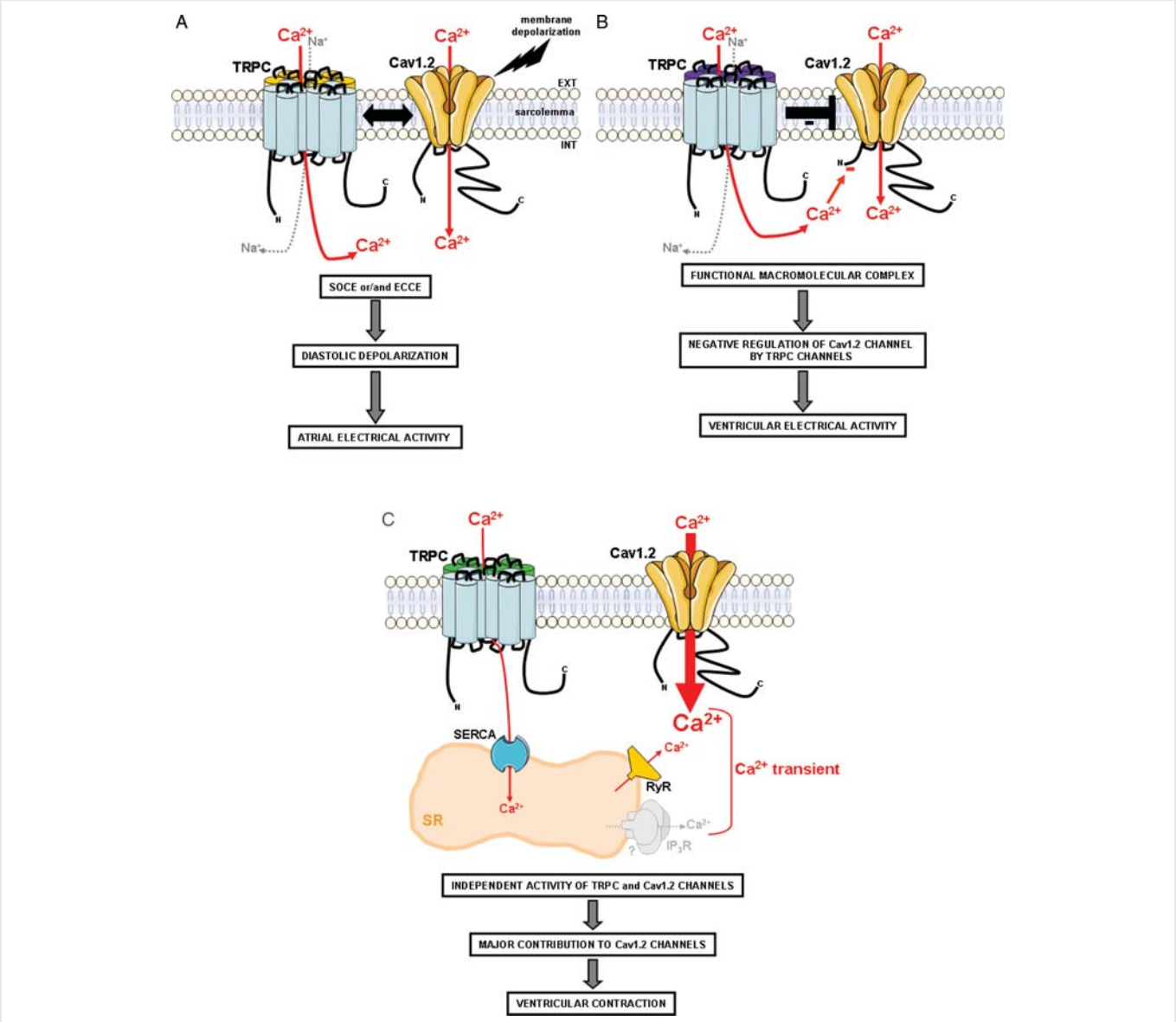


Figure 6 Model based on the present findings illustrating the regulation of electrical and mechanical activities by TRPC and Cav1.2 channels in atria and ventricle of the developing heart. (*A*) TRPC channels can act as SOCs and can form a functional unit with voltage-sensitive Cav1.2 channel, generating inward current and contributing to diastolic depolarization during spontaneous atrial beat. (*B*) Ventricular electrical activity is controlled by the macromolecular complex TRPC/Cav1.2, where TRPC channels, directly or indirectly, negatively regulate Cav1.2 activity. (*C*) Cav1.2 channel predominantly contributes to calcium transient for contraction, whereas TRPC channels play a role in refilling continuously the modest calcium stores of the SR. C, C-terminal; ECCE, excitation-coupled Ca^{2+} Entry; ext, exterior; int, interior; IP_3R, inositol 1,4,5-triphosphate receptor; N, N-terminal; RyR, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SOCE, store-operated Ca^{2+} Entry; SR, sarcoplasmic reticulum; TRPC, transient receptor potential canonical.

inhibition of TRPC channels in our model correspond well to sinus bradycardia, first-degree and second-degree atrioventricular blocks observed in the suffering foetal human heart (as reviewed in Strasburger and Wakai[40] and Sonesson[41]).

4.2 Involvement of TRPC channels in ventricular electrical activity

The possibility that spontaneous and rhythmic activity of the isolated ventricle was essentially attributable to LCC can be ruled out since NIF abolished contraction without affecting EG. As expected, NIF also reduced QTp interval reflecting a shortening of the plateau duration of the action potential in ventricular myocytes.

On the contrary, inhibition of TRPC channels led to the prolongation of QTp interval, widening of QRS complex and ventricular tachycardia. A longer QTc is known to put adult heart at an increased risk for ventricular arrhythmias; it seems also to be the case in the embryonic heart at stage 24HH when TRPC channels are inhibited. Interestingly, ventricular arrhythmias induced by inhibition of TRPC channels in our model correspond well to ventricular tachycardia and long QT syndrome observed in the suffering foetal human heart (as reviewed in Strasburger and Wakai[40]).

SKF acted selectively on TRPC channels and not on LCC or TCC since NIF and mibefradil instead reduced QTp interval and had no effect on the QRS and ventricular beating rate. The prolongation of the QT interval could result from increased inward LCC current as described in human type 8 Long QT syndrome (as reviewed in Hedley *et al.*[42]). Similarly, the ventricular tachycardia associated with prolonged QT interval and widened QRS, which finally led to arrest, could be related to LCC-dependent calcium overload as shown in the human and mouse heart model.[43,44] Indeed, we showed that the prolongation of QT interval is predominantly due to LCC activity since exposure to SKF and NIF in combination reversed the effect of SKF alone on QTp interval (*Table 1*). However, NIF did not prevent the SKF-induced QRS widening, which seems to be independent of LCC. Thus, TRPC channels may play a direct role in the control of intra-ventricular conduction as well as in atrioventricular conduction as discussed already.

Co-immunoprecipitation experiments demonstrated that all TRPC channels interact with the α1C subunit of the Cav1.2 channel in the ventricle. Additionally, TRPC channels are known to be regulatory elements for other channels forming macromolecular complex such as cystic fibrosis transmembrane conductance regulator in airway epithelial human cells and BKCa potassium channel in vascular smooth muscle cells.[45,46] These observations lead to the proposal that TRPC channels act as elements regulating negatively Cav1.2 channel by a direct or indirect interaction (*Figure 6B*). Indeed, calcium entry through TRPC channels may lead to Ca^{2+} accumulation in subsarcolemmal area close to Cav1.2 channel controlling the Ca^{2+}-dependent inactivation of Cav1.2 channel. Thus, the inhibition of TRPC channels by SKF could induce conformational changes in the ionic pore structure of Cav1.2 channel and/or could delay Ca^{2+}-dependent inactivation of the α1C subunit leading to its overactivation and cellular Ca^{2+} overload.

4.3 Modulation of ventricular contractility by TRPC channels

Ca^{2+}-free medium rapidly suppresses most of contractions of the embryonic chick heart indicating that contraction depends largely on extracellular Ca^{2+} influx. It is also well known that TRPC channels participate to contraction activity in smooth muscle and mesangial cells.[47–49]

Our findings show for the first time that specific blockade of LCC by NIF stopped contractions in the developing heart but did not affect ventricular electrical activity, revealing an electromechanical dissociation, whereas inhibition of TRPC channels by SKF diminished only partially the myocardial shortening. In contrast, Pyr3 inhibiting exclusively TRPC3 channel had no effect on contraction, suggesting that, at least, this TRPC isoform may not be involved in contractility of the embryonic myocardium. Thus, the Ca^{2+} transient necessary for contraction depends essentially on Ca^{2+} entry through LCC. TRPC channels, acting as cationic channels independently of LCC, may have a complementary role in preventing rapid depletion of the modest Ca^{2+} SR stores of the embryonic heart. This mechanism could provide a continuous store refilling during repetitive electromechanical activity ensuring Ca^{2+} homeostasis and optimal EC coupling gain (*Figure 6C*). Recently, it has been shown that TRPC1 channel modulates Ca^{2+} entry during repeated muscle contractions and allows mouse skeletal muscle to maintain force production during sustained stimulations and cope with fatigue.[50]

4.4 Conclusion

Our findings indicate that, in the developing chick heart, which possesses an early and highly complex calcium signalling network, LCC and TRPC channels appear to form a functional macromolecular complex capable of subtle and efficient regulation of pacemaking generation and conduction. Furthermore, Ca^{2+} transient triggering myofilaments interaction may depend predominantly on LCC with a complementary role for TRPC channels to refill the relatively limited sarcoplasmic Ca^{2+} stores and insure the sustained cardiac activity of the embryo.

The types of arrhythmias provoked by TRPC channels inhibition in our model are also observed in the suffering foetal human heart. Despite such similarities, there is a limitation to translate findings from one species to another. However, in the context of the recent advances in foetal cardiology, our experimental data give new insights into the underlying mechanisms of the dysfunction of the developing heart, a rather unexplored but promising domain.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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